Investigation of the Activation of Tumour-Specific Immune Responses by Gene Therapy Strategies Using a Model Tumour Antigen

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To my lovely parents,

Without your support and care nothing would have been possible,

Thank you for everything.

Abstract

Gene directed enzyme prodrug therapy using *E.coli* the enzyme nitroreductase (NR) to activate the prodrug CB1954, is being developed as an attractive targeted chemotherapy for eradication of localized tumours. In addition to direct killing of NR-expressing tumour cells and potentially also their immediate neighbours via local spread of the activated prodrug, the consequent release of tumour antigens from dying tumour cells has the potential to induce antitumour immune responses. The present study investigates the capacity of NR/CB1954-mediated tumour cell death to activate $CD8^+$ T cell responses using ovalbumin (OVA), as a model tumour antigen. The transgenic adenocarcinoma mouse prostate tumour cell line (Tramp-C1) was modified to stably express the therapeutic NR gene together OVA. These modified tumour cells were used to seed tumours in mice and OVA-specific T cell responses to gene therapy were investigated. Treatment of mice bearing NR-expressing tumours with CB1954 enhanced expansion of endogenous OVA-specific $CD8^+$ T cells and marginally enhanced OVA-specific cytotoxic T lymphocyte (CTL) activity, however long-term $CD8^+$ T cell dependent immunity was insignificant.

The possibility of enhancing NR/CB1954-mediated long-term antitumour immune responses by combining with other immunogene therapies namely, 4-1BB costimulatory ligand (4-1BBL) or granulocyte macrophage colony stimulation factor (GM-CSF) was further explored. These combined therapies notably increased the frequency of memory OVA-specific $CD8^+$ T cell and CTL response in some lymphoid tissue relative to NR/CB1954 monotherapy.

One of the obstacles to cancer immunotherapy is the development of T cell anergy early in the course of tumour progression, therefore it was of interest to investigate

the potential of NR/CB1954 and 4-1BBL combined tumour therapy to reverse $CD8^+$ T cells anergy *in vivo*. This study describes preliminary results showing the effect of this combined therapy on the proliferative and functional responsiveness of anergic $CD8^+$ T cells.

In conclusion, these findings indicate that NR/CB1954-mediated tumour cell death is a weakly immunogenic process that facilitates short-term antitumour $CD8^+$ T cell responses. Combining NR/CB1954 with intratumoural GM-CSF or 4-1BBL immunotherapy can enhance the frequency and effector function of memory tumour antigen-specific $CD8^+$ T cells; and thus has the potential to provide long-term antitumour immunity.

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Abbreviations

Ab	Antibody
ADEPT	Antibody directed enzyme prodrug therapy
AICD	Activation induced cell death
AINR	Activation induced non-responsiveness
APC	Antigen presenting cells
cDNA	Complementary DNA
CFSE	Carboxy Fluorescein diacetate, Succinimidyl Ester
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen 4
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
DLN	Draining lymph node
DMEM	Dulbecco's modified Eagle medium
E. coli	Escherichia coli
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorter
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
GDEPT	Gene directed enzyme prodrug therapy
GM-CSF	Granulocyte-macrophage colony stimulating factor
Gy	Gray
hr	Hour
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
i.p.	intraperitoneal
i.v.	Intravenous
kb	Kilobase
kDa	Kilodalton
kg	Kilogram
LN	Lymph node
M	Molar
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatability complex
mg	Milligram
min	Minutes
ml	Millilitre
mM	Millimolar
NaCl	Sodium chloride
N-DLN	Non draining lymph node
n.d	Not determined
ng	Nanogram
NK	Natural killer cells
NR	Nitroreductase
OVA	Ovalbumin
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
RAG	Recombination activating gene
RNA	Roswell Park Memorial Institute
RT	Room temperature
s.c.	Subcutaneous
SDS	Sodium dodecyl sulphate
TAA	Tumour associated antigen
TAP	Transporters associated with antigen processing
TCR	T cell receptor
Tg	Transgenic
Th1	Type 1helper T-cell
Th2	Type 2 helper T-cell
TIL	Tumour infiltrating lymphocytes
TNFR	Tumour necrosis factor receptor
TRAF	TNF Receptor Associated Factor
TRAIL	TNF-related apoptosis-inducing ligand
Treg	T regulatory cells
Tris	Tris(hydroxymethyl) methylamine
TSA	Tumour specific antigen
v/v	Volume/volume
V	Volts
wt	Wild type
w/v	Weight/volume
µg	Microgram
µM	Micromolar
µm	Micrometer

1 Introduction

1.1 Overview

Cancer is one of the leading causes of death in the world. In the next decades, the number of cancer cases and mortality is expected to double due to increased life expectancies**,** posing a major public health challenge (World Cancer Report 2008-IARC). Exposure to several environmental and genetic factors contributes to transformation of normal cells to acquire autonomous hyperproliferative and limitless survival capacities capable of forming cancer. Thus far, cancer are mainly treated with conventional therapies including surgery, chemotherapy or radiation, however these treatments may have undesirable adverse effects and limited efficacy. The negative impact of these options and treatment failure promoted the search for novel approaches with reduced systemic toxicity**,** enhanced efficacy to improve patients' survival and quality of life.

Among the different research areas for treatment of cancer is the use of targeted therapies. Ideally, these treatments endeavour to selectively kill cancerous cells while sparing normal cells from the harmful effects and complications of treatment. There are multiple types of targeted approaches available for treatment of cancer, including molecularly targeted therapies e.g. tyrosine kinase inhibitors and biologically targeted therapies such as antibodies and gene therapy. Another promising research field involves the use of vaccines or immunomodulatory agents to harness both the innate and adaptive immune system of patients to reject cancer. More recently there is a growing interest in the use of combined approaches for treatment of tumours owing to the complex nature of cancer as a disease. Indeed, combined therapies will offer the opportunity for tailoring of treatment according to the type of the tumour; also assist in overcoming resistance and recurrence of cancer. One of the interesting combined approaches is to eradicate tumour cells via

targeted chemotherapy therapy together with stimulating the host immune system using immunotherapeutic agents to augment the antitumour immune response. The synergistic potential of these two different modalities holds potential for eliciting improved therapeutic results and it is likely applicable to many types of cancer (Baxevanis et al., 2009).

This thesis is focussed on assessing the generation of antitumour immune response to one of the gene therapy approaches, which utilize *E.coli* nitroreductase enzyme (NR) to activate the prodrug CB1954 thereby inducing localized tumour cell death. Also, investigating improved immune response by combining with immunomodulatory genes. This introduction will therefore briefly review some key aspects of the immune system and cancer development and immunosurveillance, before discussing some examples of immune and gene therapy for treatment of cancer.

1.2 Cancer development

Historically, cancer development has long been considered as a multistage and a multifactorial process that depend on mutational events. Each mutation provides selective growth advantage of mutated cells to escape from their normal regulatory controls and become self-sufficient in survival towards tumour progression. In the early stages of cancer development, normal cells acquire irreversible DNA damage in critical genes regulating normal cell growth, differentiation and death, favouring uncontrolled proliferation of initiated clones (Coleman and Tsongalis, 1995). This clonal expansion can be triggered in somatic tissues not only by external factors but also by inherited mutations. During tumour progression, increased genomic instability enables the initiated cell population to override checkpoints and control mechanisms by developing self-sufficiency in growth signals, insensitivity to

growth inhibitory signals, and evasion of programmed cell death leading to limitless replicative potential. Eventually, tumour expands obligating growth of new blood vessels for nutrient supply and facilitating invasion and distant settlement – metastasis– of tumour cells in other tissues (Hanahan and Weinberg, 2000).

In parallel to cell-intrinsic mechanisms of tumour suppression, there is compelling evidence that the immune system surveys for and eliminates newly transformed cells (Zitvogel et al., 2006); however eradication of transformed cells occurs based on antigenic differences and before clinical presentation (Shankaran et al., 2001). In contrast, there are other studies that support the role of chronic inflammation as an indispensable factor in the initiation and promotion of malignant disease (Balkwill et al., 2005; Coussens and Werb, 2002). Failure of tumour immunosurveillance is associated with increasingly aggressive tumour growth, further resistance to immune destruction and subversion of the immune response (Prestwich et al., 2008).

1.3 The immune system

The host's immune system is the main defence mechanism against pathogenic micro-organisms and non infectious foreign substances. It provides a rapid and specific means of protection against a broad range of pathogens, toxic, and allergenic substances using a complex network of molecules, interdependent haematopoietic cells and lymphoid organs. Host protection against infection is provided by two types of immunity: The innate (or *non specific*) and the adaptive (or *specific*) immune response. A key feature of the immune system is its ability to discriminate self from non-self and to avoid harmful immune response to self (Chaplin, 2003).

1.3.1 Innate immunity

The innate immune response represents the first line of defence against invading pathogens. It functions through (1) physical barriers such as, epithelial layers and mucous membranes that produce mucus to trap pathogens, (2) phagocytic cells (neutrophils, monocytes, macrophages and dendritic cells) and natural killer cells (NK), (3) biochemical mediators including members of the complement system, acute phase reactants and cytokines that coordinate and activate cells of the innate immune system. In contrast to the adaptive immune response, innate immunity is rapid, short-lived and does not confer protective immunity; it helps at early times to limit infection and acts to regulate the adaptive immune system (Beutler, 2004).

1.3.2 Adaptive immunity

The adaptive immune system evolved exclusively in higher vertebrates. It is so called adaptive due to the ability of its cellular component to recognize or identify pathogen-specific antigens and respond by clonal expansion of initially rare precursors with antigen recognition receptors of appropriate specificity. This process is usually slow and it takes several days to mount an effective immune response and up to three weeks to eliminate these antigens. Another fundamental feature of the adaptive response is the diversity of antigen receptors on lymphocytes generated by random rearrangement of gene segments encoding each subunit of the antibodies from B cell and T cell receptor (TCR) of T cells (Chaplin, 2010).

The key cellular components of the adaptive immune system are B lymphocytes and T lymphocytes; however their functions are mainly dependent on the antigen presentation process that is mediated by members of the innate immune cells, namely dendritic cells (DCs). Mature DCs therefore serve as a major link between

innate and adaptive immunity through priming of T cells which in turn play an important role in regulating B cell proliferation and differentiation (Fig 1-1) (Reis e Sousa, 2004).

B cells are involved in the production of antibody-secreting cells that can directly recognise native antigen through their B cell receptors and hence constitute the humoral immune response. T lymphocytes, on the other hand, are responsible for cell-mediated immunity and can only recognize peptides displayed by antigen presenting cells (APCs) in the context of major histocompatability complex molecules. Antibodies and cytokines produced during the adaptive immune response not only form the second line of immune defence against antigenic challenge, but are also involved in activation and modulation of innate immunity via recruitment of innate cells and enhancement of antibody-mediated opsonization and cytotoxicity (Delves and Roitt, 2000).

Central to the adaptive immune system's exquisite specificity for antigens is its ability to form memory and life long immunity. This feature stimulated extensive research towards designing strategies to harness the immune system to generate protective antitumour responses. Employing the host's immune system represents a more natural and safer mean to fight cancer (Steer et al., 2010).

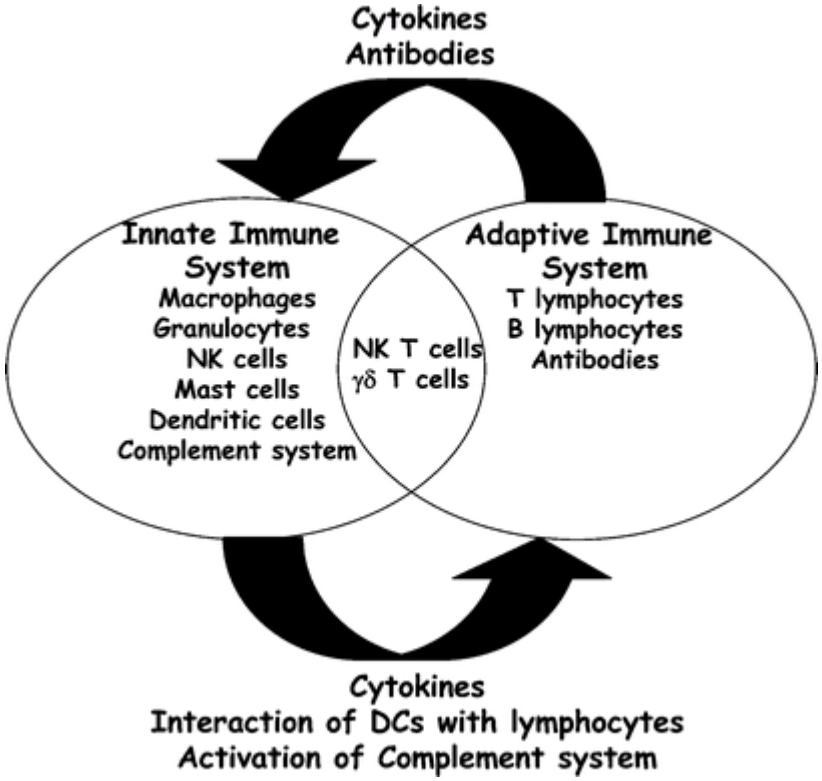


Figure 1-1: The interplay between innate and adaptive immunity
A schematic representation of the innate and the adaptive immune system, showing the complex interactions between cells of both subsets and their soluble factors. (Visser and Coussens 2005).

1.4 Recognition of cancer by the immune cells

In normal cells intracellular proteins are continuously being synthesized and degraded to maintain several important cellular homeostatic functions. Tumour cells, in contrast to normal cells, acquire several genetic and epigenetic alterations during the carcinogenesis process resulting in generation of novel proteins and over-expression of pre-existing proteins. Consequently, changes in the antigenic profile of tumour cells can allow multiple different epitopes from tumour-related proteins to be presented on the cell surface, thereby increasing the chances of eliciting specific immune responses (Pardoll, 2003). Evidence for the involvement of tumour antigens in tumour protection was demonstrated by the pioneering work of Boon and co-workers using tumour rejection mouse models (Boon and Van Pel, 1978).

This was further extended to show that these antigens were recognized by cytotoxic T lymphocytes (CTL) but were not capable of inducing a detectable antibody response (Sibille et al., 1990; Van Snick et al., 1982). There is some evidence regarding generation of humoral immune response in tumour rejection models and cancer patients, however this did not correlate with tumour protection or the disease state (Disis et al., 1999; Qin et al., 1998). Yet other experimental studies demonstrated the importance of the cellular immune response rather than the humoral element as the mediator of tumour immunity during tumour rejection (Rosenberg, 2001; Townsend and Bodmer, 1989).

Tumour cells express normal and altered proteins that are degraded into numerous antigens; few of these molecules are recognized by the immune system and can be broadly divided into two categories: tumour-associated antigens (TAA) and tumour-specific antigens (TSA). TAA are molecules expressed by both normal and tumour cells, however in tumour cells they are expressed in an immunogenic form due to abnormal level of expression, altered post translational modification, or wrong location. This distinguishes them from TSA that are only expressed on tumour cells and are products of mutated genes (Finn, 2006).

1.4.1 T cell antigens

To date, different approaches were utilised for detection of tumour antigens, aiming at identification of peptides recognised by T-cells. The genetic approach entail transfection of complementary DNA from tumour cells into target cells to examine recognition by autologous tumour-specific CTLs (van der Bruggen et al., 1991). This strategy led to the identification of MAGE, BAGE, and GAGE gene products referred to as tumour rejection antigens (Miles et al., 2006). A different biochemical

approach involve the elution of peptides bound to major histocompatability class I (MHC I) complex expressed on tumour lysates followed by further fractionation and identification (Hunt et al., 1992). More recently, serological analysis of antigens by recombinant expression cloning (SEREX) has emerged as an effective approach for identification of tumour antigen in a wide range of tumour types. This method relies on the reactivity of Immunoglobulin G (IgG) antibodies in serum of cancer patient against proteins encoded by prokaryotic cDNA libraries from tumour cells, assuming that humoral immune response is generated concurrent with T cell help and for which CTL may exist (Chen et al., 1997; Rosenberg, 2001).

According to the pattern of expression and potency in eliciting tumour rejection, T cell antigens were classified into four groups (Gilboa, 1999). Group I comprises patient-specific tumour antigens arising from somatic mutations in normal gene products due to the genetic instability of tumour cells during the oncogenic process. Group II represents tumour-specific antigens that are specially expressed only in tumour cells; however they could be shared among cancer patients. These antigens result from mutations in oncogenes or tumour suppressor proteins (e.g. ras and p53) or present in cancers of viral aetiology (epstein-barr viral antigens). Group III includes shared tumour antigens that correspond to normal gene products with highly restricted tissue distribution (e.g. cancer testis antigens; MAGE). Group IV also comprises shared tumour antigens, but correspond to normal tissue specific gene products, namely, "differentiation antigens" (such as melanocyte differentiation antigens; MelanA/MART-1). This group of antigens is expected to induce some degree of tolerance and represents the least potent tumour rejection antigens among the previously mentioned groups.

1.4.2 Antigen processing and presentation

Initiation of an adaptive immune response begins when immature DCs, being the most potent APCs, capture protein antigens and process them for presentation in the context of MHC. Immature DCs are unable to present these antigens efficiently unless they become activated in response to "danger signals" resulting from reaction to tissue damage or recognition of invading pathogens. DCs can sense these maturation stimuli through pattern recognition receptors (e.g. Toll like receptors) that detect pathogen-associated molecular patterns (e.g. lipopolysaccharide or double stranded RNA) and damage-associated molecular patterns (e.g. heat shock proteins or uric acid), also via cytokine receptors. Influenced by the maturation stimulus, DCs upregulate costimulatory molecules and increase surface expression of adhesion and chemokine receptors to enable them to migrate through afferent lymphatic vessel to sentinel lymph nodes (Guermonprez et al., 2002; Steinman, 2001). Meanwhile, naïve B and T cell clones capable of recognizing specific antigens are confined to secondary lymphoid tissues (lymph nodes, Peyer's patches and spleen and other organs) and are continually recirculating via lymph and blood in search of antigens. Once in the lymph nodes (LNs), mature DCs provide naïve T cells with immunological signals required for their activation and differentiation. The first stimulus is initiated by T-cell receptor (TCR) recognition of antigen presented in the context of MHC molecules. Accordingly, the type of MHC molecule determines the type of adaptive immune response (Joffre et al., 2009; Lammermann and Sixt, 2008).

MHC I molecules, expressed by all nucleated cells, display normal and abnormal peptide antigens on their surface for $CD8^+$ T cells mediating cellular immunity. On the other hand MHC II molecules are only expressed by APCs including

macrophages, B cells and DCs that present peptides from endocytosed proteins to $CD4^+$ T cells stimulating helper T cell-dependent humoral immune response. The pathway for protein degradation associated with antigen processing and presentation are mainly determined by the subcellular localization of the antigen processing and can be broadly categorized into the cytosolic and the endocytic pathway (Brodsky and Guagliardi, 1991).

1.4.2.1 The cytosolic pathway

Foreign antigens present in the cytosol are products of pathogenic organisms infecting host cells or mutated or overexpressed genes as in tumour cells. The majority of these proteins are generally degraded into peptides via the ubiquitin-proteosome system. Initially cytosolic proteins are tagged with multiple ubiquitin molecules in preparation for recognition and proteolysis by the proteosomes (Fig 1-2a). The generated peptides are translocated to the endoplasmic reticulum (ER) by a heterodimeric peptide transporter associated with antigen processing protein (TAP-1 and TAP-2). These proteins are members of the superfamily of ATP-binding cassette (ABC) transporters that utilize ATP to drive the transport of structurally diverse molecules. Translocated peptides bind to newly synthesized MHC class I molecules under the control of several ER resident chaperons (tapasin, calnexin, calreticulin). Stable MHC-peptide complexes are rapidly exported to the cell surface via the Golgi compartment for possible recognition by $CD8^+$ T cells (Guermonprez et al., 2002; Lankat-Buttgereit and Tampe, 2002).

1.4.2.2 The endocytic pathway

Extracellular proteins are internalized by phagocytosis or endocytosis by specialized APCs. After internalization proteins are degraded into peptides within highly acidic

compartments of the endocytic pathway. After being synthesized in the ER, MHC class II αβ heterodimers are translocated in association with the invariant chain (Ii) protein to late endosomal compartments called the (MIIC) (Fig 1-2b). Inside this compartment Ii is cleaved by proteolytic enzymes leaving a short peptide from Ii, the MHC class II–associated invariant-chain peptide (CLIP), bound to the peptide-binding groove. A non polymorphic protein human leukocyte antigen (HLA)-DM and (HLA)-DO catalyzes the dissociation of CLIP and facilitates peptide exchange. The MHC II-peptide complexes are then transferred to the surface of APCs for recognition by $CD4^+$ T cells (Jensen, 2007; Landsverk et al., 2009).

1.4.2.3 Cross presentation

Classically, peptides from cytosolic proteins are presented by MHC-I molecule, while proteins internalized from external sources are expected to access the endocytic pathway for presentation in association with MHC-II molecules. This dichotomy was challenged by Bevan's work showing that exogenous cellular antigens could be captured by APCs and used to prime $CD8^+$ T cells in a process termed 'cross-priming' (Bevan, 1976). Carbone and colleagues, subsequently, defined the cellular processes involved in presentation of exogenous antigens associated with cross priming as 'cross-presentation' (Fig 1-2c). Further studies identified B cells, endothelial cells, macrophages and in particular DCs to possess a unique ability to process exogenous protein through TAP-dependent and TAP-independent mechanisms for cross presentation and stimulation of $CD8^+$ T cells (Heath and Carbone, 2001; Jensen, 2007) . Evidence from several studies suggested that entry of exogenous protein into the cross-presentation pathway is largely influenced by the nature of the antigen, its stability, level of expression, mechanism of internalization by APCs and the type of APCs involved (Rock and Shen, 2005).

The process of cross-presentation of cell-associated antigens has evolved to permit tissue immune surveillance and is required for generation of cellular immunity against intracellular infection and tumours. In some instances, the physiological outcome of cross presentation can lead to cross-tolerance which involves CTL deletion or inducing a state of T cell anergy. This process is essential for the maintenance of peripheral tolerance and prevention of autoimmunity (Steinman et al., 2003).

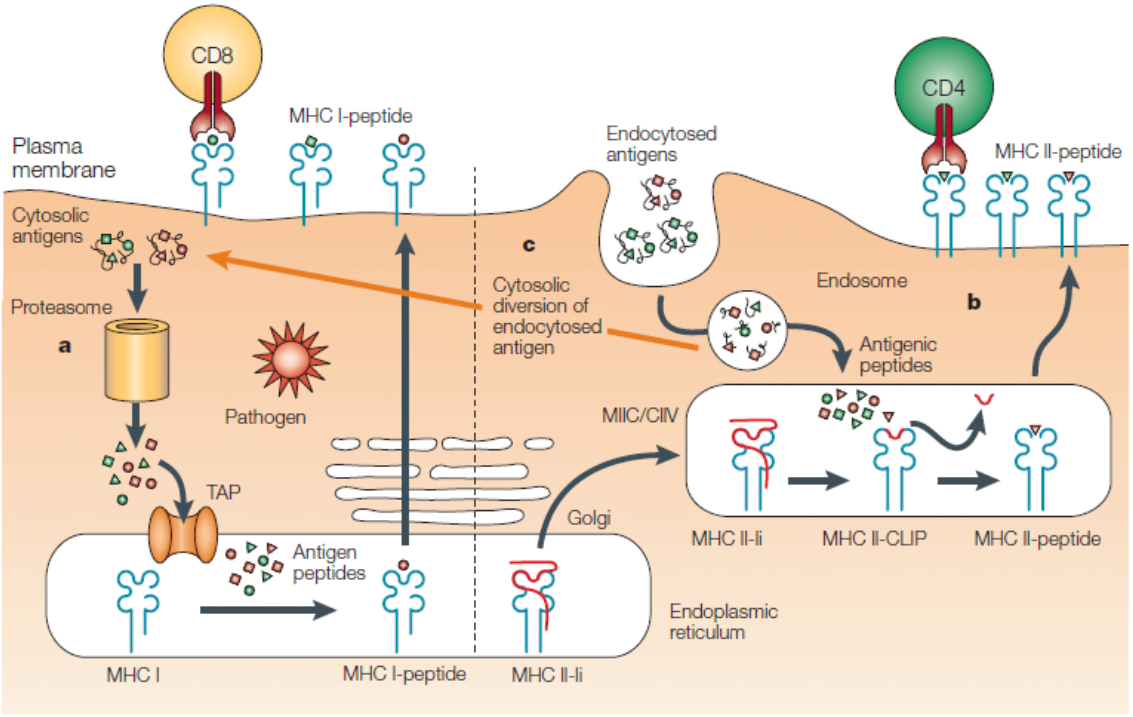


Figure 1-2: Antigen processing and presentation
Schematic representation of antigen processing **a**, via the MHC class I; **b**, MHC class II; and **c**, cross-presentation pathways (Heath and Carbone, 2001).

1.5 T cells

1.5.1 Overview

Based on the type of the chain involved in the T cell receptor (TCR) that is present on the surface of T cells and is responsible for antigen recognition, T lymphocytes

can be divided into two types: alpha beta T cells (αβ T cells) and gamma delta T cells (γδ T cells). The heterodimeric αβ T lymphocytes account for the majority of circulating T cells and reside mainly in lymphoid tissue; whereas γδ T cells constitute 1-5% of spleen and lymph nodes T cells but are highly abundant in epithelial layers of various organs, such as the small intestine, liver and reproductive tract (Chien and Bonneville, 2006). As αβ T cell predominate in most mammals and more information is available regarding their functions compared to γδ T cells, this discussion will therefore only consider the αβ T cell subset.

The αβ TCR polypeptides have very short cytoplasmic tails that lack signalling capacity; however they contain positively charged amino acids important for associations between TCR and the negatively charged amino acids within the transmembrane regions of the CD3 signal transduction complex. The CD3 coreceptor is composed of three invariant dimers: CD3-epsilon/gamma and CD3-epsilon/delta heterodimers and a TCR-zeta homodimer, which are non-covalently associated with the TCR heterodimer (Guy and Vignali, 2009). The intracellular domains of each of the CD3 chains contain immunoreceptor tyrosine-based activation motifs (ITAMs) that mediate downstream signalling events. Phosphorylated ITAMs following antigen recognition, promote the recruitment and subsequent activation of the protein tyrosine kinase ZAP-70 thereby initiating a signalling cascade which leads to T-cell activation and differentiation (Pitcher and van Oers, 2003).

Antigen recognition by T cells is a selective and restricted process that involves engagement of the TCR/CD3 complex to antigenic peptide in association with major histocompatability proteins expressed on the surface of target cells or APCs in a phenomenon termed “MHC restriction”. Antigens presented by MHC class I

molecules are recognized by cytotoxic $CD8^+$ T lymphocytes (CTL), whereas peptides displayed by MHC class II molecules are identified by helper $CD4^+$ T cells (Th).

In general, T helper cells are a major source of cytokine production that provide help for other effector cells of the immune system and are particularly important for B cell proliferation and maturation into antibody secreting cells; while cytotoxic T cells are programmed to destroy cells expressing foreign antigens bound to class I MHC molecules. However, in reality, the functions of these cells are much more diverse and highly integrated.

1.5.2 T cell receptor diversity

T cell receptor is a heterodimer composed of transmembrane polypeptides α and β chains. The two chains are covalently linked to each other by disulphide bonds. Each chain of the heterodimer contains an invariant membrane proximal domain termed the constant or C region and a highly variable membrane distal domain known as V region. The variable domains are encoded in the germline by a multiple variable (V) regions, joining (J) regions, and in some cases diversity (D) regions. Within these variable regions of both α and β chains there are three hypervariable loops corresponding to most of the variability and complementary in structure to the antigenic determinant or epitope; these are therefore known as complementarity-determining regions (CDRs) (Nikolich-Zugich et al., 2004; Schatz et al., 1992).

TCR diversity is generated during T cell development by combinatorial rearrangement of the V, D, and J gene segments initiated by two Recombination Activating Gene products known as RAG-1 and RAG-2. In the V(D)J recombination process, RAG proteins excise the DNA between a pair of specific recombination signal sequences (RSSs) adjacent to the V(D)J gene segments.

Additional TCR diversity is generated by random insertion or deletion of non-germline encoded nucleotides into the junctions before joining during the break repair process, thus the TCR diversity estimate is expected to be more than 1×10^{13} TCR- αβ receptors (Davis and Bjorkman, 1988; Krangel, 2009).

1.5.3 T cell activation

T cells play a major role in regulating the adaptive immune response; however their specialized functions are only prominent once they become activated. The events of T cell activation is initiated by the interaction of TCR complex with foreign antigens bound to MHC molecules presented on the surface of APCs. This signal alone evokes antigen-specific unresponsiveness (termed T cell anergy) or apoptotic T cell death, and needs to be complemented by other receptor/ligand interaction between T cells and APCs for optimal T-cell activation and acquisition of effector functions. This second signal is provided by specialized costimulatory molecules such as B7-1 (CD80) and B7-2 (CD86), expressed by the DCs, and which trigger CD28 expressed on naive T cells. Moreover, a third signal mediated by the cytokine milieu provides instructive signals for full differentiation of T cells into various effector subsets (Bretscher, 1999; Corthay, 2006; Prlic et al., 2007).

T cell activation is a multistep dynamic process that involves establishment of initial T cell/APC intimate physical contact to allow scanning for foreign peptide-MHC (p-MHC) complexes. In absence of antigen, T cells continue to crawl around the APCs or leave for another target cell. This initial contact is mainly dependent on loose adhesion forces generated by appropriate ligand/receptor interactions such as CD28/B7, CD2/CD58, or LFA-1 (lymphocyte function–associated antigen1)/ICAMs (immunoglobulin superfamily ligand). Once T cells encounter immunogenic p-MHC complex, the scanning process will halt resulting in

formation of the immunological synapse (IS) (Acuto and Cantrell, 2000; van Der Merwe and Davis, 2002). At this stage TCR activation initiates T cell cytoskeletal reorganisation that bring about microclustering of the TCR and segregation of other molecules including the coreceptor CD4 or CD8, costimulatory receptor CD28, the adhesion molecule CD2 and various associated signalling molecules to form the central supramolecular activation complex (cSMAC). The peripheral edge of the SMAC is surrounded by a ring of the cognate LFA-1 integrin and its cognate ligand ICAM- 1, defined as the pSMAC (peripheral SMAC). The region outside the pSMAC is enriched with large molecules such as CD43, CD45 and CD148, and referred to as the dSMAC (distal SMAC) (Freiberg et al., 2002; Valitutti, 2008). Although the main role of Immunological synapse is generally controversial, several studies suggest that it serves in stabilization of the T cell–APC conjugation to sustain signalling through the TCR for the long duration required for productive T-cell activation and execution of effector functions (Davis and van der Merwe, 2001; Rodriguez-Fernandez et al., 2010).

The T cell response to an antigenic stimulus can be divided into three successive phases: expansion, contraction and memory. The expansion phase involves the initial encounter of antigen by antigen-specific T cells that undergo antigen-driven clonal proliferation and differentiate into effector cells. Activated T cells are characterized by their ability to secret cytokines, mediate cytotoxic activity and upregulate tissue homing receptors to extravasate into non lymphoid tissues. This enables activated T cells to survey peripheral tissues and execute their effector function where antigens on the surface of infected cells or tumour cells are present. T cell expansion usually plateaus within 1-2 weeks after the initial antigen challenge and is followed by a contraction phase, whereby 90–95% of the effector T cells

present will undergo apoptosis over the next 2-4 weeks. During the memory phase, the remaining 5-10% of T cells survives for extended periods as differentiated memory T cells. Unlike naïve T cells, memory T cells are more readily activated in the presence of low antigen levels and have less stringent requirement for costimulatory molecules to undergo proliferation. Memory T cells can also rapidly acquire effector functions such as the ability to kill target cells and/or secrete inflammatory cytokines upon re-exposure to antigens (Ahmed and Gray, 1996; Kaech et al., 2002; Tan and Surh, 2006).

1.5.3.1 T cell activation and costimulation

Optimal activation of naïve T cells requires recognition of a p-MHC complex and is particularly dependent on the CD28 costimulation to augment initial clonal expansion by increasing the strength of signalling and protection against cell death. This initial costimulatory signal is triggered within the early hours after encountering an antigen and seems to be insufficient to provide long-term survival of T cells; however additional sustained or at least periodic costimulatory signalling is required to support longevity of effector or memory cells. This explained the existence of a diverse range of costimulatory molecules with distinct and overlapping functions that is tightly regulated by the different activation status of the T cells and the associated inflammatory environment (Croft, 2003; Lenschow et al., 1996; Song et al., 2004).

The receptor-ligand couples providing costimulatory signal for T cells, belong to a broad array of proteins and are involved in various cellular functions regulating T cell activation and tolerance. These molecules can be classified based on their molecular structure into two main groups namely: immunoglobulin (Ig) family members such as CD28 and the inducible costimulator (ICOS), and the tumour

necrosis factor receptor (TNFR) superfamily members including 4-1BB receptor. The cytokine receptors possessing the common γ-chain such as IL-2R, IL-4R, IL-7R, and IL-15R could also be considered as a group of costimulatory molecule, since they play an important role in regulating T cell survival (Croft, 2003; Vinay and Kwon, 2009).

At the same time, T cell responses are negatively regulated by inhibitory signals via programmed death 1 (PD-1) and cytotoxic T lymphocyte antigen (CTLA-4) receptors that have been described to down modulate ongoing T cell responses thereby preventing T-cell hyperactivation and maintaining self tolerance (Driessens et al., 2009).

The next section will briefly cover some key aspects of the two main families of the costimulatory molecules before discussing 4-1BB receptor/4-1BB ligand costimulatory system in more detail.

1.5.3.1.1 Immunoglobulin gene superfamily–costimulatory molecules

Costimulatory receptors of this group, also called the B7/CD28 family, are type I transmembrane proteins with a single IgV extracellular domain while the ligands are type I transmembrane proteins with both IgV and IgC extracellular domains. There are currently several known ligand members for this family mainly: B7.1 (CD80), B7.2 (CD86), inducible costimulator ligand (ICOS-L), programmed death-1 ligand (PD-L1), programmed death-2 ligand (PD-L2), B7-H3, and B7-H4.

The best characterized costimulatory pathway of this family involves B7-1(CD80) and B7-2 (CD86), which bind to two structurally related but functionally distinct receptors, CD28 and CTLA-4 (CD152). CD28 is constitutively expressed on the surface of naïve T cells and interaction with its ligand on antigen-presenting cells induces cell proliferation, interlukin-2 (IL-2) production and provides a critical

survival signal via activation of antiapototic factor Bcl-X_L. CD28 mediated costimulation also enhances the expression of other costimulatory molecules (CD40L) in addition to other inducible costimulatory receptors OX-40 and 4-1BB essential for Th-independent CTL immunity (Diehl et al., 2002; Walker et al., 1999; Yang and Wilson, 1996).

In contrast to CD28 receptor, CTLA-4 expression is rapidly upregulated within the early hours following T cell activation and is dependent on initial TCR engagement and CD28 costimulation. Moreover, CTLA-4 exhibit higher affinity for B7-1 and B7-2 than CD28, facilitating preferential engagement of CTLA-4 receptor on activated T cells and blockade of the B7/CD28 costimulatory pathway. Ligation of CTLA-4 delivers inhibitory signals that antagonize the initial stimulatory effects of CD28/B7 interaction and dampens the T cell response. In this way, CTLA-4 acts as a cell intrinsic inhibitor of inappropriate activation of T cells and for preservation of homeostasis. Indeed, CTLA-4 polymorphisms in humans have been associated with several autoimmune disease including Graves' disease, autoimmune hypothyroidism and type 1 diabetes (Ueda et al., 2003).

1.5.3.1.2 Tumour necrosis factor receptor (TNFR) superfamily

Different types of TNF family receptors can be classified based upon their cytoplasmic sequences and signalling properties into three major groups, namely, death domain (DD)–containing receptors, decoy receptors (DcR), and TNF receptor-associated factor (TRAF) binding receptors. Those with the death domain are known as death receptors (DRs) such as DR1 (also known as TNF-R1), DR2 (Fas), DR3, DR4 (also known as TNF-related apoptosis-inducing ligand receptor 1; TRAIL-R1), DR5 (also known as TRAIL-R2) and DR6. Crosslinking of these receptors by their corresponding ligands stimulate recruitment of DD-containing

signalling intermediates such as Fas associated death domain (FADD) and TNFR associated DD (TRADD) to activate caspase cascades, leading to apoptosis. Interestingly decoy receptors, DcR1 and DcR2 do not contain functional intracellular signalling domains or motifs and are capable of competing with DR4 or DR5 receptors for binding to the ligand (TRAIL), thereby inhibiting activation of signal transduction pathways by other TNF receptors (Aggarwal, 2003; Watts, 2005). Several members belonging to the third group of receptors, TRAF binding receptor, e.g. CD27, CD134 (OX-40), CD137 (4-1BB) herpes virus entry mediator/ (HVEM) T, CD30, and Glucocorticoid-induced TNFR family related receptor (GITR) has been reported to serve as costimulatory molecules for T-cell activation. Although, the cytoplasmic tail of this group lacks the DD, they contain TRAF-interacting motifs (TIMs) that function to recruit TRAF proteins. Mammalian TRAFs are a family of intracellular adaptors proteins (TRAF1 to 6) primarily involved in activation of signal transduction pathways such as NF-κB, JNK, ERK, p38 and PI3K, which regulate several cellular developmental and differentiating processes (Dempsey et al., 2003).

The expression of TNFR family members that provide costimulatory signals are generally inducible following antigen recognition except for CD27 and HEMV that are constitutively expressed by naïve T cells at a low level and are modulated after T cell activation. Similarly, the ligands for these receptors are not constitutively expressed by resting or immature APCs, but are activation induced, possibly in parallel with the upregulation of the expression of their receptors by T cells (Croft, 2003, 2009).

1.5.3.1.2.1 4-1BB Receptor/4-1BB Ligand

4-1BB (CD137) is a type I membrane protein, containing a cysteine rich extracellular domain, and a member of the TNFR gene family that provides another T cell co-stimulatory signal. It was originally identified by Kwon and coworker as an inducible molecule expressed by murine helper and cytolytic T cells (Kwon and Weissman, 1989). In addition to activated T cells, 4-1BB has been detected on activated NK, DCs and neutrophils from mice, whereas in humans 4-1BB expression was extended to include other cells e.g. follicular DCs, monocytes, and hepatoma cells (Cheuk et al., 2004). A soluble form of 4-1BB has also been identified in sera of patients with rheumatoid arthritis (Michel et al., 1998). 4-1BB expression by T cells in response to antigen stimulation was reported to reach its peak by 60 hr and decline within 4-5 days in mice; however the response in human is more rapid reaching a maximal level of expression by 8 hr and diminishes by 48 hr (Cheuk et al., 2004). During primary response, the expression of 4-1BB receptor coincides with the upregulation of the early activation antigen CD69 and precedes the transition to the $CD44^{hi}$ effector phenotype (Dawicki and Watts, 2004).

Ligand binding and aggregation of 4-1BB on the surface of T cells is associated with recruitment of TRAF1 and TRAF2 leading to downstream activation of mitogen-associated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K) and NF-κB signalling which contribute to cell division, enhanced cell survival and cytokine production. The NF-κB signalling pathway is known to be important for cell survival via regulation of prosurvival members of the Bcl-2 family including Bcl-XL and BFL1, and also promotes the production of cytokines, including IL-2, IL-4, IL-5 and interferon-γ (IFN-γ). Activation of the extracellular signal-regulated kinase (ERK) acts in synergy and down-regulates the pro-apoptotic molecule Bim;

while activation of other MAPK including c-Jun N-terminal Kinases and p38-MAPK promote production of cytokines (Croft, 2009; Wang et al., 2009).

The ligand for 4-1BB receptor, 4-1BBL (CD137L), is an inducible type II transmembrane glycoprotein with an extracellular carboxyterminal domain. The mouse 4-1BBL gene encodes 309 amino acids, which is poorly conserved with the human 4-1BBL protein, having only 36% amino acid identity with its human counterpart (Alderson et al., 1994; Goodwin et al., 1993). 4-1BB ligand is largely expressed following stimulation on professional APC such as DC, macrophages, and B-cells. In addition, 4-1BBL was reported to be expressed on tumour cells of myeloid, lymphoid and solid origin as well as by non-haematopoietic cells during inflammation (Cheung et al., 2007; Salih et al., 2000).

1.5.3.1.2.2 Costimulation of T cells by 4-1BB/4-1BBL

Evidence for a costimulatory role of 4-1BB/4-1BBL interaction on T cells comes from several studies using ligand transfected cells, antibodies to block receptor/ligand interactions or a soluble form of the 4-1BB fusion protein. 4-1BB ligation was reported to synergize with B7 molecule to stimulate proliferation, cell cycle progression and promote the overall cytokine secretion of naïve CD4 and CD8 T cells (Gramaglia et al., 2000; Vinay and Kwon, 1998). Studies using human and murine systems demonstrated that 4-1BB triggering preferentially augments the production of Th1 type cytokines (IFN-γ), as compared to that of the Th2 type (IL-4) (Maerten et al., 2006; Wen et al., 2002). In addition, 4-1BB costimulation protects activated T cells from activation induced cell death (AICD) contributing to an enlarged memory population (Hurtado et al., 1997). In relation to this effect, Takahashi et al. (1999) showed that agonistic anti-4-1BB antibody (mAb) preferentially inhibited peripheral death and deletion of super-antigen stimulated

$CD8^+$ T cells relative to $CD4^+$ T cells (Takahashi et al., 1999). The ability of 4-1BB signalling to promote proliferation and cytokine production of CD28 deficient T cells in response to TCR stimulation is also well-documented in the literature. A study by Maus et al. (2002) demonstrated that 4-1BBL engagement can replace CD28 signal during early T cell expansion *in vivo* (Maus et al., 2002). 4-1BB costimulation however can induce similar level of IL-2 production to that of the CD28 signal only when a strong TCR stimulus is provided, while it is less effective than CD28 mediated costimulation under conditions of limited antigenic stimulation (Saoulli et al., 1998).

In vitro, studies using anti-4-1BB mAb to stimulate $CD4^+$ and $CD8^+$ T cells demonstrated that 4-1BB signalling resulted in a four fold increase in proliferative capacity of $CD4^+$ T cells, while increasing the sensitivity for TCR stimulus and expansion of $CD8^+$ T cells by 100 fold (Shuford et al., 1997). These studies provided initial evidence for the preferential role of 4-1BB stimulation on $CD8^+$ T cells. This was further substantiated *in vivo* using 4-1BB and 4-1BBL deficient mice (DeBenedette et al., 1999; Kwon et al., 2002). Although, these animals showed normal development of lymphocytes and lymphoid organs, T cell effector functions, mainly CTL-mediated immune response were diminished in various viral and graft versus host rejection models (Bertram et al., 2002; Shedlock et al., 2003; Tan et al., 1999). Furthermore, tumour suppression observed in response to anti-4-1BB mAb treatment is believed to be mediated via enhanced $CD8^+$ T cell-mediated immunity (Miller et al., 2002; Taraban et al., 2002). Nevertheless, selective depletion of $CD4^+$ T cells in vivo resulted in loss of the antitumour effect (Melero et al., 1997). This was further explained by Giuntoli et al. (2002) demonstrating that helper T cells are

essential for maintenance and potentiation of the proliferative response and effector function of CTLs (Giuntoli et al., 2002).

In support of the role of 4-1BB signalling in augmenting secondary CTL response, Tan et al. (2000) showed that agonistic anti-4-1BB mAb can restore $CD8^+$ T cell responses in 4-1BBL$^{-/-}$ mice to a level similar to those in 4-1BBL$^{+/+}$ mice following immunization with lymphocytic choriomeningitis virus peptide (LCMV), however secondary CTL response was abrogated in 4-1BBL$^{-/-}$ mice upon viral rechallenge and in the absence of the agonistic anti-4-1BB mAb. The lack of secondary CTL response was attributed to lower numbers of viral-specific T cells with impaired ability to eliminate the infection compared to 4-1BBL$^{+/+}$ mice upon viral challenge (Tan et al., 2000). Similarly, Zhu et al. (2007) reported that 4-1BB deficient memory T cells failed to respond to CD137 mAb and that 4-1BB engagement selectively triggers memory but not naive T-cell proliferation *in vivo* (Zhu et al., 2007).

Interestingly, the 4-1BB receptor/ligand system mediates bidirectional transduction of signals for both interacting T and APCs. Binding of 4-1BBL on APCs provides a proliferative signal for B cells synergistically with anti-IgM antibody, while stimulating secretion of pro-inflammatory cytokines by macrophages such as IL-6, IL-8 and TNF-α, facilitating recruitment to the site of inflammation (Kang et al., 2007; Lippert et al., 2008; Vinay and Kwon, 1998). In addition, reverse signalling into lymphocytes through 4-1BB ligand appears to regulate T cell proliferation, increase expression of CD95 and induce apoptosis (Kwon et al., 2000; Suzuki and Fink, 1998).

1.5.4 T cell tolerance

The stochastic nature of the TCR rearrangement process may inevitably results in the generation of T cell that recognize self components , and thus having the potential to attack healthy tissues and posing a threat of developing autoimmune diseases. Different T cell tolerance mechanisms, however, have evolved as a fine balancing act to ensure maintenance of tolerance to self-antigens while retaining the capacity to mount robust immune responses against diverse antigenic stimuli during an adaptive immune response. These control mechanisms are broadly categorized into central and peripheral tolerance.

1.5.4.1 Central tolerance

Originally, T lymphocytes are generated from bone marrow progenitor cells that migrate and mature in the thymus. During αβ T cell maturation, T lymphoid progenitor cells undergo sequential rearrangement of the β and the α chains of the T cell receptor and initiate expression of both the CD4 and CD8 coreceptors, generating $CD4^+CD8^+$ double-positive (DP) thymocytes that express low levels of CD3/TCR complex (Lacorazza and Nikolich-Zugich, 2004; Rothenberg, 2002). In the thymic cortical epithelium, DP thymocytes capable of low affinity recognition of peptides bound to MHC I and MHC II molecules receive survival signals resulting in positive selection of these thymocytes and further differentiation into $CD4^+$ or $CD8^+$ single-positive (SP) T cells. In contrast, high-affinity interactions induce apoptotic death signals and negative selection of self reactive thymocytes. Only 3–5% of DP thymocytes survives these checkpoints and migrates as SP thymocytes to the medulla for further screening to ensure deletion of autoreactive thymocytes that have escaped negative selection. After maturation $CD4^+$ or $CD8^+$ T

cells specialized as helper and cytotoxic T cells, respectively, are then exported from the thymus and enter the peripheral circulation (Palmer, 2003; Takahama, 2006)

Remarkably, medullary thymic epithelial cells (m-TECs) posses the capacity to express a diverse range of tissue-restricted antigens (TRAs) in a process known as promiscuous gene expression. Although epigenetic mechanism and other transcriptional factors might be involved in TRAs expression, the transcriptional autoimmune regulator (AIRE) was reported to largely modulate the expression of a large proportion of TRAs in mTECs and function to induce negative selection of autoreactive thymocytes (Kont et al., 2008; Kyewski and Derbinski, 2004). In humans, as in mice, mutations in the AIRE gene results in autoimmune diseases that are characterized by the presence of autoantibodies to multiple self antigens and lymphocytic infiltration in target organs (Bjorses et al., 1998; Ramsey et al., 2002).

Despite the highly efficient negative selection process for thymocytes that recognize self-peptide–MHC complex with high affinity, some of the medium to high-affinity self-reactive T cells (mainly $CD4^+$ T cells) escape this process and undergo non-deletional central tolerance to give rise to natural T regulatory cells (nTregs). This subpopulation of T cells is essential for maintenance of immunologic self-tolerance and T cell homeostasis in non-inflammatory settings (Bluestone and Abbas, 2003; Sakaguchi, 2004).

Thymic derived Tregs arise from deletion resistant $CD4^+$ T cells that preferentially express the interleukin (IL) 2 receptor α chain (CD25) and the Forkhead box P3 (Foxp3) transcription factor. The importance of Foxp3 expression in development and function of Tregs was established by the observation that mutations in Foxp3 constitute the molecular cause of Treg deficiency resulting in fatal autoimmune

lymphoproliferative disease in both humans and in mice (Fontenot and Rudensky, 2005). While Foxp3 is selectively expressed in nTreg cells, peripheral naïve and effector T cells remain Foxp3-negative unless activated in the presence of immunosuppressive cytokines e.g. IL-10 and TGF-β, which stimulate their development into cells with Treg activity. These induced Treg cell population is termed adaptive Treg cells that function predominantly during self damaging inflammatory immune activation and autoimmunity (Bluestone and Abbas, 2003; Rothstein, 2006).

1.5.4.2 Peripheral tolerance

Although central tolerance is the main mechanism for elimination of autoreactive T cells, it remains incomplete where some T cells fail the intrathymic selection process and escape to the periphery. Therefore, the immune system provides other extrathymic control mechanisms to regulate peripheral self-reactive T cells and maintenance of immune tolerance to self. Arnold and colleagues described peripheral tolerance as a multistep mechanism that depends on the tolerogenic signal in inducing different levels of T cell tolerance, and that deeper states of tolerance can be achieved by sustained tolerogenic stimulation (Arnold et al., 1993). There are several mechanisms by which peripheral tolerance is achieved, however these can be broadly classified into clonal ignorance, death by deletion and functional unresponsiveness (Fazekas de St Groth, 2001). These mechanisms of peripheral tolerance are likely to be operating during the course of tumour development and progression contributing towards tumour-induced T cell tolerance and inefficient generation of antitumour immune response (Swann and Smyth, 2007).

1.5.4.2.1 Clonal ignorance

According to the affinity-based selection of thymocytes, autoreactive T cells entering the periphery have self-specific TCR of low affinity and hence can remain naïve even when auto-antigens are presented in the periphery. In support of this notion, Girgis et al. demonstrated that low avidity self-reactive T cells did not induce any signs of autoimmunity in transgenic mice expressing the cognate neo-self antigen; however high avidity T cells transferred into these animals proliferated and differentiated in response to the neo-self antigens. They also showed that low avidity T cells exhibit an anergic phenotype, which is responsible for maintenance of functional tolerance *in vivo* (Girgis et al., 1999).

The majority of tumour antigens identified to date are self-non mutated proteins that were found to be expressed within the thymus. As a result, many potentially high avidity tumour-reactive T cells are often eliminated from the T cell repertoire early during T cell development in the thymus (Sarma et al., 1999). The residual low affinity tumour-specific T cells were shown to lack the ability to recognise tumour cells or trigger suboptimal T cell activation contributing to the failure of the immune system in controlling tumour growth (Carrabba et al., 2003; Iero et al., 2007).

Peripheral high affinity/avidity T cells can also remain ignorant when the antigens are sequestered in immune-privileged sites such as the testis, central nervous system and the eye. However, there are pathological conditions when these sequestered antigens are presented in the presence of the right stimulatory conditions resulting in deleterious inflammatory immune responses. In such sites peripheral tolerance is normally maintained by other mechanisms including local production of immunosuppressive cytokines such as interleukin 10 (IL-10) and transforming

growth factor β (TGF-β), or expression of the inhibitory ligands e.g. FasL (Harber et al., 2000).

1.5.4.2.2 Peripheral deletion

Although clonal deletion of self- reactive T cells was thought to occur mainly in the thymus, evidence for extrathymic deletion in response to self-antigens was provided from earlier studies using mice that constitutively express self-superantigen derived from the Minor lymphocyte stimulating locus (Mls) (Webb et al., 1990). In addition to endogenous superantigens, peripheral deletion of T cells was also demonstrated for bacterial superantigens and other conventional viral and peptide antigens in both naïve $CD4^+$ and $CD8^+$ T cells (Fazekas de St Groth, 2001).

Subsequent studies using adoptive transfer of naive antigen-specific TCR transgenic (Tg) T cells into mice, containing defined antigens expressed under the control of tissue specific promoters, verified that deletion of autoreactive $CD8^+$ T cells was the primary mechanism by which these potentially damaging cells are regulated in the periphery (Garza et al., 2000; Kurts et al., 1997).

Deletional tolerance was also observed in tumour setting, over-expression of the p53 tumour antigen was reported to induce functional deletion of $CD8^+$ T cells carrying TCRs with the highest affinity for p53, resulting in selection of p53-specific, low avidity effector CTL (Hernandez et al., 2000).

Mature lymphocytes destined for deletion usually undergo passive apoptotic cell death due to the lack of survival stimuli, such as costimulators and cytokines, resulting in loss of expression of antiapoptotic proteins, mainly the Bcl family. In other instances apoptotic death occurs actively and is termed activation-induced cell death (AICD) that is mediated by molecules of TNFR superfamily e.g. Fas receptor. Activation of T cells causes upregulation of the death receptor, Fas (CD95), and its

ligand, FasL, stimulating T cells to kill each other through downstream components of Fas-mediated death signalling (Van Parijs and Abbas, 1996, 1998).

Another possible mechanism responsible for T cell deletion was recently proposed and involves the homeostatic cytokine IL-7. In the absence of the cognate antigen, naïve T cells express IL-7 receptor (IL-7R), which inhibits programmed cell death by the up-regulation of members of the anti-apoptotic bcl-2 family, and therefore maintain long-term survival of T cells in the peripheral compartment (Fry and Mackall, 2001). Stimulation of T cells with the Ag/MHC complexes promotes proliferation of T cells coupled with downregulation of the IL-7R which in turn results in stimulation of the proapoptotic Bim molecule implicated in clonal deletion (Bouillet et al., 2002). Redmond and Sherman proposed that weak antigenic stimulation under tolerogenic conditions induces elimination of the majority of T cells through a TCR-mediated proapoptotic signal (Fig 1-3). Upon chronic antigen exposure, the T cell population surviving the initial AICD would face either deletion or anergy which is mainly dependent on the strength of the antigenic stimulus. In situations of weak TCR stimulation, cells downregulate IL-7R and are unable to maintain expression of anti-apoptotic proteins and are therefore steered into the path of clonal deletion. In the second scenario, strong TCR signalling is associated with inhibition of Ras/ERK pathway, involved in T cell activation, leading to the emergence of an anergic phenotype (Redmond and Sherman, 2005).

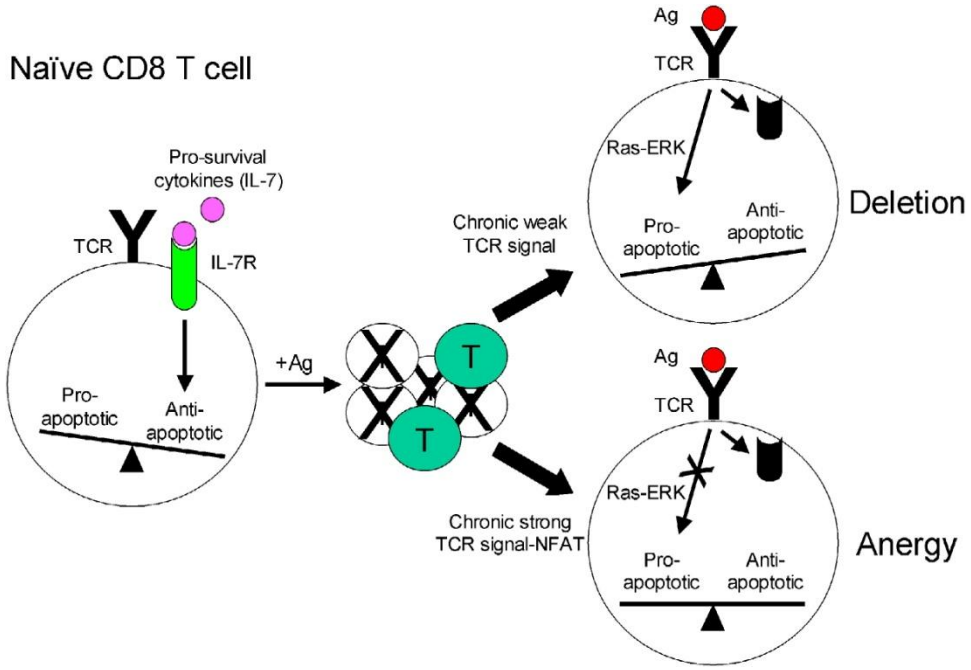


Figure 1-3: A model of CD8$^+$ T cells fate during peripheral tolerance: Anergy versus Deletion
In tolerogenic conditions, chronic exposure to antigen induces CD8$^+$ T cell proliferation and promotes downregulation of the IL-7R coupled with inhibition of anti-apoptotic genes that triggers deletion of activated cells. A small population of activated T cells can survive this deletional phase, however, if the antigenic stimuli continue these cells face different fates that are mainly determined by the strength of the antigenic stimulus. When T cells are exposed to low level of antigen, the balance is tipped towards death by deletion via a TCR-mediated proapoptotic signal. While in presence of a strong TCR-antigen interaction, inhibition of the Ras/ERK signalling pathway but not the proapoptotic signal is involved in induction of CD8$^+$ T cell anergy (Redmond and Sherman, 2005).

1.5.4.2.3 Functional inactivation

The term anergy was initially defined for B cells to describe a state of functional inactivation observed following administration of the relevant antigen *in vivo*. As the phenomenon of B cell unresponsiveness was antigen-specific, the description for this state was further refined to clonal anergy (Nossal and Pike, 1980). Subsequently, this term was used to describe murine and human T cell responses following tolerogenic stimulation that is characterized by impairment of the T cell ability to proliferate and produce IL-2 upon restimulation in the presence of a

costimulatory signal *in vitro* (Schwartz, 1990). In some cases clonal anergy is also associated with reduced production of IL-3, granulocyte macrophage colony stimulating factor and IFN-γ; however T cell responsiveness can be restored by addition of IL-2 (Beverly et al., 1992; Hollsberg et al., 1996). Since then any form of limited T cell proliferation, differentiation or cytokine production observed in a variety models of suboptimal T cell activation is generally termed 'anergy'.

1.5.4.2.3.1 $CD8^+$ T cell anergy

The first observation of anergy in $CD8^+$ T cells was reported using murine T cell clones stimulated with antigen and APC lacking costimulatory molecules. Anergic $CD8^+$ T cells were defective in IL-2 production and failed to proliferate but effector function including IFN-γ production and CTL activity were normal. Otten and Germain termed this form of unresponsiveness as 'split anergy' as costimulation was required for only certain TCR-dependent effector functions (Otten and Germain, 1991). Another level of $CD8^+$ T cell anergy was induced by stimulation of cells with allo-antigen in the presence of IL-10 treated DC. These conditions resulted in reduced CTL response in addition to the loss of proliferative capacity of anergic $CD8^+$ T cells (Steinbrink et al., 1999). Preckel et al. also found that impaired proliferative and cytolytic $CD8^+$ T cell responses resulting from presentation of altered hapten ligands by DC can be reversed upon addition of interleukin IL-2 or IL-12 plus IL-18 (Preckel et al., 2001).

Although full activation of $CD4^+$ T cells can be achieved by TCR and CD28 signalling, a third signal that can be provided by IL-12 or type I IFN seems to be required for productive activation of naïve $CD8^+$ T cells. The Mescher group showed that stimulation of $CD8^+$ T cells with high level of peptide/MHC complex and B7 protein immobilized on microtitre wells induced T cell proliferation but they

failed to develop cytolytic activity (Curtsinger et al., 2003a). Thus, at high levels of TCR engagement, optimal proliferation requires only IL-2, but development of cytolytic function requires IL-12; whereas low TCR engagement levels requires both cytokines for proliferation and acquisition of effector function. However, addition of IL-12 to the culture restored the cytolytic function of $CD8^+$ T cell induced in absence of the signal 3 stimulus (Curtsinger et al., 2003b).

Some of the coinhibitory receptors upregulated following engagement of the TCR by p-MHC present on APCs seems to play a role in induction of T cell anergy. Although CTLA-4 has been implicated in $CD4^+$ T cell anergy, $CD8^+$ T cell from CTLA-4 deficient mice were not susceptible to anergy similar to those observed in T cells from wild-type mice (Frauwirth et al., 2001; Greenwald et al., 2001). In contrast, PD-1 receptor was involved in establishment of tolerance in both $CD4^+$ and $CD8^+$ T cells. PD-1/PD-L1 interactions play an important role in regulation of autoimmune disorders observed in experimental models and inhibition of antitumour immune response (Srinivasan and Frauwirth, 2009). PD-1 and PD-L1 deficient $CD8^+$ OT-I Tg T cells proliferated vigorously upon tolerogenic stimulation and resisted induction of anergy, while wild-type OT-I T cells were rendered tolerant (Tsushima et al., 2007). Upregulation of PD-1 following antigen encounter suppresses IL-2 production in $CD8^+$ T cells during anergy induction. However, the proliferative response of anergic $CD8^+$ T cells was restored by addition of IL-2, irrespective of PD-1 expression by T cells (Chikuma et al., 2009).

Interestingly, effector $CD8^+$ T cells that initially received a full set of signals can develop a form of anergy termed activation-induced non-responsiveness (AINR). These cells are characterized by rapid decline in their proliferation rate and inability to produce IL-2; however they retain their capacity to mediate CTL response and

produce IFN-γ in response to antigenic stimulation (Deeths et al., 1999). This form of $CD8^+$ T cell anergy has been also observed *in vivo* and accounted for defective $CD8^+$ T cell response against tumour and virus models (Mescher et al., 2007).

1.6 Cancer immunosurveillance and immunoediting

The notion that the immune system can recognize and eliminate cancerous and/or precancerous cells before they develop into clinically apparent tumours was first proposed by Ehrlich in 1909. After fifty years, Burnet and Thomas formulated the concept of cancer immunosurveillance based on *in vivo* studies that promoted better understanding of tumour immunity. However, initial experimental examination of this hypothesis provided inadequate evidence to support suppression of cancer cells by the immune system and thus challenging the existence of the concept of cancer immunosurveillance. The comeback of the immunosurveillance concept was initiated by providing evidence that IFN-γ and lymphocytes control development of transplanted tumour and protect against chemically induced tumourogenesis. Since then, other studies have demonstrated the role of immune effector cells, such as B, T, NK and natural killer T (NKT) cells, and of type I and II IFNs in tumour immune surveillance (Dunn et al., 2002; Reiman et al., 2007). Supportive clinical information for involvement of the immune system in cancer control was concluded based on compelling evidence in three research areas including: the observation that immunocompromised patients are at higher risk of development viral cancers than the normal population; generation of endogenous immunity to autologous tumour cells; the presence of immune effector cells infiltrating tumour tissues that is positively correlated with patient survival (Dunn et al., 2004b).

Despite of the continuous cancer immune surveillance process, tumours can still develop in individuals with functioning immune system. Experimental studies

however provided evidence demonstrating the capacity of the immune system in selection of less immunogenic tumour variants capable of developing into cancer. The scope of this process was suggested to encompass three phases namely: elimination, equilibrium and escape (Dunn et al., 2004a; Zitvogel et al., 2006).

The classical concept of cancer immunosurveillance was modified to represent the elimination phase occurring in the early stages of tumour development, during which the innate and adaptive immune system work together to recognize and destroy tumour cells. In the elimination phase, the innate immune system responds to the proinflammatory signal –produced by tumour cells or the surrounding microenvironment– by migration to the developing tumour site. The innate immune cells including NK, NKT and γδ T cells may recognize tumour cells via NKG2D ligands resulting in production of IFN-γ and maturation of DCs. Mature DCs ingest tumour antigens released from dying tumour cells, induced by INF-γ dependent processes or NK-mediated cell killing, and migrate to lymph nodes to activate and recruit the adaptive immune cells. The generated tumour-specific T lymphocytes orchestrate the elimination of tumour cells and generation of antitumour immunity.

Although direct evidence for this process was difficult to find, the observation that immunodeficient mice lacking B, T, NK or NKT cells or cytokines are more susceptible to tumour development; also studies comparing tumour initiation, growth, and metastases in wild-type versus immunodeficient mice revealed the importance of the immune components in elimination of developing tumours (Kim et al., 2007).

As the elimination process continues, sculpting of the tumour cells yields tumour cell clones with a less immunogenic phenotype capable of evading immune destruction. This will initially lead to a state of equilibrium between tumour cell

death and escape. The duration of this phase is expected to occur over a period of many years and which would be mainly dependent on the efficiency of the individual immune system in controlling tumour cells. In support of this notion, Mackie et al. reported a case of two patients that received kidney transplants from a donor that has been treated from primary melanoma and was in remission for 16 years. The allograft recipients developed metastatic melanoma 1-2 years post transplantation suggesting that the donor's immune status maintained the tumour growth within the equilibrium phase; however transplantation in recipients with compromised immune system favoured progressive clinical metastatic disease (Dunn et al., 2004b; MacKie et al., 2003).

Eventually, tumour cells acquire multiple genetic and epigenetic alterations enabling them to evade and suppress antitumour immune responses and consequently develop into clinically detectable tumours. Tumour escape can be achieved on the tumour cell level by several mechanisms including loss of tumour-specific antigen recognition by the immune system –due to alteration in antigen processing and presentation–, and reduced susceptibility to tumour cell death. Additionally tumour cells can regulate antitumour immune responses by direct secretion of immunosuppressive cytokines e.g. IL-10 and transforming growth factor β (TGF-β) or indirectly via regulatory T cells, and hence contributing to induction of immunological tolerance (Prestwich et al., 2008).

1.7 Immunotherapy for cancer

Over the last two decades immunotherapy has evolved as a promising therapeutic modality for treatment of cancer. The current established therapies employ a broad range of approaches that exploit components of the adaptive and the innate immunity to selectively attack and eliminate tumour cells. These approaches can be

broadly characterized as passive or active therapies. In passive interventions, effector molecules or cells are administered to the patients to mediate direct tumour cell killing, e.g. monoclonal antibodies and Tg TCR T cells targeted at specific tumour antigens. By contrast active therapies involve harnessing the individual's immune system to elicit antitumour immune response e.g. vaccines, cytokines, costimulatory molecules and others (Dougan and Dranoff, 2009; Murphy, 2010). In some instances, different active immunotherapeutic strategies are combined together to potentiate tumour-specific immune responses for example, gene transfer of cytokines (Granulocyte Macrophage Colony-Stimulating Factor; GM-CSF, IL-2) into allogeneic, or autologous tumour cells in the form of cytokine gene-tumour cell vaccine (Jaffee et al., 2001; Palmer et al., 1999). Another form of combined immunotherapeutic approach involves co-administration of mAb against costimulatory receptors e.g. anti-OX-40 or anti-4-1BB mAbs together with vaccination with tumour antigen–loaded DC (Sharma et al., 2006).

There are an overwhelming number of clinical and preclinical studies available in the field of cancer immunotherapy; however the next section will focus on immunotherapies relevant to the present study.

1.7.1 Cytokines– Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF)

Cytokines are a large family of low-molecular weight proteins, released mainly by cells of the immune system, that are involved in several biological processes from cell proliferation to inflammation, immunity, migration, fibrosis, repair, and angiogenesis. These molecules act as a short-range chemical mediator between different immune cells and bind to high affinity receptors that require low receptor occupancy for optimal immune stimulation. During immune responses, cytokines

are secreted within the tissues at high concentration with a short-half life thereby reducing the amount reaching the circulation (Feldmann, 2008; Khawli et al., 2008). However, in severe inflammatory conditions aberrant cytokine secretion is associated with increased cytokine level in the blood resulting in inflammation and tissue damage. Therefore, systemic administration of cytokines is often associated with adverse effects including fever, hypotension, headache, malaise, weakness and capillary leaky syndrome (de Gast et al., 2000). This makes it difficult to achieve a clinically relevant dose without having unfavorable reaction; and hence limits the therapeutic application of systemic cytokines. Alternatively, other targeted rationales are adopted including direct intratumoural cytokine application, the use of cytokine-antibody fusion proteins with specificity for tumour-associated antigens, or cytokine-gene based approaches (Hornick et al., 1999; Jinushi and Tahara, 2009; Sone and Ogura, 1994).

It is a well established that DCs play an essential role in tumour surveillance by enhancing the cross-presentation of immunogenic tumour antigens and triggering adaptive immune T and B cell responses. Given the importance of DC in induction of antitumour immune response, targeting DCs at the tumour microenvironments represent a potential therapeutic strategy for a wide range of tumours.

Among the different cytokines that promote activation and differentiation of DC, GM-CSF was identified as the most potent cytokine mediating long lasting antitumour immunity following vaccination with cytokine-secreting tumour cells (Dranoff et al., 1993). GM-CSF is a member of a large family of glycoprotein growth factors that regulate the growth and differentiation of haematopoietic progenitor cells from various lineages, including granulocytes, macrophages, eosinophils and erythrocytes. It is produced by macrophages, T cells, mast cells,

endothelial cells, and fibroblasts in response to different immune and inflammatory stimuli (Gasson, 1991; Liu and Grundstrom, 2002).

The initial comparative analysis conducted by Dranoff et al. demonstrated the superiority of GM-CSF-secreting murine melanoma tumour cells in inducing tumour rejection relative to a variety of cytokines tested including IL-1, IL-2, IL-5, IL-6, INF-γ and TNF-α (Dranoff et al., 1993). The potency of GM-CSF at the vaccine site was related its ability to recruit and activate DCs to cross present tumour antigens for priming of $CD4^+$ T cells, $CD8^+$ T cells, and CD1-restricted invariant natural killer T (iNKT) cells to mediate antitumour immune response (Gillessen et al., 2003). Consistently, histological analysis of biopsies from the sites of injection with GM-CSF gene-modified tumour cell vaccine (GVAX) in patients with metastatic renal cell carcinoma and pancreatic cancer undergoing phase I clinical trial revealed dense macrophage, dendritic cell, eosinophil, neutrophil infiltrate up to 3 days from vaccination that was replaced by T-cell infiltrates by day seven (Jaffee et al., 2001; Simons et al., 1997).

In a dose-response preclinical study, the minimum level of GM-CSF secretion required for effective vaccination was 35 ng/10^6 cells/24 h, while higher doses did not improve the antitumour immune response (Jaffee et al., 1996). Serafini and colleagues further found that high doses of GM-CSF substantially diminish antigen-specific T cell mediated antitumour immune responses by recruitment of myeloid-derived suppressor cells (Serafini et al., 2004). In addition to the level of cytokine, distribution of the vaccine inoculum to different site can significantly modulate the therapeutic index of the vaccine. Indeed, vaccination of mice with a single vaccine dose that is divided over three different limbs provided improved vaccine-induced immunity compared to administration of the same dose at a single location. The

vaccine potency was also improved when tumour cell expressing lower levels of GM-CSF that showed reduced antitumour immune response –on single site administration– was distributed to several sites (Jaffee and Pardoll, 1997). Interestingly, the use of GM-CSF-secreting allogeneic tumour cell vaccine in preclinical model enhanced specific-antitumour immunity by increasing the immunogenicity of tumour cells thereby providing an alternative mean to overcome the technical difficulty of expanding primary autologous human tumour cells (Li et al., 2009; Thomas et al., 1998).

Promising results observed with GM-CSF-secreting cancer vaccine in preclinical studies led to phase I and phase I/II clinical testing of both autologus and allogeneic GM-CSF-secreting tumour cells. These clinical trials established safety and bioactivity –inferred from distinctive infiltrates of $CD4^+$ T cells, $CD8^+$ T cells, DCs and other inflammatory cells– following different dose escalation schemes in patients with metastatic renal cell carcinoma (RCC), metastatic melanoma, metastatic prostate carcinoma and early or advanced non-small cell lung cancer carcinoma (NSCLC) (Emens, 2009). The major side effects observed in some of the clinical trials using autologus tumour cells included erythema, swelling, pruritus and pain with systemic low grade fevers, chills and fatigue, while local grade 1 and 2 erythema, indurations and tenderness at the vaccine site was observed with allogeneic tumour cell vaccine.

The level of GM-CSF secretion by tumour cell vaccine varies between clinical trials (Table 1-1); however most clinical trials uses a dose cell range of $1x10^6$ -1×10^7 cell per vaccine. A phase II clinical trial conducted in patient with stage II or III pancreatic cancer received escalating high doses in the range of 1×10^7- 50×10^7 cells of allogeneic GM-CSF secreting tumour cells (220ng/10^6 cells/24 hr) demonstrated

that the highest dose of $5x10^8$ cell vaccine was well tolerated by patients and showed preliminary efficacy (Borrello and Pardoll, 2002; Eager and Nemunaitis, 2005).

Clinical evidence from phase I and II trials provided the base for further testing of GM-CSF-secreting tumour cell vaccination to phase III trials in prostate cancer. However, results from a completed phase III trial in hormone-refractory metastatic prostate cancer patients immunized with irradiated allogeneic GM-CSF-secreting tumour cells failed to provide evidence for clinical benefit compared to the standard docetaxel anticancer drug. These disappointing results led to the proposition of integrating GM-CSF-secreting tumour cell vaccination with other approaches e.g. anticancer drugs and immunostimulatory molecules (Gupta and Emens, 2010; Jinushi and Tahara, 2009).

Cancer (Stage)	Vaccine	Vector	Number of patients	Dose (number of irradiated cell)	GM-CSF production (ng/10^6 cells/24 h)
Renal cell (IV)	Autologus	Retrovirus	16	$4x10^6$ - $4x10^8$	17 -149
Melanoma (IV)	Autologus	Retrovirus	5	$1x10^7$	56 -100
Melanoma	Autologus	Retrovirus	29	ND	84 - 965
Pancreatic cancer (I,II,III)	Allogeneic	Plasmid	14	$1x10^7$ - $5x10^8$	ND
Prostate	Autologus	Adenovirus	8	$1x10^7$ or $5x10^7$	143 - 1403
NSCLC	Autologus	Adenovirus	35	$1x10^6$ - $1x10^7$	Mean 233
Melanoma (IV)	Autologus	Adenovirus	9	$2x10^6$ - $1x10^7$	80 - 424
Melanoma (IV)	Intratumoural injection	Vaccinia virus	7	N/A	N/A
Solid tumour	Intratumoural injection	Herpes simplex type 1	15	N/A	N/A
Melanoma (IV)	Autologus	Adenovirus	35	$1x10^6$ - $1x10^7$	745
Prostate	Allogeneic	Adenovirus	34	$1x10^8$ - $3x10^8$	-
Prostate	Allogeneic	Adenovirus	65	$1x10^8$ - $3x10^8$	-
NSCLC	Autologus	Adenovirus	83	$5x10^6$ - $1x10^8$	50 - 1871

Table 1-1: Summary of clinical trials testing GM-CSF gene-transduced vaccine (Eager and Nemunaitis, 2005)
ND, not described; N/A, not available

1.7.2 Costimulatory molecules – 4-1BBL

The role of 4-1BB costimulatory molecule in T cell activation was extensively studied as discussed in section 1.5.3.1.2.2. These studies have identified 4-1BB costimulation as a potential target for immunotherapeutic strategies aiming at enhancing the activation and maintenance of tumour-specific T cells.

In preclinical tumour models and clinical studies, provision of a costimulatory signal to tumour-specific T cells can be achieved using agonistic anti-4-1BB mAb that would enhance direct activation of tumour infiltrating lymphocytes (TIL) by tumour antigens without the need for APC costimulation. Systemic administration of agonistic anti-4-1BB mAbs eradicated established large tumours in mice, including the poorly immunogenic Ag104A sarcoma, B10.2 fibrosarcoma and EL4E7 lymphomas as well as the highly tumorigenic P815 mastocytoma. Tumour rejection was associated with marked augmentation of tumour specific CTL response (Melero et al., 1997; Miller et al., 2002; Wilcox et al., 2002). Furthermore, enhanced systemic antitumour immune response was observed in mice bearing 3 days intracranial MCA 205 sarcoma or GL262 glioma; however agonistic anti-4-1BB mAbs failed to demonstrate beneficial impact on metastasized poorly immunogenic tumours, including B16/D5 melanoma, C3 tumour, TC-1 lung carcinoma and B16-F10 melanoma (Kim et al., 2001; Wilcox et al., 2002). Wilcox and coworker's reported that failure of anti-4-1BB mAbs in treatment of these tumours was due to that immunological ignorance, rather than anergy or deletion of tumour-specific CTLs during the progressive tumour growth. T cell ignorance can be overcome by immunization with a tumour antigen–derived peptide together with anti-4-1BB mAbs treatment; however this was still insufficient for complete eradication of tumours (Wilcox et al., 2002).

Another approach of 4-1BB costimulation targeted cancer immunotherapy is genetic modification of tumour cells to express 4-1BBL. Tumour cells transduced with 4-1BBL cDNA showed reduced tumourogenicity and increased survival of rechallenged mice. The observed antitumour effect have been ascribed to production of Th1 $CD4^+$ T cell cytokines including IFN-γ, IL-2 and TNF-α and increased CTL activity. The same Th1 helper response and tumour rejection following vaccination was reported in mice immunized with K1735 melanoma transduced with membrane bound single-chain Fv fragments (scFv) of anti-4-1BB, however tumour regression was $CD4^+$ T cell and NK cell dependent, but $CD8^+$ T cell independent (Li et al., 2008; Ye et al., 2002).

Despite the efficiency of anti-4-1BB monoclonal in inducing antitumour immunity, Sun and colleagues demonstrated that anti-4-1BB antibodies can ameliorate the incidence and severity of experimental autoimmune encephalomyelitis autoimmunity and to suppress humoral immunity in mice (Sun et al., 2002a; Sun et al., 2002b). 4-1BB-mediated inhibition of immune response and blocking of disease progression has been reported in other autoimmune and inflammatory models in mice including experimental lupus, collagen-induced arthritis, graft-versus-host disease (GVHD), and allergic asthma (Lee et al., 2009). Controversially, 4-1BB costimulation can have a dual outcome by either stimulation or suppression of immune responses in tumour and autoimmune models, respectively. This dichotomy is apparently dependent on the type of lymphocyte involved, cellular activation status, and the nature of the immune response and thus targeting 4-1BB pathway for immunotherapy requires careful understanding of the pathogenesis, cellular component involved in the target disease, and the health situation of individual selected for therapy (Sun et al., 2004).

In other experimental studies, *ex vivo* generation of tumour-specific T cells using anti-4-1BB mAbs or tumour cell expressing 4-1BBL intended for adoptive therapies has been found to mediate superior antitumour immunity in different mouse tumour models (Strome et al., 2000; Yi et al., 2007).

These promising results were recently translated into a phase I study to examine tolerability and clinical activity of an anti-4-1BB mAb, designated BMS-663513, in melanoma, RCC, ovarian and prostate cancer patients. BMS-663513 was proven tolerable over a wide dose range (0.3-15 mg/kg) and showed increased levels of circulating activated $CD8^+$ and $CD4^+$ T cells, also increased expression of IFN-γ in post treatment tumour biopsies (Sznol M, 2008).

1.8 Gene therapy for cancer

Gene therapy is the delivery of functioning genes into cells or tissues with the aim to restore normal gene function or to provide new cellular function for treatment or slowing down the progression of a particular disease or for prophylactic purposes. The concept of gene therapy emerged early in 1960's and was pioneered following the progress in molecular cloning of mammalian genes into prokaryotic plasmids together with the development of viral vectors as a mean for gene transfer (Cotrim and Baum, 2008; Flotte, 2007). Early in the 1990's the first clinical gene therapy trial was started in patients with severe combined immunodeficiency (SCID) due to adenosine deaminase (ADA) deficiency. Since then, gene therapy is being considered as a potential new approach for treatment of a variety of disease such as monogenic disorders, cardiovascular and neurological diseases and cancer (Blaese et al., 1995; Culver et al., 1991). By 2010, the number of clinical trials for gene therapy reached 1703 study, 64.7% of which were for treatment of cancer. These cancer gene therapy trials encompassed a broad range of strategies that involve

mainly immunological targeted therapies (some examples were mentioned in section 1.6), tumour suppressor gene replacement, oncogene inactivation, suicide gene (gene directed enzyme prodrug), inhibition of angiogenesis, and viral oncolysis (www.wiley.co.uk/genmed/clinical).

In the next part of the introduction will briefly review the different types of vector that are available for gene therapy and then will focus on gene directed enzyme prodrug therapy as another form of targeted strategy, with greatest emphasis on the Nitroreductase/CB1954 system.

1.8.1 Gene delivery vectors

The transfer of genetic material to cancer cells or the tumour microenvironment represent a crucial step in gene therapy, thus several delivery systems have been developed to accommodate different experimental and clinical settings. The ideal delivery system for a successful gene therapy protocol would specifically target tumour cells, tissue or organ of interest, achieve transgene expression at a level sufficient for therapeutic benefit over a desired time course and capable of delivering large size therapeutic gene as well as being immunologically inert and safe for use in humans. Transgene delivery systems falls into two broad categories: viral and non-viral based vectors (Pfeifer and Verma, 2001).

1.8.1.1 Viral vectors

Viral vectors are genetic shuttles generated by replacing essential viral sequences – dispensable for replication, assembly or virulence– with therapeutic genes and transcriptional regulatory elements. These vectors are replication deficient and can only be replicated in packaging cell lines engineered to provide the deleted viral gene product for the production of recombinant virus. Some viral vectors have the

ability to integrate into the human genome and thus achieve long-term gene expression (such as retroviruses). The other non-integrating vectors remain episomal (e.g. adenoviral vector) and are prone to loss during cell division contributing to transient gene expression (Verma and Weitzman, 2005). Because of the efficiency of retroviral and adenoviral vectors in transgene delivery and the improved vector design, these vectors are the most commonly used delivery systems in gene therapy clinical trials and account together for nearly 45% of the gene transfer methods used in humans (www.wiley.co.uk/genmed/clinical).

Retroviruses are lipid enveloped viruses with single-stranded RNA genome, and can be classified into oncoretroviruses, lentiviruses, and spumaviruses. The simplest retroviral genome contains three essential genes, *gag*, *pol*, and *env* that are flanked by long terminal repeats (LTR); however complex retroviruses contain other genes encoding for accessory proteins required for viral life cycle. Early retroviral vectors for gene therapy have been derived from the simple MuLV by removal of viral proteins and insertion of up to 8 kb therapeutic gene downstream the 5′ LTR together with the packaging signal (Ψ). Transgene expression is regulated from the promoter/enhancer elements present in the U3 region of the 5' LTR or from an exogenous promoter (Hu and Pathak, 2000). However viral regulation of transgene expression under the enhancer/promoter elements of LTRs can lead to insertional activation of cellular oncogenes located adjacent to the vector integration site thereby increasing the risk for initiation of cancer. To increase the safety of retroviral vectors, self inactivating (SIN) vectors were engineered to contain an additional internal promoter to drive transgene expression but lack the U3 region of the 3' LTR and thus abolishing LTR driven transcriptional activity (Yu et al., 1986).

Although MuLV retroviral vectors can provide long-term gene expression, they have relatively low vector titre and can transduce only dividing cells. The latter limitation is a significant disadvantage in cancer gene therapy since typically minor fraction of the tumour cell will only be dividing at the time of gene therapy. This hurdle was overcome by generation of lentiviral vectors derived from human immunodeficiency virus 1 (HIV-1) that are capable of targeting both dividing and quiescent tumour cells in addition to improved virus titre level (Miyoshi et al., 1998).

Currently, adenoviral-based viral vectors are the most commonly used gene delivery system in gene therapy clinical studies. Although more than 52 human serotypes have been identified, the most commonly used adenoviral (Ad) vectors for gene delivery are generated from human adenoviruses of serotype 2 and 5. Human adenoviruses are a family of non-enveloped, double stranded DNA viruses. The adenoviral genome is composed of various transcriptional regions which include early regions (E1, E2, E3, E4), two delayed early units (IX and IVa2), and a late region (L1 through L5). Deletion of the E1 region of the adenoviral genome renders the virus replication-deficient and eliminates the potential for oncogenicity. The first generation of replication-deficient adenoviral vectors were E1 and often E3 deleted with a transgene cassette being inserted into the E1 deleted region. Further deletion of the E3 locus increases the capacity of Ad vectors to accommodate large transgene cassettes of up to 7.5 kb in size (Volpers and Kochanek, 2004). However, several reports recorded generation of humoral and cellular immune response against adenoviral proteins predisposing patients to severe immune and inflammatory symptoms and accounting for short-term transgene expression (Bessis et al., 2004; Ritter et al., 2002). More recently gutless or helper-dependent Ad was generated by

deleting all essential viral coding sequences to increase the safety and transgene capacity of Ad vectors (Alba et al., 2005).

Overall, adenoviral vectors are promising delivery system for gene therapy due to their high *in vivo* gene transfer efficiency, their ability to infect a wide spectrum of both dividing and non dividing cells of a variety of cell types and their capacity to deliver relatively large segments of foreign DNA. In clinical application, the relatively short-lived transgene expression renders adenoviral vectors more suitable for treatment of cancer but not monogenic disorders that require long-term gene expression provided by the DNA integrating viral vector (Bouard et al., 2009).

1.8.1.2 Non viral vectors

Another successful approach for the delivery of therapeutic transgene system is the use of non-viral vectors, which are increasingly used in clinical trials. These systems were developed to avoid some of the critical problems observed with viral vectors, such as the immune response, and limited packaging capacity. Potentially, their low toxicity and immunogenicity will allow repeated safe administration of gene therapy to achieve efficient long-term expression of therapeutic genes. Non viral gene therapy vectors are also an attractive tool due to their relatively simple manipulation and lower production cost. There are three general non viral vector systems currently being studied: cationic liposomes, DNA polymer conjugates, and naked DNA (Edelstein et al., 2007; Hughes, 2004).

1.8.2 Gene directed enzyme prodrug therapy (GDEPT)

Classical chemotherapy is an invaluable treatment for controlling and delaying the progress of many types of cancers. However, it is limited by insufficient therapeutic index, a lack of specificity, and the emergence of drug-resistant cell subpopulations,

resulting in reduced efficacy of drug therapies. This is particularly more pronounced when treating solid tumours with poor internal vascularization, imposing difficulty in systemic delivery of the drug and thus rendering solid tumours refractory to the therapy (Greco and Dachs, 2001; Niculescu-Duvaz and Springer, 2005). Moreover, many available chemotherapeutic agents are designed to selectively target rapidly dividing cells, resulting in considerable side effects to highly mitotic normal tissues such as bone marrow and the lining of the gastrointestinal tract.

One potential area to improve the selectivity of cancer chemotherapy for solid tumours is the promising field of enzyme prodrug gene therapy. In such a strategy, a non-mammalian enzyme (viral, bacterial or yeast) is delivered intratumorally, followed by systemic administration of enzyme-specific non-cytotoxic prodrugs, resulting in conversion of the prodrug into active cytotoxic substance that causes direct tumour cell death (Fig 1-4). In the literature, this strategy is known by different names: gene directed enzyme prodrug therapy (GDEPT); suicide gene therapy (SGT); virus directed enzyme prodrug therapy (VDEPT); and gene prodrug activation therapy (GPAT) (Niculescu-Duvaz et al., 1998).

The GDEPT approach offers several appealing features over the chemotherapy in treatment of cancer patients. Most importantly, the prodrug is activated only within the vicinity of tumour cells and thus sparing off-target organs from the adverse effects of toxic metabolites. Tumour selectivity can be also increased by expressing the prodrug-activating enzyme from a tumour or tissue-specific promoter, thereby delivering the therapeutic gene preferentially to the tumour cells. However, in some localized tumours such as locally recurrent prostate cancer or gliomas direct delivery of the gene can be achieved via localized injection.

Although, the therapeutic benefit of this approach is limited to the number of cell transduced with the transgene at the injection site, the generated metabolite can diffuse to neighbouring untransduced tumour cells inducing tumour cell death. This phenomenon is termed local bystander effect and helps overcome the problem of suboptimal gene delivery. In addition, the release of tumour antigens from dying cells can activate the host immune system via T and NK cells to eliminate local and distant untransduced tumour cells; this is known as the immune bystander effect. Both the local and immune bystander effect greatly contributes towards the observed therapeutic benefit observed in many studies (Freeman et al., 1996; Portsmouth et al., 2007; Saukkonen and Hemminki, 2004).

An ideal prodrug should be of high affinity to enzymes encoded by the transgenes used in therapy, metabolically stable in its prodrug state and able to diffuse efficiently through tissues. Moreover, the generated metabolite should be cell cycle independent and have satisfactory half life to allow for diffusion and mediation of a bystander effect (Portsmouth et al., 2007).

There are several enzyme and prodrug combinations that were proposed as promising GDEPT; however the most common enzyme-prodrug systems studied will be described below.

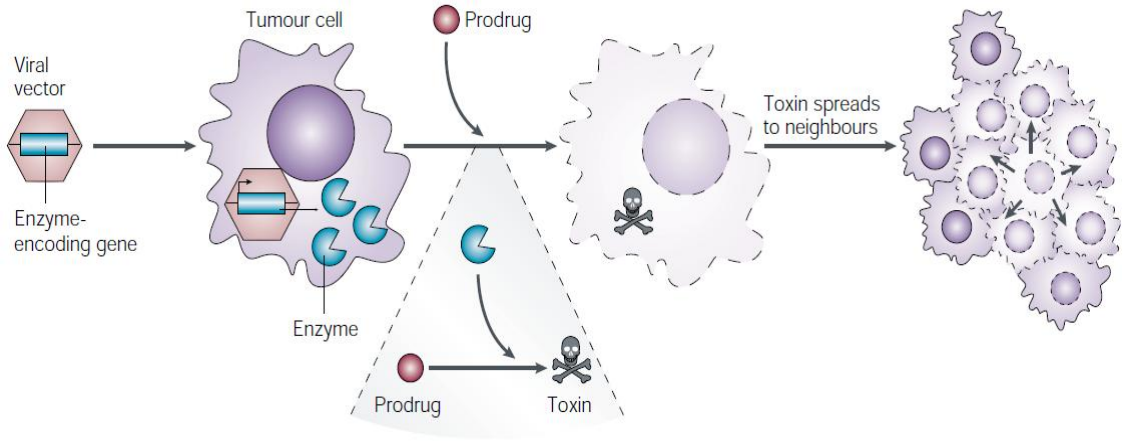


Figure 1-4: Schematic diagram of gene directed enzyme prodrug therapy (GDEPT).
A transgene encoding an enzyme is delivered the tumour using viral or non-viral vectors. Only a fraction of the target cells will express the foreign gene and synthesise the enzyme. After systemic administration of the prodrug, local prodrug activation takes place within transduced tumour cells inducing cell death. Toxic metabolites diffuse between tumour cells allowing for bystander eradication of neighbouring untransduced cells (McCormick, 2001).

1.8.2.1 Herpes simplex virus–thymidine kinase

The most widely studied GDEPT system is the herpes simplex virus (HSV) thymidine kinase (*tk*) enzyme in combination with the prodrug ganciclovir (GCV). Moolten first reported the potential value of this system by demonstrating chemosensitization of *tk*-transduced murine cell to a nucleoside analogue *in vitro* and *in vivo* (Moolten, 1986). HSV-*tk* is capable of phosphorylating purine nucleoside derivatives e.g. ganciclovir, acyclovir and bromovinyl-deoxyuridine to monophosphates. These analogues are usually used for the treatment of herpes simplex virus infections in humans and have poor affinity for the mammalian *tk*. The generated monophosphates are further phosphorylated by cellular guanylate kinase into to diphosphate and triphosphate forms. GCV-triphosphate is a nucleotide analogue which competes with deoxyguanosine triphosphate (dGTP) for

incorporation into elongating DNA by DNA polymerase α, causing premature strand termination, replication failure and cell death (Elion, 1983; Ilsley et al., 1995).

The HSV-*tk*/GCV system was reported to mediate S/G2-phase cell cycle arrests associated with apoptotic cell death; however the mode of cell death is seemingly dependent on the specific cell type used in the study (Wei et al., 1998). Given that GCV is cell cycle specific, this makes it particularly appropriate for targeting rapidly dividing tumour cells such as glioblastoma, medulloblastoma, and anaplastic astrocytoma (Chen et al., 1994). However, the proportion of dividing cells in rapidly growing tumours does not exceed 20% and thus the HSV-*tk*/GCV system has been considered less effective for slowly dividing tumours (Hoshino et al., 1986). Nevertheless, tumour regression was observed in subcutaneous adenocarcinoma and fibrosarcoma preclinical models when 10% of the tumour cells expressed HSV-*tk*. This was attributed to the transfer of activated GCV through gap junctions or via uptake of apoptotic vesicles by the surrounding cells and hence mediating bystander cell killing. The transfer of cytotoxicity to adjacent non-transduced cells requires tight cell to cell contact to allow for intracellular gap junction communication, since the highly charged ganciclovir triphosphate is lipid-insoluble and can not diffuse freely across the lipid bilayer of cell membranes (Culver et al., 1992; Mesnil and Yamasaki, 2000).

A number of gene delivery systems were shown suitable and efficient in the transfer of the HSV-*tk* including retroviral and adenoviral vectors, direct intratumoural naked DNA adminstration and liposomal polymers. Successful delivery of HSV-*tk* gene was also translated into promising preclinical results in animal models with established glioblastomas, liver metastases, hepatocellular carcinomas, human head and neck carcinomas, and human mesotheliomas (Greco and Dachs, 2001). The

system progressed further into clinical studies demonstrating initial efficacy and tolerability in patients with brain, colorectal, ovarian, prostate, head and neck, and malignant glioma (Fillat et al., 2003). In a phase III clinical trial, non significant benefit was observed in glioma patients receiving HSV-*tk*-expressing retrovirus producer cells coupled with GCV. This was explained by inefficient transgene delivery through the non-migratory vector-producing cells (Rainov, 2000). However, injection of an adenovirus expressing HSV-*tk* into the wound bed following resection of glioma, in combination with GCV administration, significantly increased median survival from 37.7 to 62.4 weeks (Immonen et al., 2004).

1.8.2.2 Cytosine deaminase

The system consisting of cytosine deaminase (CD) and 5-fluorocytosine (5-FC) has been considered particularly useful for the treatment of patients with metastatic colorectal carcinoma, since CD catalyzes the deamination of the antifungal agent 5-FC to 5-fluorouracil (5-FU), which is a chemotherapeutic agent widely used in the treatment of colorectal cancer (Palmer et al., 2002). Although, cytosine deaminase has been found in both bacteria and yeast, previous studies have reported that CD obtained from yeast is more efficient in converting 5-FC into 5-FU than its bacterial counterpart. This was also coupled with improved therapeutic outcome when using yeast CD compared to the bacteria enzyme in *in vivo* tumour models (Kievit et al., 1999).

The bioactivated 5-FU is further converted by cellular enzymes into 5-fluoro-deoxyuridine-5´-monophosphate (5-FdUMP) or phosphorylated to 5-fluorodeoxyuridine-5´-triphosphate (5-FdUTP). The earlier derivative is a thymidylate synthase inhibitor capable of inducing thymidine starvation leading to

cell death; while 5-FdUTP can replace UTP in RNA synthesis resulting in the inhibition of nuclear mRNA transport and ultimately DNA damage (Kerr et al., 1997).

CD/5-FU has been studied successfully as a potential gene therapy strategy for treatment of cancer from different origin in *in vitro* and *in vivo* tumour models. However, the therapeutic effect of the CD/5-FU system could be further enhanced by fusion of the CD gene to uracil phosphoribosyltransferase (UPRT) that catalyzes the direct conversion of 5-FU into its active metabolites, resulting in a more effective treatment of colorectal cancer in a preclinical model. This approach also markedly increased the bystander effect of CD/5-FU when examined *in vitro* (Chung-Faye et al., 2001b). A significant bystander effect was also reported in nude mice bearing colorectal tumour xenografts that express CD as few as 2% of tumour cells (Huber et al., 1994). Unlike HSV-*tk*/GCV, the local bystander effect of CD/5-FU does not require direct cell to cell contact or functional intracellular gap junction, since 5-FU can diffuse freely across the cell membrane by non-facilitated diffusion suggesting improved bystander effect over HSV-*tk*/GCV (Lawrence et al., 1998). Indeed, CD/5-FU showed higher *in vitro* bystander effect and therapeutic benefit than HSV-*tk*/GCV in Epstein Barr virus-associated lymphomas (Rogers et al., 1996), renal cell carcinoma (Shirakawa et al., 1999), and thyroid carcinomas (Nishihara et al., 1998).

The safety of CD/5-FU system as GDEPT was first examined in a phase I clinical trial in patients with breast cancer by direct intratumoural injection of a plasmid construct (Pandha et al., 1999). Subsequently, CD/5-FU was used in a phase I clinical trial in combination with HSV-*tk* gene therapy using replication-competent Ad viral vector for the treatment of prostate cancer (Freytag et al., 2002). The

double suicide gene therapy was again examined in prostate cancer patients but using replication-competent Ad viral vector with oncolytic properties concomitant with radiation therapy (Freytag et al., 2003). This Phase I/II clinical trial demonstrated absence of dose-limiting toxicities and of treatment-related severe adverse effects of the replication-competent adenovirus-mediated double-suicide gene therapy when combined with conventional radiotherapy. A different delivery system involving intratumoural injection of an attenuated strain of *Salmonella typhimurium* expressing the *E. coli* CD gene in refractory cancer patients, showed safety and promising efficacy in 2/3 patients (Nemunaitis et al., 2003).

1.8.2.3 *E. coli* Nitroreductase

In the GDEPT systems mentioned so far, the prodrug is converted by the enzyme to activated metabolites that are only toxic to actively dividing cells. However, the system using the wild-type *E. coli* nitroreductase, encoded by the *NfsB* gene, and the prodrug 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB1954) generates very potent DNA cross linking agents whose effects are largely cell cycle-independent (Kerr et al., 1997).

CB1954 is a weak monofunctional alkylating agent that was found to have a high therapeutic index against Walker rat carcinoma tumour model but was inactive against other tumours used for screening of anticancer drugs (Niculescu-Duvaz et al., 1998). The sensitivity of the Walker carcinoma cells was subsequently reported to be due to a high level expression of Walker rat DT diaphorase, a dehydrogenase enzyme that can use either NADH or NADPH as a cofactor. This enzyme catalyses the reduction of CB1954 to the bifunctional alkylator 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide, which after further reactions is converted to a more potent electrophile capable of forming permanent DNA interstrand cross-links

(Knox et al., 1988a; Knox et al., 1988b). However, the use of CB1954 against human cancer was limited by the poor efficiency of human DT diaphorase in conversion of CB1954 into its toxic metabolite (Boland et al., 1991).

Nitroreducatase (NR) isolated from the *E. coli* bacteria was found to bioactivate CB1954 up to 90 times faster than Walker rat DT diaphorase, suggesting that the nitroreducatase enzyme and the prodrug CB1954 (NR/CB1954) have the potential as directed enzyme/prodrug therapy (Anlezark et al., 1992). Subsequently, cloning and integration of the *nfsB* gene product, NR enzyme, in a retroviral vector allowed its evaluation in the GDEPT approach. NR expression in murine fibroblast NIH3T3 cells and several human cancers including melanoma, ovarian carcinoma or mesothelioma cells were rendered sensitive to CB1954 killing (Bridgewater et al., 1995; Michael et al., 1994). Also, NR/CB1954 showed promising results in preclinical studies using human tumour xenografts and syngeneic tumour models (Clark et al., 1997; Dachs et al., 2009; Djeha et al., 2000; Weedon et al., 2000). The toxic CB1954 metabolite induces p53 independent apoptosis resulting from inhibition of DNA function due to formation of DNA adducts and interstand cross links at higher rates than other potent DNA cross linking agents (Cui et al., 1999; Drabek et al., 1997; Friedlos et al., 1992) . This is reflected by the rapid cell death of NR expressing cells when treated with CB1954 following a short exposure time of 4 hours *in vitro*. Importantly, the DNA crosslinking agents are cell cycle independent and are capable of killing both proliferating and non-proliferating cells (Bridgewater et al., 1995).

GDEPT using NR/CB1954 system was reported to mediate an efficient bystander effect in a number of murine and human cell lines, since the primary CB1954 toxic metabolites are membrane permeable prodrugs and can diffuse freely through cell

membranes, regardless of the presence or absence of gap junctions or tight cell to cell contact (Bridgewater et al., 1997; Djeha et al., 2000; Green et al., 2003; McNeish et al., 1998). *In vitro* studies using mixed unmodified and NR-expressing ovarian carcinoma or pancreatic cancer cells showed that a significant bystander effect was observed when just 5-10% of total cell population express NR (Green et al., 1997). Similarly, a potent bystander effect was reported in *in vivo* tumour models with hepatocellular carcinoma and burkitt lymphoma when only 5-30% of tumour cells express the therapeutic enzymes (Djeha et al., 2000; Westphal et al., 2000).

On the basis of these promising preclinical studies, the safety and tolerability of CB1954 was examined in patients with gastrointestinal malignancies; this study determined a recommended an i.v dose of 24 mg/m^2. Based on *in vitro* studies, such a dose is likely to be associated with a clinical benefit in the presence of adequate NR expression. A phase I clinical trial was conducted using replication-defective adenovirus encoding nitroreducatase (CTL102) in patients with primary and secondary liver cancers prior to resection. This trial reported that up to 5×10^{11} particles of CTL102 can be administered by direct intratumoural injection resulting in high levels of NR expression in tumours with minimal side effects (Chung-Faye et al., 2001a; Palmer et al., 2004). The combination of CTL102 and CB1954 was subsequently tested in a phase I/II clinical trial for treatment of prostate cancer demonstrating safety and tolerability of this system. In addition, the trial also provided preliminary evidence suggesting partial biological efficacy represented, by a change in prostate specific antigen (PSA) kinetics in some patients (Patel et al., 2009).

In an attempt to increase the therapeutic efficacy of NR/CB1954, NR was subjected to modification based on structural activity relationship studies, designed to generate mutant NR with improved prodrug activation. Recently two NR mutants (T41L/N71S and T41L/F70A) were engineered with superior efficiency (14-17-folds) than wild-type NR at sensitising the cancer cells to CB1954 (Jaberipour et al., 2010). Also, several highly promising new prodrugs has been developed showing increased potency and greater bystander effect relative to the original CB1954 (Helsby et al., 2004; Jiang et al., 2006; Singleton et al., 2007).

1.8.2.4 GDEPT – Immune bystander effect

The notion that GDEPT systems can induce antitumour immune response was first suggested following the observation of intense inflammatory infiltrates of macrophages, $CD4^+$ and $CD8^+$ lymphocytes in tumours from immunocompetent mice treated with HSV-*tk*/GCV or CD/5-FC systems (Barba et al., 1993; Caruso et al., 1993; Consalvo et al., 1995). Ramesh and colleagues also detected proinflamatory cytokine TNF-α, IL-1, and IL-6 within HSV-*tk* expressing tumours 24 hours following GCV treatment, while INF-γ and GM-CSF was upregulated after 3-4 days, indicating that HSV-*tk*/GCV treatment initiates an intratumoural cytokine cascade that is favourable for generation of antitumour immune responses (Pope et al., 1997; Ramesh et al., 1996). The same study also showed that an intact host immune system is required for mediation of *in vivo* bystander effect, since athymic mice succumb to tumour development when only 50 % of tumour cell populations express HSV-*tk*. In contrast, an efficient bystander cell killing led to tumour rejection when tumours were established in immunocompetent animals.

An immune bystander effect helps improve the efficiency of the GDEPT by promoting the development of a systemic antitumour immunity. Indeed, a number

of studies demonstrated that HSV-*tk* or CD expressing tumours were eliminated in vivo following the systemic administration of prodrug. Treated animals remained tumour-free and were able to reject tumour cells following a rechallenge with a tumourigenic dose of untransduced parental tumour cells (Kuriyama et al., 2004). The protective immunity observed with CD-induced tumour regression in an adenosarcoma tumour model was found to be tumour specific and does not confer immunity against a rechallenge with wild-type fibrosarcoma (Mullen et al., 1994).

Classical preclinical studies to examine the distant bystander effect showed that treatment of subcutaneous HSV-*tk* or CD expressing tumours on one flank of immunocompetent mice had no effect on the progression or the size of unmodified parental tumour cells established on the contralateral flank (Freeman et al., 1993; Mullen et al., 1994). However, other studies reported the regression of anatomically distant hepatic, lung, and colorectal metastases as well distant ovarian tumours following ablation of localized HSV-*tk* or CD tumours (Misawa et al., 1997; Nagy et al., 2000; Pierrefite-Carle et al., 2002; Pierrefite-Carle et al., 1999). This discrepancy could be due to different transgene expression levels, immunogenicity of tumour cells or the anatomical location of tumour deposits.

In vivo depletion studies showed that $CD8^+$ T-cells or granulocytes but not $CD4^+$ T cells are critical for initial tumour regression in CD/5-FC treated mice; however long-term antitumour immunity was dependent on both $CD4^+$ and $CD8^+$ T cells (Consalvo et al., 1995). Yamamoto and colleagues reported that growth inhibition of a second tumour challenge in tumour-cured mice, treated with HSV-*tk* /GCV, was associated with efficient tumour-specific CTL response that was mainly mediated by $CD8^+$ T cells (Yamamoto et al., 1997). However, other studies

indicated NK cells are essential for the antitumour effects induced by HSV-*tk* /GCV system (Hall et al., 1998).

As described so far, an immune bystander effect play an important role in enhancing regression of localized and metastatic tumours treated by HSV-*tk* /GCV or CD/5-FC in many experimental system, however few studies have addressed the ability of NR/CB1954 system to mediate an antitumour immune responses in preclinical studies.

Green et al. demonstrate that AB22 murine mesothelioma tumours stably expressing NR failed to grow in 3/6 immunocompetent mice after treatment with CB1954, and the surviving animals were protected from a subsequent rechallenge with unmodified AB22 cells and remained free for the duration of the experiment (120 days) However this study was conducted with a small number of mice. Also, combining GM-CSF with NR/CB1954 was shown to delay tumour outgrowth and enhance survival of mice bearing colorectal or prostate tumours indicating that the therapeutic benefits of NR/CB1954 could be enhanced by stimulation of the immune system using cytokine gene therapy (Green et al., 2003; Young et al., 2008). However, animals that were tumour-free following treatment with NR/CB1954 plus GMCSF failed to reject tumour cells upon rechallenge with a tumourigenic dose of parental prostate tumour cells. Other studies also demonstrated that mice immunized with a low and a high dose of NR expressing tumour cell implant and treated with CB1954, remained tumour-free; but only mice implanted with initial high dose of tumour cells were able to resist tumour formation following a second tumour rechallenge. Improved tumour protection and long-term antitumour immunity was achieved by combining NR/CB1954 with GM-CSF or high-level expression of heat shock protein 70 (Djeha et al., 2005).

1.9 Aims of the thesis

The development of a specific, tumour specific antitumour immunity following treatment of cancer with NR/CB1954 would enhance tumour regression and ideally promote eradication of metastatic tumours and prevent tumour recurrence in cancer patients. However, little evidence has been reported to support generation of an immune bystander effect to NR/CB1954 system. To clearly investigate the effect of NR/CB1954 therapy on the immune response, the present study was designed to examine tumour-specific $CD8^+$ T cell responses to this enzyme/prodrug activation therapy, since $CD8^+$ T cells have been shown to play a critical role in tumour immunity. It also aims to explore $CD8^+$ T cell response to combined therapies of NR/CB1954 with immunostimulatory gene therapy involving 4-1BBL and GM-CSF.

To achieve these aims, the research worked with the following specific objectives:

- Establish an *in vivo* model tumour system to study NR/CB1954 mediated immune responses by modification of transgenic adenocarcinoma murine prostate cell line (Tramp-C1) to stably express the therapeutic NR gene together with ovalbumin (OVA) as a model tumour antigen.
- Study the capacity of NR/CB1954-mediated cytotoxicity to activate antitumour $CD8^+$ T cell responses using OT-I T cell adoptive transfer experiments in immunodeficient and immunocompetent mice or relying on the endogenous OVA-specific $CD8^+$ T cell responses in immunocompetent mice.

- Explore the possibility of augmenting NR/CB1954-mediated tumour-specific $CD8^+$ T cell responses by using the immunomodulatory genes 4-1BBL or GM-CSF.
- Establish $CD8^+$ T cell anergy in OT-I transgenic mice and further investigate the capacity of a combined tumour gene therapy of NR/CB1954 and 4-1BBL to reactivate adoptively transferred anergic OT-I T cells.

2 Materials and Methods

2.1 Suppliers of the materials

Unless otherwise stated all materials were supplied by Sigma-Aldrich (Poole, UK). Deionised water (ddH20) was obtained from a Maxima Ultrapure Water (ELGA, HighWycombe, UK).

2.2 Molecular biology

2.2.1 PCR amplification for molecular cloning

Polymerase chain reaction (PCR) was used for the amplification of DNA for cloning purposes, and diagnostic determination of *E. coli* transformants. Optimal primers and annealing temperatures were worked out and a PCR reaction containing 1µl of forward and reverse primer (50pmol/µl) (synthesised by Alta Biosciences, University of Birmingham, UK), 50ng DNA template, 1µl of 40 mM dNTP 5µl 10x expand high fidelity buffer (Roche), and double deionized water (ddH_2O) to adjust the total volume to 49µl, was heated to 93ºC. Pfu Taq enzyme (2.5U/µl) (Roche) was then added to the reaction as a hot start reaction. Reactions were allowed to cycle in a Whatman thermal cycler (Biometra, Whatman) under the conditions described in Table 2.1. For cloning purposes PCR products were purified by agarose gel electrophoresis and recovered DNA content was determined.

Primer description	Oligonucleotide sequence (5'-3')	PCR cycle
OVA-F1	GTA GCC ACC ATG GCT GCA GAT CAA GCC AGA GAG	94ºC for 5 min; (94ºC for 45 sec, 54ºC for 30 sec, 72ºC for 90 sec) x 25; 72ºC, 5 min
OVA-R1	GTC TGG ATG CAG CAG AGA AC	
OVA-F2	GAC TGA ATG GAC CAG TTC TAA TG	Sequencing PCR cycles
OVA-R2	CCT CCA TCT TCA TGC GAG G	
OVA-R3	GGA TGA AGA CAC ACA AGC AAT	94ºC for 5 min; (94ºC for 45 sec, 58ºC for 30 sec, 72ºC for 90 sec) x 25; 72ºC, 5 min
OVA-F3	TCT CTG CCT GCT TCA TTG ATT T	
SV40-F1	TCA ACC TGA CTT TGG AGG C	94ºC for 5 min; (94ºC for 45 sec, 55ºC for 30 sec, 72ºC for 90 sec) x 25; 72ºC, 5 min
SV40-R1	TTC CTC TGC TTC TTC TGG	
mGM-CSF-F1	CTT TTC CTG GGC ATT GTG G	Sequencing PCR cycles
mGM-CSF-R1	ATG CGG ATA GGT AAC	

Table 2-1: Oligonucleotide sequences used in molecular cloning

2.2.2 Restriction endonuclease digestion of DNA

For cloning and diagnostic DNA digestions, DNA restriction enzymes (Boehringer Mannheim or New England Biolabs) were used in accordance with the reaction conditions described in the manufacturer's instructions using the appropriate colour coded enzyme buffers. An analytical scale restriction enzyme digest was usually performed in a volume of 20µl on 0.5µg of substrate DNA with 2 µl of appropriate 10x enzyme buffer and 1 unit (U) of restriction endonuclease. This was incubated at 37°C (or other recommended temperature) for a minimum of one hour. In situations where the use of multiple enzymes was required, an appropriate buffer was selected according to the manufacturer's instructions (New England Biolabs catalogue)

which allowed for high levels of activity for all enzymes being used. For digestion of larger quantities of DNA (e.g. for fragment purification), the restriction endonuclease digestion was scaled up and carried out in a similar way except for the use of 1.5 U of enzyme per μg DNA in a total reaction volume of 100-200 μl.

2.2.3 Agarose gel electrophoresis

Agarose powder (Invitrogen) was mixed with TAE buffer (40 mM Tris, 20 mM acetate, 2mM EDTA) to prepare 0.7% gel mixture and dissolved by heating in a microwave, then poured into a casting tray containing a sample comb and allowed to solidify at room temperature. The casting tray was inserted horizontally into the electrophoresis chamber filled with TAE buffer and DNA samples (2-10 μl) were mixed with 6x Ficoll loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll Type 400) then loaded onto the gel. Gels were then run at 80 V, generally until the bromophenol blue had migrate ≈80% the length of the gel. DNA fragments were visualised on a U.V. trans-illuminator (Spectroline TVC-312A) after staining the DNA with 0.5μg/ml ethidium bromide (EtBr) in TAE buffer and images were captured using a Kodak EDAS290 digital camera.

2.2.4 Purification of DNA from agarose

DNA fragments obtained from restriction enzyme digest were resolved on a low melting point 1 % agarose gel (SeaKem GTG, Cambrex Bio Science Wokingham, Ltd.). Samples were prepared by mixing the DNA digests with 6x Ficoll loading buffer (1:6 v/v) and 1/10,000 dilution of stock SYBR® Gold nucleic acid gel stain (Molecular Probes, Eugene, Oregon USA) before loading onto the gel. After running the gel, DNA bands were visualized under blue light (in order to avoid UV damage to the DNA that would occur with EtBr visualisation) with a Dark Reader™

(Clare Chemical Research), and the required bands were excised. The DNA was subsequently recovered from the gel slice using a QIAquick Gel Extraction kit (Qiagen) following the manufacturer's protocol.

2.2.5 DNA extraction using phenol/chloroform

Phenol/chloroform extraction was routinely performed to remove enzymes and other contaminating proteins from DNA samples. An equal volume of aqueous phenol was added to DNA samples prepared in 0.2-3 ml 10 mM Tris 1 mM EDTA (T10E1) buffer. The tube was vortexed for 15 seconds and then the phases were separated by centrifugation in a microfuge for one minute at 13,000 rpm. The lower organic phase was discarded and an equal volume of a 1:1 mixture of phenol and chloroform was added to the remaining supernatant. This process was repeated twice, and then chloroform was added to the aqueous phase followed by vortexing and centrifugation. The upper aqueous phase was carefully removed and transferred to a clean tube.

2.2.6 DNA precipitation

To concentrate and further purify DNA preparations, DNA was precipitated by the addition of either 0.1 volumes of 3 M sodium acetate, or 1/50 volume of 5 M NaCl followed by the addition of 2 volumes of absolute ethanol or 1 volume of propan-2-ol. If small amounts of DNA were to be precipitated 10-20μg linear polyacrylamide (LPA) from 10mg/ml stock was also added as a carrier. The solution was then mixed and incubated at -20°C for 30 minutes followed by centrifugation in a microfuge at 16000g for 15 minutes. The supernatant was aspirated and the pellet was then air dried before dissolving in an appropriate volume of $T_{10}E_1$ buffer.

2.2.7 Quantitation of DNA

DNA was quantified using the DNA binding reagent PicoGreen (Molecular Probes). DNA standards ranging from 0-100ng were made in duplicate from a serial two fold dilutions of a known standard DNA stock solution (New England Biolabs). DNA samples (100 µl) were loaded in triplicate on a 96 well plate. Stock solution of PicoGreen reagent was diluted 1 to 200 in $T_{10}E_1$ and 100 µl was added to each well. Samples were mixed by pipetting and incubated in the dark for 2 minutes. Fluorescence was determined using an absorption wavelength of 485 nm and emission wavelength of 535nm using a Victor plate reader (PerkinElmer, Monza, Italy; Formerly Wallac). DNA concentration of the unknown samples was determined by interpolation from the standard curve.

2.2.8 Ligation of DNA fragments

The required vector and insert DNA fragments from restriction digests were purified from the gel using a QIAquick gel extraction kit (Qiagen) according to the manufacturer's instructions. The 5' ends of the cut vectors were dephosphorylated using calf intestinal alkaline phosphatase (CIAP; Roche Diagnostics) and incubated at 37ºC for 1 hr. The enzyme was then heat inactivated at 75ºC for 10 minutes before recovering the DNA via phenol/chloroform extraction and ethanol precipitation. Ligations were performed by mixing 0.2 pmoles of the insert DNA to 100 ng, 200 ng or 300 ng of the required vector fragment in the presence of 1U T4 DNA ligase (Roche Diagnostics) in a 20 µl ligation volume for 16 hr at 14ºC. After ligation, a potion of the DNA ligated product was examined by agarose gel electrophoresis and the remainder was further cleaned up by phenol/chloroform extraction and ethanol precipitation.

2.2.9 Bacterial cell transformation and amplification

Chemically competent *E. coli* XL-2 Blue (XL-2) was prepared according to Molecular cloning manual (Sambrook and Russell, 2001). Ligated DNA was transformed mixing 5 to 10 ng of recombinant plasmid DNA or ligation mixture with 100 µl competent XL-2 bacteria. After incubation on ice for 30 minutes, the bacterial mixture was subjected to a 90 second heat shock in 42°C water bath and was then transferred to 900µl of room temperature sterile SOB media [8.4g SOB mixture (Difco) in 300ml ddH_2O)] supplemented with 20mM filter sterilised glucose. The culture was incubated at 37ºC for one hour before spreading on sterile SOB agar [15 gram of bacto-agar (Difco) in 1 litre SOB medium] plates containing 100 µg/ml ampicillin or other appropriate antibiotic, and incubated overnight at 37°C. Individual colonies were selected and grown in 3 ml sterile LB-Broth [6g LB broth base (Invitrogen) in 300 ml ddH_20] containing the appropriate antibiotic and incubated overnight at 37°C in a shaking incubator.

2.2.10 Small scale plasmid DNA preparation from bacteria (Mini-prep)

Bacterial culture was pelleted by centrifugation for 5 minutes at 5000 rpm in a Sorvall SM-24 rotor. The supernatant was discarded and the sample was maintained on ice throughout the procedure. The pellet was resuspended in 100 µl of ice-cold solution I (50 mM glucose, 10 mM EDTA pH 7.5, 25 mM Tris.Cl pH 8.0) and vortexed for a few seconds. Then, 50 µl of freshly prepared lysozyme solution (10 mg/ml in solution I) was added with vortexing to lyse bacterial cell wall. After incubation for 10 minutes on ice, 300 µl of solution II (0.2 M NaOH, 1% w/v SDS) was added with vortexing to denature proteins and chromosomal DNA and the mixture was kept on ice for 10 minutes. This was followed by addition of 225 µl of

ice-cold solution III (5 M acetate and 3 M potassium, pH 4.8-5.0) to precipitate the protein and single stranded DNA. The tube was mixed by inversion before leaving on ice for 10 minutes. Samples were centrifuged at 9000 rpm for 10 minutes and clear supernatants were transferred to a clean tube before precipitation with 675 µl isopropanol for 20 – 30 minutes at 4ºC. DNA was pelleted and redissolved in 200 µl of T100E5N100 (100 mM Tris.Cl, 10 mM EDTA, 100 mM NaCl, pH8) containing 10 µg/ml RNAase A, heat-treated to inactivate contaminating DNAase. After 1 hr at 37ºC, DNA was purified via phenol/chloroform extraction and ethanol precipitation before DNA pellets were resuspended in 50 µl T10E1. The correct DNA plasmid constructs was checked by digestion of approximately 5 µl of the DNA with an appropriate restriction enzyme, before analysis by agarose gel electrophoresis. If restriction digests confirmed the desired clone, large scale production of the plasmid DNA was carried out.

2.2.11 Large scale plasmid DNA preparation from bacteria (Bulk-prep)

The desired bacterial colonies were used to inoculate 200 ml of sterile LB-Broth medium supplemented with an appropriate antibiotic and grown overnight at 37°C in a shaking incubator. The bacterial cultures were pelleted in a centrifuge pot by centrifugation for 5 minutes at 5000 rpm in a Sorvall GSA rotor at 4°C. The supernatant was discarded and the bacterial pellet was maintained on ice throughout the procedure. The pellet was thoroughly resuspended in 4 ml ice-cold solution I followed by the addition of 1ml of freshly prepared 10 mg/ml lysozyme (in solution I) where the suspension was left on ice for 10 minutes. Then, 10ml of solution II was added and the pot was gently inverted a few times to ensure complete mixing before being left on ice for a further 10 minutes. This was followed by addition of

7.5 ml of solution III and vortexing for a few seconds to ensure complete mixing. After storage on ice for a further 10 minutes, bacterial debris was pelleted by centrifugation at 8000 rpm for 15 minutes at 4°C in a Sorvall GSA rotor. The supernatant was carefully transferred to a clean 250 ml centrifuge bottle and all nucleic acids were precipitated by the addition of 22.5 ml of propan-2-ol for 30 minutes at -20°C. The suspension was centrifuged at 8000 rpm for 20 minutes at 4 °C and plasmid DNA pellets were air dried for 1-3 hr before dissolving in 2.5 ml of $T_{50}E_{10}$ (50 mM Tris.Cl, 10 mM EDTA) overnight at room temperature.

2.2.12 Purification of plasmid DNA by caesium chloride density gradient centrifugation

Crude plasmid DNA from bulk prep was purified by caesium chloride (CsCl; Invitrogen) density gradient centrifugation. In a 15 ml tube, 3.03 g of Ultrapure caesium chloride was weighed before adding crude plasmid solutions. The liquid weight of the plasmid solution was then made up to 2.75 ml by the addition of $T_{50}E_{10}$ on followed by addition of 275 µl EtBr (10 mg/ml) and incubation on ice for 10 minutes. The solution was centrifuged 10000 rpm for 10 minutes in a Sorvall SM24 rotor to precipitate contaminating RNA. The supernatant was then transferred into 3.9 ml Beckman Quick-Seal™ centrifuge tubes, and any gap between the liquid and neck of tube was filled with isopycnic CsCl solution (1 g CsCl:1ml $T_{50}E_{10}$). Tubes were heat sealed and ultra centrifuged at 100000 rpm for 4 hours followed by 95000 rpm for 4 hours, followed by a 65000 rpm gradient relaxation step for 30 minutes in a Beckman Optima™ TLX bench top ultracentrifuge using a TLN-100 near vertical tube rotor. The lower DNA plasmid lower band was carefully removed by piercing the side of the tubes with a 21 gauge needle and 2 ml syringe and

diluted with 3 volumes of $T_{10}E_1N_{100}$ (solution in a 15 ml tube. The DNA was extracted twice with phenol/chloroform to remove ethidium bromide followed by DNA precipitation with one volume of propan-2-ol for 30 minutes at -20ºC. DNA was pelleted by centrifugation at 10000 x rpm for 10 minutes in a Sorvall SM24 rotor at 4 °C. The pellet was resuspended in 400 µl of $T_{10}E_1N_{100}$ (10 mM Tris.Cl, 1 mM EDTA, 100 mM NaCl, pH 8) and transferred to a clean Eppendorf tube before precipitation with 2 volumes of absolute ethanol for 30 minutes at -20ºC. Finally, the pellet was then resuspended in 200 µl of $T_{10}E_1$ solution.

2.2.13 DNA sequencing

PCR based sequencing reactions were performed with an appropriate primer in a 20 µl reaction volume using the ABI Prism Big Dye Terminator cycle sequencing ready reaction kits (PE Biosystems), according to the manufacturer's protocol. Briefly, sequencing reactions were set up using 5-20 ng of the DNA of interest, 4 µl of the supplied 2.5x buffer, 4 pmol sequencing primer, 4 µl terminator mix, and the appropriate volume of water in 0.2 ml PCR tubes. The reactions were run in a thermocycler (GeneAmp 9700) for 25 cycles of: 96°C for 30 seconds, 50°C for 15 seconds, 60°C for 4 minutes. The products were transferred to Eppendorf tubes and precipitated by addition of 10 % (v/v) 3 M sodium acetate (pH 4.6) and 3 volumes of cold 100% ethanol. The mixtures were well mixed, left at room temperature for 15 minutes and pelleted in a microfuge at 13000 rpm for 20 minutes. The pellet was washed with 250 µl 70% ethanol, followed by centrifugation at 13000 rpm for 5 minutes. The final DNA pellets were air-dried for 15 minutes and stored at -20°C for subsequent sequencing.

Prior to loading to the sequencer, the pellets were resuspended in 5 µl of loading

dye (5:1 mixture of deionised formazide and 50 mg/ml dextran blue in 25 mM EDTA, pH 8), vortexed and briefly spun down. The samples were denaturated at 90°C for 2 minutes in a heat block and then kept on ice. A volume of 5 μl from each sample was loaded onto a 96 well plate and run on an ABI PRISM® 377 DNA sequencer, and analyzed using Sequencher 4.5 software (Gene codes Corporation, Ann Arbor, Michigan, USA).

2.3 Cell culture

2.3.1 Cell lines

Cell line	Origin	Reference
Tramp-C1 cells	Transgenic adenocarcinoma murine prostate tumour cell line (Tramp) derived from TRAMP C57BL/6 male mice that develop histological prostatic intraepithelial neoplasia which progress to adenocarcinoma with distant metastases by 24-30 weeks of age (ATCC)	Foster et al., 1997
FLYA13 cells	Human fibrosarcoma cell line-based amphotropic packaging cell line (ATCC).	Cosset et al., 1995
Phoenix-A cells	Human embryonic kidney HEK 293-based cell amphotropic packaging cell line (Dr. Steve Lee, University of Birmingham, UK)	Swift et al., 2001
B16 cells $B16_{OVA}$ cells	Mouse melanoma B16 cell line and its counterpart expressing ($B16_{OVA}$) full length secreted ovalbumin (Dr. Richard Vile, Mayo Clinic, USA)	Hu and Lesney, 1964
B3Z T cell hybridoma	Murine T-cell hybridoma specific for the OVA/MHC class I complex. It induces intracellular accumulation of lacZ protein upon TCR activation (Dr. Neil Blake, University of Liverpool, UK)	Karttunen et al., 1992

Table 2-2: Different cell lines used in the present study

2.3.2 Maintenance of mammalian cell lines

Murine Tramp-C1 prostate adenocarcinoma cells and their transduced counterparts were maintained in Dulbecco's Modified Eagles Medium (D-MEM) with 20mM HEPES supplemented with 2mM glutamine, 10% foetal calf serum (FCS) (Sigma-Aldrich), 2mM L-glutamine, 1mM pyruvate, 100 U/ml penicillin, 100μg/ml streptomycin, 5μg/ml insulin and 1nM/ml Dihydrotestosterone (DHT).

FLYA13 packaging cells, B16 and $B16_{OVA}$ melanoma cells were maintained in Dulbecco's Modified Eagles Medium (D-MEM) with 20mM HEPES supplemented with 2mM glutamine, 10% foetal calf serum (FCS) (Sigma-Aldrich), 2mM L-glutamine, 1mM pyruvate, 100 U/ml penicillin and 100μg/ml streptomycin.

Phoenix packaging cells were maintained in Dulbecco's Modified Eagles Medium-high glucose (4500 mg/L) supplemented with 2mM glutamine, 10% foetal calf serum and 1mM pyruvate, 100 U/ml penicillin and 100μg/ml streptomycin.

B3Z T cell hybridoma cells were maintained in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% foetal calf serum, 50μM 2-Mercaptoethanol, 2mM L-glutamine, 1mM pyruvate, 100 U/ml penicillin and 100μg/ml streptomycin.

Cell were cultured in vented 25-75cm^2 flasks (Iwaki) at 37ºC in a humidified atmosphere containing 5% CO_2, and routinely passaged when 90% confluent. The media was removed, cells washed once with Phosphate Buffered Saline (PBS) and diluted trypsin-EDTA (0.05% w/v trypsin, 0.02% w/v EDTA) added to cover the cells. Cells were collected in 10 ml medium to inhibit trypsin, counted, and pelleted by centrifugation at 1200 rpm for 5 minutes. The pellet was resuspended in fresh complete media and plated at appropriate dilutions into 75cm^2 flasks.

2.3.3 Cell number quantitation

The number of viable cells in cell suspension was determined by diluting 1:1 with 0.4% Trypan blue. One drop of the dilute solution was applied on a haemocytometer (Improved Neubauer) and unstained viable cells were counted manually under microscope. The number of cells per ml was calculated using the formula: number of cells per ml = [(number of cells in 5 grids (4x4 squares) x 10^4)/5]/2 (dilution factor)

2.3.4 Cryopreservation of cell lines

For long-term storage of cells, cells were grown to approximately 75% confluence, trypsinised as usual, and re suspended in 10 ml medium. Cells were transferred to a 15 ml Falcon tube and centrifuged at 1000 x rpm for 5 minutes (Beckman GS-6R bench top centrifuge). The supernatant was decanted, and the pellet resuspended in 1 ml ice cold FCS containing 10% (v/v) DMSO. Cells were transferred into cryovials (Nunc), and frozen to -80ºC overnight prior to long-term storage in a -180ºC liquid nitrogen freezer.

For culturing of cryopreserved cells, cryovials were quickly defrosted in a 37ºC water bath, followed by a drop wise addition of 10 ml pre-warmed medium. Cells were then centrifuged at 1000 rpm for 5 minutes before resuspension of the cell pellet in fresh medium and plating in tissue culture flasks.

2.3.5 Microscopy

A Zeiss Axiovert 25 inverted microscope was used for all phase-contrast and fluorescence microscopy using 5, 10, 20 and 40x objectives.

2.3.6 Stable transfection of virus packaging cells

2.3.6.1 Transfection of cell with plasmid DNA using calcium phosphate co-precipitation

One day prior transfection, FLYA13 virus packaging cells were seeded at a density of approximately 5 x 10^5 per 60 mm dish or 25 cm^2 flask. The appropriate quantities of the required plasmids were ethanol precipitated in Eppendorf tubes with 0.1 M NaCl and 2.5 volumes of absolute ethanol. The DNA was pelleted at 13000 x rpm in a microfuge and the supernatant removed in a sterile laminar airflow cabinet

using a sterile syringe and fine needle. DNA pellet was redissolved in 175 µl 10 mM Tris.HCl pH 7.5 and approximately 5-20 µg DNA was added to 25 µl 2 M $CaCl_2$ and mixed and then 200 µl of 2x HEBS (50 mM HEPES NaOH pH 7.05, 0.25 M NaCl, 1.5 mM NaH_2PO_4; adjusted to pH 7.05 with NaOH, then brought up to 200 ml and filter sterilised) was placed in a plastic bijou, and the DNA/$CaCl_2$ solution added drop wise to the HEBS, whilst mixing gently on a vortex mixer. This mixture was left to stand for 20 to 30 minutes, during which time a faint, milky precipitate formed. During the DNA incubating period, the medium was removed from the cells and sterile fresh medium was added followed by addition of chloroquine to a final concentration of 100 µM (i.e. 1/100 volume of 10 mM stock). The DNA/calcium phosphate coprecipitate was added to the medium over the cells, mixed gently, and returned to the incubator for 4 to 5 hours. The medium was then removed from the culture plates and 2 ml of 20% glycerol in serum free medium was added for 90 sec. After the glycerol shock the medium was removed and fresh complete culture medium was added and incubated in a humidified atmosphere containing 5% CO_2 at 37ºC. After two days, transduced FLYA13 cells were cultured in Puromycin (5µg/ml) for 2 weeks. When most untransduced cells had died, the remaining antibiotic resistant cells were passaged and grown to produce a stock of retroviral producer cells.

2.3.6.2 Transfection of cells with plasmid DNA using Fugene®6

FLYA13 or Phoenix cells were transfected using Fugene®6 according to the manufacturer's instructions. Briefly, 3µl Fugene®6 was carefully added to 97 µl serum free medium in an Eppendorf tube and incubated for 5 minutes at room temperature. The appropriate volume of plasmid DNA (1-2µg) was added to the

transfection mixture, vortexed for few seconds and incubated for 15 minutes at room temperature. The transfection reagent-DNA complex was added to FLYA13 cells in a drop-wise manner and the solution was swirled over the cells to ensure even distribution over the entire plate/flask surface. After two days, transduced FLYA13 cells were cultured in G418 selection (500 μg/ml) for 2 weeks. When most untransduced cells had died, the remaining antibiotic resistant cells were passaged and grown to produce a stock of retroviral producer cells.

For Phoenix cell transfection, the culture supernatant containing viral particles were harvested 48 hr later, filtered through 0.45-μm pore-sized membranes, prior to usage for transduction of tumour cells.

2.3.7 Stable transduction of tumour cell lines with retroviral supernatant

Tramp-C1 cells were plated at a density of 1 x 10^6 in 60 mm plate and allowed to adhere overnight. Up to 5 ml of 0.45 μm filtered supernatant from transduced FLYA13 cells or Phoenix cells was added to the Tramp-C1 cells in the presence of 8 μg/ml polybrene (Sigma). This was done twice daily for a period of one week, before cells were subjected to G418 selection (500 μg/ml). When most untransduced cells had died, the remaining G418 resistant cells were plated in 96 well plates at a limiting dilution to produce clonal transduced cell lines. These were then bulked up before characterisation.

To increase the efficiency of Nitroreductase retroviral transduction of Tramp-C1 cells, Combimag transfection reagent (OZ Biosciences) was used according to the manufacturer's protocol. Cells were plated at a density of 2 x 10^5/well in a 6 well plate and allowed to adhere overnight. A volume of 4 μl/well of Combimag solution was mixed by pipetting in with cell culture medium prior to placing on top of the

magnetic plate for 20 minutes. The magnetic plate was removed and culture plates were incubated in a humidified atmosphere containing 5% CO_2 at 37ºC. After 48hr, cells were subjected to G418 selection (500 μg/ml) as described earlier.

2.3.8 Generation of single-cell clones from transduced Tramp-C1 using limiting dilution

Transduced Tramp-C1 tumour cells were trypsinized, washed with PBS and then and resuspensed at $1x10^4$ cells/ml complete D-MEM culture medium. The cell suspension was filtered through a 40 μm cell strainer to eliminate clumps and to obtain a single cell suspension. The cells were then diluted 100x by transferring precisely 200 μl of cell suspension to 20 ml of medium, the suspension was mixed well by inverting twice, followed by further 10x and 50x dilution to achieve cell densities of 10 cells/ml and 2 cells/ml, respectively. For inoculation, the cell suspension was then inverted several times and transferred to a conical medium reservoir. Using an 8-channel multi-pipette (Gilson), 100 μl was transferred to the wells of sterile 96-well plates. The plates were then covered with aluminium foil or and placed at 37ºC in a humidified atmosphere containing 5% CO_2. After 24 hr, the plates were checked for wells containing 1 cell and were marked for further observation. Cell clones arising from single-cells were passaged as they grew, in sequence from the 96-well plate into a 48-well, 24-well, 12-well, and 6-well plates and finally in tissue culture flasks. Single-cell clones were then bulked up and cryopreserved before characterisation.

2.4 Cellular assays

2.4.1 Preparation of protein extracts from mammalian cells

To extract protein from mammalian cells, cells were washed three times with ice cold PBS. The supernatant was discarded and cell pellets were lysed with a suitable volume of NP-40 lysis buffer (150mM NaCl, 1% NP-40, 50mM Tris.HCL pH 8) (e.g. 300 µl buffer for 25 cm^2 flask). Crude cell lysates were transferred into a clean prechilled Eppendorf and left on ice for at least 20 minutes until complete lysis. Lysates were clarified by centrifugation at 13,000 rpm for 5 minutes at 4ºC and stored at –70°C.

2.4.2 Determination of protein concentration

Protein concentration was determined using the Bio-Rad protein assay kit (BioRad Laboratories) according to the manufacturer's micro-titre assay protocol. The dye reagent was prepared by diluting 1 part dye reagent concentrate with 4 part ddH_2O followed by filtration to remove particulates. Mammalian cell lysates were diluted in PBS and 1 µl diluted lysates were added to 200 µl diluted dye reagent in wells of a 96-well plate. The protein concentration was determined from a standard calibration curve constructed by a two-fold dilution of 100µg/ml bovine serum albumin (BSA). Absorbance was measured at 450 nm using a Victor2 1420 Multilabel Counter (PerkinElmer, Monza, Italy; Formally Wallac).

2.4.3 Western blot analysis of proteins separated by SDS-PAGE electrophoresis

2.4.3.1 Denaturating SDS polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis

SDS-PAGE was performed using a Mini-Protean III tank (BioRad) by the method of Laemmli (Laemmli, 1970). Glass plates were cleaned with ethanol and the apparatus assembled according to the manufacturer's instructions. A volume of 10 ml resolving gel (375mM Tris.HCl (pH 8.8), 0.1% SDS, 0.1% ammonium persulfate, 30% (w/v) acrylamide/0.8% (w/v) bis mix (National Diagnostics, Geneflow Ltd) and TEMED depending on the desired percentage of gel) was poured. Water saturated butanol was layered on top of the resolving gel to remove air bubbles and gels were allowed to set at room temperature until solidified. Saturated butanol was thoroughly washed out with distilled H2O followed by addition of 4ml stacking gel (125mM Tris.HCl (pH 6.8), 0.1% SDS, 0.1% ammonium persulfate, 0.01% TEMED and 0.67ml 30% (w/v) acrylamide/0.8% (w/v) bis mix) on top of the set resolving gel. A comb was inserted in the stacking gel before it was allowed to set at room temperature. Prior to gel loading, the comb was removed and the wells were washed extensively with x1 SDS-PAGE running buffer (10x stock solution; 0.25M Tris.HCl pH 8.3, 1.92M glycine and 1% (w/v) SDS). Samples containing 15-100μg of total protein were denatured by boiling with 3x concentrated gel sample buffer (GSB; 187.5mM Tris.HCl pH 6.8, 6% w/v SDS, 30% glycerol, 0.03% w/v bromophenol blue, 150mM DTT) for 5 minutes. Denatured samples were cooled on ice, pulse centrifuged to remove condensation and loaded onto the assembled gel. Gels were electrophoresed at 120-150V (constant voltage) for 90-120 minutes.

2.4.3.2 Immunoblotting/western blotting

Following SDS electrophoresis, proteins were transferred onto methanol soaked 0.45μM PVDF membranes (Millipore, Watford, UK) using the BioRad Mini Trans-Blot tank. Tanks containing transfer buffer (50 mM Tris.HCl, 190 mM glycine, 20% (v/v) methanol) and assembled mini gel holder cassettes were placed on a magnetic stirrer to prevent the build up of heat and electrophoresed for 1 hour at 100V. Membranes were washed in TBS-T buffer (1x TBS; 20 mM Tris.HCl pH 7.6, 136 mM NaCl, supplemented with 0.1% Tween-20) for 5 minutes and blocked in TBS-T containing 10% low-fat milk or 10% of BSA for 1 hour at room temperature. Blocked membranes were then washed three times for 5 minutes in TBS-T and incubated overnight at 4ºC with the relevant primary antibody (Table 2-3). Membranes were washed three times for 5 minutes each in TBS-T, incubated for 1 hour at RT with the relevant peroxidase conjugated secondary antibody (Table 2-3) diluted in TBS-T, and then washed a further 3 times for 5 minutes each in TBS-T. Proteins were visualised by exposure to film following detection using enhanced chemiluminescence reagent (ECL; Amersham). The films were developed with Kodak GPX developer and fixer (Kodak, Hemel Hempstead, UK)

Antibody	Dilution	Supplier
Polyclonal rabbit anti-chicken ovalbumin	1/4000	AbD Serotec
Polyclonal sheep anti-NTR	1/8000	Dr. Peter Searle
Monoclonal mouse anti-β-tubulin	1/10,000	Sigma-Aldrich, UK
Anti-rabbit secondary antibody	1/10,000	Sigma-Aldrich, UK
Anti-sheep secondary antibody	1/10,000	Sigma-Aldrich, UK

Table 2-3: List of primary and secondary antibodies used in western blotting

2.4.4 ß-galactosidase assay for activation of B3Z hybridoma

B3Z is a T-cell hybridoma which recognizes the SIINFEKL peptide in the context of H-2K^b and expresses β-galactosidase (β-Gal) upon activation. B3Z T cells (1x 10^5/well) were cocultured with control or OVA-expressing cells (1x10^4) in wells of a 96-well plate. After an overnight incubation, ß-galactosidase activity of the B3Z T cells was determined using Luminescent β-galactosidase Detection Kit II (Clontec) following the manufacturers' instructions. Briefly, plates were centrifuged at 1800 rpm for 5 minutes and then washed twice with 200 µl/well PBS. Cells were lysed by adding 50 µl/well ice cold lysis buffer and then freezed and thawed for three cycles. The plates were centrifuged down at 1800 rpm for 5 minutes at and the supernatant (25-40 µl) were transferred to new wells of a white background 96 well plate (PerkinElmer). Reaction buffer mixture (160-175 µl/well) was added to cell lysates and incubated in the dark for 1 hr. Relative light emission was recorded using a benchtop microplate luminescence counter (Topcount.NXT, Packard).

2.4.5 Chromium release cytotoxicity assay

Tramp-C1 cells or splenocytes from C57BL/6 mice (target cells) were prepared in suspension at $1x10^6$/ ml cell. Cells were pelleted by centrifugation at 1000 rpm for 5 minutes at 4ºC. The media was removed with a transfer pipette and the pellet was resuspended in 500 µl medium before pulsing with 5 µg/m SIINFEKL peptide (Innovogen, Sweden). After incubation for 1 hr, cells were washed twice with PBS and pelleted with centrifugation. The pellet was then resuspended in 0.25 mCi ^{51}Cr (Sodium Chromate) (Amersham) and incubated at 37ºC for 1 hr. Target cells were washed twice with complete RPMI medium and then resuspended at $5x10^4$/ml in complete RPMI before addition of 100 µl target cell suspension in triplicates to appropriate wells of a 96 well V bottom plate (Nunc). Effector cells were also counted, resuspended and added to targets to give Effector:Target ratios (E:T) of 33:1, 10:1, 3:1 and 1:1 or 0.3:1. Targets were plated out with the addition of media only (to determine spontaneous release) and with RPMI+0.1% SDS (to give maximal release). The plates were then centrifuged at 1000 rpm for 5 minutes then incubated at 37ºC for 5 hr.

A volume of 100µl of the cell supernatant was then removed from each well and placed into individual LP2 tubes. These were then loaded onto a Packard Cobra II D5010 Ten Detector Scintillation Gamma Counter. Results are expressed as percentage specific lysis which was calculated using the following formula:

% Specific lysis= [(Sample release-Spontaneous release)/(Maximum release-Spontaneous release)] x 100

2.4.6 *In vitro* cytotoxicity assay: MTT test

The sensitivity of transduced tumour cell lines to prodrug was assayed by measuring of mitochondrial metabolic activity using MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide) which indirectly reflects viable cell numbers. Cells were seeded on day one at 1 x 10^4 cells/ per 150 µl medium in wells of a 96 well flat bottom plate and allowed to adhere overnight. On day one, medium was aspirated from wells using a Vacusafe Comfort (INTEGRA Biosciences AG, Chur, Switzerland) and the prodrug CB1954 was added at a range of concentrations in 180 µl medium to quadruplicate well for 4 or 24 hr at 37ºC. Following the incubation period, prodrug was removed and 180 µl/well fresh medium was added. After 48 hr, medium was removed and replaced with 150 µl fresh culture medium containing 0.5 mg/ml MTT, diluted fresh from a 5 mg/ml stock solution. Plates were incubated for 4hr at 37 °C before the MTT solution was aspirated from the wells. Plates were dried for 30 minutes at room temperature before the addition of 150 µl/well DMSO. Absorbance was measured at 490 nm using a Victor2 1420 Multilabel Counter.

2.4.7 Granulocyte-macrophage colony-stimulating factor enzyme-linked immunosorbent assay (ELISA)

Murine granulocyte-macrophage colony-stimulating factor (mGM-CSF) was determined in the supernatant of mGM-CSF-expressing Tramp-C1 cells using Mouse GM-CSF ELISA Ready-SET-Go (ebiociences) following the manufacturers' guidelines. Briefly, cells were resuspended at 5x10^5/5 ml 2% FBS culture medium and then cultured in 6 cm culture dishes (Nunc). After 48 hr, supernatant was transferred into 15 ml centrifuge tubes (Falcon) and then centrifuged at 3000 rpm for 5 minutes at 4ºC. One day before collecting the supernatant, capture mGM-CSF

antibody was diluted in coating buffer and 100 µl/well was added to 96-well immunoplates plates overnight at 4ºC. The plate was then washed three times with wash solution (PBS + 0.05 % Tween-20) and then the test supernatants and standards were added to the wells for 2 hr at 37ºC. The plate was washed 5x with wash solution and Horse-radish peroxidase (HRP)-conjugated detection reagent was added to the plate (100 µl/well) for 60 minutes. The plate was again washed 5x and then 100 µl of Avidin-HRP substrate was added and incubated in the dark at room temperature for 15-20 minutes. The reaction was terminated using 50 µl/well 1M hydrochloric acid and the absorbance was measured at 450nm by a Wallac Victor2 1420 plate reader. Colour change was proportional to m-GM-CSF concentration and unknowns were determined using standard calibration curves of known concentration.

2.5 *In vivo* experiments

2.5.1 Mice

All animal experiments were conducted in accordance with the updated guidelines issued by the UK Coordinating Committee on Cancer Research (1998; Workman et al., 2010); and covered by Home Office Project and Personal licenses under the Animals (Scientific Procedures) Act 1986. Animals were maintained under specific pathogen-free conditions according to Home Office regulations at the Biomedical Services Unit of the University of Birmingham. Animals used in this study were either bred at the BMSU or obtained from Harlan UK (Dodgeford Lane, Loughborough, Leicestershire, LE12 9TE). Breeding pairs of OT-I BoyJ mice were a kind gift from Dr. Caetano Reis e Sousa, Immunobiology Laboratory, London Research Institute, Cancer Research UK.

Different strains of only male mice were used during the course of this study including:

- Athymic nude (*nu/nu*) C57BL/6 mice (B6)

Nude mice carry a loss of function mutation in the *Foxn1* gene (forkhead box N1) resulting in the characteristic hairless phenotype and abnormal development of the thymus (Balciunaite et al., 2002; Wortis et al., 1971). Consequently, they have severely reduced numbers of immature and mature T-cells and are therefore profoundly deficient in mediating T cell dependent immune responses including: antibody formation (Kaushik et al., 1995) that requires $CD4^+$ helper T cells, and cell-mediated immune responses, which require $CD4^+$ and/or $CD8^+$ T cells. Nude mice however do have a minimal amount of extra-thymic T-cell maturation occurring in spleen (Palacios and Samaridis, 1991), partial defects in B cell

development (Szabo et al., 1998), normal number of macrophages and NK cells, normal APC function, and normal complement activity.

- Wild-type (wt) C57BL/6 mice (B6).

  Both wild-type and nude B6 mice naturally express the CD45.2 allele of the CD45 leukocyte common antigen on all leukocytes.
- OT-I CD45.1^{+} TCR-transgenic C57BL/6 mice have transgenic Vα2Vβ5 TCRs specific for recognition of ovalbumin derived epitope $OVA_{257–264}$ (SIINFEKL) in the context of H2-K^{b} (Clarke et al., 2000) and carry the allelic variant, CD45.1.
- In adoptive transfer experiments C57BL/6 mice with the CD45.2 alloantigen served as recipients for donor OT-I lymphocytes carrying the alternate CD45.1 allele; using this system we were able to monitor tumour specific-immune responses in terms of their frequency and effector functions in different lymphoid tissues.

2.5.2 Harvesting lymph nodes and spleen

Mice were culled by cervical dislocation, laid on their backs and sprayed with 70% alcohol. The four limbs were stretched and fixed to the dissection board using pins. The skin in the lower groin was then lifted up to make a small incision where the scissor was inserted longitudinally to detach the skin form the abdominal wall. This was followed by a medial midline skin incision starting from the lower groin region up to the neck. Again the intervening connective tissues between the skin and the muscle in the hind limbs and forelimbs area were loosened where vertical incision along each limb was made. The skin was pulled back to the sides and secured with pins to expose all peripheral lymph nodes. Then, the inguinal, axillary and brachial

lymph nodes (LNs) from both sides of the animal were removed from the surrounding tissues using curved forceps. The LNs were separated into draining LNs (DLNs) and non draining LNs (N-DLNs) based on their relative distance from the tumour implantation site. After cutting open the abdominal wall, the mesenteric lymph node was exposed by unfolding the small intestine to allow for its separation; while the spleen was easily dissected by pushing the stomach and liver on the left side. The lymphoid tissues were transferred to 6 or 12 well plate containing 2 ml sterile culture medium.

2.5.3 Preparation of single-cell suspension from lymph nodes, spleen or blood

Freshly removed lymph nodes and spleen were homogenized using the back of a 3 ml syringe plunger until mostly fibrous tissue remains. The clumps were further dispersed in the suspension by pipetting several times. Cell suspension was filtered through 250 μm mesh nylon screen (Cadish Precision Meshes, UK) placed on top of a 5 or 50 ml tube. An additional 1 ml medium was used to wash the culture plate and the filter. The suspension was centrifuged at 1000 rpm for 5 minutes at 4ºC then the supernatant was discarded. The pellet was resuspended in 2 ml red cell lysis buffer (150 mM NH_4Cl, 10mM $KHCO_3$, 0.1 mM EDTA pH8) then incubated at 37ºC for 5 minutes with occasional shaking (blood requires longer incubation for 15 minutes). Lysis buffer was diluted by filling the tube with medium, and then lymphocyte cell suspension was filtered again and centrifuged at 1000 rpm for 5 minutes at 4ºC. The supernatant was discarded and the pellet was washed once then resuspended in lymphocyte culture medium. The cells were counted, and the viability was then determined using the trypan blue dye to exclude dead cells.

Blood samples were collected either via tail bleeds or terminal heart puncture into 0.5 ml Eppendorf tubes containing 100-200 μl 0.1% EDTA. The samples were then

transferred into 5 ml tubes filled with 3 ml red cell lysis buffer and processed similar to lymph nodes and spleen.

2.5.4 *Ex-vivo* OT-I T cell expansion for *in vivo* administration

OT-I lymphocyte were cultured at a density of $5x10^6$ per wells of a 6-well plate in complete RPMI medium. Cells were stimulated with 5μg/ml SIINFEKL peptide in the presence of 100U/ml IL2 for 5 days. Cells were harvested and surface stained as will be discussed in section 2.4.8 for the percentage of $CD3^+CD8^+$ T cells and CD69 activation marker prior to adoptive transfer.

2.5.5 Adoptive transfer of transgenic OT-I T cells

Single-cell suspensions from spleen and lymph nodes from OT-I BoyJ mice (6-8 weeks of age) were prepared as described in section 2.4.3. Lymphocytes containing the indicated number of transgenic OT-I T cells were injected intravenously (i.v.) in 200μl PBS at the appropriate time in the experimental design.

2.5.6 CB1954 prodrug administration *in vivo*

CB1954 used *in vivo* was of a clinical grade, sourced from OSI Pharmaceuticals, Inc. Aston Molecules Ltd. (10 Holt Court South, Aston Science Park, Birmingham, B7 4EJ). CB1954 was diluted in PBS and administered intraperitoneally (i.p.) using 1ml insulin syringes to mice at 20 μg/10 μl/g body weight (20mg/kg).

2.5.7 Subcutaneous tumour inoculation in mice

Tumour cells were grown until approximately 90% confluence was reached in 150 cm^2 flasks. Cells were then trypsinized, harvested in fresh culture medium and pelleted by centrifugation for 5 minutes at 1200 rpm. The cell pellet was washed twice with PBS and resuspended at $5x10^6$ cells/100 μl PBS. For matrigel-tumour

cell mixture, tumour cells were resuspended at $5x10^6$ cells/150 µl serum free medium and mixed before injection with 150 µl of ice-cold matrigel matrix (Becton Dickinson). For subcutaneous injection of tumour cells, a 1 ml insulin syringe was normally used to inoculate tumour cells in the right flank of the shaved skin of 6-8 week-old male C57BL/6 mice. Alternatively, a 3 ml syringe was used to inoculate matrigel-tumour cell preparations. Once tumours become palpable the greatest longitudinal diameter (length) and the greatest transverse diameter (width) were measured using a digital calliper twice a week. Tumour volume was calculated using the modified ellipsoidal formula (Tomayko and Reynolds, 1989):

Tumour volume = $(\text{length} \times \text{width}^2)/2$

2.5.8 Isolation of tumour cells from subcutaneous tumours in mice

Subcutaneous tumours were excised and placed in DMEM medium containing 100 U/ml penicillin and 100µg/ml streptomycin, minced using a scalpel into small pieces and then centrifuged at 1,800 rpm for 5 minutes. The supernatant was discarded and the pellet was incubated in a digestion solution (5 ml/tumour) containing 1 mg/ml trypsin, 1 mg/ml collagenase D (Boehringer Mannheim), and 0.25 mg/ml DNase I (Boehringer Mannheim) in Hank's balanced salt solution and incubated at 37°C for 1 hr with occasional shaking every 15 minutes. Tumour cell suspension was filtered through a 250 µm mesh nylon screen to remove tissue aggregates and then centrifuged at 1,800 rpm for 5 minutes. The pellet was then washed with PBS, resuspended in complete culture medium and cultured in wells of a 6-well plate.

2.5.9 Irradiation of mice

On the day of s.c implantation of tumour cell, mice were exposed to a single dose of 1, 2.5, or 5 gray (Gy) total body irradiation delivered from ^{60}Cobalt γ-radiation source (Gammatron, Siemens, Germany) in a specially designed well-ventilated metallic canisters. The dose rate (Gy/minute) from the source was calculated taking in consideration source decay rates.

2.5.10 Flow cytometric analysis

2.5.10.1 Surface cell staining

Single-cell suspensions from tumour cell lines or lymphoid tissues were phenotypically analyzed by a single step labelling procedure. Briefly, cells ($1x10^6$/100 µl) were washed twice in 1% FCS in PBS (1% FCS/PBS) and labelled with the appropriate fluorochrome-conjugated anti-mouse monoclonal antibodies (Table 2-4) for 30 minutes at 4ºC. Thereafter, cells were washed twice and resuspended in 500 µl 1% FCS/PBS or fixed in 1% paraformaldehyde for later analysis. The samples were run on a four-colour Beckman Coulter XL flow cytometer or a 16 colour Beckman Coulter LSR II and $1x10^4$ or $5x10^5$ events were collected. Isotype-matched antibodies were used to define marker settings and live cell gating was analyzed by 5 µg/ml propidium iodide staining of cells. Flow cytometry data was analysed using FlowJo software (Version 7.5.5; Tree Star Inc.).

Antibody	Concentration/100µl staining buffer	Supplier
Anti-mouse CD3 PE-Cy5	1 µl	BD Biosciences
Anti-mouse CD3 Pacific Blue	1.25µl	eBiosciences
Anti-mouse CD8 APC	0.5µl	BD Biosciences
Anti-mouse CD8 Alexa Fluor 700	0.5 µl	eBiosciences
Anti-mouse Vα2 TCR FITC	0.5 µl	eBiosciences
Anti-mouse CD4 APC	1µl	BD Biosciences
Anti-mouse CD45.1 APC	1µl	eBiosciences
Anti-mouse B220 PE	1 µl	eBiosciences
Anti-mouse IFN-γ Pacific Blue	2 µl	eBiosciences
Anti-mouse CD69 PE-Cy7	1 µl	BD Biosciences
Anti-mouse CD44 PE	0.65 µl	eBiosciences
Anti-mouse CD62-L PE	0.65 µl	eBiosciences
Anti-mouse CD107a PE	2.5 µl	eBiosciences
Anti-mouse IL-2 FILTC	2.5 µl	eBiosciences
Anti-mouse 4-1BBL PE	0.5 µl	eBiosciences
Anti-mouse H-2Kb APC	0.65µl	eBiosciences
Anti-mouse H-2Kb/OVA (SIINFEKL) Pro5® MHC Pentamer	2.5 µl	ProImmune
Rat IgG2b,κ Isotype control PE-Cy5		eBiosciences
Rat IgG2b,κ Isotype control Pacific Blue		eBiosciences
Rat IgG2a,κ Isotype control Alexa Fluor		eBiosciences
Rat IgG2a,κ Isotype control APC		eBiosciences
Rat IgG2a Isotype control FITC		eBiosciences
Rat IgG2a,κ Isotype control PE		eBiosciences
Rat IgG2b Isotype control PE		eBiosciences

Table 2-4: List of antibodies used in flow cytometric analysis
Isotype control concentration was used at the same concentrations of the specific antibodies of interest.

2.5.10.2 Pentamer staining

Freshly prepared single-cell suspensions were incubated with appropriate PE-labelled MHC pentamer reagent at 37ºC for 20 minutes. After washing with cold buffer, cells were resuspended and incubated with the appropriate fluorochrome-conjugated anti-mouse monoclonal antibodies for 20 minutes at 4ºC. The stained cells were washed twice with cold 1% FCS/PBS buffer and re-suspended in 300 µl of buffer for analysis by flow cytometry.

2.5.10.3 Intracellular cytokine cell staining

To detect cytokine-producing cells, intracellular staining was performed using BD Cytofix/Cytoperm Golgi stop Kit following the manufacturers' instructions. Briefly cells were stimulated with 5μg/ml SIINFEKL peptide overnight or for 4 hr in a humidified incubator with 5% CO_2 at 37ºC. Golgi stop was added 4 hr before cells were washed with cold 1% FCS/PBS and stained with surface antibodies as described in 2.4.8. The cells were then fixed with Cytofix/Cytoperm solution for 20 minutes at 4ºC and washed with 1% FCS/PBS. Following fixing, cells were washed and permeabilised with Perm/Wash Solution for 15 minutes at 4ºC. The cells were then pelleted and stained with the appropriate fluorochrome-conjugated anti-mouse monoclonal antibodies in 100μl for 20 minutes at 4ºC. The stained cells were washed twice with cold 1% FCS/PBS buffer and re-suspended in 300μl of buffer for analysis by flow cytometry.

2.5.10.4 CD107a staining

Lymphocyte cell suspension were stimulated with 5μg/ml SIINFEKL peptide in the presence of Golgi stop and 2.5 μg/100 μl anti-CD107-PE antibody in wells of a round bottom 96-well plates for 5 hr in a humidified incubator with 5% CO_2 at 37°C. The cells were washed twice with 1% FCS/PBS and labelled with the appropriate fluorochrome-conjugated anti-mouse monoclonal antibodies (Table 2-3) for 30 minutes at 4ºC. The stained cells were washed twice with cold 1% FCS/PBS buffer and re-suspended in 300 μl of buffer for analysis by flow cytometry.

2.5.11 In vivo cytotoxicity assay

Stock carboxy fluorescein diacetate, succinimidyl ester (CFSE) (Molecular Probes) in DMSO was diluted to 5 or 2 μM in PBS. Target cell populations were prepared

from naive C57BL/6 male mice using RBC-lysed splenocytes. The residual white cells ($2x10^7$ cells/ml PBS) were divided into two populations and equal volume from high (5 μM) or low (2 μM) diluted CFSE solutions were added to the cells at a ratio of 1:1 (vol:vol) and incubated for 10 minutes at 37°C with frequent shaking. The reaction was stopped by adding equal volume of cold complete RPMI medium for 1 minute. CFSE labelled cells were washed twice with complete RPMI medium and PBS as another final wash. Cells labelled with 5 μM CFSE ($CFSE^{Hi}$) left unpulsed and those labelled with 2 μM CFSE ($CFSE^{Lo}$) were pulsed with 5 μg/ml $OVA_{257-264}$ (SIINFEKL) peptide for 1 hr at 37ºC with periodic agitation. Cells were then washed twice with PBS and pellets were resuspended in $5x10^7$ cells/ml PBS. Labelled splenocytes were mixed together in a 1:1 ratio for i.v. injections, where each mouse received 5 x 10^6 $CFSE^{Lo}$ pulsed and 5 x 10^6 $CFSE^{Hi}$ cells in 200μl PBS. After 16 hr, lymphoid tissues were removed and single-cell suspensions were generated as described in section 2.4.3 before acquisition of data on the flow cytometer. CFSE donor target splenocytes were differentiated from host cells and the percentage of target cell killing was determined as:

100 – [(percentage of peptide-pulsed targets in treated recipients/percentage of unpulsed targets in treated recipients)/(percentage of peptide-pulsed targets in naive recipients/unpulsed targets in naive recipients) x 100].

2.5.12 Analysis of cell proliferation

2.5.12.1 Analysis of cell proliferation using CFSE dilution

CFSE is a fluorescein related dye consisting of a fluorescent molecule containing a succinimydyl ester functional group and two acetate moieties. CFSE diffuses freely into cells where intracellular esterases cleave the acetate groups converting it to a

fluorescent, membrane impermeable dye which binds to intracellular proteins. As cells divide the dye is partitioned equally between mother and daughter cells, the number of cell divisions can therefore be determined according to the number of equally spaced peaks of CFSE fluorescence, typically up to six divisions can be discerned from CFSE dilution. The area under each peak was determined to calculate the percentages of dividing lymphocytes in each round of cell division or simply as a measure of dividing and undivided populations.

RBC-lysed splenocytes were washed three times with PBS, centrifuged at 1200 rpm for 10 minutes and resuspended at 2×10^7cells/ml in PBS. Stock CFSE in DMSO was diluted with PBS to 5 μM prior to addition to the cells at a ratio of 1:1 (vol:vol). After incubation at 37ºC for 10 minutes with frequent shaking, the reaction was stopped by adding equal volume of cold complete RPMI medium for 1 minute. CFSE labelled cells were washed twice with complete RPMI medium and PBS as another final wash before resuspension at the appropriate cell number in PBS or medium for adoptive transfer in mice or *in vitro* cell culture.

2.5.12.2 Analysis of OT-I T cell proliferation in response to target cells using thymidine incorporation

Transgenic OVA-specific $CD8^+$ T cells (OT-I) were obtained from spleen and lymph nodes of 8-10 week old OT-I BoyJ transgenic mice. A single-cell suspension from RBC-lysed lymphocytes was counted and then surface stained with anti-CD3 and -CD8 to determine the percentage of $CD3^+CD8^+$ T cell population in each sample. Lymphocytes were adjusted to 1 x 10^6 $CD8^+$ T cells/ml in complete RPMI medium. Target Tramp-C1, $Tramp_{OVA}$, $Tramp_{OVA}$-4-1BBL cells were exposed to 120 gray (Gy) from cobalt γ-radiation source then washed in PBS and resuspended at 1 x 10^5 cells/ml in complete lymphocytes medium. $CD8^+$ T cells at a ratio of 10:1

or 15:1 were cocultured with a constant number of 1 x 10^4 irradiated target cells/100µl in wells of a 96-well U-shaped tissue culture plates at 37ºC in a humidified incubator with 5% CO_2. After 72 hr, cells were pulsed with 1 µCi/20µl ^{3}H thymidine (Amersham) followed by an overnight incubation. The plate was then washed 3 times and cells were harvested by vacuum filtration (Filtermate Harvester, Packard) onto a glass fibre filters (Packard). The air-dried filter was flitted onto a 96-well Omnifilter template (PerkinElmer) and 50 µl of Microscint20 scintillation fluid (Packard) was added per well. The level of thymidine (count per minute; c.p.m) per well was detected using a benchtop scintillation counter (Topcount.NXT, Packard).

2.6 Graph plotting and statistical analysis

All graphs were plotted and statistical analysis performed using Graphpad Prism version 4 software (Graphpad software Inc., La Jolla, CA, USA).

Dose response curves for MTT assay were analysed using sigmoidal dose response non linear regression (curve fit). Where appropriate, limits were set for the top and bottom of the curve, and 95% confidence intervals were determined.

3 Results: Generation and characterization of Tramp$_{OVA}$ and Tramp$_{OVA}$-NR cells

3.1 Introduction

The experimental design to study the immune responses to NR/CB1954-mediated cytotoxicity is based on modifying Tramp-C1 tumour cells to stably express the model tumour antigen ovalbumin (OVA) together with the therapeutic NR gene. These modified tumour cells will serve in establishing a model system to study the immunological response to treatment of subcutaneous tumours in syngeneic mice. Upon treatment with the prodrug CB1954, dying tumour cells are likely to increase the availability of tumour antigens to antigen processing cells. Therefore, immune response to NR/CB1954-cell death and the consequent release of OVA tumour-antigen can be monitored by evaluating OVA-specific T cell responses *in vivo*.

Ovalbumin was chosen as model antigen, since it is a well-defined protein and has been used as a model antigen in several immune-based studies. It contains two well defined epitopes $OVA_{257–264}$ (SIINFEKL) and $OVA_{323–339}$ (ISQAVHAAHAEINEAGR) that are presented in the context of MHC class I and class II, respectively (Davies et al., 2009). These peptides are recognized by OT-I $CD8^+$ and OT-II $CD4^+$ T cells derived from transgenic mice expressing the TCR specific for $OVA_{257–264}$ and $OVA_{323–339}$ in the context of $H\text{-}2K^b$ and $H\text{-}2A^b$ molecules, respectively (Barnden et al., 1998; Clarke et al., 2000).

OVA is a 45 kDa secreted protein and its full length coding sequence encodes a signal sequence (1-45 a.a) that directs the protein to the endoplasmic reticulum, and from there it progresses through the secretory pathway to the outside of cell membrane (Meek et al., 1982). Secreted proteins are normally endocytosed, processed and presented on MHC class II molecules by APCs. However, they can also enter the MHC class I presentation pathway of APCs via a process termed cross-presentation (Shakushiro et al., 2004). Therefore, deletion of the signal

sequence of OVA protein will limit protein expression to the cytosol and to the MHC class I antigen presentation pathway, thus mimicking the expression of tumour associated antigens. Also, this modification was expected to prevent significant levels of antigen presentation except upon death of the tumour cells.

This chapter describes, cloning of cytoplasmic OVA and generation of $Tramp_{OVA}$ and $Tramp_{OVA}$-NR single cell clones. After characterization and selection of promising single cell-derived clones, the tumourigenicity of $Tramp_{OVA}$ and $Tramp_{OVA}$-NR cells was further characterised following subcutaneous injection in mice. Ultimately, the growth characteristics of $Tramp_{OVA}$ tumours were examined together with the OT-I $CD8^+$ T cell response to the tumour.

Figure 3-1: Full length chicken ovalbumin cDNA
Schematic diagram showing the ovalbumin coding region (solid blue) and non-coding region (stipple pattern) within the ovalbumin full length cDNA. The position of the $OVA_{257–264}$ (SIINFEKL) and $OVA_{323–339}$ (ISQAVHAAHAEINEAGR) epitopes that are presented in the context of MHC class I and class II, respectively.

3.2 Generation of $Tramp_{OVA}$ clones

3.2.1 Construction of p-BABE-OVA-puro retroviral vector

For construction of the pBABE-OVA-puro vector, the cytoplasmic cDNA of OVA was generated by amplifying the DNA fragment encoding 138 to 386 a.a from pBS-OVA plasmid (Shastri and Gonzalez, 1993) using the following specific primers

OVA-F1 forward: 5’ –GTA GCC ACC ATG GCT GCA GAT CAA GCC AGA GAG-3’ and OVA-R1 reverse: 5’-GTC TGG ATG CAG CAG AGA AC-3’ (Fig 3-2). A Kozak (underlined) sequence preceding a newly introduced initiation codon of OVA gene was integrated to increase the efficiency of protein translation. The PCR product (≈ 800 bp) was gel purified, then ligated into alkaline phosphatase treated and *SnaB*I linearized pBABE-puro vector. The ligated product was used to transform competent *E. coli* XL2 bacteria. Purified plasmids recovered from different colonies were designated as pAS09B1 to pAS09B10 and were analyzed by restriction enzyme mapping for the presence of the insert and the correct insert orientation using *Nco*I, *Pst*I, and *Xba*I enzymes. Plasmid digestion was expected to generate two fragments of 4642 and 1112 bp with *Nco*I enzyme, four fragments of 4197, 872, 339, and 176 bp with *Pst*I digest, and two fragments of 3424 and 2605 bp with *Xba*I digestion. Gel electrophoresis analysis of the products of three plasmid digestion (pAS09B3, pAS09B4 and pAS09B10) showed the exact number of DNA bands that corresponded with the expected sizes. This indicates that a single copy of the insert was present and in the right orientation (Fig 3-3A).

DNA sequencing of the full length of ovalbumin were also performed using OVA-F2 forward: 5’-GAC TGA ATG GAC CAG TTC TAA TG-3’ and OVA-R2 reverse: 5’ –CCT CCA TCT TCA TGC GAG G-3’ primers to ensure correct coding region. When comparing the OVA DNA sequence results for pAS09B3, pAS09B4 and pAS09B10 with that obtained from the GenBank, analysis revealed that a single adenine nucleotide in the GenBank OVA sequence located at amino acid (a.a) position 188 (OVA_{188}) was changed into a guanine nucleotide in p-Babe-OVA-puro (Fig 3-3B). This change resulted in replacement of a threonine codon with an adenine codon, however this point mutation has no effect on the main two MHC

class I and II restricted epitopes recognized by OT-I $CD8^+$ T cells and OT-II $CD4^+$ T cells, respectively.

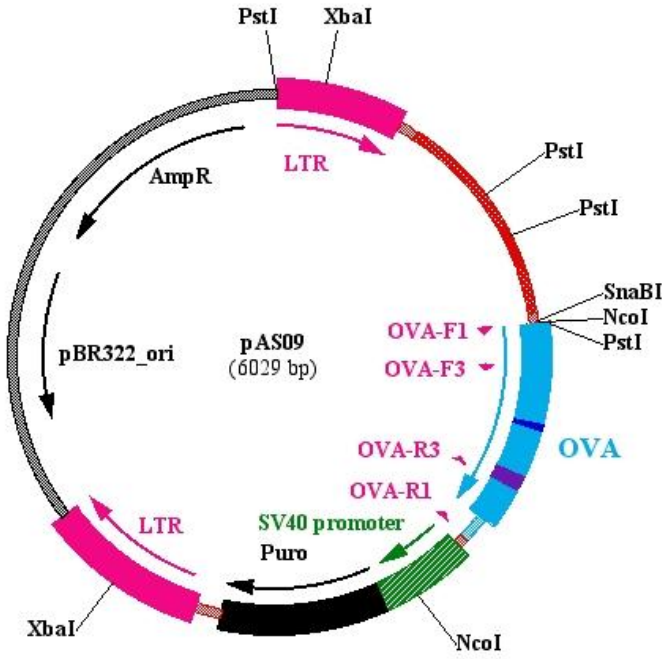
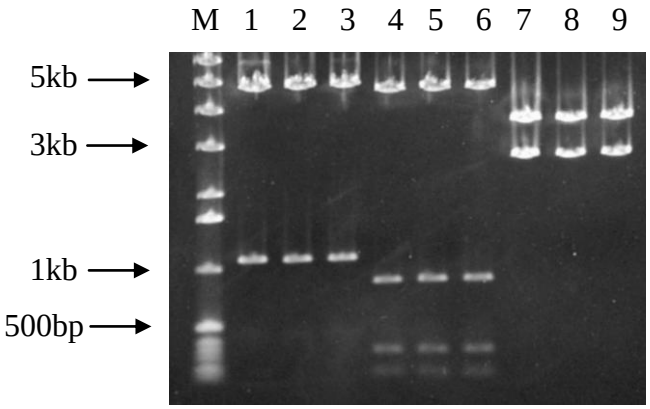


Figure 3-2: p-BABE-OVA-puro (pAS09) retroviral plasmid map
The diagram shows the positions of primers and restriction enzyme sites used in the cloning process. Also shown are the two retroviral LTRs, the OVA gene under the transcriptional control of the 5' LTR, the puromycin resistance gene (Puro) driven by the SV40 promoter, and the plasmid vector replication origin and ampicillin resistance gene.

A.



B.

GenBank OVA sequence	GAG	AAA	**A**CA	TTT	AAG
	E	K	**T**	F	A
p-Babe-OVA-puro	GAG	AAA	**G**CA	TTT	AAG
	E	K	**A**	F	A

Figure 3-3: Characterization of pBABE-OVA-puro (pAS09) retroviral vector
A, gel electrophoresis analysis of pAS09B3 (lane 1, 4, 7), pAS09B4 (lane 2, 5, 8) and pAS09B10 (lane 3, 6, 9) plasmid digests. M: size marker (1kb DNA ladder); lanes (1-3): *Nco*I digests; lanes (4-6): *Pst*I digests; lanes (7-9): *Xba*I digests of pAS09B3, 4 and 10 plasmids. **B**, comparison of OVA DNA and encoded amino acid sequences in the GenBank and p-BABE-OVA-puro. The threonine amino acid in pBS-OVA was replaced by alanine in p-BABE-OVA-puro. The difference between the two sequences is highlighted in red.

3.2.2 Generation of single cell-derived Tramp$_{OVA}$ clones

Initially, FLYA13 retrovirus packaging cells were transfected with pAS09B3 or pBabe-puro plasmids using Fugene 6 (Roche) following the manufacturer's instructions, to allow entry of plasmid DNA into the cells. After 48 hr, the cells were selected in 5µg/ml puromycin for 2 weeks until colonies of antibiotic resistant cells were clearly visible. The transfected pool of FLYA13$_{OVA}$ was propagated further into large cultures and the supernatant containing retrovirus particles was

collected and kept at -20ºC. To generate $Tramp_{OVA}$ clones, Tramp-C1 cells were transduced with supernatant from $FLYA13_{OVA}$ packaging cells supplemented with 6 μg/ml polybrene every 12 hr for 2 days. The cultures were then selected in puromycin for 2-3 weeks. To select single cell clones, puromycin resistant colonies were plated using the limiting dilution method in 96 well plates and left for 3-4 weeks until individual colonies were apparent. Transduced colonies that originated from the lowest plated cell densities and were likely to originate from single cells were picked from the plates and expanded further under puromycin selection. To screen for the integration of the OVA gene into the genome of $Tramp_{OVA}$ clones, genomic DNA was extracted from each clone and used as a template for PCR using OVA-F3 forward: 5'-GGA TGA AGA CAC ACA AGC AAT-3' and OVA-R3 reverse: 5'-TCT CTG CCT GCT TCA TTG ATT T-3' (reverse) PCR primers. Integration of OVA gene into the genomic DNA of $Tramp_{OVA}$ single cell clones should be indicated by amplification of a ~450 bp product (Fig. 3-4). Among the different single cell clones screened, six are shown demonstrating a clear PCR product at the expected size compared to the PCR product from the original pBabe-OVA-puro plasmid in lane 2.

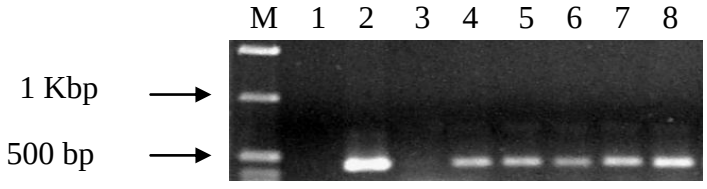


Figure 3-4: Gel electrophoresis analysis of PCR products from single cell derived $Tramp_{OVA}$ clones
PCR reactions in lanes 1-8 used the following as templates: lane 1: water; lane 2: plasmid AS09B3; lane 3: DNA from parental Tramp-C1; lane 4: clone 1; lane 5: clone 3; lane 6: clone 5; lane 7: clone 6; lane 8: clone 8. Lane M: size marker.

3.2.3 Detailed characterization of $Tramp_{OVA}$ clones

3.2.3.1 Detection of ovalbumin protein expression

To confirm OVA protein expression in $Tramp_{OVA}$ cells, lysates from parental Tramp-C1 and $Tramp_{OVA}$ polyclonal cells, as well as the previously –described OVA expressing melanoma cell line ($B16_{OVA}$) (Gough et al., 2005), were analyzed by western blotting using anti-OVA antibody. In this analysis parental Tramp-C1 and B16 cells served as negative controls, whereas 10-50 ng of crude ovalbumin and $B16_{OVA}$ melanoma cells were used as reference and positive control, respectively. Figure 3-5 shows that crude OVA protein displayed two clear bands at the predicted molecular weight of 45 KDa and that the level of OVA protein could be detected down to 10 ng. However, OVA expression in 50 µg cell lysates from both $B16_{OVA}$ cells, expressing full length OVA (45 KDa), and $Tramp_{OVA}$ cells, expressing truncated OVA (27 KDa) was below the 10 ng detection level (Fig 3-5). Therefore, it was difficult to verify OVA expression in $Tramp_{OVA}$ cells by western blot analysis.

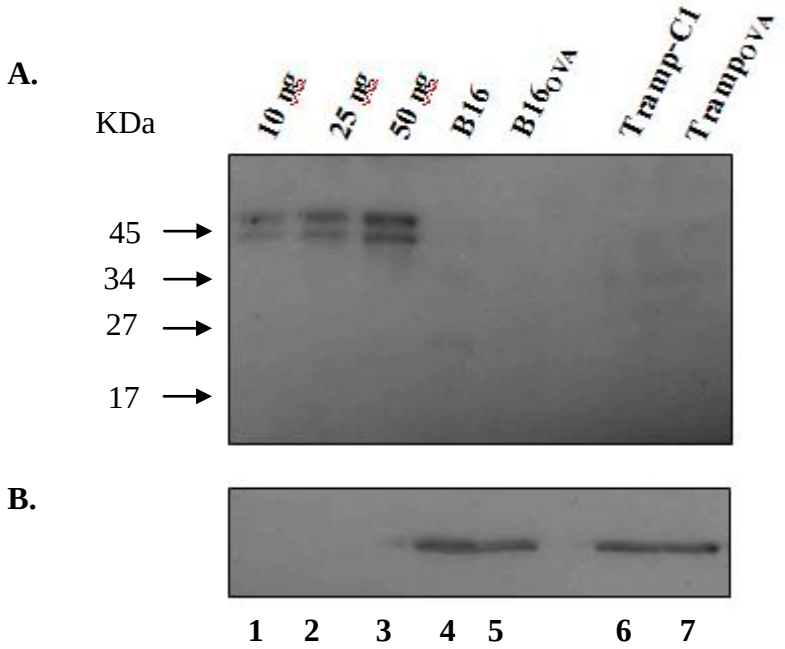


Figure 3-5: Western blot analysis for ovalbumin protein in polyclonal $Tramp_{OVA}$ cells

B16 melanoma, $B16_{OVA}$, Tramp-C1 and $Tramp_{OVA}$ cells were harvested for protein lysate preparation. An amount of 30 μg of total protein lysate and 10-50 ng purified OVA protein as a reference standard was examined for OVA expression by western blot analysis using specific antibodies (**A**). β-tubulin was used as an internal loading control (**B).** The samples were as follow: purified OVA protein as indicated (lanes 1, 2, 3); B16 melanoma cells (lane 4); $B16_{OVA}$ cells (lane 5); Tramp-C1 cell (lane 6); and $Tramp_{OVA}$ polyclonal population (lane 7).

3.2.3.2 Activation of OVA-specific (B3Z) $CD8^+$ T cell hybridoma by $Tramp_{OVA}$ clones

As an alternative approach to confirm and compare the level of OVA expression between different $Tramp_{OVA}$ clones, we examined whether sufficient OVA antigen is processed and displayed in the context of MHC molecules to permit recognition and activation of B3Z T cells.

The B3Z mouse cell line is a $CD8^+$ T cell hybridoma expressing a TCR that specifically recognizes $OVA_{257–264}$ peptide (SIINFEKL) in the context of MHC class I (H-$2K^b$). It contains an Escherichia coli *lacZ* reporter gene (encoding β-galactosidase enzyme) under the transcriptional control of the nuclear factor of

activated T cells (NFAT) responsive element of IL-2 promoter. Selective recognition of SIINFEKL/MHC I complex by the TCR of B3Z T cells correlates with the level of expression of β-galactosidase enzyme, which can then be evaluated using a variety of chromogenic and fluorogenic substrates (Karttunen et al., 1992).

To assess the ability of $Tramp_{OVA}$ clones to activate B3Z hybridoma T cells, cells were cultured in complete media supplemented with (IFN-γ) overnight, to induce MHC class I expression, followed by coculturing with B3Z T cells for 16 hr. In this assay, parental Tramp-C1 cells were used as a negative control while Tramp-C1 pulsed with SIINFEKL peptide provided a positive control. Figure 3-6 shows the different levels of β-galactosidase enzyme activation in response to $Tramp_{OVA}$ clones, likely reflecting the variation in the levels of SIINFEKL peptide presented by the different clones. Clone 6 stimulated B3Z T cells by 1.4 times the level seen in peptide pulsed cells, while clones 3 and 5 were 70% as effective as the peptide-pulsed cells. The highest level of B3Z T cell activation was induced by clone 8; however this clone showed abnormal cellular morphology and delayed growth rate compared to other clones and thus was excluded from further analysis. Likewise clone 1 was also excluded due to minimal B3Z T cell activation. In conclusion, the efficient activation of B3Z hybridoma T cells by clones 3, 5 and 6 indicated competent presentation of the SIINFEKL peptide, and therefore these clones were selected for further analysis.

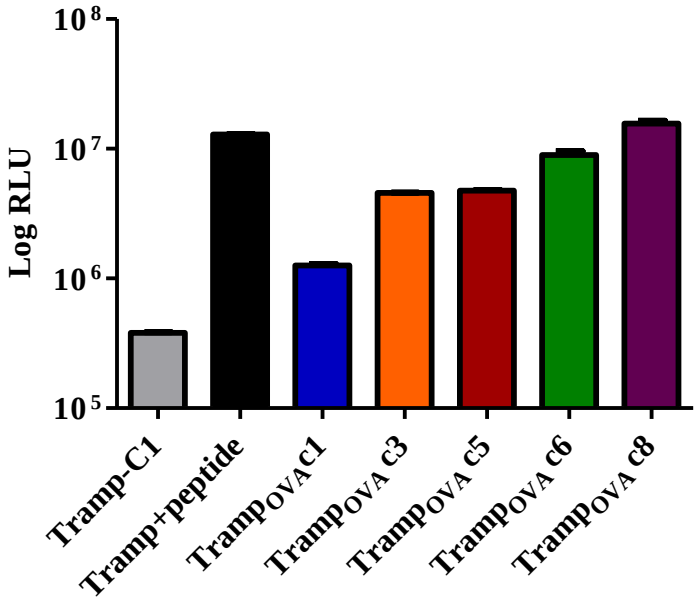


Figure 3-6: B3Z T cell activation by $Tramp_{OVA}$ clones
Cells were treated with 100U/ml IFN-γ overnight, then $1x10^4$ Tramp-C1, Tramp-C1 loaded with peptide (1μg/ml) or $Tramp_{OVA}$ clones were cocultured in wells of a 96 well plate with $1x10^5$ B3Z T cell hybridoma cells for 16 hr. β-galactosidase activity in B3Z T cells was assayed in total culture lysates after incubation with β-galactosidase luminescent substrate for 1 hr at 37ºC. Data are presented as the mean relative luminescence unit of triplicate wells (±SEM) on a log scale.

3.2.3.3 Lysis of $Tramp_{OVA}$ clones by effector OT-I T cells

To further study the characteristics of the selected $Tramp_{OVA}$ clones, their ability to stimulate cytotoxicity of OVA-specific effector $CD8^+$ cells (OT-I T cells) was assessed. The cytotoxicity was examined using a chromium release assay. Briefly, a single cell suspension of spleen and lymph nodes from male OT-I mice was prepared and activated *in vitro* with 1μg/ ml SIINFEKL peptide for 5 days to differentiate into effector cells. These *ex vivo* stimulated cells were then incubated with chromium labelled $Tramp_{OVA}$ clones at the indicated ratios for 5 hr at 37ºC. As shown in Figure 3-7, there was negligible lysis of the parental Tramp-C1 cells, while maximal cytotoxicity was evident at all ratios with Tramp-C1 cells loaded with the SIINFEKL peptide. Sensitivity to OVA-specific lysis of the $Tramp_{OVA}$

clones was most prominent at effector/target (E/T) ratio of 33:1, where the highest specific lysis (65%) was observed with clone c6 while c3 and c5 were less sensitive and induced 36% and 13% specific lysis, respectively. However, at lower E/T ratios, cytotoxicity levels were reduced to below 21% with marginal differences between clones at each ratio.

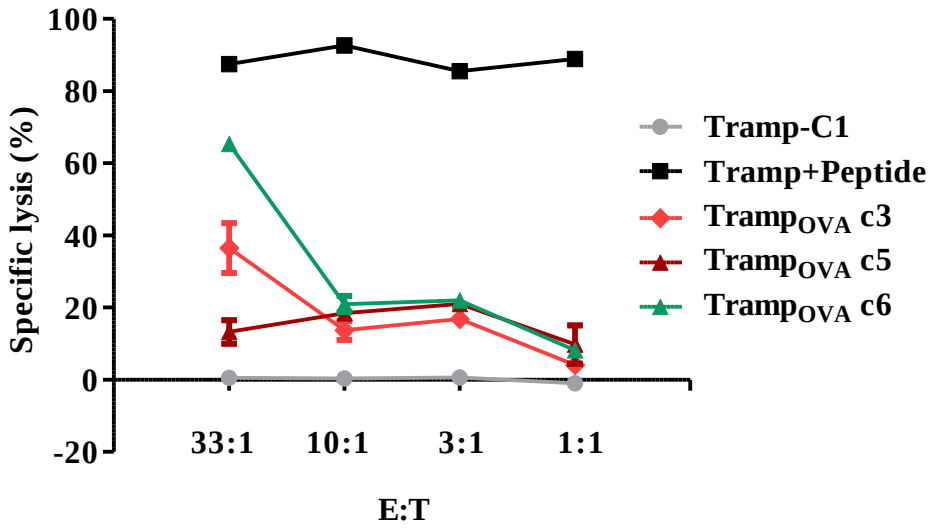


Figure 3-7: *In vitro* CTL assay to compare OVA-specific cytolytic sensitivity of $Tramp_{OVA}$ clones
Effector OT-I T cells were prepared from single cell suspension from spleen and lymph node from OT-I mice and stimulated with 1 µg/ml SIINFEKL peptide in complete media with 100U/ml IL-2 for 5 days. effector OT-I T cells (E) were cocultured with ^{51}Cr-labeled Tramp-C1, Tramp-C1 loaded with peptide or $Tramp_{OVA}$ clone as target cells (T) at the indicated ratios for 5 hr. Each point represents the mean of triplicate cultures (±SEM).

3.2.3.4 MHC class I surface expression by $Tramp_{OVA}$ clones

The level of MHC class I expression is a key determinant of the immunogenicity of tumour cells and rejection of tumour cells *in vivo* (Nanni et al., 1996). Previous studies reported that early passages of Tramp-C1 cells express low level of surface MHC class I molecules that decline on *in vitro* culturing (Grossmann et al., 2001). Therefore, it was of interest to evaluate MHC class I expression by $Tramp_{OVA}$ clones and whether treatment with IFN-γ would affect the level of expression. To

study MHC class I expression, parental Tramp-C1 and $Tramp_{OVA}$ clones either untreated or treated with interferon- γ (IFN-γ) were stained using anti-MHC class I antibody and analyzed by flow cytometry. The results in Figure 3-8A revealed that parental Tramp-C1 cells expressed low levels of MHC I molecules and even lower levels were observed in clones c6 and c5, however clone 3 showed higher levels of MHC class I expression than parental Tramp-C1 cells. On treatment of cells with IFN-γ, MHC class I expression was vastly induced in parental Tramp-C1 and $Tramp_{OVA}$ clones (Figure 3-8B). Interestingly, the order of MHC class I expression among IFN-γ treated clones and parental cells was the same as that observed in untreated cells with clone 3 expressing higher levels than IFN-γ-stimulated parental Tramp-C1 cells.

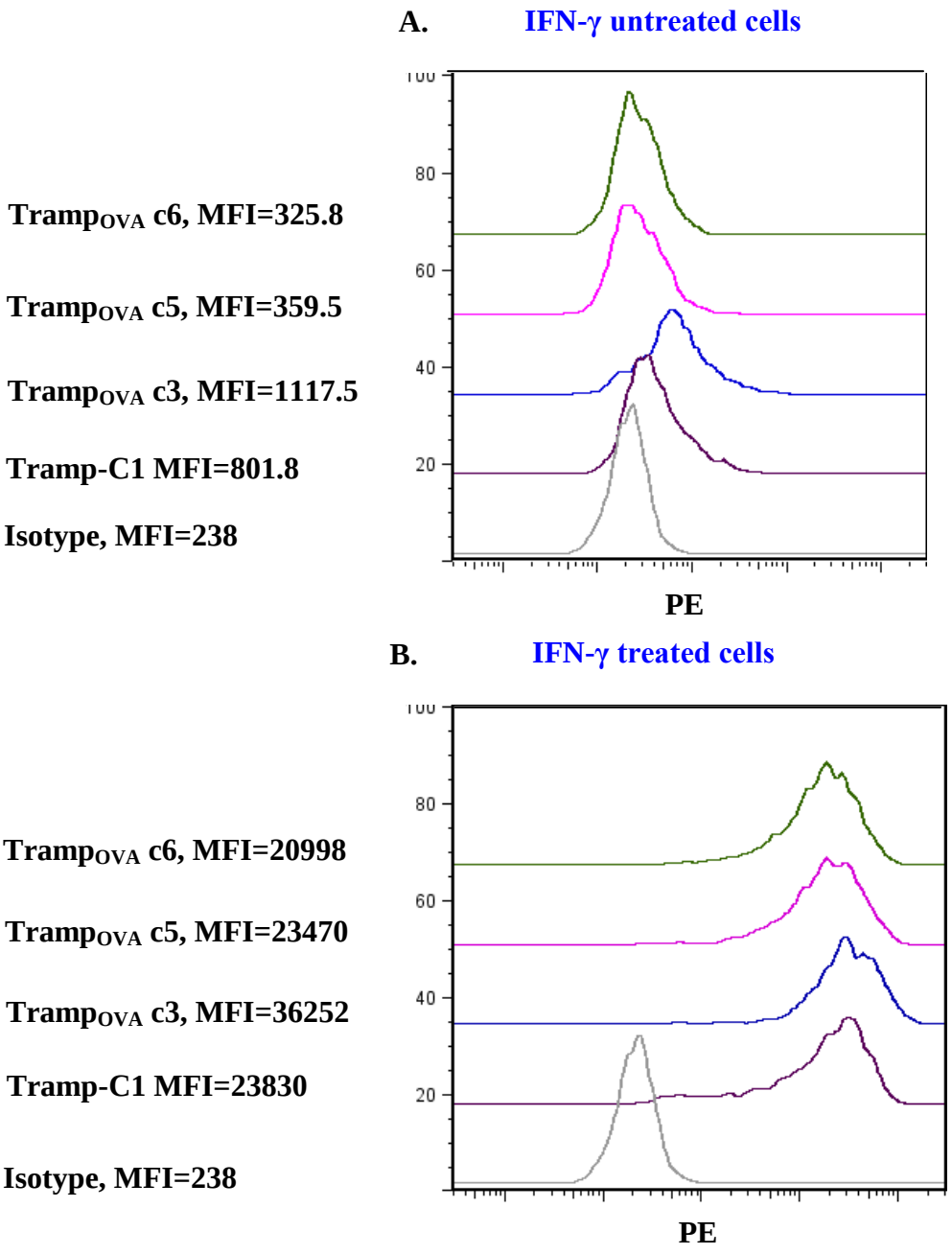


Figure 3-8: Expression of MHC class I molecule by different Tramp$_{OVA}$ clones
Tramp-C1 cells and Tramp$_{OVA}$ clones were cultured: **A** without or **B** with 100U/ml IFN-γ overnight. 1x10^5 cells/100 µl were stained with MHC class I-PE or isotype control antibodies for 20 min at 2-8ºC before analysis by flow cytometry. The different cell lines are indicated to the left of each horizontal series of the histograms and the numbers represent the mean fluorescence intensity (MFI) of MHC class I expression.

3.2.4 Choice of $Tramp_{OVA}$ clone 3 for establishment of the model tumour cell line

Section 3.2.3 has described the characterisation of clones transduced with pBABE-OVA-puro in order to identify a clone suitable in which OVA would serve as a model tumour antigen. This clone would ideally express low level of OVA-antigen but sufficient for recognition by tumour-specific T cells.

As indicated above, during the screening process, some of the clones with slow growth rate *in vitro* or inefficient OVA presentation were excluded. Thus, only 3 promising clones (c3, c5 and c6) were identified. The c6 clone was rejected from the selection as it is more immunogenic than the c3 and c5 clones to B3Z and OT-I cells (Fig 3-6 and 3-7). Then, c3 clone was chosen as it showed inducible MHC class I expression (Fig 3-8) and reasonable level of OVA-antigen presentation as demonstrated by its ability to induce OVA-specific OT-I cell responses that should allow proper tracking of the immune response.

3.3 Generation of $Tramp_{OVA}$ clones expressing nitroreducatase enzyme

To introduce NR gene to $Tramp_{OVA}$, viral supernatant from FLYA13-NR retrovirus producer cells previously generated in our laboratory (Jaberipour et al., 2010) were used to transduce $Tramp_{OVA}$ cells (clone 3). However, initial attempts failed to generate any G-418 resistant transduced cells, suggesting that the virus titre in the FLYA13-NR cells was too low. Combimag beads were therefore used in addition to 6 µg/ml polybrene to improve the efficiency of virus delivery. Transduced cells were selected using puromycin and G-418 antibiotics. After 2-3 weeks, antibiotic resistant colonies were pooled and tested for CB1954 prodrug sensitivity and by western blot analysis to confirm NR expression. Clonal cell lines of $Tramp_{OVA}$-NR

cells were then generated by limiting dilution of the cell pool, and a number of $Tramp_{OVA}$-NR single cell-derived clones were expanded in large cultures for further screening.

3.3.1 Nitroreductase expression in $Tramp_{OVA}$-NR clones

To confirm that $Tramp_{OVA}$-NR clones express NR protein, cell lysates from Tramp-C1, as a negative control, $Tramp_{OVA}$-NR polyclonal cells and single cell clones were immunoblotted for NR and β-tubulin proteins.

Figure 3-9 shows that, unlike Tramp-C1 cells, the $Tramp_{OVA}$-NR polyclonal line and single cell clones successfully expressed NR protein. However, $Tramp_{OVA}$-NR polyclonal cells and c2, c4, c6 and c15 clones demonstrated relatively lower levels of NR protein expression compared to other clones.

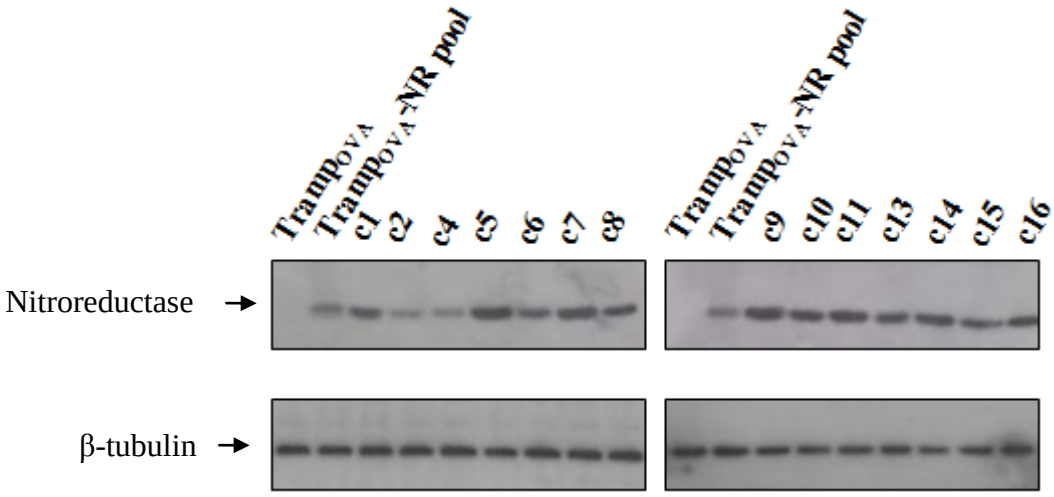


Figure 3-9: Nitroreductase expression in $Tramp_{OVA}$-NR cells
$Tramp_{OVA}$ cells, $Tramp_{OVA}$-NR pool of cells or single cell clones were harvested for protein lysate preparation. An amount of 5 μg of total protein lysate preparation was examined for nitroreducatase enzyme expression and β-tubulin as a loading control by western blot analysis using specific antibodies.

3.3.2 $Tramp_{OVA}$-NR clones sensitivity to CB1954 prodrug

In order to choose a single cell-derived clone with suitable prodrug sensitivity for further use *in vivo* experiments, the sensitivity of single cell clones to CB1954 was

determined *in vitro* using the MTT cytotoxicity assay. The CB1954 prodrug concentration showing 50% reduction in viability (IC_{50}) was obtained from dose response curves and was used to compare between various clones. Figure 3-10 shows the sensitivity of different clones to CB1954 plotted as dose response curves where the IC_{50} values varied markedly between clones ranging from 7.5 to 133 µM CB1954 (Table 3-1). Five (c4, c6, c11, c13 and c15) clones showed IC_{50} values below that of the original pool of cells (29 µM) for which the IC_{50} were determined as 24.6, 8.5, 7.4, 8.4, 20.8 µM CB1954, respectively.

On the second attempt for determining the IC_{50} values of the different clones that involved sub-culturing of cells for 3 passages which is equal to ≈15 population doublings, the IC_{50} values were increased to more than two fold in some of the clones (c5, c6, c9, c13 and c14) (Table 3-1). This suggested that the clones may have not originated from a single cell, or that the level of NR expression was reduced during continued *in vitro* growth of the cells. Thus, we choose to select clone 11 which shows the best stability for NR expression.

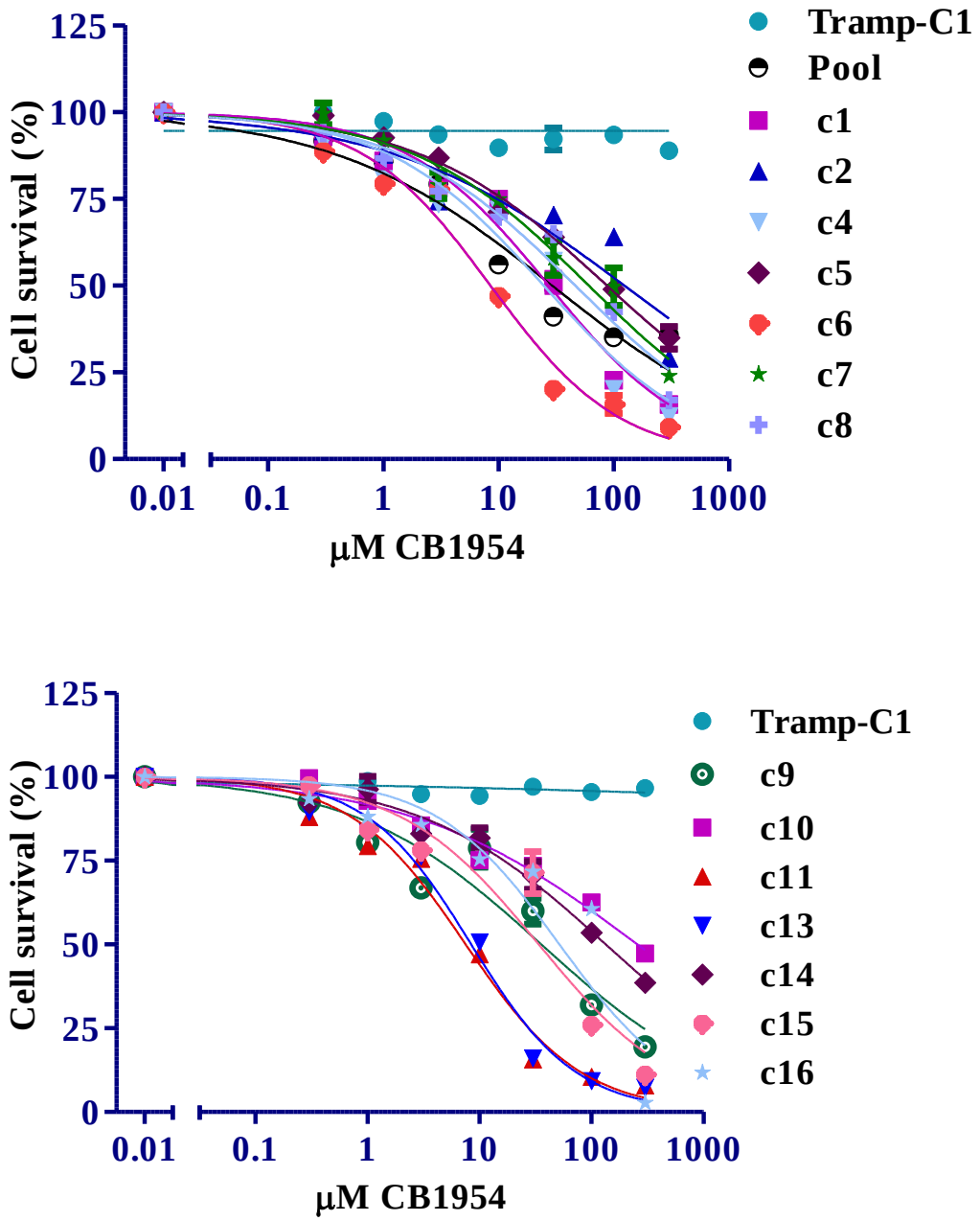


Figure 3-10: Sensitization of Tramp$_{OVA}$-NR single cell-derived clones to CB1954

Tramp, Tramp$_{OVA}$-NR pool, or Tramp$_{OVA}$-NR single cell-derived clone ($1x10^4$ cell/180 µl) were treated with prodrug at the indicated concentrations for 4 hr. Cell viability was determined 48 hr after prodrug addition. Data points show mean (±SD) of quadruplicate wells, and are normalised to 100% survival in absence of prodrug.

Clone	I.C_{50} value µM CB1954 First round	I.C_{50} value µM CB1954 Second round
Tramp-C1	> 300	n.d
Pool	29.2	n.d
c1	36	37.8
c2	93	126.6
c4	24.6	47.7
c5	38.1	86.84
c6	8.5	23.8
c7	52.2	60.5
c8	43.2	46.2
c9	35.8	188.2
c11	7.5	8.8
c13	8.4	37.7
c14	133.2	300
c15	20.8	33.7
c16	29.52	51.7

Table 3-1: Tramp$_{OVA}$-NR single cell-derived clones IC$_{50}$ range of CB1954
The IC$_{50}$ ranges were graphically determined from the dose response curves (as shown on Fig. 3-11) plotted from two different experiments. Clones with the lowest IC$_{50}$ values were marked in red colour.
n.d (not determined)

3.3.3 Presentation of ovalbumin epitope by Tramp$_{OVA}$-NR clone 11

To confirm that Tramp$_{OVA}$-NR clone 11 has not lost the OVA transgene its ability to stimulate B3Z T-hybridoma cells was assessed as described previously (section 3.2.3.2. and Fig 3-6). Overnight incubation of B3Z hybridoma-T cells with parental Tramp-C1 cells induce similar background levels of β-galactosidase enzyme to that observed with B3Z hybridomas alone (Fig. 3-11). In contrast, both Tramp$_{OVA}$ and

Tramp$_{OVA}$-NR activated B3Z T cells and induced accumulation of β-galactosidase enzyme; however Tramp$_{OVA}$ cells showed ≈6 fold higher levels of β-galactosidase expression compared to Tramp$_{OVA}$-NR cells. This indicated that Tramp-$_{OVA}$-NR cells possess reduced ability to present OVA-epitope relative to their parental Tramp$_{OVA}$ cells.

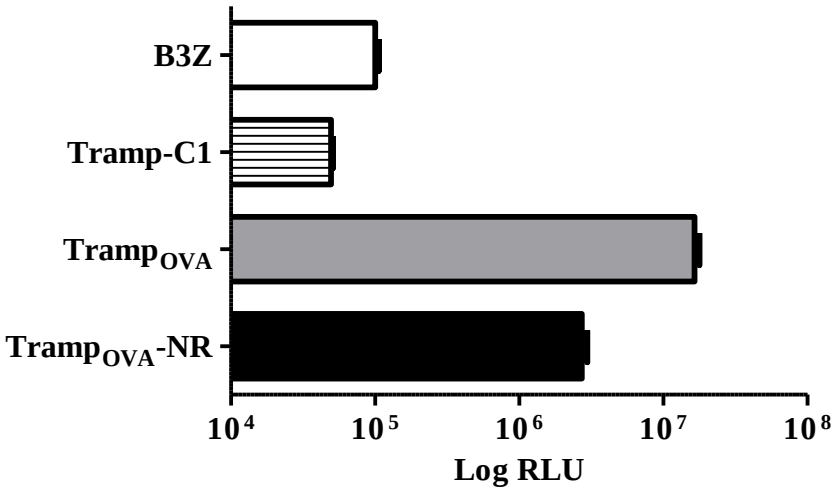


Figure 3-11: B3Z T cell activation by Tramp$_{OVA}$-NR cells
B3Z T-hybridoma cells (1x10^5) were left alone or cocultured in a 96 well plate with 1x10^4/well Tramp-C1, Tramp$_{OVA}$, or Tramp$_{OVA}$-NR cells for 16 hr. β-galactosidase activity in B3Z T cells was assayed in total culture lysates after incubation with β-galactosidase luminescent substrate for 1 hr at 37ºC. Data are presented as the mean relative luminescence (RLU) of quadruplicate wells (±SEM) on a log scale.

3.3.4 Bulk growth of Tramp$_{OVA}$ and Tramp$_{OVA}$-NR cells in preparation for *in vivo* experiments

Since the IC$_{50}$ values of Tramp$_{OVA}$-NR single cell-derived clones were increasing on sub-culture of cells, it was decided to generate a single large batch of Tramp$_{OVA}$-NR clone 11 and to freeze it down into multiple cryotubes as a uniform seed stock for *in vivo* experiments. This should minimize potential variability by ensuring equal OVA or NR expression in tumour cell implants between *in vivo* experiments.

The schematic diagram on figure 3-12A shows the strategy. $Tramp_{OVA}$ clone 3 or $Tramp_{OVA}$-NR clone 11 cells were expanded in large 150 cm^2 flasks and the IC_{50} for CB1954 was determined after passage 1 (P1) and passage 2 (P2). Then, the cells were frozen down in multiple aliquots of seed stock when the P2 cultures were 100% confluent. Before each *in vivo* experiment, one cryotube of the seed stock was thawed and cultured in preparation for subcutaneous injection of 10 mice with $5x10^6$ cells/animal. Also, in most experiments, the IC_{50} of cell surplus to inoculation requirements designated as passage 4 (P4) was determined on the day of seeding the mice. As shown in Figure 3-12B and C, the IC_{50} value of $Tramp_{OVA}$-NR cells at P1 increased considerably from 21.5 to 244 µM at P4 when the cells were exposed to CB1954 for 4 hr, whereas the difference in the IC_{50} values between P1 and P4 was less pronounced (from 2.6 to 12.3 µM) when extending CB1954 exposure to 24 hr.

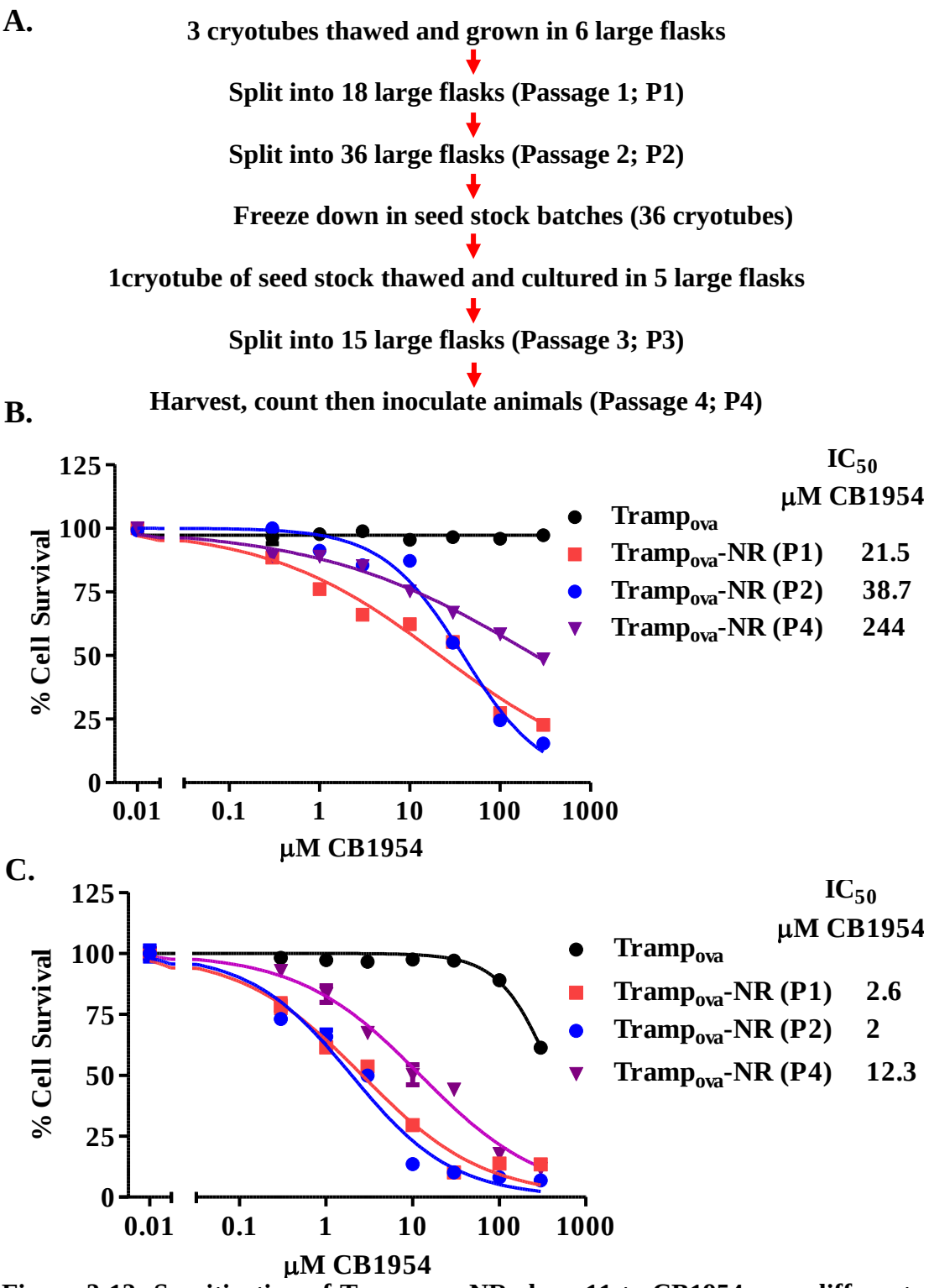


Figure 3-12: Sensitization of $Tramp_{OVA}$-NR clone 11 to CB1954 over different cell passages

A, schematic representation of bulk growth of $Tramp_{OVA}$-NR cells. **B** and **C**, $1x10^4$ $Tramp_{OVA}$ or $Tramp_{OVA}$-NR clone11 from different passages were treated with prodrug at the indicated concentrations for 4 hr or 24 hr, respectively. Cell viability was determined 48 hr after prodrug addition. Data points show mean (±SD) of quadruplicate wells, and are normalised to 100% survival in absence of prodrug. The IC_{50}'s determined from the data are indicated to the right of graph.

3.4 Tumourigenicity of $Tramp_{OVA}$ and $Tramp_{OVA}$-NR cells in nude C57BL/6 mice

To examine the growth of $Tramp_{OVA}$ and $Tramp_{OVA}$-NR cells *in vivo*, a previously established tumourigenic dose of $5x10^6$ Tramp cells was chosen (Kwon et al., 1999). Parental Tramp-C1 or $Tramp_{OVA}$-NR cells were s.c seeded on the right flank of C57BL/6 nude male mice and tumour volumes were assessed over the following weeks. In group of mice seeded with parental Tramp-C1 cells, palpable tumours of 45.2 mm^3 mean volume were detected by day 14 in 3/3 mice (Fig 3-13A). These tumours progressively developed to a mean volume of 476 mm^3 on day 33. In contrast, mice inoculated with $Tramp_{OVA}$-NR cells showed relatively smaller tumour volumes compared to Tramp-C1 group at all time points and achieved a 74 mm^3 tumour volume after 26 days (Fig 3-13B).

In a similar experiment, seeding of $Tramp_{OVA}$ cells s.c. in nude mice resulted in the appearance of visible tumours between 14 to 17 days that developed into 58.6 mm^3 mean volume by 24 days (Fig. 3-13C).

These results show that s.c injection of $5x10^6$ $Tramp_{OVA}$ or $Tramp_{OVA}$-NR cells is tumorigenic in C57BL/6 nude male mice and that both $Tramp_{OVA}$ and $Tramp_{OVA}$-NR tumours exhibit almost similar growth rates. However, the growth rate of $Tramp_{OVA}$ and $Tramp_{OVA}$-NR was slower compared to parental Tramp-C1 tumours (Fig 3-13D).

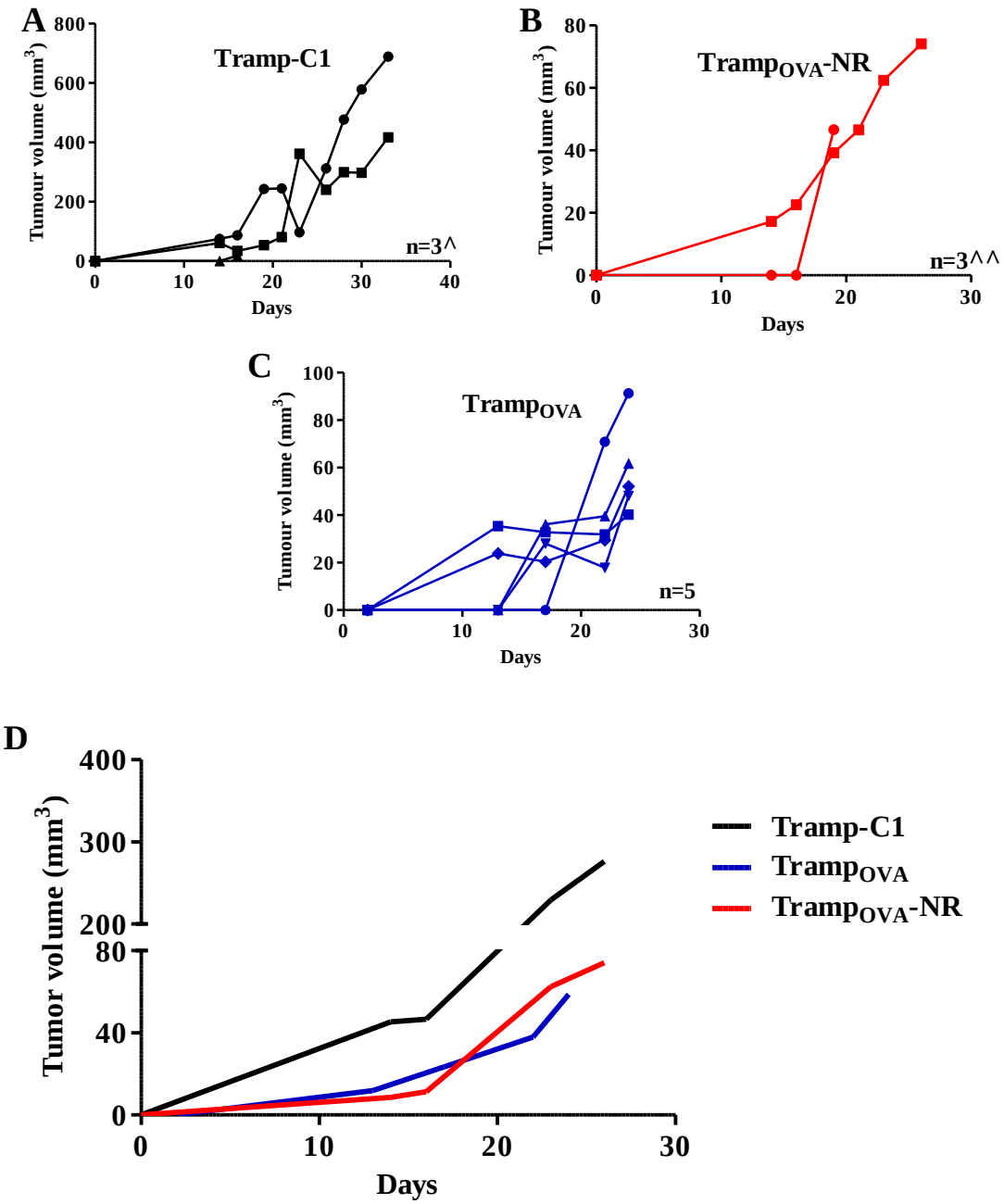


Figure 3-13: Growth characteristics of subcutaneous tumours initiated by parental Tramp-C1 and the subclones $Tramp_{OVA}$ and $Tramp_{OVA}$-NR cells

Male nude C57BL/6 mice, 16-20 weeks old, were injected s.c. in the right flank with $5x10^6$ of the indicated tumour cells. Tumour volume was measured manually with a calliper three times a week. **A**, **B** and **C** show tumour growth of individual mice in the indicated group. **D**, shows an overlay of mean tumour growth curves for each group are shown.

n=the initial number of mice per group.

^ One mouse was lost due to eye abscess on day 15.

^^ Two mice were lost due to eye abscesses on day 7 and 20

3.5 Dose titration of OT- I T cells in nude C57BL/6 mice with established $Tramp_{OVA}$ tumours

Adoptive transfer of OVA-specific OT-I $CD8^+$ T cells may affect the growth of OVA expressing tumours in mice and this effect is expected to be dose dependent. Therefore, OT-I T cell dose titration was adopted to determine a sub-therapeutic dose of OT-I T cells for *in vivo* studies. The ideal dose would have minimal effect on OVA expressing tumour growth in the absence of additional stimulation, and thus would permit monitoring of $CD8^+$ T cell activation in response to NR/CB1954-mediated tumour cell killing *in vivo*.

3.5.1 Effect of 10 million naïve OT-I T cells

In a pilot study, two groups of nude C57BL/6 mice were seeded on the right flank of the back of the mice by s.c. injection of $5x10^6$ $Tramp_{OVA}$ cells, while the third group received $5x10^6$ Tramp-C1 cells s.c. At day 0, defined when mean tumour diameter reached 5 mm, to provide a negative and a positive control respectively, one group of mice bearing parental Tramp-C1 tumours and another one bearing $Tramp_{OVA}$ tumours received $10x10^6$ effector OT-I lymphocytes ($\approx 9\ x10^6$ $CD8^+$ T cells) to provide a negative and a positive control, respectively. The third group of mice with $Tramp_{OVA}$ tumours received $10x10^6$ naïve OT-I lymphocytes ($\approx 4\ x10^6$ $CD8^+$ T cells) served as test group. As described in the scheme in Figure 3-14A tumour diameter was followed over the experimental time and 2 mice from each group were culled at day 5 and 10 for analysis of $CD8^+$ T cells in blood, spleen and lymph nodes pool (axillary, brachial, inguinal and mesenteric).

In control group, mice inoculated with Tramp-C1 cells that received effector OT-I cells, the mean tumour volume slightly increased overtime and none of the mice

were tumour-free. Conversely, groups of mice seeded with $Tramp_{OVA}$ tumours and receiving either naïve or effector OT-I T cells showed complete tumour regression in 2/4 and 2/5 of the mice, respectively, by day 10 (Fig 3-14 B and C). This indicates that adoptive transfer of $10x10^6$ naive or effector OT-I T cells can result in inhibition of $Tramp_{OVA}$ tumour growth.

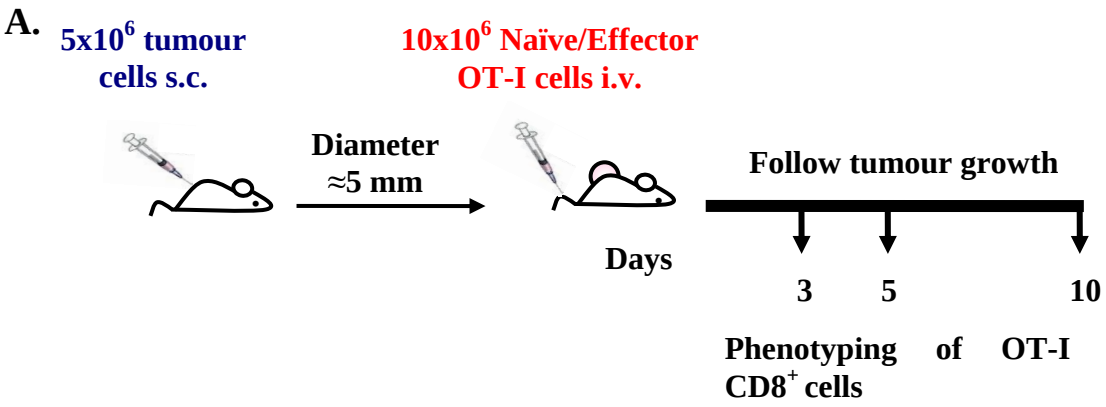


Figure 3-14: Effect of $10x10^6$ naïve or effector OT-I T cells on subcutaneous tumour growth of $Tramp_{OVA}$ cells in syngeneic nude C57BL/6 mice.
A, schematic representation of the experimental design. Male nude C57BL/6 mice, 16-20 weeks old, were injected s.c. in the right flank with $5x10^6$ Tramp-C1 or $Tramp_{OVA}$ cells. At day 0 (defined when tumour diameter reaches 5 mm), mice received either $10x10^6$ naïve or effector OT-I T cells i.v. as indicated in each treatment group. Effector OT-I T cells were prepared from single cell suspension from spleen and lymph node from OT-I mice and stimulated with 1 µg/ml SIINFEKL peptide in complete media with 100U/ml IL-2 for 5 days. Tumour diameter was measured manually using a calliper three times a week. Blood samples were collected via tail vein on day 3, 5 and 10 and the red blood cells were lysed to facilitate lymphocytes analysis by flow cytometry. At day 5 and 10, two mice were culled from each group for isolation of spleen and lymph nodes.

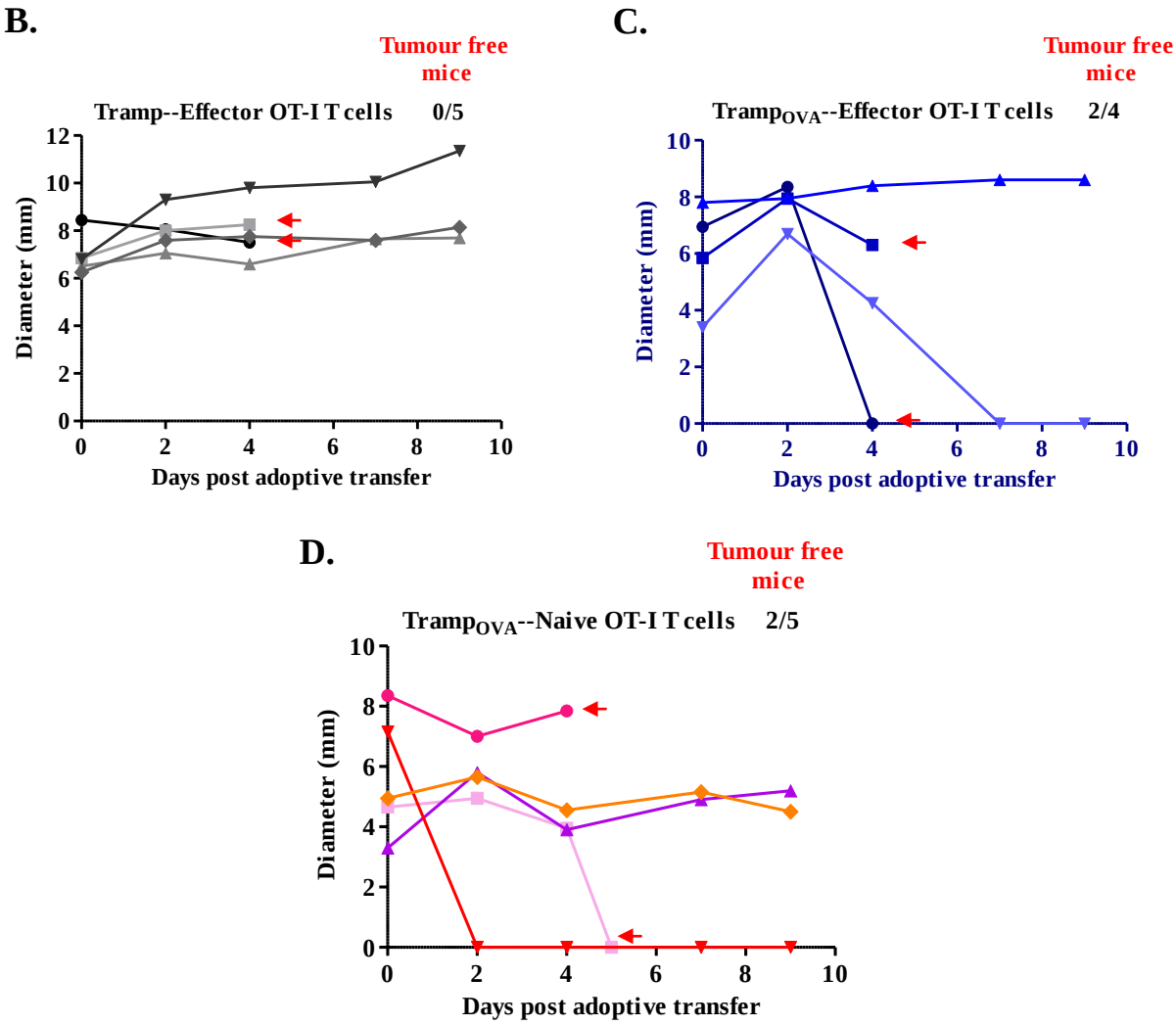


Figure 3-14 ...cont. B, **C** and **D** show data points of tumour diameter for individual mice following adoptive transfer in the indicated groups.
→ Small red arrows indicate animals culled on day 5 for immunological analysis.

To further follow the OT-I CD8$^+$ T cell responses to Tramp-C1 and Tramp$_{OVA}$ tumours, the percentages and phenotype of adoptively transferred cells were examined in blood, spleen and lymph nodes in different groups. Activation of CD8$^+$ T cells due to OVA-tumour antigen recognition was monitored using the CD69 early activation marker, since it is only up-regulated by T cells following antigen-induced activation and not due to homeostatic proliferation in immunodeficient mice (Marleau and Sarvetnick, 2005).

In blood, the percentage of $CD8^+$ T cells in groups of mice bearing Tramp-C1 or $Tramp_{OVA}$ tumours and received effector $CD8^+$ T cells were nearly comparable (≈ 4%) by day 3 (Fig. 3-15A). However it was reduced to ≈ 2% on day 5 and was maintained at the same level till day 10. In contrast, the percentage of $CD8^+$ T cells in mice bearing $Tramp_{OVA}$ tumours and receiving naive cells was very low (0.22%) by day 3 and markedly increased (10 folds) by day 10, indicating antigen-driven proliferation.

In spleen, as in LNs, the percentage of $CD8^+$ T cells in mice that received effector T cells and Tramp-C1 tumours was almost the same on day 5 and 10, while those inoculated with $Tramp_{OVA}$ cells dropped by approximately 60% by day10. Conversely, mice receiving naive T cells and seeded with $Tramp_{OVA}$ tumours demonstrated a 2.6 fold increase in the percentage of $CD8^+$ T cells (Fig. 3-15B and C).

Analysis of CD69 expression by adoptively transferred $CD8^+$ T cells revealed that the level of CD69 was markedly increased in blood and lymphoid tissues from mice that received naive T cells relative to groups of mice receiving effector T cells that expressed negligible levels on day 5 (Fig. 3-15D, E and F). Interestingly, groups receiving naive or effector T cells and bearing $Tramp_{OVA}$ tumour cells expressed similar levels of CD69 in blood and spleen but not in LNs by day 10. Intriguingly, CD69 was notably upregulated in mice seeded with Tramp-C1 tumours and receiving effector cells only in blood (Fig. 3-15D).

Collectively, these results demonstrated that a T cell dose of $10x10^6$ OT-I lymphocytes can affect progression of $Tramp_{OVA}$ tumours and would therefore interfere in analyzing the effect of NR/CB1954 therapy on tumour growth. Also,

this shows the capacity of $Tramp_{OVA}$ tumours to inherently activate naive OT-I T cells into effector $CD8^+$ T cells capable of mediating tumour regression *in vivo*.

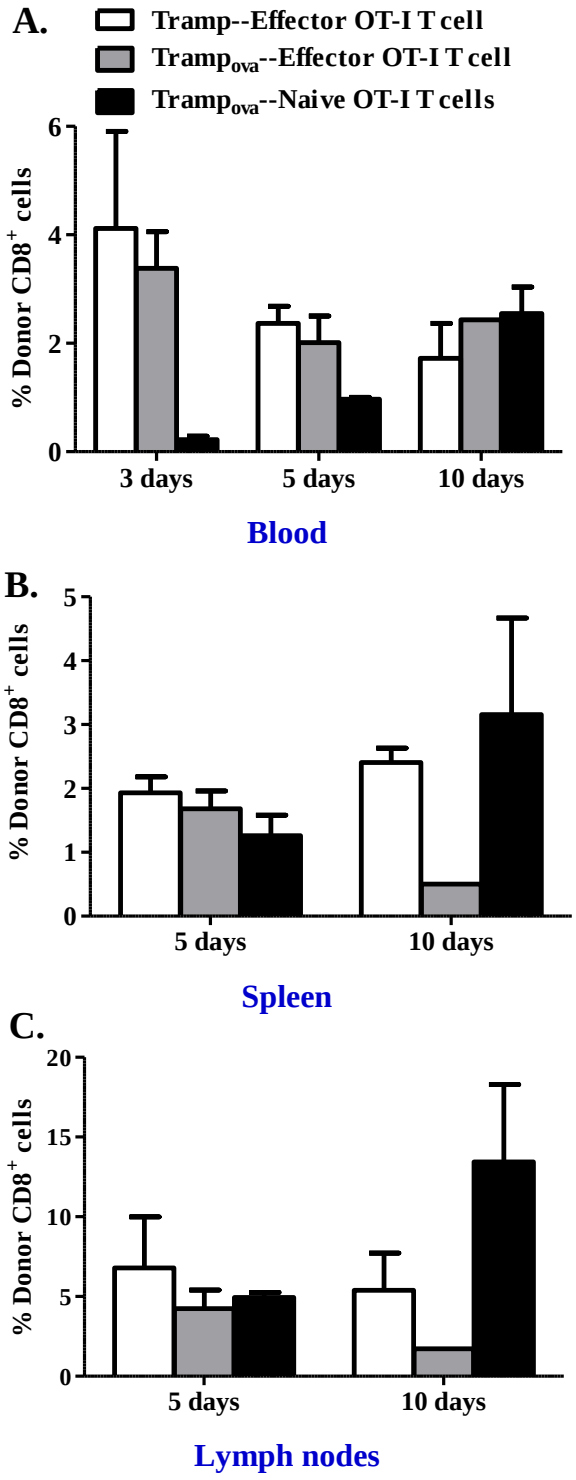


Figure 3-15: Donor CD8$^+$ T cell proliferation and activation in nude C57BL/6 mice bearing Tramp-C1 or Tramp$_{OVA}$ tumours

Single cell suspension from blood, LNs and spleen were stained with anti-CD45.1, -CD3, -CD8 and -CD69 before analysis by flow cytometry. **A**, **B** and **C**, percentage of donor CD8$^+$ T cells in blood, spleen and LNs from the indicated groups of mice, respectively. Graphs represent mean values (±SEM) for each group. donor CD8$^+$ T cells were analyzed by flow cytometry using triple staining for CD45.1, CD3 and CD8 antibodies in blood, spleen and lymph nodes at the indicated times.

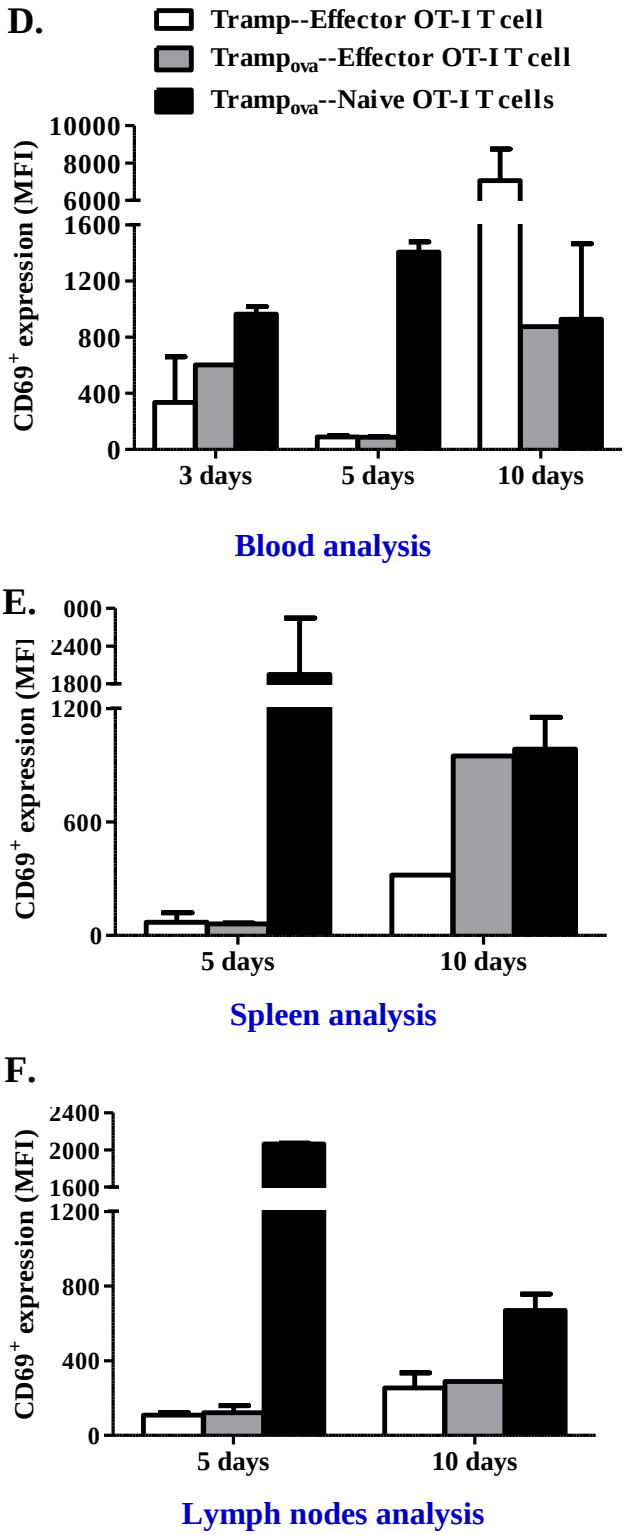


Figure 3-15 …cont. D**,** E and F**,** CD69 expression by OT-I CD8$^+$ T cells in blood, spleen and LNs from the indicated groups of mice, respectively. Graphs represent mean fluorescent intensities (MFI) (±SEM) of each group as calculated using FlowJo software.

3.5.2 Effect of 2.5 million OT-I T cells

A lower dose of 2.5x 10^6 naïve OT-I lymphocytes (≈ 1.6 x10^6 $CD8^+$ T cells) was chosen to examine its effect on $Tramp_{OVA}$ tumour growth in nude C57BL/6 mice. As can be seen in Fig 3-16, there was no significant difference between tumour growth of the control group that received PBS and those receiving OT-I lymphocytes i.v. In addition, no signs of tumour regression were observed and the tumour growth rates of both groups were indistinguishable. This experiment suggested that adoptive transfer of 2.5x 10^6 naïve OT-I lymphocytes has minimal influence on tumour growth and therefore would provide a suitable window in which the effect of immunostimulation arising from NR/CB1954 gene therapy could be investigated.

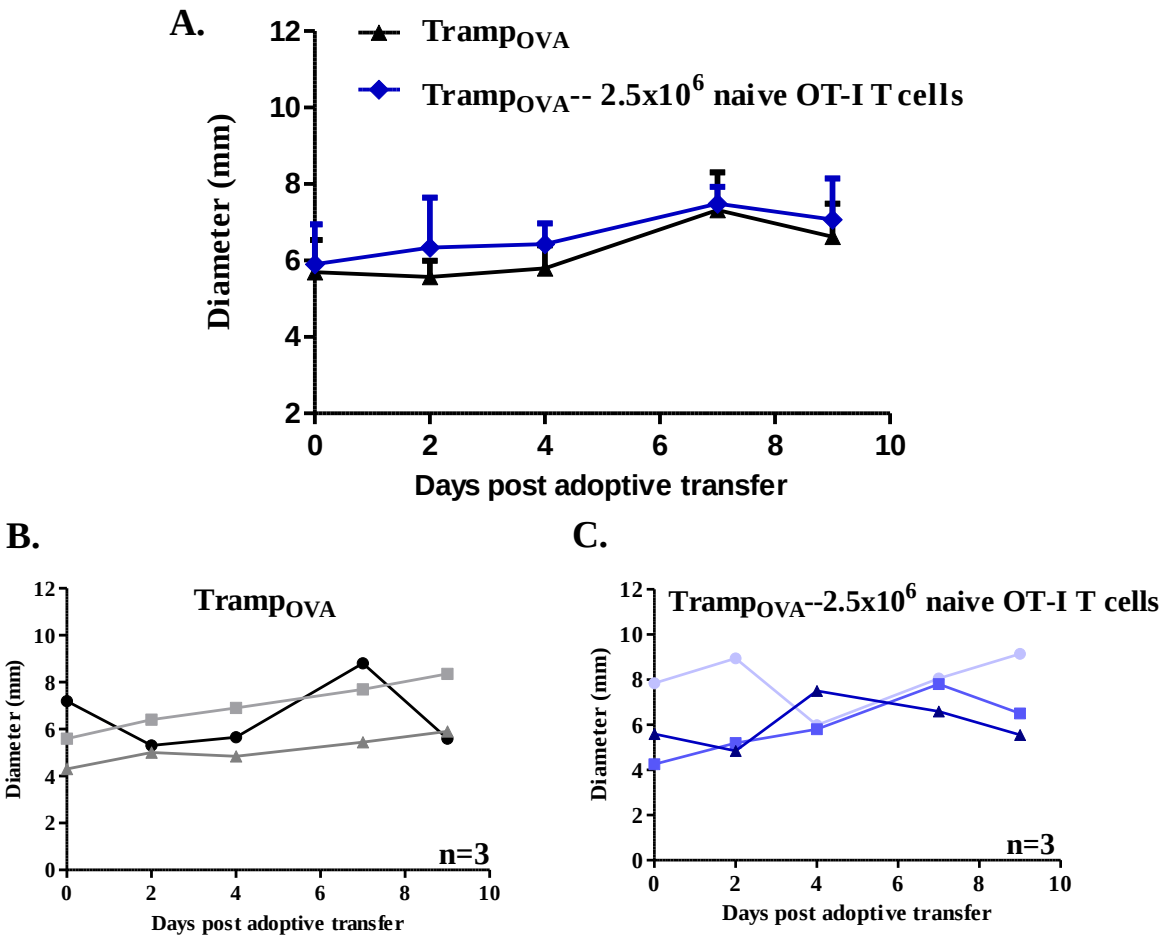


Figure 3-16: Effect of 2.5x10^6 naïve OT-I T cells on subcutaneous tumour growth of Tramp$_{OVA}$ cells in syngeneic nude C57BL/6 mice

Male nude C57BL/6 mice, 16-20 weeks old, were injected s.c. in the right flank with 5x10^6 Tramp$_{OVA}$ cells. At day 0 (defined when tumour diameter reaches 5 mm); mice receive either PBS or 2.5x10^6 naïve OT-I T cells i.v. as indicated in each treatment group. Tumour diameter was measured manually using a calliper three times a week. **A**, data points show the mean (±SEM) of tumour size from individual mice in each group. **B** and **C** show tumour growth of individual mice in the indicated group.

n=the initial number of mice per group

3.6 Discussion

Model antigens have been proven to be useful tools in understanding the immune response to a variety of pathological conditions, and also in the development of immunotherapeutic strategies. These model antigens contain epitopes critical for eliciting T cell responses and for which TCR specificity for these immunodominant epitopes was identified. This facilitated the generation of antigen-specific TCR transgenic T cells and other genetic tools to monitor immune response to infectious agents or tumour cells expressing model antigens. Normally, the nature of the model antigen dictates the location of the expressed protein and subsequently the development of either humoral or cellular immune responses.

A truncated cytoplasmic version of OVA protein served in this study as a model tumour antigen, thereby mimicking the cellular localization of tumour antigens and ensuring similar route of antigen processing and presentation. Gene transfer of cytoplasmic OVA to Tramp-C1 tumour cells resulted in generation of polyclonal $Tramp_{OVA}$ cells. The level of OVA expression in 50 μg cell lysate was below that of 10 ng crude OVA protein as shown by western blot analysis (Fig 3-5). Therefore, we opted for an indirect quantification of protein expression via assessing OVA-epitope/MHC I complex presentation using the B3Z hybridoma T cells that can detect OVA-epitope on a single cell level. Polyclonal and single cell $Tramp_{OVA}$ cells stimulated varying levels of inducible β-galactosidase in B3Z hybridoma T cell following activation, demonstrating OVA-epitope presentation and also confirming that OVA is expressed by $Tramp_{OVA}$ clones (Fig 3-6). This allowed further screening of different clones and selection of $Tramp_{OVA}$ single cell clone 3 expressing low levels of OVA. Thereafter, $Tramp_{OVA}$-NR cells were generated through transduction of $Tramp_{OVA}$ cells with NR retroviral particles. As seen for

OVA expression, $Tramp_{OVA}$-NR cells derived from single cell clones expressed different levels of NR protein (Fig 3-9 and 10). The difference in protein expression between individual transformants could be due to several factors, mainly: the site of integration of transgenes relative to cellular transcription signals, the number of transgene copies integrated within the genomic DNA, and other epigenetic related effects (Pannell and Ellis, 2001; Yao et al., 2004). $Tramp_{OVA}$-NR clone 11 was selected for further studies use due to its high level of NR expression and sensitivity to the prodrug CB1954; however reduced $Tramp_{OVA}$-NR sensitivity to CB1954 was observed following sub-culturing of these cells (Fig 3-12). Reduced level or complete loss of transgene expression was also reported in a number of tissues transduced with retroviral vectors (Challita and Kohn, 1994; Challita et al., 1995; Skarpidi et al., 1998). It was found that transcription from the retroviral LTR is altered by DNA methylation which affects sustained expression of retrovirally transduced genes. In addition, CpG-hypermethylation in the transgene promoter is associated with altered chromatin structure contributing to loss of transgenes expression or gene silencing (He et al., 2005).

Preclinical animal tumour models, using subcutaneous implants, represent a simple and preliminary approach to acquiring clinically relevant data on the therapeutic efficacy of cytotoxic agents. It can also provide insight into the cellular and molecular mechanism of action of cancer therapies on different types of tumours. Antitumour immune responses to cancer therapies, in particular, are more favourably studied in syngeneic mouse system to minimize non-therapeutic immune responses and rejection of tumour growth. In the present study, genetically modified syngeneic $Tramp_{OVA}$ and $Tramp_{OVA}$-NR cells were tumourigenic in athymic C57BL/6 mice at a cell dose of $5x10^6$ cell/mouse; however they exhibited slower

tumour growth rate compared to parental Tramp-C1 (Fig 3-13). A similar observation was reported by McNeish et al. (1998) showing that NR expressing ovarian SKOV3 tumours derived from single cell clones, showed slower growth kinetics than parental cells in nude Balb-c mice. The apparent difference in tumour growth suggested that gene transfer of OVA, NR or the antibiotic resistant transgenes into the tumour cells have affected the tumour initiating potential of $Tramp_{OVA}$ and $Tramp_{OVA}$-NR cells. Antigens encoded by these transgenes might have been released from dying cells probably present in the initial tumour cell inoculum or due to spontaneous tumour cell death thereby stimulating an immune response. Given that athymic mice have significant B cell function and normal phagocytic and NK activity, potentially humoral and NK-mediated immune response against these transgenes products could possibly interfere in the progression of $Tramp_{OVA}$ and $Tramp_{OVA}$-NR cells (Hanna, 1980; Orengo et al., 2003; Zhou et al., 2004).

The use of immunodeficient mice in conjunction with adoptive transfer of single-avidity TCR transgenic T cells facilitates easy, reliable and reproducible monitoring of specific-immune responses to therapeutic agents. However, the T cell dose in different models varies according to the experimental questions and the model design. In the present study, dose titration of OT-I lymphocytes against OVA expressing tumours demonstrated that tumour growth was markedly affected by adoptive transfer of high numbers of activated or naïve OT-I lymphocytes ($10x10^6$) leading to complete regression in some of the mice (3-14). Furthermore, up-regulation of CD69 marker by $CD8^+$ T cells in groups of mice bearing $Tramp_{OVA}$ tumours and receiving naïve OT-I T cells indicates priming and activation of OT-I $CD8^+$ T cells by the OVA-expressing tumour cells suggesting that proliferation was

mainly antigen-driven and not due to homeostatic reconstitution in lymphopenic hosts (Fig 3-15). By reducing the T cell dose to $2.5x10^6$ OT-I lymphocytes, the fine balance between tumour growth and the presence of OT-I T cells could be achieved, and thus providing a window for studying OVA-specific T cell responses (3-16). These results describe establishment of an adoptive transfer tumour model system designed for examination of $CD8^+$ T cell responses to NR/CB1954 gene therapy.

4 Results: $CD8^+$ T cell responses stimulated by NR/CB1954-mediated tumour cell killing *in vivo*

4.1 Introduction

The antitumour effect of NR/CB1954 targeted gene therapy is well established *in vitro* and *in vivo*. Its capacity to kill not only transduced cells but also neighbouring untransduced cells is known as the local bystander effect. Additionally, an immune related bystander effect was shown to be involved in tumour rejection and generation of antitumour immunity in response to other enzyme/prodrug systems: HSV-*tk*/GCV and CD/5-FC. However, the ability of NR/CB1954-mediated tumour cell killing to generate cellular immune response that could protect the animals from rechallenge needs further investigation.

The aim of this chapter is to study the efficiency of NR/CB1954 system in controlling tumour growth using the tumour model system (described in chapter 3), and to monitor whether tumour cell killing results in activation of OVA-specific T cells and generation of effector CTL. This chapter will also describe experiments that investigate variant model systems with the aim of improving the characterization $CD8^+$ T cell responses in immunodeficient and immunocompetent hosts.

4.2 Donor $CD8^+$ T cell responses to NR/CB1954-mediated cytotoxicity in immunodeficient mice

4.2.1 Sensitivity of established $Tramp_{OVA}$-NR tumours in nude C57BL/6 mice receiving sub-therapeutic OT-I cell dose

To investigate the sensitivity of $Tramp_{OVA}$-NR tumours to CB1954, $Tramp_{OVA}$-NR cells were seeded s.c in nude C57BL/6 mice. When mean tumour volume reached 30 mm^3 (day 0), all mice received 2.5 million naïve OT-I T cells, followed 1 day later by daily doses of 20 mg/kg CB1954 or vehicle for 3 consecutive days. Tumour

volume was recorded over time and as shown in Figure (4-1A and B), CB1954 treatment resulted in a significant reduction in mean tumour volume ($p=0.0039$) by day 14 compared to the vehicle treated group. CB1954 treated tumours continued to regress and all four animals were tumour-free by day 23. In addition, median survival was prolonged from 28 days in the vehicle group to more than 40 days with CB1954 group (Fig 4-1D). All the mice in the CB1954-treated group remained tumour-free for 40 days, after which they were killed in order to examine the differentiation of OT-I T cells into effector memory T cells (see section 4.1.3).

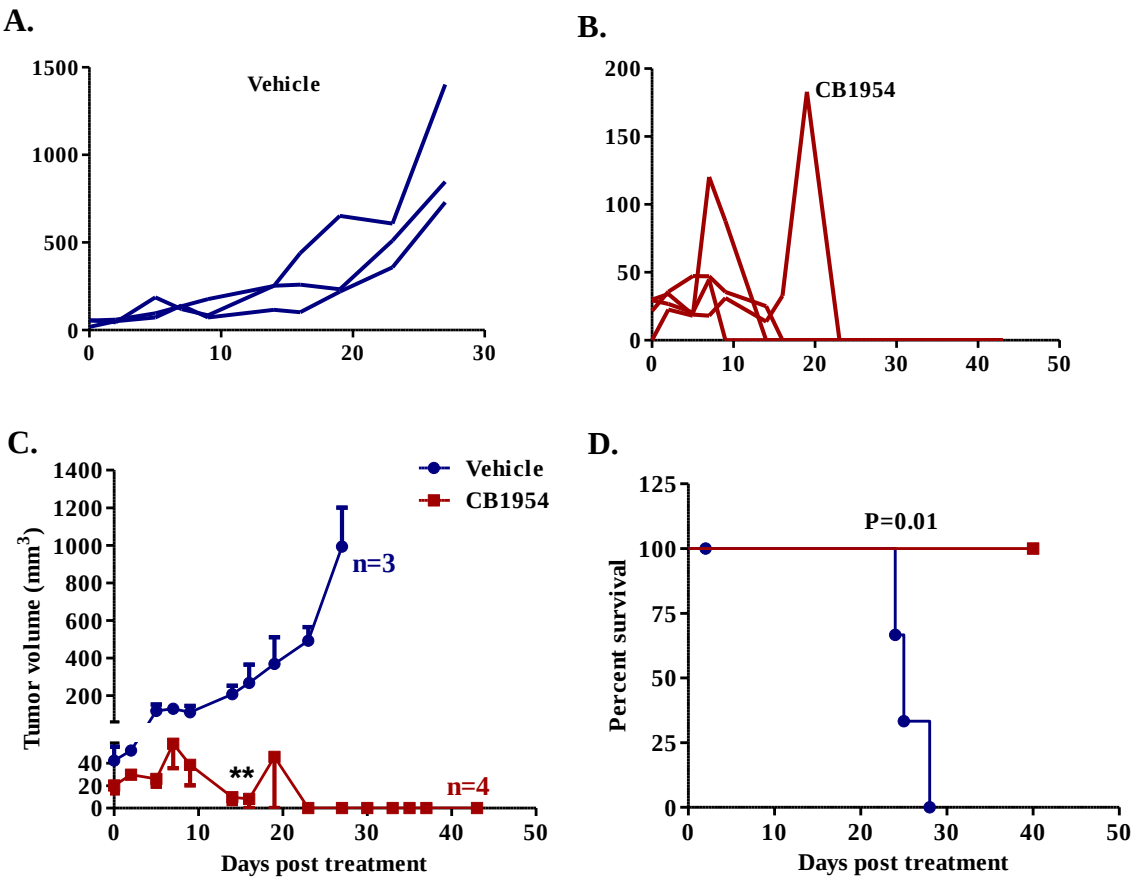


Figure 4-1: Tramp$_{OVA}$-NR tumour growth in nude C57BL/6 mice treated with CB1954

Male C57BL/6 mice bearing established Tramp$_{OVA}$-NR tumours of 30 mm^3 volume received 2.5x10^6 OT-I T cells followed one day later by treatment with 20 mg/kg CB1954 or vehicle once a day for 3 consecutive days. Tumour volume was measured manually with a caliper three times a week. **A** and **B**, tumour growth of Tramp$_{OVA}$-NR of individual mice in vehicle and CB1954 group, respectively; **C**, Mean (±SEM) volume of Tramp$_{OVA}$-NR tumours in CB1954 and vehicle treated groups; **D**, Kaplan-Meier survival analysis of time between treatment and reaching 1000 mm^3.

4.2.2 Testing for generation of CD8$^+$ T cell immunity following NR/CB1954 gene therapy

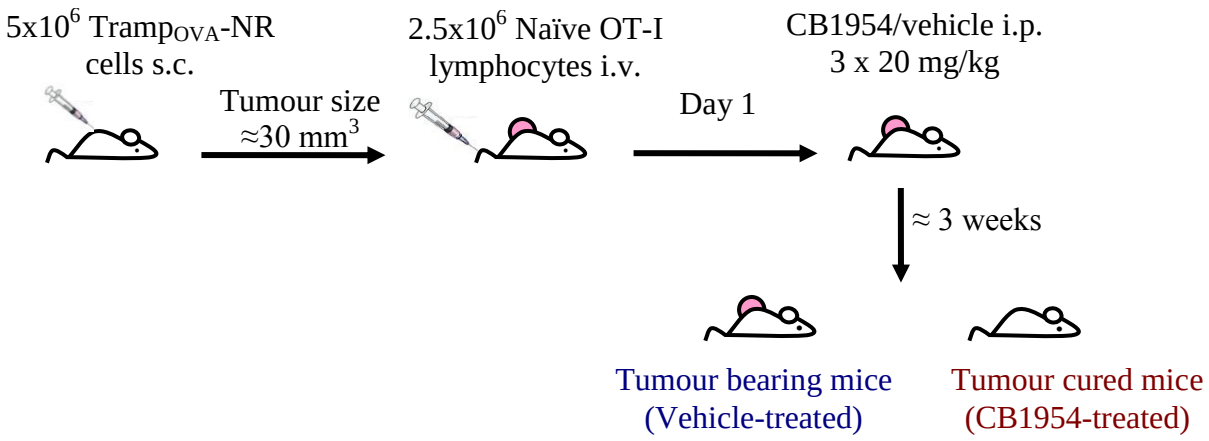
The capacity of CB1954 treatment to induce complete tumour regression of Tramp$_{OVA}$-NR tumours in nude C57BL/6 mice receiving a sub-therapeutic OT-I cell dose was confirmed. The next question was to examine whether NR/CB1954-mediated tumour cell death can stimulate antitumour CTL response. For this experiment, mice from the same treatment groups described in the previous section

(4.1.2) showing complete tumour regression for 3 weeks (Fig 4-2A) were simultaneously challenged with two populations of splenocytes. One population was loaded with $OVA_{257-264}$ (SIINFEKL) peptide and labelled with a high concentration of CFSE ($CFSE^{Hi}$); the other was untreated and labelled with a low concentration of CFSE ($CFSE^{Lo}$). The latter served as a negative internal control to monitor *in vivo* OVA-specific cytotoxicity (Fig 4-2B). After 16 hr, animals were killed and single cell suspensions from LNs and spleen were analysed by flow cytometry, the frequency of $CFSE^{Hi}$ and $CFSE^{Lo}$ target cells was monitored to determine the percentage of OVA-specific lysis. The ratio of $CFSE^{Hi}$ to $CFSE^{Lo}$ target cells representing non-specific lysis in the *in vivo* cytotoxicity assay was determined in naïve control mice that did not receive any prior treatment.

As shown in Fig 4-2C, control mice showed negligible background levels of OVA-specific lysis in both spleen and lymph nodes. However a surprisingly high OVA-specific lysis of ≈94% was observed in lymph nodes from vehicle-treated mice, indicating that the presence of $Tramp_{OVA}$-NR tumours alone promoted differentiation of OT-I T cells to functional effector CTLs. However, these CTLs were clearly unable to prevent tumour growth in these animals. The lysis of SIINFEKL-loaded cells in mice receiving only a sub-therapeutic OT-I cell was not determined in the present experiment due to limitations in the numbers of the mice. Nevertheless, CB1954 treatment of $Tramp_{OVA}$-NR tumours bearing mice induced a slight, but statistically significant increase (4%) in target cell killing. Unlike LNs, the difference in OVA-specific lysis in spleen from vehicle and CB1954 treated groups was insignificant, however high levels (> 94%) of cytotoxicity was observed in both groups supporting the hypothesis of $Tramp_{OVA}$-NR cells-induced T cell activation.

These data imply that NR/CB1954 treatment may slightly enhance the generation of antitumour CD8+ T cell response. However the magnitude of OVA-specific T cell activation could possibly be affected by treatment-unrelated expansion of OT-I T cells driven by homeostatic proliferation and OVA antigen presentation in our model. Other experimental factors that may have also reduced the difference between treatments includes: a) pooling of tumour-draining and non draining lymph nodes together with the mesenteric lymph node for analysis, and b) the ratio of effector/target cells.

A.



B. *In vivo* cytotoxicity assay

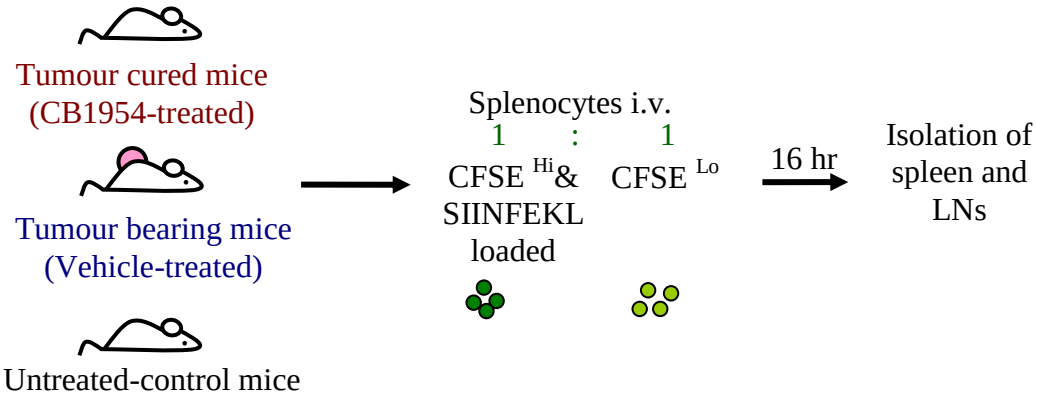


Figure 4-2: OVA-specific CTL response following long-term tumour regression of CB1954 treated Tramp$_{OVA}$-NR tumours in nude C57BL/6 mice
A, Groups of Tramp$_{OVA}$-NR bearing mice that were previously treated with either CB1954 or vehicle mice and showed tumour rejection or progression, respectively, were examined in this experiment. **B**, *In vivo* cytotoxicity assay. Splenocytes from wt C57BL/6 mice were labelled with 1 µM CFSE (CFSE^{Lo}) as an internal control population or peptide pulsed with 2.5 µg/ml SIINFEKL and labelled with 2.5 µM CFSE (CFSE^{Hi}) to serve as the target population. A mixture of 5x10^6 cells from each population (1x10^7 total cells) was transferred i.v into all groups of mice. After, 16 hr, spleen and LNs (axillary, brachial, inguinal and mesenteric) were harvested and single-cell suspensions were prepared for flow cytometric analysis.

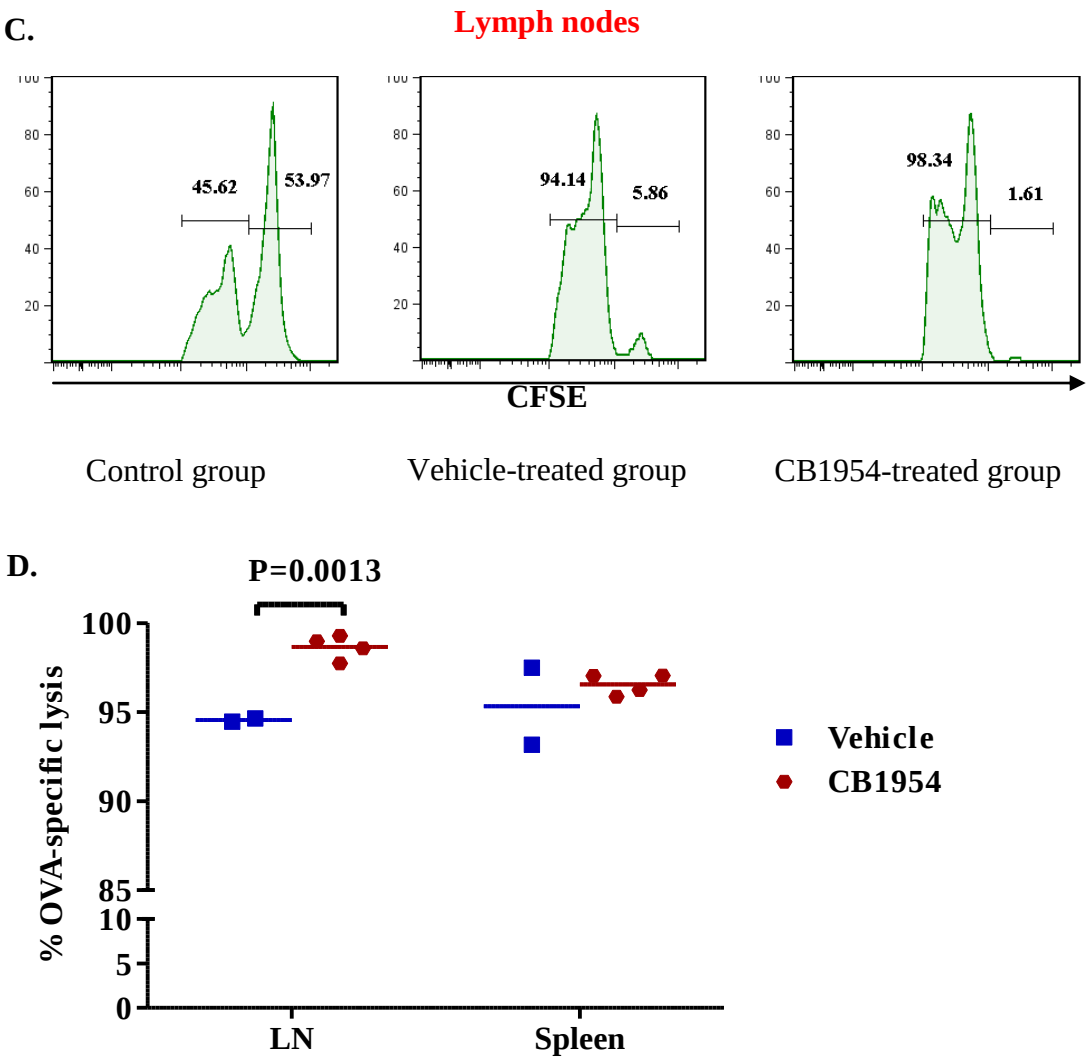


Figure 4-2 …cont. C, histograms from pooled cytometric data showing the frequency of SIINFEKL-pulsed and $CFSE^{Hi}$ labelled splenocytes (right gate) and peptide-unpulsed and $CFSE^{Lo}$ labelled splenocytes (left gate) in LNs from the indicated groups. **D**, *in vivo* OVA-specific CTL response. Target cells labelled with $CFSE^{Hi}$ and $CFSE^{Lo}$ were analyzed by flow cytometry and the percentage of target cell killing was determined. Data points represent the percentage of lysed target cells in different animals and the horizontal line represents the mean of lysed target cells.
The P value indicates the statistical significance as analyzed by Student's t-test

4.2.3 Examining the capacity of lymphocytes from CB1954/vehicle treated $Tramp_{OVA}$-NR tumour bearing mice to provide protective antitumour immunity in secondary nude C57BL/6 hosts

To examine the potential significance of the generated tumour-specific CTLs in response to CB1954 treatment, the capacity of these effector cells to provide protection against parental tumour cells was further investigated. This was addressed by establishment of 30 mm^3 $Tramp_{OVA}$-NR tumours in nude C57BL/6 mice followed by adoptive transfer of $2.5x10^6$ Naïve OT-I lymphocytes i.v and vehicle/CB1954 treatments as previously described in Figure 4-2A. After 3 weeks, mice were culled and lymphocytes from individual mice in each group were pooled and examined by flow cytometry. The percentage of OVA-specific T cells was estimated as 1.4% and 1.025% in vehicle and CB1954 treated mice, respectively (this was an approximate estimate of the percentage of $CD8^+$ T cells due to a technical problem on the day of the analysis).

Subsequently, lymphocytes pool containing $\approx$ 1x 10^5 OVA-specific T cells from vehicle or CB1954 treated tumour bearing mice (referred to as vehicle or CB1954 primed T cell group, respectively from here on) were adoptively transferred to new groups of mice prior to inoculation with a tumourigenic dose of $Tramp_{OVA}$ cells (Figure 4-3A).

Figure 4-3B and C shows that palpable tumours were evident by day 35 post-adoptive transfer in recipients of CB1954-primed T cells. The appearance of initial tumour growth was observed somewhat later in recipients of vehicle-primed T cells (day 44). Although tumours of vehicle-primed T cell recipients grew more rapidly and were nearly double the volume of those in CB1954-primed T cell recipients by

day 52, due to the small group size this did not reach statistical significance (Fig 4-3D).

Comparing the mean tumour volumes of the two treatment groups (Fig4-3E), it appears that the growth rate of tumours in recipients of CB1954-primed T cells is slower than those of vehicle-primed T cells recipients, possibly consistent with greater immune protection; although the shorter lag period to tumour development in this group could argue against this.

(Note that mouse 3 and 4 in vehicle-primed T cell recipients were culled earlier in the course of the experiment due to gastrointestinal infection)

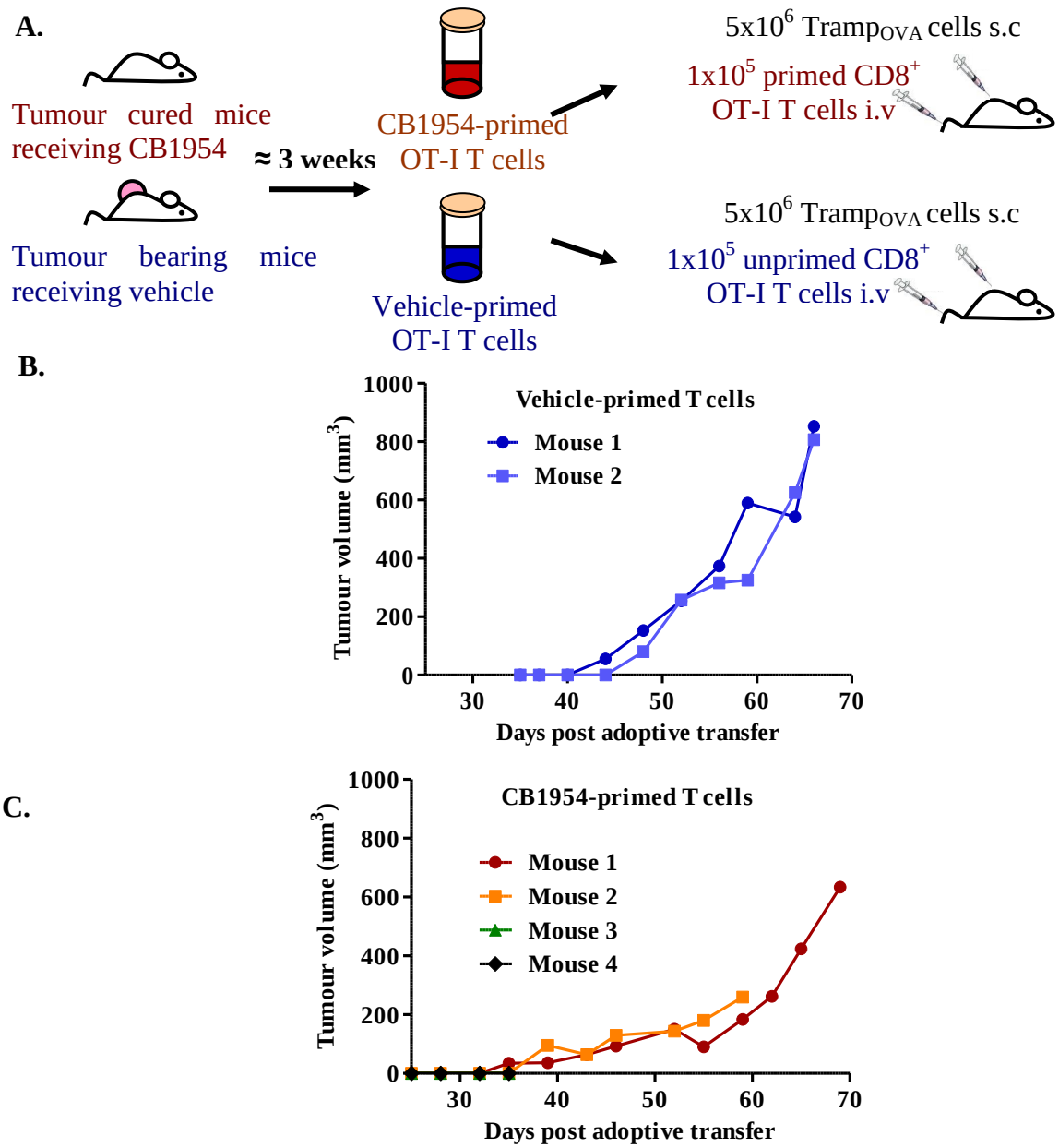
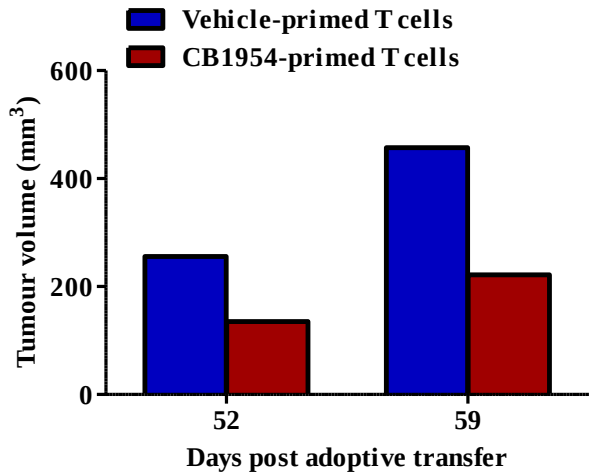


Figure 4-3: Prophylactic efficacy of lymphocytes from CB1954/vehicle treated Tramp$_{OVA}$-NR tumour bearing mice against Tramp$_{OVA}$ tumour growth in secondary nude C57BL/6 mice

Single cell suspensions from the lymph nodes and spleen of tumour-free mice (culled ≈ 3 weeks after complete tumour regression) and tumour bearing mice (culled 30 days post adoptive transfer) were prepared and surface stained with anti-vα-2 and -CD8 for flow cytometric analysis. A dose of 1x10^5 OVA-specific T cells from the lymphocytes pools of the previous groups designated vehicle and CB1954-primed T cells were transferred to secondary nude groups of mice followed by s.c seeding of 5x10^6 Tramp$_{OVA}$ cells on the same day. Tumour volume was measured manually using a calliper three times a week. **A**, schematic representation of the experimental design. **B** and **C**, growth curves of Tramp$_{OVA}$ tumours in individual mice receiving vehicle or CB1954 primed T cell, respectively.

Mouse 3 and 4 in vehicle-primed T cell recipients were culled earlier in the course of the experiment due to gastrointestinal infection.

D.



E.

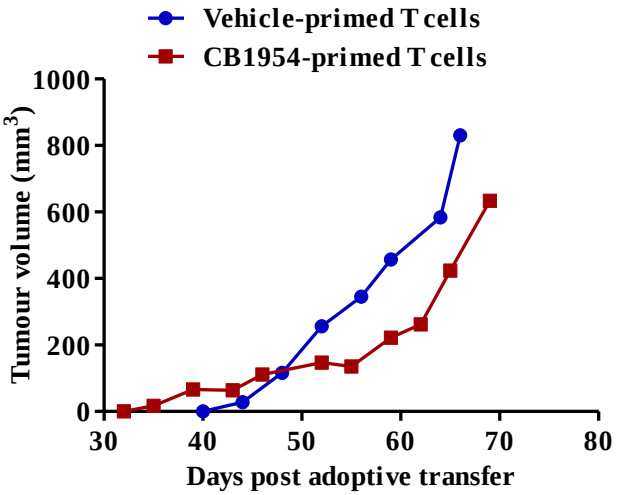


Figure4-3 …cont. D, mean tumour volume of vehicle or CB1954 primed T cell recipients at 52 and 59 days; **E**; An overlay of the growth curves of the mean volume of $Tramp_{OVA}$ tumours post adoptive transfer of vehicle or CB1954-primed T cell.

4.2.4 Characterization of tumour cells derived from $Tramp_{OVA}$ tumour bearing mice after secondary adoptive transfer of vehicle or CB1954 primed T cell

Previous studies using OVA as a model tumour antigen and OT-I T cells in a therapeutic setting found that tumour out growth was due to selection of tumour variants that produce insufficient amount of the tumour antigen. (Bathe et al., 2003; Dalyot-Herman et al., 2000) It was therefore of interest to examine whether tumour

cells retrieved from recipients of vehicle- or CB1954-primed T cells retained their ability to express OVA antigen. In addition, simian virus 40 large tumour antigen (SV40 Tag) gene was also detected to verify that tumours excised from mice originated from $Tramp_{OVA}$ tumour cells and not from any other epithelial origin. SV40 gene was chosen since the Tramp-C1 cell line was derived from mouse prostate cancer developed in SV40 tag-induced transgenic mouse models (Foster et al., 1997). To accomplish this, tumours from groups of mice described in Figure 4-3E were excised and dissociated to single cell suspensions using an enzyme cocktail of collagenase, DNase and trypsin, followed by culturing in complete media for a few weeks.

4.2.4.1 Detection of SV40 Tag and OVA genes in $Tramp_{OVA}$ tumour

Genomic DNA from parental Tramp-C1, $Tramp_{OVA}$ cells or tumour cells derived from the mice was isolated and analysed by PCR using specific primers to amplify fragments from SV40 Tag and OVA genes. For SV40 Tag, the following primers were used SV40- F1 forward: 5'-TCA ACC TGA CTT TGG AGG C-3' and SV40-R1 reverse: 5'- TTC CTC TGC TTC TTC TGG-3' (reverse). For OVA gene the two primers were OVA-F3 forward: 5'-GGA TGA AGA CAC ACA AGC AAT-3' and OVA-R3 reverse: 5'-TCT CTG CCT GCT TCA TTG ATT T-3'. The resulting PCR products were compared by agarose gel electrophoresis. Figure 4-4A shows a clear single band consistent with the expected size (546 bp) of SV40 Tag amplified fragment, which was equally positive in parental Tramp-C1, $Tramp_{OVA}$ cells and tumour derived cells. As expected a fragment of ≈450 bp from the OVA gene was amplified only from parental $Tramp_{OVA}$ and tumour rederived cells (Figure 4-4B). This confirmed that the cells that grew out of the explanted tumour cells were indeed derived from the original $Tramp_{OVA}$ cells.

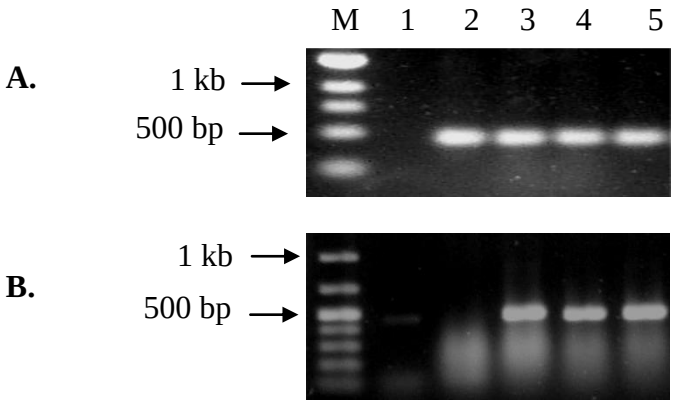


Figure 4-4: Gel electrophoresis of PCR product of OVA and SV40 genes in genomic DNA extracted from tumour cells.
A and **B**, PCR products of SV40 and OVA transgenes, respectively. The amplified PCR products were separated by 0.7% agarose gel and photographed under UV after staining with ethidium bromide. The size of the PCR products was checked against a 1kb DNA ladder (M). PCR reactions in Lane 1-5 used the following as templates: lane1, water; lane2, Tramp-C1; lane 3, $Tramp_{OVA}$ cells; lane 4: tumour cells from vehicle-primed T cells recipients; lane 5: tumour cells from CB1954-primed T cells recipients.

4.2.4.2 Activation of ovalbumin-specific B3Z T cell hybridoma by tumour rederived cells

To determine whether these tumour-derived cells retained the ability to present $OVA_{257-264}$ peptide (SIINFEKL) to T cells, their ability to activate B3Z T cells was tested. Tumour cells were pretreated with IFN-γ (to upregulate MHC class I) for 16 hr or left untreated; followed by an overnight incubation with B3Z T cell hybridoma. As shown in Figure 4-5, parental Tramp-C1 stimulated negligible B3Z cells activation whereas control in vitro-cultured $Tramp_{OVA}$ cells clearly activated B3Z hybridoma cells and the level of activation was increased by IFN-γ pretreatment of tumour cells. In contrast, $Tramp_{OVA}$ cells recovered from tumours failed to activate the B3Z cells with or without IFN-γ pretreatment. Since PCR analysis showed that OVA gene was still present, these tumour cells might have been selected variants of $Tramp_{OVA}$ cells that had down-regulated OVA expression

perhaps due to epigenetic gene silencing. Another possibility could be that these tumour cells do express OVA but have down-regulated expression of one of the component of the antigen processing machinery such as TAP proteins or MHC class I molecules.

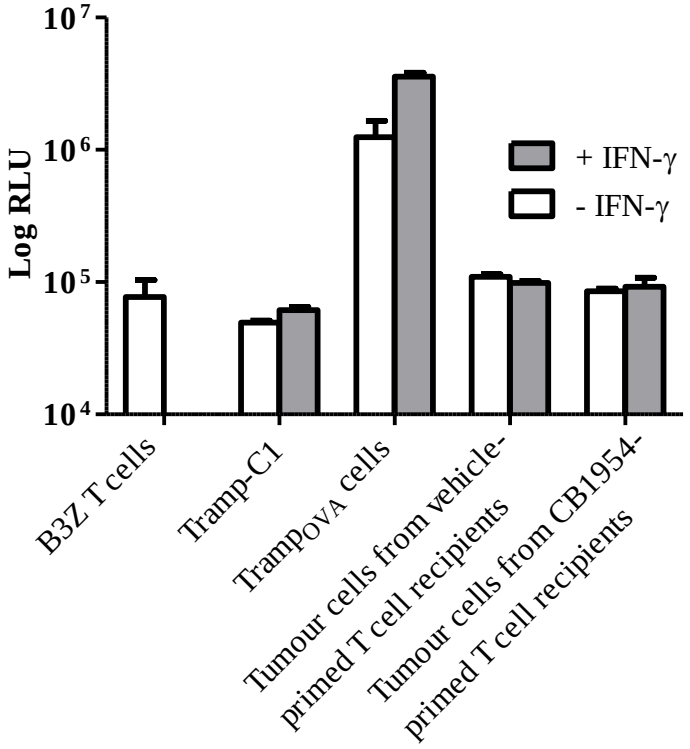


Figure 4-5: OVA antigen presentation by $Tramp_{OVA}$ tumour cells derived from vehicle- or CB1954-primed T cell recipients
Parental Tramp-C1, $Tramp_{OVA}$ and $Tramp_{OVA}$ tumour cells derived from vehicle or CB1954-primed T cell recipients were innoculated with or without 100U/ml interferon. After 16 hr, $1x10^4$ tumour cells were cocultured with $1x10^5$ B3Z T cell hybridoma in wells of a 96 well plate overnight. β-galactosidase activity in B3Z T cells was assayed in total culture lysates after incubation with β-galactosidase luminescent substrate for 1 hr at 37ºC. Data are presented as relative luminescence unit (RLU) from quadruplicate wells (±SEM) on a log scale.

4.2.5 Donor $CD8^+$ T cell responses to NR/CB1954-mediated cytotoxicity in a modified model

In an effort to reduce the experimental time scale and to minimize loss of animals due to intrinsic health issues prior to analysis, the experimental design was modified

by starting the adoptive transfer of OT-I T cells one day after seeding of tumour cells, one day prior to CB1954/vehicle treatment, rather than waiting for tumours to grow in mice and then OVA-specific cytotoxicity was examined in vivo, 4 weeks after the last injection of vehicle/CB1954 (Fig 4-6A). In addition some experimental factors that were thought to influence $CD8^+$ T cell response was adjusted; mainly by analysis of draining lymph nodes (DLN) separately from non-draining lymph nodes (N-DLN) and increasing the number of target cells in *in vivo* cytotoxicity assay to $6x10^6$ splenocytes.

As shown in Figure 4-6B, vehicle treated group showed high level of OVA-specific lysis ranging from 67 to 81% in lymphoid tissues and blood similar to that seen in previous experiment (Fig 4-2B), whereas administration of CB1954 resulted in negligible increase in cytotoxicity (range, 71- 85%) in different lymphoid tissues and blood compared to vehicle treated group.

In the same experiments the frequencies of OVA-specific $CD8^+$ T cells were analysed by flow cytometry using CD45.1, CD3, and CD8 antibodies to differentiate between adoptively transferred donor OVA-specific $CD8^+$ T cells ($CD45.1^+$) and recipient cells ($CD45.1^-$). Interestingly, CB1954 treatment increased the mean frequencies of $CD8^+$ T cells by 1.5 fold in DLN, 1.4 fold in N-DLN and 1.7 fold in blood while there was negligible difference between CB1954 and vehicle treated groups in the spleen (Fig 4-6C).

These results and that observed in figure 4-2D indicate that the high CTL response is probably due to $CD8^+$ T cell activation due to the presence of $Tramp_{OVA}$-NR cells rather than the treatment with vehicle or CB1954, however CB1954 treatment may improve tumour-specific $CD8^+$ T cells expansion as suggested by the increased frequencies

of memory $CD8^+$ T cells. Nevertheless, minimal if any increase in OVA-specific lytic activity could be achieved by CB1954 treatment.

A.

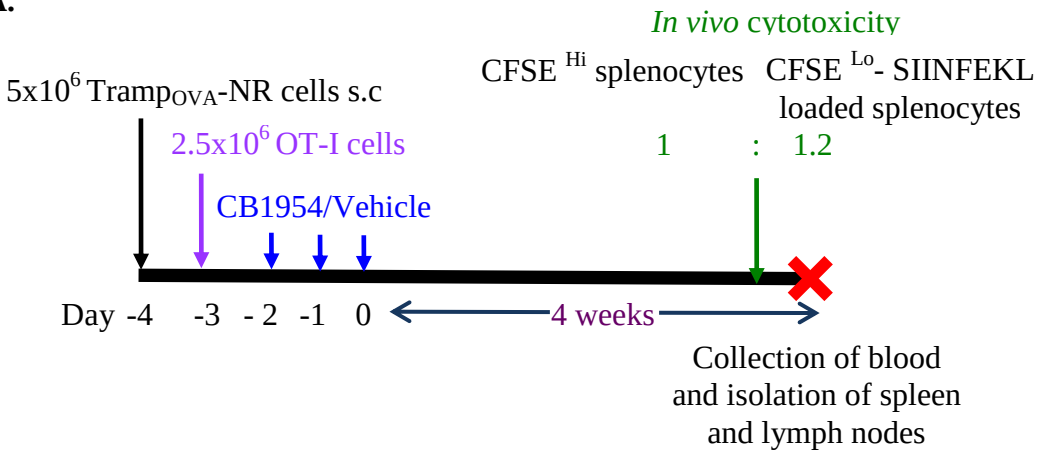


Figure 4-6: Donor $CD8^+$ T cell responses to NR/CB1954-mediated cytotoxicity in nude C57BL/6 mice

A, Modified experimental design. Male nude C57BL/6 mice, 16-20 weeks old, were injected s.c in the right flank with $5x10^6$ $Tramp_{OVA}$-NR cells. One day later, mice received $2.5x10^6$ OT-I lymphocytes i.v followed by 3 consecutive doses of 20 mg/kg CB1954 or vehicle for 3 days. After 4 weeks, a mixture of $5x10^6$ control splenocytes labelled with 2.5 µM CFSE ($CFSE^{Hi}$) and $6x10^6$ target splenocytes labelled with 1 µM CFSE ($CFSE^{Lo}$) and pulsed with 5 µg/ml SIINFEKL (total $1x10^7$ cells) was administered i.v into all groups of mice. After 16 hr, spleen, draining and non-draining axillary, brachial and inguinal LN from vehicle or CB1954 group were harvested and single-cell suspensions were prepared for flow cytometric analysis.

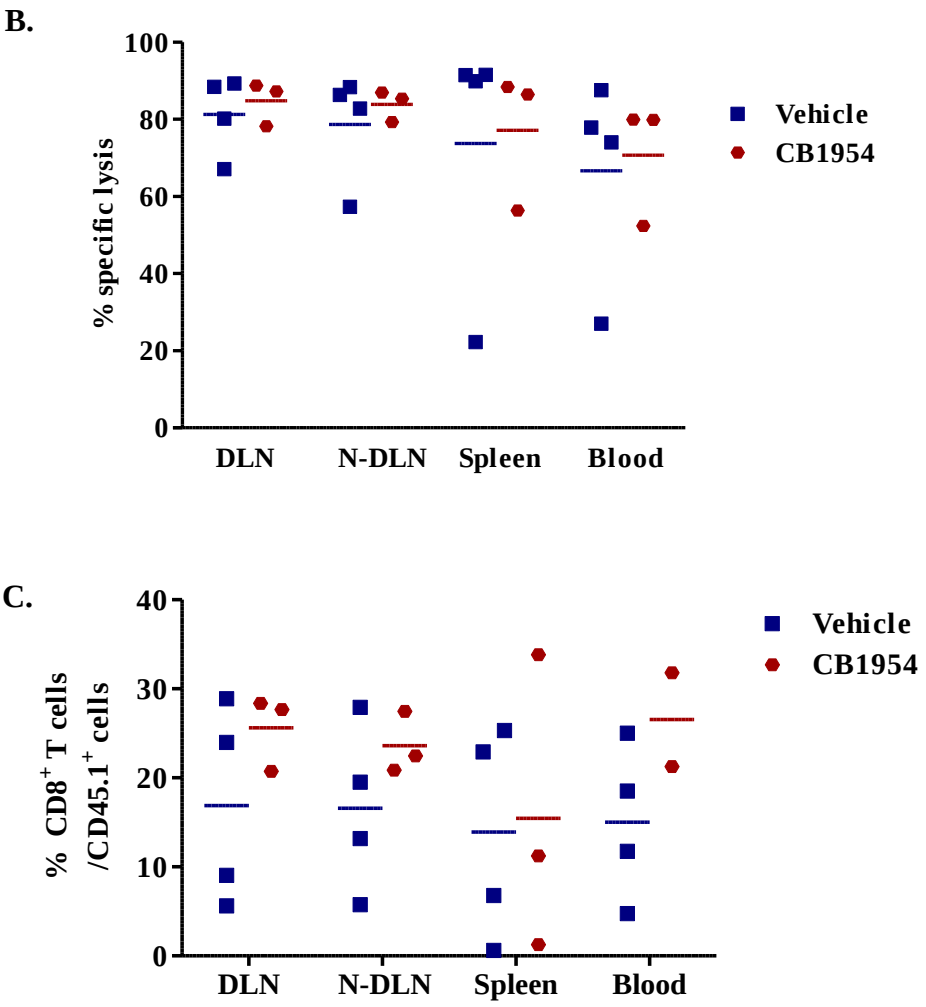


Figure 4-6 …cont. B, *in vivo* OVA-specific CTL response. Target cells labelled with $CFSE^{Hi}$ and $CFSE^{Lo}$ were analyzed by flow cytometry and the percentage of target cell killing was determined. Data points represent the percentage of lysed target cells in different animals and the horizontal line represents the mean of lysed target cells. **C**, the percentage of OVA-specific T cells relative to total donor $CD45.1^+$ lymphocytes. Single-cell suspensions form DLN, N-DLN, spleen and blood were stained with anti-CD45.1, -CD3 and -CD8 before analysis by flow cytometry. Data points represent the numbers of live $CD45.1^+CD8^+CD3^+$ cells relative to total donor $CD45.1^+$ cells in different animals and the horizontal line represents the mean.

4.3 Donor $CD8^+$ T cell responses to NR/CB1954-mediated cytotoxicity in immunocompetent mice

4.3.1 Introduction

It was clear from the previous results that the CTL activity of adoptively transferred OT-I T cells in nude C57BL/6 mice was quite high irrespective of whether OT-I T cells were adoptively transferred after tumour establishment or just one day following tumour cell implantation, and was shown to be at least largely unrelated to treatment with vehicle or CB1954. Also, increasing the number of target cells did not show detectable improvement in the sensitivity of the *in vivo* CTL assay. This suggested that other experimental factors were responsible for such high response; one possible explanation is the inherent capacity of cytoplasmic OVA-tumour antigen in the present model tumour cells to specifically activate OT-I T cells and upregulate CD69 activation marker following 3 days of adoptive transfer as seen in Fig 3-14. Also, it could be related to the space filling phenomenon "homeostatic expansion" observed following adoptive transfer of T cells in lymphopenic hosts; thus increasing the numbers of OVA-specific T cells and consequently increasing the numbers of effector cells and lowering the detection threshold of the assay to detect slight differences especially when using small group numbers. Together these factors may have contributed to the high cytolytic activity in all treatment groups and therefore it was of interest to monitor antitumour $CD8^+$ T cell responses through the adoptive transfer of OT-I T cells into immunocompetent wt C57BL/6 to limit effects due to homeostatic expansion. This system will additionally provide $CD4^+$ T cell helper responses required for optimal CTL-mediated antitumour immunity (Marzo et al., 2000). Also, the use of wt C57BL/6 would allow evaluation of both short- and long-term $CD8^+$ T cell responses post NR/CB1954 therapy, and to avoid

the intrinsic health issues and availability problems encountered with nude C57BL/6 mice.

4.3.2 Tumourigenicity of $Tramp_{OVA}$ cells in wild-type C57BL/6 mice

To investigate the ability of $Tramp_{OVA}$ cells to establish tumours in immunocompetent mice, the same tumourigenic dose of $Tramp_{OVA}$ cells ($5x10^6$) used with nude mice was chosen for initiation of s.c tumour growth in wt C57BL/6 mice. However, only 1/15 mice showed tumour growth after 76 days with mean tumour volume of 732 mm^3 in a 3 month follow-up period indicating that this dose is immunogenic in wt C57BL/6 mice. In an attempt to attain the tumourigenic threshold of $Tramp_{OVA}$, another dose of 10 $x10^6$ cells were used to inoculate s.c tumours in mice. The lag phase before the appearance of tumour growth was slightly shortened to 45-67 days in 4/5 mice; and then the tumours rapidly progressed into invasive tumours which made external measurements with callipers less reliable as indicator of tumour size, nevertheless one mouse still remained tumour-free at 90 days (Fig 4-7).

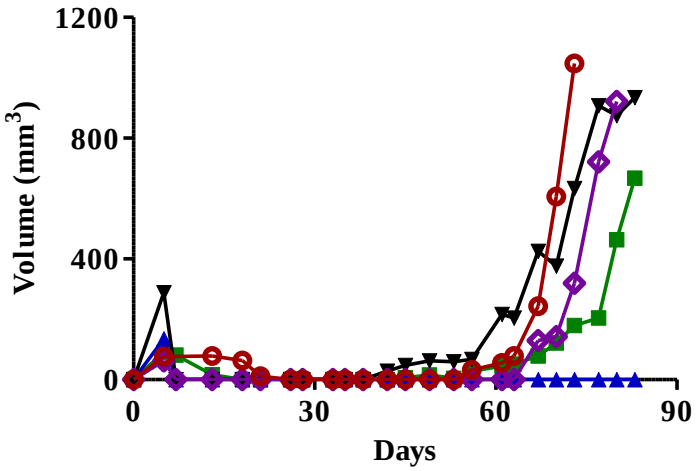


Figure 4-7: Growth characteristics of subcutaneous $Tramp_{OVA}$ tumours in wild-type C57BL/6 mice using $10x10^6$ cell inoculum
Male C57BL/6 mice, 6-8 weeks old, were injected s.c in the right flank with $10x10^6$ $Tramp_{OVA}$ cells. Tumour volume was measured manually using a calliper three times a week. Each line represents tumour growth in an individual mouse. n=5

4.3.3 Tumourigenicity of $Tramp_{OVA}$ tumours using matrigel in wild-type C57BL/6 mice

In an attempt to reduce the time required by injected $Tramp_{OVA}$ tumour cells to develop into visible tumours, a solubilized basement membrane preparation rich in extracellular matrix proteins (Matrigel) was used to promote tumour growth *in vivo*. As shown in Figure 4-8, a cell suspension of $5x10^6$ $Tramp_{OVA}$ cells admixed with Matrigel resulted in initial palpable mean tumour volume of 130 mm^3 in 3/3 mice for nearly 2 weeks; however tumour volumes were unstable and showed phases of growth and shrinkage, before finally all tumours showed complete regression by 82 days.

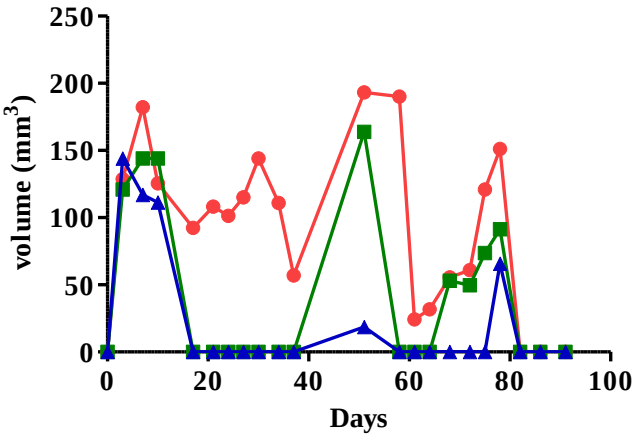


Figure 4-8: Growth characteristics of subcutaneous $Tramp_{OVA}$ tumours in wild-type C57BL/6 mice in the presence of matrigel
Male C57BL/6 mice, 6-8 weeks old, were injected s.c in the right flank with a mixture of $5x10^6$ $Tramp_{OVA}$ cells/150 µl serum free medium and ice-cold matrigel matrix at 1:1 volume ratio. Tumour volume was measured manually using a calliper three times a week. Each line represents tumour growth of an individual mouse. n=3

4.3.4 Tumourogenicity of Tramp$_{OVA}$ and Tramp$_{OVA}$-NR tumours in irradiated wild-type mice C57BL/6 mice

The alternation of tumour growth and regression seen with Tramp$_{OVA}$-matrigel implants suggested that an immune mediated response might be involved in tumour rejection in wt C57BL/6 mice. To examine this hypothesis, C57BL/6 mice were irradiated with different doses of gamma-irradiation followed by seeding Tramp$_{OVA}$ tumour cells s.c. At a dose of 1.5 Gy total body irradiation, the mice remained tumour-free for 45 days (Fig 4-11A), while with 2.5 Gy an initial tumour growth was evident in 2/4 mice which decreased in size after 2 weeks and remained stationary with tumour size below 25 mm^3 tumour in 2/4 for 3 weeks, however 1/4 mice remained tumour-free for 45 days (Fig 4-11B). A similar tumour growth pattern was initially observed with 5 Gy; however the stationary tumour phase was shortened to 10 days followed by evident increase in tumour growth in 4/4 mice (Fig 4-11C). These results implied that lymphodepletion using irradiation facilitated tumour establishment and that 5 Gy would be the minimal dose to permit appreciable tumour growth within 3-4 weeks time.

It was therefore reasonable to examine the tumourigenic capacity of Tramp$_{OVA}$-NR tumour cells in 5 Gy total body irradiated wt C57BL/6 mice. As shown in Figure 4-9, Tramp$_{OVA}$-NR tumour cells achieved a transient tumour growth in 3/4 mice, however these gradually regressed by 35 days and 3/4 mice remained tumour-free at 75 days. Only one mouse showed progressive tumour growth after a 24 day tumour-free period, thus demonstrating the inability of Tramp$_{OVA}$-NR to routinely establish stable tumours in 5 Gy irradiated animals.

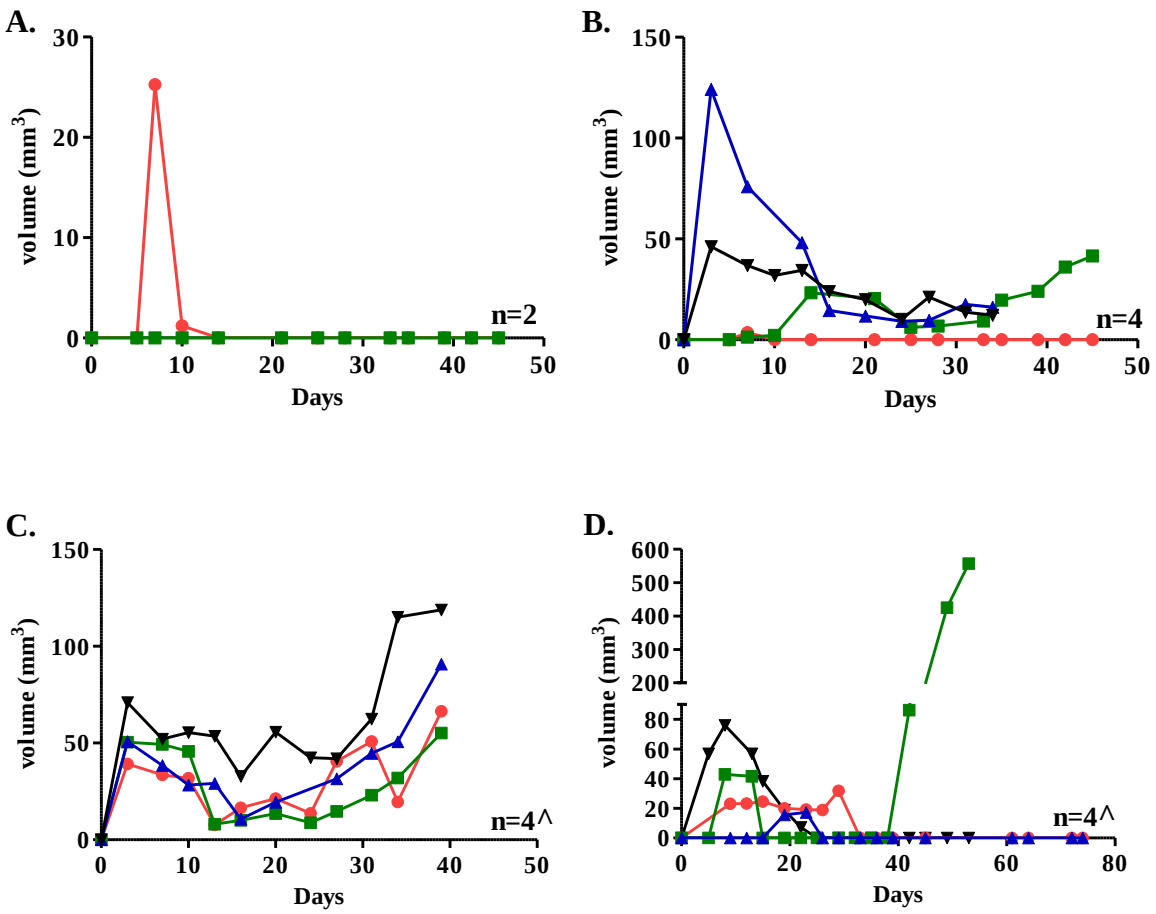


Figure 4-9: Growth characteristics of subcutaneous $Tramp_{OVA}$ and $Tramp_{OVA}$-NR tumours in irradiated wild-type C57BL/6 mice
Male C57BL/6 mice, 6-8 weeks old, were irradiated using a ^{60}Cobalt γ-radiation source followed by s.c injection of $5x10^6$ $Tramp_{OVA}$ cells in the right flank on the same day. Tumour volume was measured manually using a calliper three times a week. **A**, **B** and **C** $Tramp_{OVA}$ tumour in C57BL/6 mice irradiated at 1.5, 2.5 and 5 Gy, respectively. D, $Tramp_{OVA}$-NR tumour growth in C57BL/6 mice irradiated at 5 Gy. Each line represents tumour growth of an individual mouse.
^ represents two separate experiments.

4.3.5 Donor $CD8^+$ T cell responses to NR/CB1954-mediated cytotoxicity in a short-term model

Having tried several methods to establish $Tramp_{OVA}$-NR tumours in wt mice that proved unsatisfactory, we chose to study immune responses to the NR/CB1954 gene therapy in wt mice using fresh s.c implants of tumour cells rather than in established tumours. Although this model would not ideally mimic the complex

immunosuppressive cellular and non-cellular components of the solid tumour microenvironment, nonetheless it could provide preliminary insight into the immunogenic potential of NR/CB1954-mediated cell death to induce $CD8^+$ T cell responses *in vivo*.

4.3.5.1 OVA-specific $CD8^+$ T cell proliferation

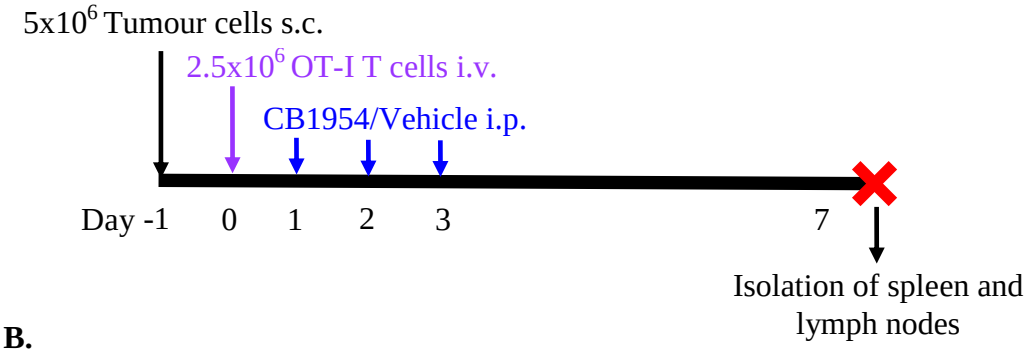
To determine whether NR/C1954-mediated tumour cell death would result in OVA-tumour antigen presentation and greater activation of donor OT-I T cells, wt C57BL/6 mice were seeded with the tumour cells followed by adoptive transfer of CFSE labelled OT-I T cells before vehicle/CB1954 treatment (Fig 4-11A).

After 4 days from the last dose of vehicle/CB1954, the percentage of donor $CD8^+$ T cells relative to total $CD8^+$ T cells were analysed in different lymphoid tissues. The data in Fig 4-10C shows that the percentage of OVA-specific $CD8^+$ T cells are approximately equal to 1% in different lymphoid tissues of control group indicating that this is the proportion at which OT-I cells can reside in the examined lymphoid compartment in the absence of OVA-antigenic stimulation. This percentage was increased by an average of 2.8 fold in DLN of mice bearing $Tramp_{OVA}$-NR implants and treated with vehicle, and by slightly greater average of 3.6 fold in DLN of CB1954 treated mice, although not reaching statistical significance. In addition, there were negligible differences in the percentage of OT-I $CD8^+$ T cells from N-DLN or spleen between vehicle and CB1954 treated groups.

To explore whether this modest increase in the percentage of OT-I T cells within the DLN in CB1954 group could be verified using an alternative approach, proliferation of donor OVA-specific $CD8^+$ T cells was analysed by tracking CFSE dilution in dividing cells. In the control group, non-specific proliferation of adoptively

transferred OT-I T cells was minimal in wt C57BL/6 mice, while $Tramp_{OVA}$-NR cells in the vehicle treated group stimulated proliferation with 48% of OT-I T cells detected having undergone at least one, and some more than 5 rounds of division indicating that OVA expression by the model tumour cells can result in priming of OVA-specific cells *in vivo*. As expected, CB1954 treatment of mice with $Tramp_{OVA}$-NR tumour cells induced greater activation of the OT-I T cells, with approximately 70% of OT-I T cells divided once or more rounds of division with increased numbers of accumulated cells in each round of division compared to vehicle treatment. This suggested that indeed NR/CB1954-induced cell death slightly increased OVA-tumour antigen release in the tumour microenvironment and increased activation of tumour antigen-specific $CD8^+$ T cells.

A.



B.

Group	Tumour cells	Treatment
Control	5x 10^6 Tramp-C1	Prodrug
Vehicle	5x 10^6 $Tramp_{OVA}$ -NR	Vehicle
CB1954	5x 10^6 $Tramp_{OVA}$ -NR	CB1954

C.

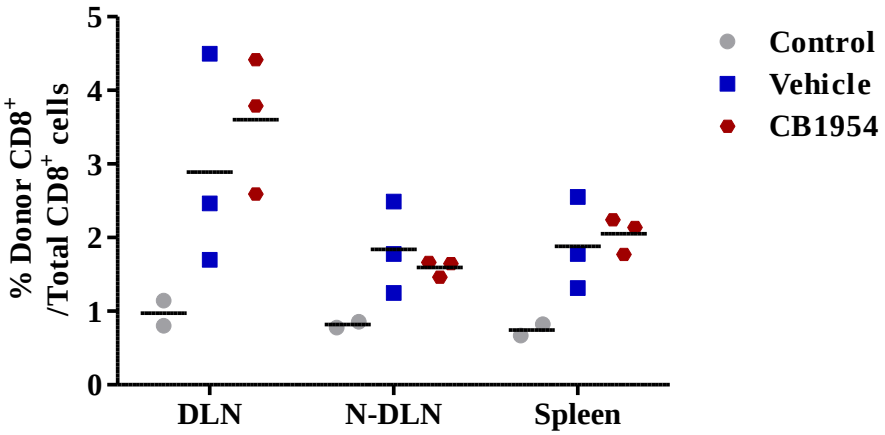


Figure 4-10: Donor OVA-specific T cell expansion in response to NR/CB1954-mediated cytotoxicity

Male C57BL/6 mice, 8-12 weeks old, were injected s.c in the right flank with 5x10^6 $Tramp_{OVA}$-NR cells. One day later, mice received 2.5x10^6 CFSE labelled OT-I lymphocytes i.v followed by 3 consecutive doses of 20 mg/kg CB1954 or vehicle for 3 days. At day 7, spleen, DLN and N-DLN were harvested and single-cell suspensions were prepared for flow cytometric analysis. A, schematic representation of the experimental design; B, experimental groups; C, The percentage of donor $CD45.1^+$ $CD3^+$ $CD8^+$ T relative to total $CD3^+$ $CD8^+$ T cells, data points represent the percentages in different animals and the horizontal line represents the mean.

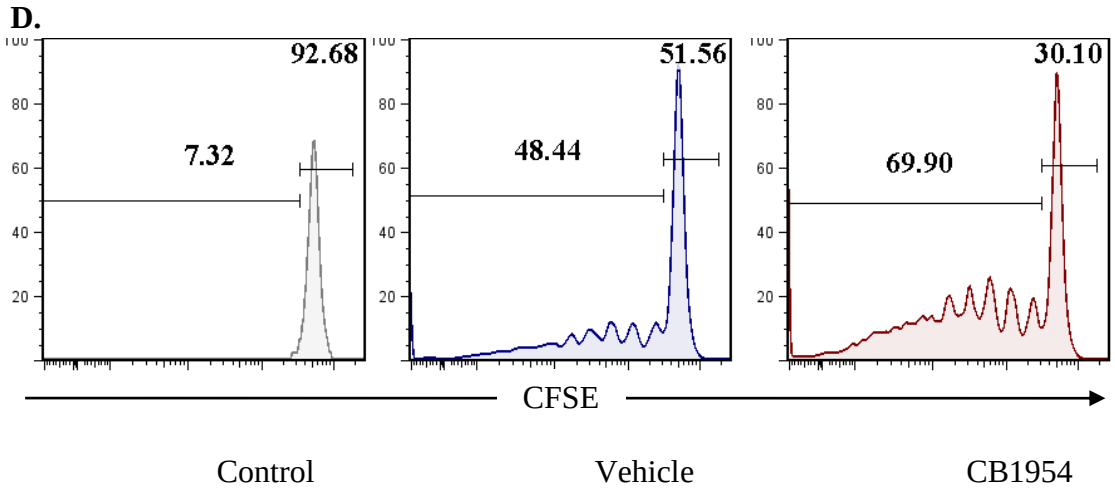


Figure 4-10…cont. Lymphocyte cell suspension from DLN of different treatment groups were stained with anti-CD45.1, -CD3 and -CD8 before analysis by flow cytometry. **D**, histograms for CFSE dilution of viable $CD45.1^+$ $CD3^+CD8^+$ cells from pooled flow cytometric data from DLN recovered from different groups of mice as indicated.

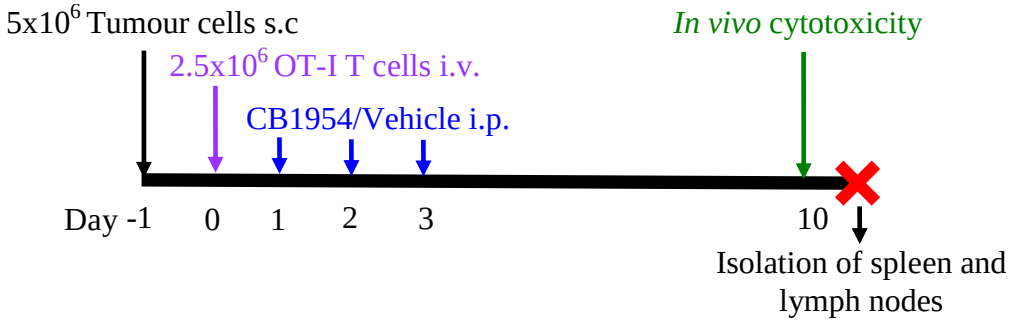
4.3.5.2 OVA-specific CTL effector function

To study whether activation and proliferation of donor $CD8^+$ T cells in DLN of NR/CB1954 treated mice is accompanied by acquisition of effector function, groups of mice were treated according to the experimental design and their treatment group illustrated in Fig 4-11A and B, respectively. After 1 week from the last dose of vehicle/CB1954, groups of mice were challenged with CFSE labelled and OVA-peptide loaded splenocytes in an *in vivo* cytotoxicity assay.

Although noticeable OVA-specific CTL responses (45%) were detected in different lymphoid tissues of mice treated with vehicle and seeded with $Tramp_{OVA}$-NR cells, administration of CB1954 increased the average level of cytotoxicity by 8%, 12% and 22% in DLN, N-DLN and spleen, respectively, although the small group size (n=2) of this pilot-scale experiment precludes attainment of statistical significance.

These results suggested that NR/CB1954-mediated tumour cell death can slightly increase the activation and cytolytic activity of donor tumour-specific $CD8^+$ T cells in this short-term adoptive transfer model.

A.



B.

Group	Tumour cells	OT-I T cells	Treatment
Control	--	--	--
Vehicle	5x 10^6 $Tramp_{OVA}$ -NR	2.5x10^6 cells	Vehicle
CB1954	5x 10^6 $Tramp_{OVA}$ -NR	2.5x10^6 cells	CB1954

Figure 4-11: OVA-specific CTL response to NR/CB1954-mediated cytotoxicity
Male C57BL/6 mice, 8-12 weeks old, were injected s.c in the right flank with 5x10^6 Tramp-C1 or $Tramp_{OVA}$-NR cells. One day later, mice received 2.5x10^6 OT-I lymphocytes i.v followed by 3 consecutive doses of 20 mg/kg CB1954 or vehicle for 3 days. At day 10, a mixture of 5x10^6 control splenocytes labelled with 2.5 µM CFSE ($CFSE^{Hi}$) and 5x10^6 target splenocytes labelled with 1 µM CFSE ($CFSE^{Lo}$) and pulsed with 5 µg/ml SIINFEKL was administered i.v. into all groups of mice. After 16 hr, lymphoid tissues were harvested and single-cell suspensions were prepared for flow cytometric analysis. A, schematic representation of the experimental design; B, experimental groups.

C.

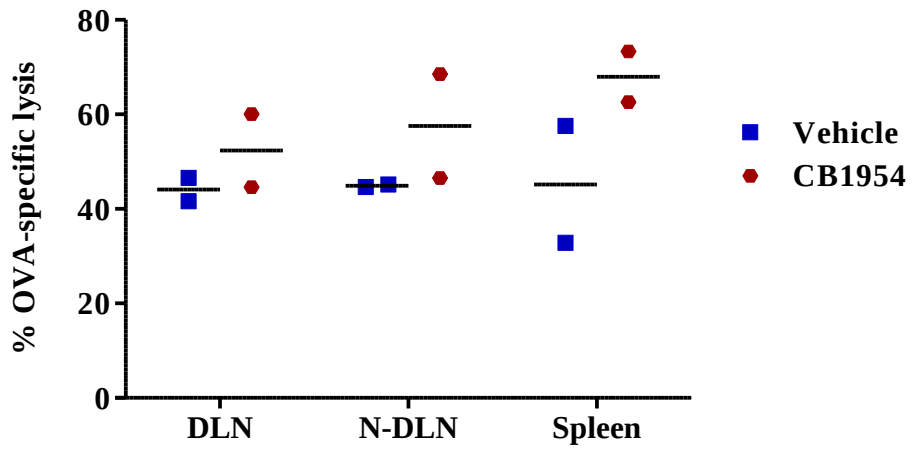


Figure 4-11 …cont. C, *in vivo* OVA-specific CTL response. Target cells labelled with $CFSE^{Hi}$ and $CFSE^{Lo}$ were analyzed by flow cytometry and the percentage of target cell killing was determined. Data points represent the percentage of lysed target cells in different animals and the horizontal line represents the mean of lysed target cells.

4.4 Endogenous antitumour $CD8^+$ T cell responses to NR/CB1954-mediated cytotoxicity in wild-type C57BL/6 mice

Although adoptive transfer of TCR transgenic T cells allows for detection of low levels of antigen-specific T cell response occurring early after immune stimulation, this experimental approach usually uses high input of single-avidity clonal TCR transgenic T cells that does not reflect the natural numbers and TCR affinity of endogenous CD8 T cell pool. Therefore, it was next sought to investigate the normal physiological endogenous antitumour $CD8^+$ T cell immune responses to NR/CB1954 treatment in wt C57BL/6 mice.

4.4.1 Expansion of endogenous OVA-specific $CD8^+$ T cell in response to NR/CB1954-mediated cytotoxicity in wild-type C57BL/6 mice

The frequency of endogenous antitumour $CD8^+$ T cell against the dominant ovalbumin epitope-SIINFEKL was monitored using OVA/MHC Class I pentamer

one week or four weeks after the last dose of vehicle or CB1954 to reflect the normal physiological immune responses during the activation/expansion and contraction/memory phase of the T cell response, respectively.

To analyze the frequency of OVA-specific $CD8^+$ T cells, groups of mice were treated according to the experimental design and their treatment group illustrated in Fig 4-12A and B. After one or four weeks from the last dose of CB1954/vehicle, mice were culled and lymphoid tissues were isolated to enumerate pentamer H-$2K^b$-SIINFEKL positive cells in different treatment groups of mice.

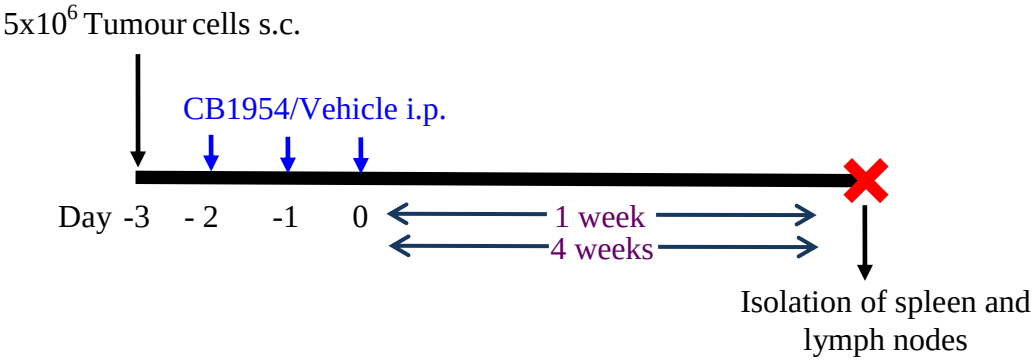
The frequency of OVA-specific $CD8^+$ T cells in DLN and N-DLN from vehicle treated group was nearly similar to background levels in control mice one week from the last dose of vehicle, while there was a 3 fold increase in the spleen of vehicle treated mice compared to that of control animals (Fig 4-12C). In contrast CB1954 treatment induced significant expansion of OVA-specific $CD8^+$ T cells by 3, 2.2 and 3.9 fold in DLN, N-DLN and spleen respectively relative to the vehicle treated group, demonstrating that NR/CB1954-mediated tumour cell death and the consequent release of greater tumour antigen could significantly stimulate priming and activation of $CD8^+$ T cells.

After 4 weeks from vehicle/CB1954 treatment, a slight difference in OVA-specific $CD8^+$ T cells could be observed in lymphoid tissues between control and vehicle treated group. Whereas the CB1954 treatment group exhibited a modest increase in the average numbers of OVA-specific $CD8^+$ T cells by 1.5 fold in DLN and 1.86 fold in N-DLN compared to vehicle treated mice (Figure 4-12D), however this difference did not reach statistical significance.

These results indicated that NR/CB1954-mediated release of OVA-tumour antigen promoted expansion of OVA-specific $CD8^+$ T cells that was evident after 7 days

from CB1954 treatment, however the reduced frequency of these cells after 4 weeks suggested that these cells are in the contraction phase.

A.



B.

Group	Tumour cells	Treatment
Control	5x 10^6 Tramp-C1	CB1954
Vehicle	5x 10^6 $Tramp_{OVA}$ -NR	Vehicle
CB1954	5x 10^6 $Tramp_{OVA}$ -NR	CB1954

C.

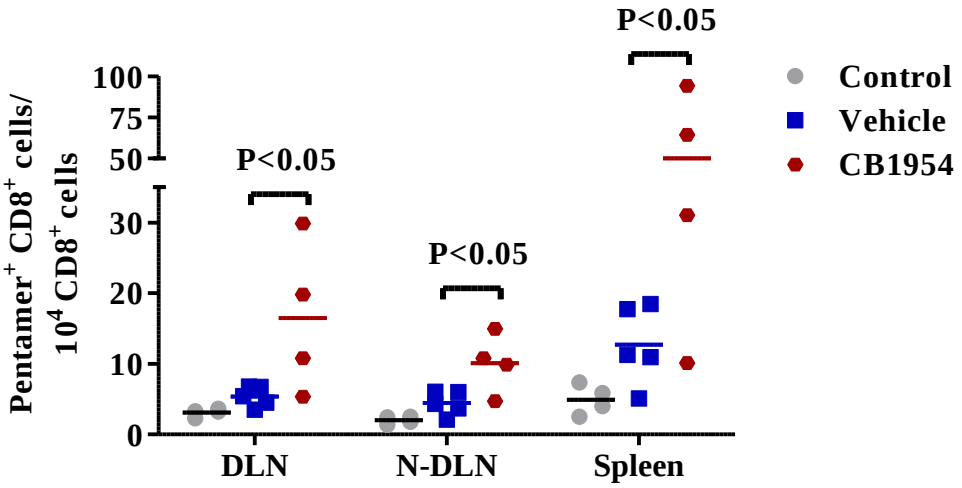


Figure 4-12: Generation of endogenous OVA-specific $CD8^+$ T cell following NR/CB1954 treatment

Male C57BL/6 mice, 8-12 weeks old, were injected s.c in the right flank with 5x10^6 of the indicated tumour cells. One day later, mice received 3 consecutive doses of 20 mg/kg CB1954 or vehicle for 3 days. After 1 or 4 weeks, spleen, draining and non-draining LN were harvested and single-cell suspensions were prepared for flow cytometric analysis. **A**, experimental design to study endogenous OVA-specific $CD8^+$ T cell response, **B**, different experimental groups of mice. **C**, flow cytometric analysis of live H-2K^b/SIINFEKL pentamer$^+$ $CD3^+$ $CD8^+$ cells in spleen, DLN and non-DLN harvested 1 week post CB1954/vehicle treatment. Data represents the individual percentages and group mean of pentamer-positive cells relative to total $CD8^+$ cells.

The P value indicates the statistical significance as analyzed by one-way ANOVA and post-hoc Bonferroni test.

D.

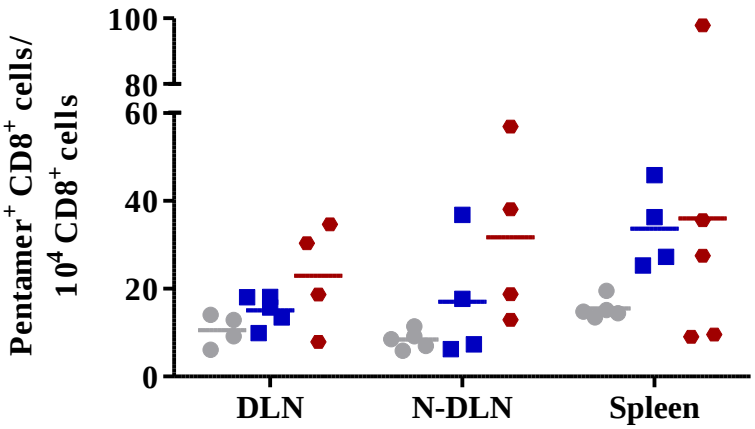


Figure 4-12 …cont. D, flow cytometric analysis of live H-2K^{b}/SIINFEKL pentamer^{+} CD3^{+} CD8^{+} cells in spleen, DLN and non-DLN harvested 4 weeks post CB1954/vehicle treatment. Data represents the individual percentages and group mean of pentamer-positive cells relative to total CD8^{+} cells.

4.4.2 Generation of OVA-specific CTLs following NR/CB1954-mediated cytotoxicity in wild-type C57BL/6 mice

To explore whether NR/CB1954-mediated expansion of tumour-specific CD8^{+} T cells was also associated with differentiation of endogenous OVA-specific CD8^{+} T cells into effector CTLs, the same experimental design and similar experimental groups shown in Fig 4-12A and B was used. After one or four weeks from the last dose of vehicle/CB1954 mice were challenged with CFSE labelled and OVA-peptide loaded target splenocytes in an *in vivo* cytotoxicity assay16 hr before the experimental endpoint.

After one week from vehicle/CB1954 treatment, negligible OVA-specific lysis was observed in different lymphoid tissues in control group, surprisingly very high OVA-specific lysis of 94%, 92% and 93% was seen in DLN, N-DLN and spleen of vehicle treated group, respectively. However, CB1954 treatment of mice inoculated

with $Tramp_{OVA}$-NR implants further increased the lysis of SIINFEKL loaded target cells by a range of 1.8-4.2% in lymphoid tissues (Fig 4-13A).

Assessment of OVA-specific lysis 4 weeks post treatment revealed that the average cytolytic activity was much reduced to a range of 6 to 14% in lymphoid tissues, and with virtually identical cytotoxicity levels in both vehicle and CB1954 treated groups at each of the tested sites.

These results shows that, in the short-term model (one week), despite the significantly increased expansion of OVA-specific $CD8^+$ T cells in CB1954 treated mice, this was translated into a marginal increase in ova-specific lysis, similar to the level of cytolytic activity seen in the adoptive transfer experiments. Whereas, four weeks post treatment, CB1954 treatment of $Tramp_{OVA}$-NR inoculated mice did not show any superior antitumour CTL response relative to the vehicle group.

Overall, these experiments indicated that mere inoculation of $Tramp_{OVA}$-NR cells induces high CTL activity and reduces the sensitivity of the assay in the short-term but not the long-term analysis. CB1954 treatment however stimulated a marginal increase in antitumour CTL response only one week post therapy.

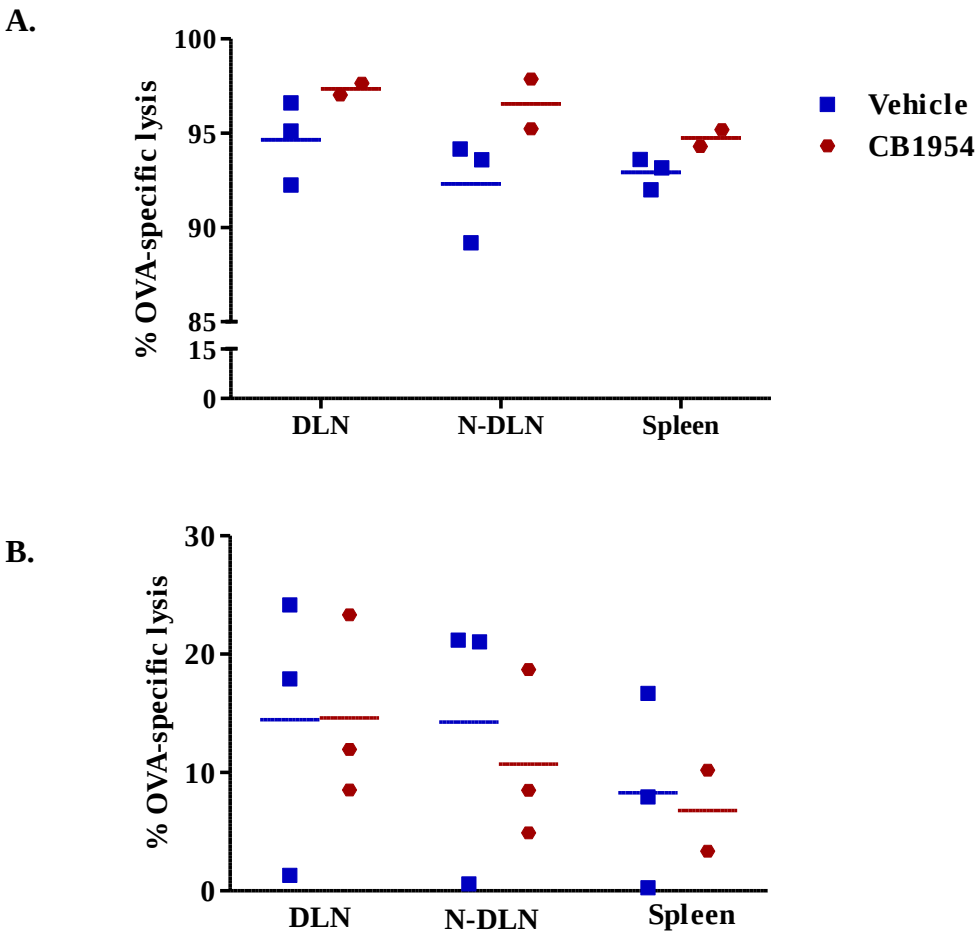


Figure 4-13: Endogenous OVA-specific CTL response to NR/CB1954-mediated cytotoxicity

A and **B**, *in vivo* OVA-specific CTL response after 1 or 4 week from CB1954/vehicle treatment, respectively. Target cells labelled with $CFSE^{Hi}$ and $CFSE^{Lo}$ were analyzed by flow cytometry and the percentage of target cell killing was determined. Data points represent the percentage of lysed target cells in different animals and the horizontal line represents the mean of lysed target cells.

4.5 Discussion

The mode by which tumour cells die in response to cytotoxic agent is the major determinant of the shape of the host immune response against tumour and consequently the efficiency of the chemotherapeutic agent. Indeed, an immunogenic cell death can enhance antitumour immunity and inhibit tumour growth. The strength of the antitumour immune response following cell death is dependent on the interaction between various cell death-associated stimuli and antigen presenting cells, and its capacity to cross-prime antitumour $CD4^+$ and $CD8^+$ T cells (Haynes et al., 2008).

Apoptotic cell death has long being considered nonimmunogenic or even tolerogenic; whereas necrosis has been deemed to be pro-inflammatory however recent studies have shown that a combination of signals arising from pre-apoptotic and late apoptotic dying cells stimulates efficient antigen presentation and cytotoxic T-cell activation (Tesniere et al., 2008). Thus, the immunogenicity of tumour cell death has shifted from the old morphological distinction of cell death to the new paradigm of whether it is immunogenic or tolerogenic death.

NR/CB1954 gene therapy was reported to induce apoptotic tumour cell death rather than necrosis (Palmer et al., 2003), nevertheless the impact of NR/CB1954-mediated tumour cell killing on the host immune response is still lacking. To elucidate the cellular immune response to NR/CB1954-mediated cytotoxicity, a model system of tumour cells expressing OVA as a tumour antigen and the therapeutic NR gene, complemented with adoptive transfer of a sub-therapeutic dose of transgenic OVA-specific T cells was established. Using this model system, the ability of NR/CB1954 to induce complete tumour regression and increase survival of NR expressing tumour bearing mice was demonstrated as a proof of

principle (Fig 4-1C and D). Similar results were reported using different tumour models either stably expressing NR cell clones or through viral transfer of NR to established tumours in both immunocompetent and immunodeficient mice (Benouchan et al., 2006; McNeish et al., 1998).

Examining the generation of memory antitumour CTL response following NR/CB1954 treatment revealed a slight increase in OVA-specific cytotoxicity (Fig 4-2C). However, the presence of OVA-expressing cells alone was capable of inducing high CTL response which may reduce the threshold of the assay to show clear differences in immune response between groups. In addition, the probable homeostatic expansion of OT I T cells in immunodeficient host may increase the numbers of OVA-specific effector cells resulting in high treatment unrelated CTL response.

In a secondary adoptive transfer experiment, the capacity of memory CB1954-primed OT-I cells to protect against $Tramp_{OVA}$ tumour growth was marginal and thus providing little evidence for the development of antitumour immunity (Fig 4-3D and E). This can be explained, in part, by the high ratio of tumour load (50 $Tramp_{OVA}$ cells) to low number CTL (one OT-I primed T cells) allowing for tumour outgrowth. Also, the loss of expression of the model OVA tumour antigen in the growing tumours might have contributed to this marginal prophylactic activity (Fig 4-5).

The present results showed that it was difficult to evaluate the magnitude of memory OVA-specific $CD8^+$ T cells in response to CB1954 in light of the treatment-unrelated high activation of OT-I T cells and the small numbers of animals in the group. However, when changing the design of the CTL assay, increasing the numbers of targets and separate analysis of DLN from N-DLN, the

same marginal increase in the frequency of memory OVA-specific OT-I T cells and CTL response was again observed (Fig 4-B and C). Thus, suggesting inefficient generation of long-term antitumour immunity following NR/CB1954 treatment. In contrast, a study by Green et al. (2003) demonstrated that mice bearing NR tumours and cured with CB1954 were able to reject a rechallenge with unmodified tumour cells indicating generation of immunological memory and that NR/CB1954 can stimulate an immune bystander effect (Green et al., 2003). The main apparent difference between our model and Green's model was the immune status of the model host. In Green's model, immunocompetent Balb/c mice were used which during the initial tumour rejection process have acquired antitumour immunity against undefined, probably multiple tumour associated antigens. Whereas in our adoptive transfer model system, the main focus was monitoring antitumour immunity to a single tumour antigen in immunodeficient hosts. Hence, it is more likely that generation of antitumour immunity against several tumour antigens in Green's model would be more superior to those generated against a single epitope in the present study. This is consistent with the notion that immunization using defined peptides has a limited therapeutic scope compared to vaccination with tumour cell lysates that can stimulate tumour specific-immune responses against both immunodominant and subdominant epitopes from tumour antigens (Chakraborty et al., 1998; Ovali et al., 2007; Soleimani et al., 2009).

Another difference between the two models is the importance of $CD4^+$ T cells in providing pre- and post-priming help to $CD8^+$ T cells to enhance recruitment and cytolytic function of tumour-specific $CD8^+$ T cells and also the generation of memory antitumour immunity (Bos and Sherman, 2010; Huang et al., 2007). It is anticipated, even with the leakiness of the immune system of the immunodeficient

nude C57BL/6 mice in our model, that $CD4^+$ T cell help to OVA-specific OT-I T cells will be minimal and would probably reduce the longevity and the efficiency of the antitumour $CD8^+$ T cells at later time points or in the secondary adoptive challenge against $Tramp_{OVA}$ cells.

As an alternative to lymphopenic hosts, immunocompetent mice were used to constrain homeostatic expansion of adoptively transferred T cells and to provide $CD4^+$ T cell help for OT-I T cells. However, in the attempt of establishing of tumour growth in wt C57BL/6 mice, the tumourigenic dose of implanted $Tramp_{OVA}$ cells was increased to 10 million cells and tumours required approximately 8 weeks to grow which makes it difficult for application as a model. Also, the use of Matrigel, tumour growth promoter, failed to enhance the tumourigenicity of $Tramp_{OVA}$ cells. These results together with an earlier observation demonstrating that 5 million $Tramp_{OVA}$ tumour cells can grow in immunodeficient hosts led us to conclude that these cells are highly immunogenic and that tumour growth might be established by short-term depletion of immune cells. The duration of lymphodepletion induced by irradiation is dose dependent and can reach up to 3 weeks following total body irradiation with a sublethal dose of 5 Gy (Jo, 1992). Previously in chapter 3 $Tramp_{OVA}$ and $Tramp_{OVA}$-NR tumour cells grew to a suitable tumour size within 2-3 weeks, therefore the duration after total body irradiation of wt mice is expected to allow for the growth of tumours to a suitable tumour size before restoration of the immune system. However, total body irradiation of wt mice with 1 Gy did not promote tumour growth (Fig 4-9A), while 2.5 Gy allowed an initial tumour growth in 2/4 of the mice, then remained stationary for 3 weeks (Fig 4-9B). Increasing the dose of irradiation up to 5 Gy permitted tumour growth in 4/4 of the mice and promoted tumour progression following a

short stationary phase (2 weeks). This suggested that recovery from lymphodepletion with 2.5 Gy occurred while the tumour is in its early stages of development resulting in the immune system being able to impede tumour formation which is reflected in a long stationary phase. In this phase equilibrium occur between the selective pressure exerted by the immune system on tumour cells and tumour progression. Conversely, 5 Gy irradiation induced a longer duration of lymphodepletion, allowing for tumour development and escape from immune recognition leading to progressive growth of $Tramp_{OVA}$ tumours (Fig 4-9C). Surprisingly, $Tramp_{OVA}$-NR tumour cells failed to grow in 5 Gy irradiated mice (Fig 4-9D) indicating either increased immunogenicity or loss of tumourigenicity of these cells. The apparent loss of tumourigenicity by $Tramp_{OVA}$-NR could be partly explained by the fact that these cells were generated from a single cell-derived clone requiring large scale *in vitro* amplification which may predispose the cells to increased genetic instability, transformation, and diminished proliferation (Schiller and Bittner, 1995; Vukicevic et al., 2010). Furthermore, generation of humoral or cellular immune response against the non-mammalian NR enzyme itself may also contribute towards increasing the immunogenicity of $Tramp_{OVA}$-NR cells and consequently tumour rejection.

The problems encountered during establishment of $Tramp_{OVA}$-NR tumour growth in immunocompetent mice led us to rely on fresh tumour cell implants as an alternative to established solid tumours as a preliminary model to study the immune response. In a short-term adoptive transfer setting, NR/CB1954-mediated tumour death stimulated a slight increase in the frequency and proliferation of OVA-specific $CD8^+$ T cells in the DLN (Fig 4-10C and D) that was associated with marginal increase in CTL response (Fig 4-11C). It is therefore suggested that

NR/CB1954 treatment can only stimulate regional priming and activation of adoptively transferred $CD8^+$ T cells.

Adoptive transfer experiments have been valuable tools in examining the immune response in a variety of normal and pathologic situations using mainly large input numbers (> 10^6) of transgenic TCR-T cells. However, recent studies demonstrated that that the number of transferred transgenic TCR-T cells affects critical aspects of an immune response like proliferation, kinetics, phenotype and memory generation (Badovinac et al., 2007; Obar et al., 2008). The altered immune response is likely due to competition of T cells for APCs, where higher numbers of T cells will compete for antigen/MHC complex, cytokines and costimulatory signals that will probably result in uneven stimulation of proliferating T cells and the generation of both terminally differentiated cells as well as intermediates (Kedl et al., 2002; Lanzavecchia, 2002). Importantly, seeding high numbers of transgenic OT-I T cells limits the expansion of effector T cells; alters the magnitude and the timing of the peak response, and also reduces Granzyme B release by effector cells suggesting that the precise numbers of TCR transgenic T cells that can mimic the endogenous response must be empirically determined for each model (Badovinac et al., 2007). Exploring the endogenous immune response to NR/CB1954 treatment was therefore of a considerable interest to verify the results obtained using adoptive transfer experiments.

By tracking endogenous $CD8^+$ T cell responses during the expansion phase (1 week post therapy), NR/CB1954 treatment induced significant expansion of $CD8^+$ T cells not only in the DLN but also in N-DLN and spleen indicating a systemic immune response (Fig 4-12A). This result is consistent with earlier data in the present study showing the NR/CB1954 treatment enhances proliferation of donor OT-I T cells 4

days post therapy (Fig 4-10C and D). Surprisingly, high OVA-specific CTL response was observed due to inoculation of OVA-expressing tumour alone (4-13A). Thus, again interfering in assessment of the CTL activity and making accurate interpretation of the results difficult. However, NR/CB1954 treatment promoted marginal increase in OVA-specific CTL response. On the other hand, a trend like increase in the frequency of OVA-specific $CD8^+$ T cells in the secondary lymphoid tissues was observed 4 weeks post NR/CB1954 suggesting that they are in the early contraction phase (4-12B). The negligible OVA-specific CTL response however indicated inefficient generation of tumour-specific $CD8^+$ T cell-mediated immunity (4-13B).

Collectively, these results indicated that NR/CB1954-mediated tumour killing is a weakly immunogenic process that facilitates development of short-lived antitumour immune response but minimal memory tumour-specific immunity.

5 Results: $CD8^+$ T cell responses to combined therapy with NR/CB1954 and 4-1BBL or GM-CSF *in vivo*

5.1 Introduction

Earlier data in this study indicated that NR/CB1954-mediated tumour cell killing is an immunogenic process that facilitates development of short-term antitumour responses; nevertheless long-lasting $CD8^+$ T cell dependent immunity was apparently insignificant. Aiming to improve the quantity and quality of activated immune effector cells, we investigated incorporation of immunomodulatory genes to increase the immunogenicity of tumour cells dying in response to NR/CB1954 gene therapy and to enhance the generation of memory antitumour immune response.

The first approach used in the present study was to deliver 4-1BB costimulatory ligand (4-1BBL) to tumour cells aiming to induce tumour-selective presentation of 4-1BBL to tumour-reactive T cells. 4-1BBL was chosen as it can provide 'signal 2' for the activation of naive T cells and can function as natural T cell adjuvant in promoting T cell division, survival, and effector function such as cytokine secretion and cytotoxicity (Du et al., 2007). In addition, enhanced 4-1BB/4-1BBL cross linking was shown to strengthen T cell-mediated antitumour immunity (Melero et al., 1998).

Another approach was to introduce the GM-CSF gene into the tumour cells to produce high local amounts of the protein and to avoid the usual systemic side effects of the cytokine. Production of GM-CSF cytokine within the tumour microenvironment would result in recruitment of APCs and creates a favorable environment for tumour antigen presentation and priming of naïve T cells.

To elucidate the potential use of either 4-1BBL or mGM-CSF immunomodulatory genes in combination with NR/CB1954 gene therapy to stimulate long-term tumour-specific $CD8^+$ T cell responses; 4-1BB ligand and mGM-CSF were introduced to

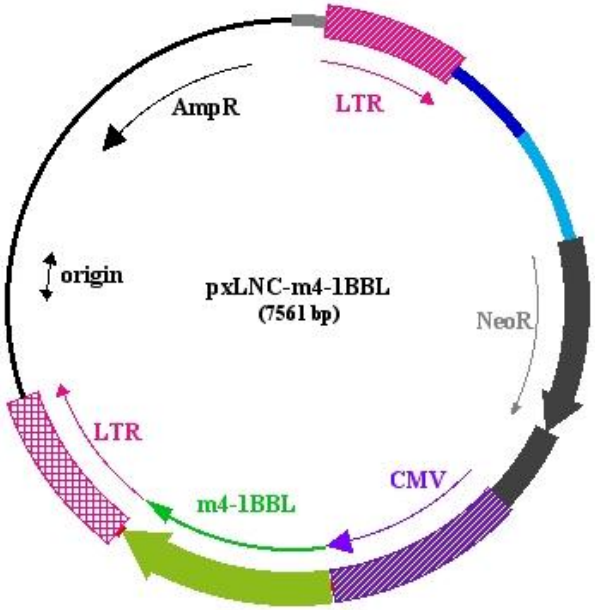
$Tramp_{OVA}$ cells to generate $Tramp_{OVA}$-4-1BBL and $Tramp_{OVA}$-GMCSF subclones. This chapter describes the use of each of these modified cell lines together with $Tramp_{OVA}$-NR as implants in wt C57Bl/6 mice to study the impact of either 4-1BBL or m-GMCSF on memory $CD8^+$ T cell responses induced by NR/CB1954 therapy.

5.2 $CD8^+$ T cell responses stimulated by combined therapy of NR/CB1954 and 4-1BB costimulatory ligand

5.2.1 Generation of single cell-derived $Tramp_{OVA}$-4-1BBL clones

The pxLNC-m4-1BBL retroviral plasmid (Fig 5-1) was previously generated in the Gene Therapy Group (University of Birmingham) by cloning of cDNA sequence encoding murine 4-1BBL into a retroviral vector downstream of CMV immediate–early promoter to produce high levels of gene expression. pxLNC-m4-1BBL was used to transfect the virus packaging FLYA13 cells and to generate pools of stably expressing FLYA13-4-1BBL cells upon selection with 500 μg/ml G-418. Retrovirus-containing supernatant along with polybrene (8 μg/ml) were used to transduce $Tramp_{OVA}$ cells followed by culturing in G-418 selective media. After verification of 4-1BB ligand expression in $Tramp_{OVA}$-4-1BBL pool of cells by flow cytometry, established cells underwent single cell cloning by limited dilution.

A.



B.

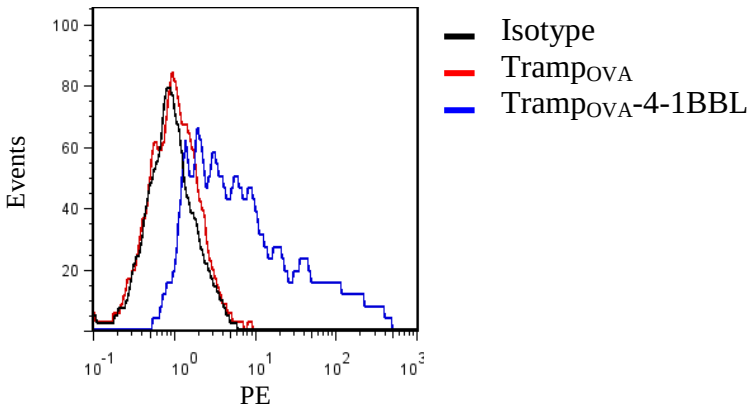


Figure 5-1: Generation of Tramp$_{OVA}$-4-1BBL cells.
A, pxLNC-m4-1BBL retroviral plasmid map showing the position of the 4-1BBL gene inserted downstream of the CMV promoter and the neomycin resistance gene driven by the 5' LTR. **B**, The level of 4-1BBL surface expression in the Tramp$_{OVA}$-4-1BBL pool of cells. Tramp$_{OVA}$ and Tramp$_{OVA}$-4-1BBL cells ($1x10^5$ cells/100µl) were stained with 4-1BBL-PE or isotype control antibodies as indicated in the key, before analysis by flow cytometry.

5.2.2 Characterization of $Tramp_{OVA}$-4-1BBL cells

5.2.2.1 4-1BB costimulatory ligand expression by $Tramp_{OVA}$-4-1BBL clones

To identify clones with high levels of 4-1BBL expression, surface staining of 4-1BB ligand in single cell-derived $Tramp_{OVA}$-4-1BBL clones was evaluated by flow cytometry. Levels of 4-1BB ligand expression were classified into: negligible, low, moderate and high levels based on MFI. Among the 18 clones screened, most showed moderate (Fig 5-2C) or high (Fig 5-2D and E) level of 4-1BBL, and only 4 had low (Fig 5-2B) to negligible (Fig 5-2A) levels of expression.

Although clone 9 showed the highest level of 4-1BBL expression, it showed abnormal spindle-like cell shape compared to the homogenous polygonal morphology of normal cells as well as slow proliferative capacity, thus this clone was excluded.

Clone 21, being one of the highly expressing clones, was selected for further usage in *in vivo* experiments. For this purpose, large scale cultures of $Tramp_{OVA}$-4-1BBL c21 were prepared and stored as explained in section 3.4.2 while monitoring the expression 4-1BBL at each passage.

As shown in Figure 5-2F, 4-1BBL expression was more or less similar among different passages (P1-4) confirming uniform stable expression of the protein over time.

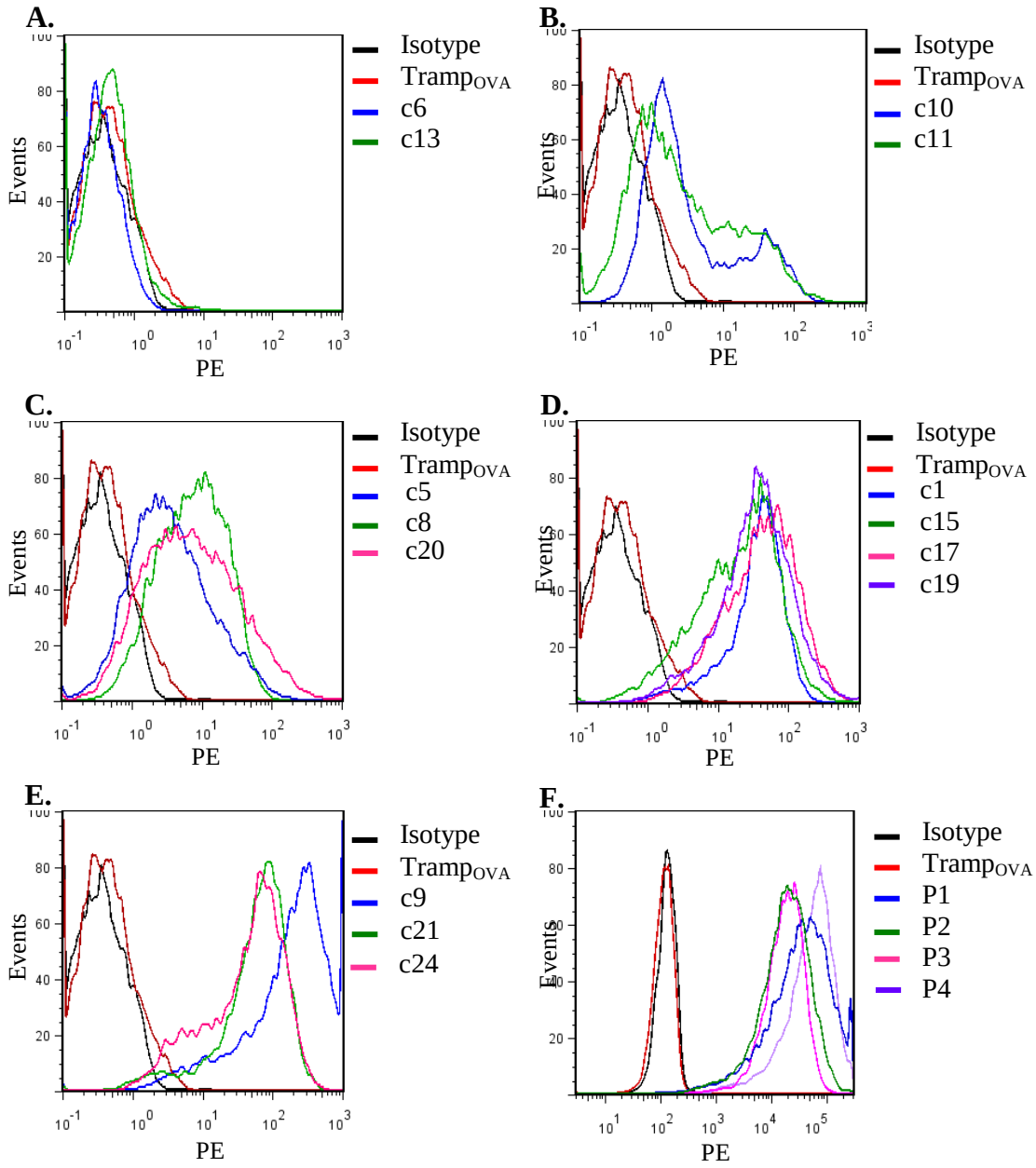


Figure 5-2: Surface expression of 4-1BB ligand by Tramp$_{OVA}$-4-1BBL clones. Tramp$_{OVA}$ cells and Tramp$_{OVA}$-4-1BBL clones ($1x10^5$ cells/100µl) were stained with 4-1BBL-PE or isotype control antibodies for 20 min at 2-8ºC before analysis by flow cytometry. Histograms of Tramp$_{OVA}$-4-1BBL clones were grouped according to the level of 4-1BBL expression into: **A**, negligible; **B**, low; **C**, moderate; and **E**, high. **F**, stable expression of 4-1BBL by clone 21 over different passages (P1 to P4). Each histogram represents as indicated to the right of each plot

5.2.2.2 Presentation of OVA-epitope by Tramp$_{OVA}$-4-1BBL clone 21

To compare OVA epitope presentation between Tramp$_{OVA}$-4-1BBL c21 and parental Tramp$_{OVA}$ cells, B3Z T cells activation was used as readout. As can be seen in Figure 5-3, B3Z T cell activation was similar to background levels with control Tramp-C1 cells. Interestingly, Tramp$_{OVA}$-4-1BBL cells showed 5.3 fold reduced capacity to prime B3Z hybridoma compared to Tramp$_{OVA}$ suggesting lower levels of H-2K^{b}-OVA $_{(257–264)}$ complexes presentation by Tramp$_{OVA}$-4-1-BBL. Nevertheless, this shows that the TrampOVA-4-1BBL cells can still present OVA antigen to activate cognate T cells.

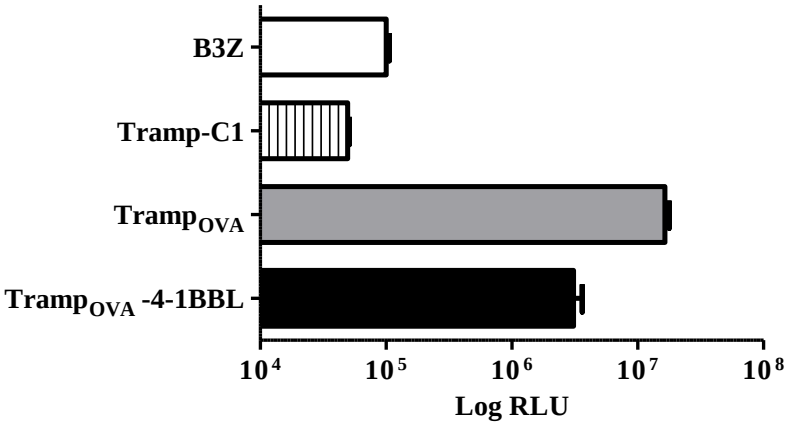


Figure 5-3: B3Z T cell activation by Tramp$_{OVA}$-4-1BBL cells.
Tramp$_{OVA}$-4-1BBL or Tramp$_{OVA}$ cells were cocultured at 1x10^4/well in a 96 well plate with 1x10^5 B3Z T cell hybridoma cells for 16 hr. β-galactosidase activity in B3Z T cells was assayed in total culture lysates after incubation with β-galactosidase luminescent substrate for 1 hr at 37ºC. Data are presented as the mean relative luminescence (RLU) of quadruplicate wells (±SEM) on a log scale.

5.2.2.3 Activation of naïve OT-I CD8$^+$ T cells by Tramp$_{OVA}$-4-1BBL cells

To examine the effect of 4-1BBL expression on expansion of OT-I T cells, CFSE labelled naïve OT-I T cells were cocultured with irradiated Tramp-C1, Tramp-$_{OVA}$ or Tramp$_{OVA}$-4-1BBL cells. After 4 days, CFSE dilution was monitored by flow cytometry. Data in Figure 5-4 shows that naïve OT-I T cells stimulated with Tramp-C1 cells did not proliferate whereas those stimulated with Tramp$_{OVA}$ cells has shifted further to the left of the CFSE plot indicating that the all OT-I T cells underwent more than 2 rounds of division. Despite Tramp$_{OVA}$ cells higher expression of OVA epitope, Tramp$_{OVA}$-4-1BBL cells stimulated OT-I T cells sufficiently to go through more than 2 rounds of divisions where some of the activated cells underwent 7 successive divisions. These results suggest that 4-1BBL expression can enhance expansion of OT-I T cells

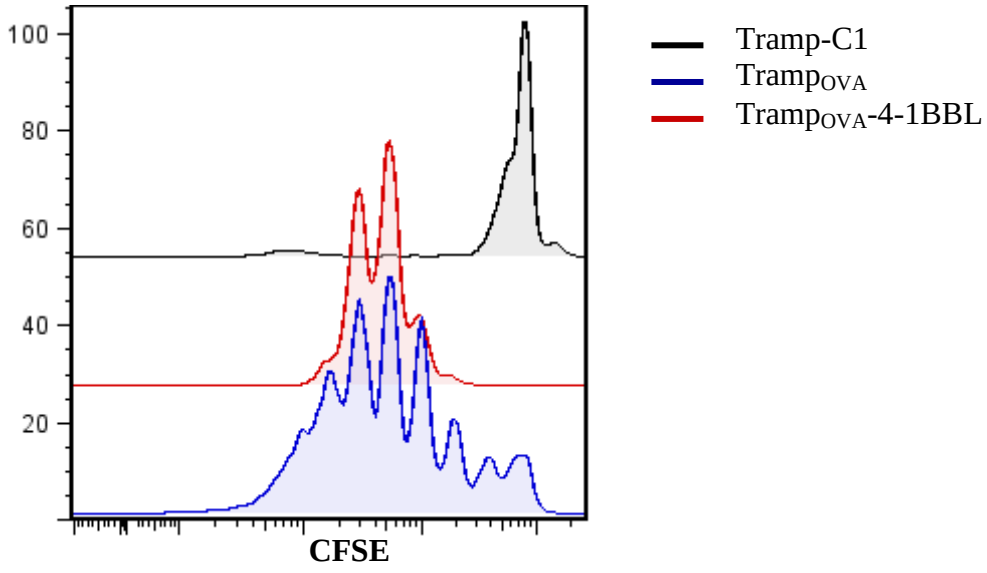


Figure 5-4: Proliferation of OT-I CD8$^+$ T cells stimulated by Tramp$_{OVA}$-4-1BBL cells.
Naïve OT-I T cells were labelled with 2.5 µM CFSE and then 5x10^6 OT-I T cells were cocultured with 5x10^5 irradiated Tramp-C1, Tramp-$_{OVA}$ or Tramp$_{OVA}$-4-1BBL cells in wells of a 6 well plate. After 4 days, OT-I lymphocytes were harvested and stained with anti-CD3 and CD8 antibodies before analysis by flow cytometry. Histograms for CFSE dilution of viable CD3$^+$CD8$^+$ cells stimulated with irradiate Tramp-C1, Tramp$_{OVA}$ or Tramp$_{OVA}$-4-1BBl cells for 4 days. Representative results are shown from 3 cultures.

5.2.2.4 Stimulation of effector OT-I CD8$^+$ T cell responses by Tramp$_{OVA}$-4-1BBL cell

To test the efficacy of Tramp$_{OVA}$-41BBL versus Tramp$_{OVA}$ cells in improving CD8$^+$ T cell effector functions, OT-I T cells were stimulated *ex-vivo* with either Tramp$_{OVA}$-41BBL or Tramp$_{OVA}$ irradiated cells. After 4 days, OT-I T cells were harvested and used as effectors in a 4 hour chromium release assay against SIINFEKL peptide pulsed target Tramp-C1 cells. The 4-1BBL stimulated OT-I T cells exhibited approximately a two fold increase in cytotoxicity against SIINFEKL loaded target cells compared to Tramp$_{OVA}$ stimulated OT-I cells at different E/T ratios (Fig 5-5).

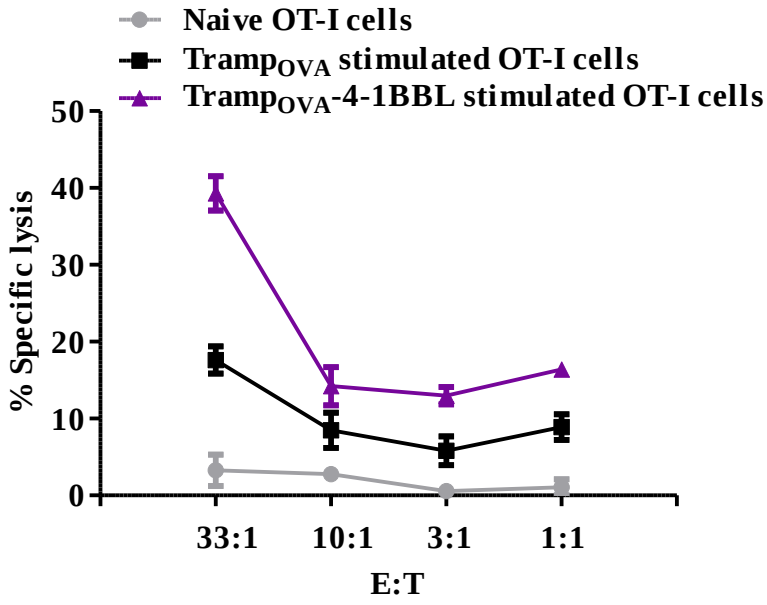


Figure 5-5: Activation of OVA-specific CTL responses following different priming conditions.
Naïve OT-I T cells ($5x10^6$) were cocultured with $5x10^5$ irradiated Tramp-C1, Tramp-$_{OVA}$ or Tramp$_{OVA}$-4-1BBL cells in wells of a 6 well plate. After 4 days, OT-I lymphocytes were harvested and washed twice to serve as effectors (E), then incubated with 5 µg SIINFEKL peptide pulsed and ^{51}Cr-labeled target Tramp-C1 cells (T) at the indicated ratios for 5 hr at 37ºC. Each point represents triplicate chromium release determinations (±SEM).

5.2.2.5 Immunogenicity of Tramp$_{OVA}$-4-1BBL cells in C57BL/6 mice

In our earlier experiments, Tramp$_{OVA}$ tumour cells were found to be immunogenic at a dose of 5 million cell s.c. inoculum in wt mice. Therefore, to increase the chance of tumour development a tumour cell dose of 10 million cells was chosen to study establishment of Tramp$_{OVA}$-4-1BBL tumours in different conditions.

In wt C57Bl/6 mice, initial tumour volumes ranging from 109 mm^3 to 278 mm^3 were evident one week following tumour injection, however these regressed completely by day 18. Mice remained tumour free for the 110 days follow-up period (Fig 5-6A). As establishment of Tramp$_{OVA}$ tumour growth seen in section 4.2.2 was done in parallel to the present experiment, it was legitimate to compare survival probabilities for Tramp$_{OVA}$ and Tramp$_{OVA}$-4-1BBL tumour bearing mice. As shown in Figure 5-6B, mice bearing Tramp$_{OVA}$-4-1BBL tumour cells showed a significant survival advantage compared with mice bearing Tramp$_{OVA}$ tumours. The median survival of mice bearing Tramp$_{OVA}$ tumours was 85 days (range 78–107 d) compared with > 124 days for Tramp$_{OVA}$-4-1BBL tumours (mice were culled at d124 for further examinations).

In an attempt to enhance tumour growth in wt mice, matrigel was admixed with tumour cell suspension for s.c. injections. However, tumour growth was erratic and showed cycles of extended phases of tumour growth followed by short periods of regression. After 82 days, the experiment was terminated and mice showing tumour growth were dissected to isolate tumour cells for further analysis (Figure 5-6C).

Ultimately, 5 Gy irradiated wt mice were used to establish tumour growth. Figure 5-6D shows similar initial tumour growth to Tramp$_{OVA}$ cells for almost 2-3 weeks. This was followed by extended complete tumour regression in 2/3 mice for 75 days. These results indicated that Tramp$_{OVA}$-4-1BBL cells are highly immunogenic and

that expression of 4-1BBL in the tumour microenvironment significantly protected against tumour growth and can prolong survival.

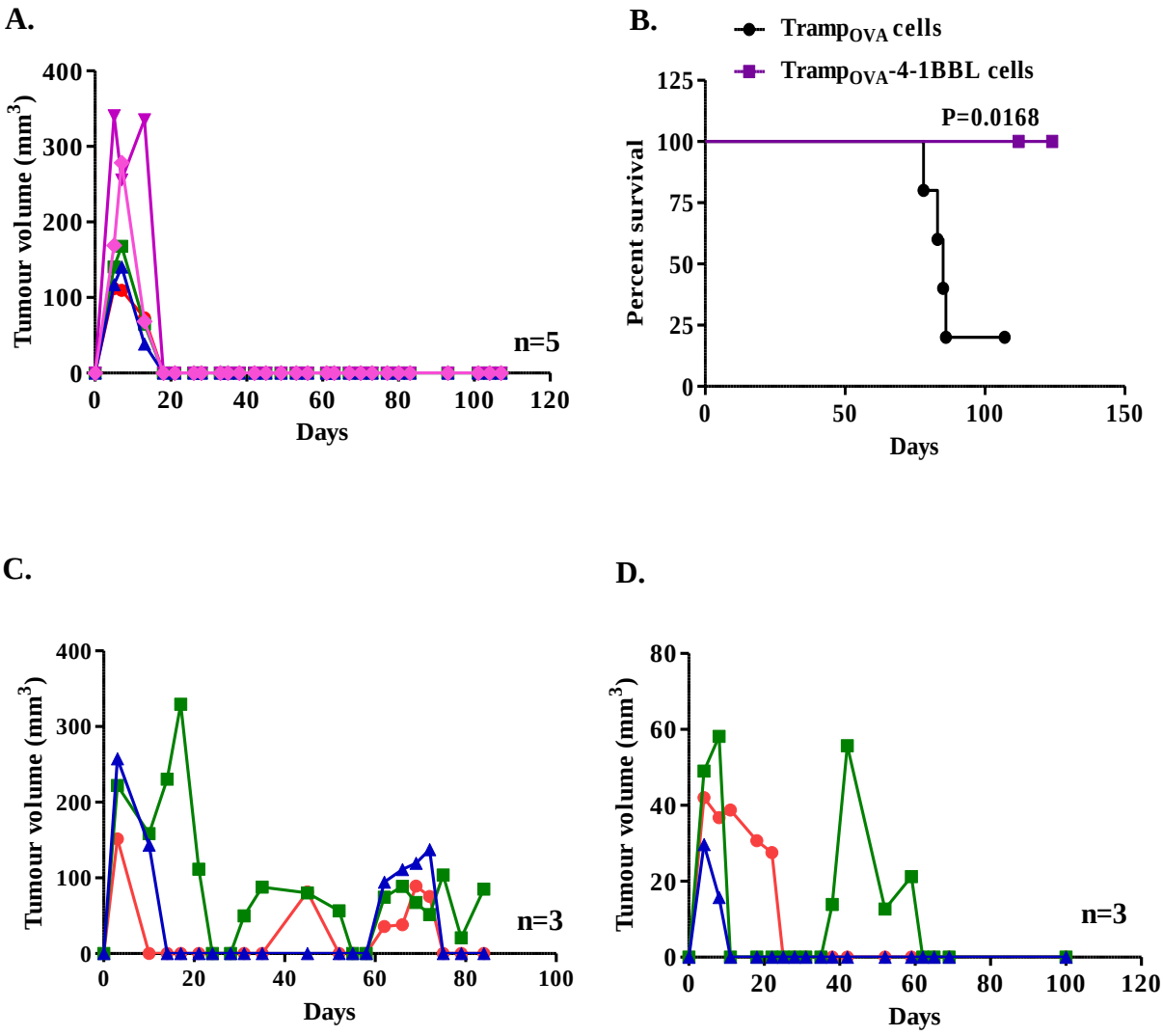


Figure 5-6: Comparison of different conditions for subcutaneous growth of 10 million Tramp$_{OVA}$-4-1BBL cells in C57BL/6 mice

Male C57BL/6 mice, 6-8 weeks old, were injected s.c. in the right flank with 10x10^6 Tramp$_{OVA}$ cells. Tumour volume was measured manually using a calliper three times a week. Each line represents tumour growth in an individual mouse. **A**, tumour growth in wt mice; **B**, Kaplan-Meier survival analysis of time between inoculation of Tramp$_{OVA}$ and Tramp$_{OVA}$-4-1BBL tumour cells and reaching 1000 mm^3 volume; **C**, tumour establishment using a mixture of 10 x10^6 Tramp$_{OVA}$-4-1BBL cells and matrigel in 1:1 volume ratio for s.c. injections; **D**, tumour growth in 5 Gy irradiated mice. N represents the number of mice in the experiment.
The P value indicates the statistical significance as analyzed by Wilcoxon test.

5.2.2.6 Characteristics of tumour cells from matrigel grown Tramp$_{OVA}$-4-1BBL tumours in C57BL/6 mice

The abnormal tumour growth pattern seen in Tramp$_{OVA}$-4-1BBL/matrigel grown tumours was intriguing. We wanted to confirm the absence of tumour cell implants underneath the skin in those mice showing regressing tumour, and to examine the cellular phenotype of the tumours in mice with evident growth. To achieve this, mice were culled and any residual tumour cells were excised. Tumour free mice showed clear s.c. surface without any signs of inflammation or evident tumour cell clusters; however the internal surface of the skin was scraped and washed to collect all adherent cells. On culturing of these cells for more than 2 weeks no evident growth was seen confirming absence of any residual tumour cells.

The tumour excised from the only mouse showing tumour growth in matrigel-grown tumours was small, soft in texture, and easily separated into single cells for culturing. After 2 weeks in culture, few clusters of cells could be detected however they were flattened with a small nucleus and large cytoplasm volume compared to tumour cells isolated earlier in section 4.2.2.5 that were relatively small with large and irregular nucleus containing several nucleoli and scanty cytoplasm (Fig5-7). In addition matrigel-retrieved tumour cells demonstrated slow growth rate and on subculturing the cells did not adhere to the surface of the culture vessel and died. This may suggest that the observed residual growth was mainly a scar tissue at the site of tumour cell injection and that the retrieved cells were fibroblast-like (mesenchymal) cells that might be involved in scar formation. Also, these cells are expected to be primary in nature and might have required different culture condition from those intended for Tramp-$_{OVA}$-4-1BBL cells for their survival and proliferation.

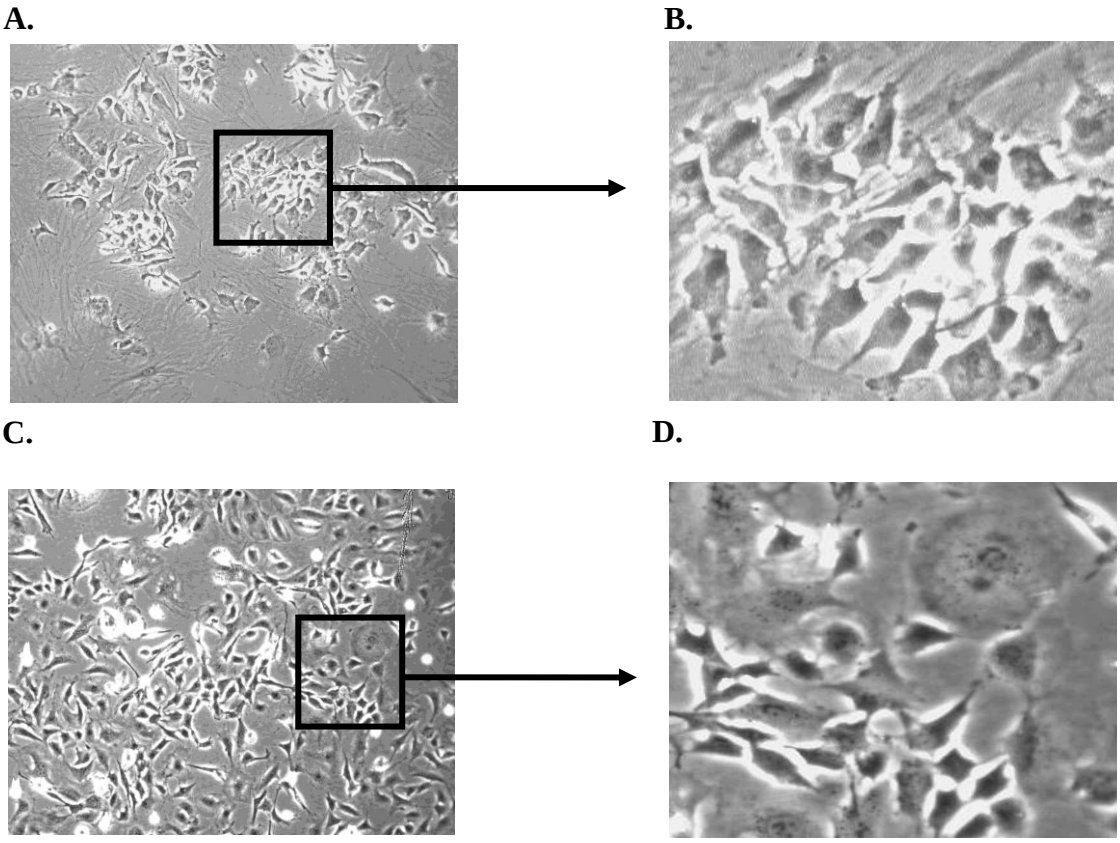


Figure 5-7: Light micrograph of tumour cells derived from Tramp$_{OVA}$-4-1BBL matrigel grown tumours in C57BL/6 mice
Tumours were excised from mice, dissociated by enzyme digestion and cultured in wells of 6 well plates for 2 weeks. **A** and **C**, Phase contrast images of tumour cells from mice bearing Tramp$_{OVA}$ and Tramp$_{OVA}$-4-1BBL tumours, respectively viewed at 5x magnification. **B** and **D** 40x magnified view of the respective tumour cells.

5.2.3 Endogenous memory OVA-specific CD8$^+$ T cell responses in Tramp$_{OVA}$-4-1BBL immunized C57BL/6 mice

As 4-1BBL can directly enhance the immunogenicity of tumours we therefore were interested in testing whether tumour regression correlated with production of long-term antitumour CD8$^+$ T cell responses. To address this, we first examined the generation of OVA-specific CD8$^+$ T cells in tumour free mice inoculated with

$Tramp_{OVA}$-4-1BBL tumour cells and $Tramp_{OVA}$ tumour bearing mice after ≈3 month from the initial tumour seed (shown in Fig 5-6B). In this experiment naïve mice that did not receive any treatment served as a control group.

OVA-specific $CD8^+$ T cells in DLN, N-DLN and spleen were identified by $OVA_{257–264}$/H-$2K^b$ pentamer staining and flow cytometric analysis. As shown in Fig 5-8B the numbers of OVA-specific $CD8^+$ T cells in the DLN from mice with $Tramp_{OVA}$ tumours were indistinguishable from background levels seen with control mice receiving no tumour inoculum, however the mean frequencies were slightly increased in the N-DLN and spleen of mice bearing $Tramp_{OVA}$ tumour cells compared to control group but did not reach statistical significance. In mice that had been inoculated with $Tramp_{OVA}$-4-1BBL cells, the number of OVA-specific $CD8^+$ T cells was significantly increased in DLN and spleen, however the increase in N-DLN did not reach significance relevant to those receiving $Tramp_{OVA}$ cells.

We proceeded to ask whether the OVA-specific memory $CD8^+$ T cells generated *in vivo* could display effector functions in response to OVA-tumour antigen challenge. Initially, we performed *in vivo* cytotoxicity assays on $Tramp_{OVA}$ and $Tramp_{OVA}$-4-1BBL mice nearly 3 months after initial tumour challenge. In groups of mice with $Tramp_{OVA}$ tumours, the average percentage of OVA-specific target lysis was ≈ 10% in different lymphoid tissues; whereas mice implanted with $Tramp_{OVA}$-4-1BBL cells significantly increased OVA-specific cytotoxicity by 3 fold in DLN. A 2.5 fold increase was observed in N-DLN but this failed to reach statistical significance. Negligible increase in OVA-specific target cell killing was observed in the spleen compared to mice receiving $Tramp_{OVA}$ cells (Fig 5-8C).

Production of IFN-γ and IL-2 cytokines in response to OVA-tumour antigen was also investigated as part of evaluating OVA-specific $CD8^+$ T cells effector

functions. Lymphocytes from spleen and lymph nodes from mice receiving no tumours, $Tramp_{OVA}$ or $Tramp_{OVA}$-4-1BBL cells were harvested and restimulated *ex-vivo* with $OVA_{257-264}$ peptide to determine cytokine production by intracellular staining. The proportion of IFN-γ^+CD8^+ T cells was similar among T cells from DLN of both control and $Tramp_{OVA}$ bearing mice, while it was slightly increased in N-DLN and spleen from $Tramp_{OVA}$ group compared to control mice. In contrast a significant population of IFN-γ^+CD8^+ T cells was observed in DLN and N-DLN of $Tramp_{OVA}$-4-1BBL inoculated mice compared to those seeded with $Tramp_{OVA}$ cells; however the response of $CD8^+$ T cells in spleen was very similar in all groups (Fig 5-8D).

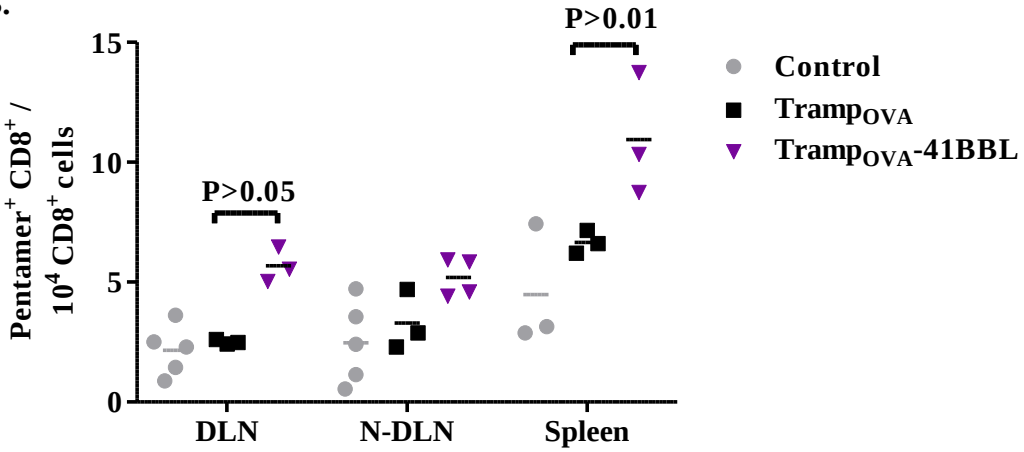
Fig 5-8E shows that IL-2 production was negligible in different lymphoid tissues in $Tramp_{OVA}$ group compared to control mice. Likewise IFN-γ, IL-2^+ producing $CD8^+$ T cells in $Tramp_{OVA}$-4-1BBL inoculated mice were significantly increased in DLN and to lesser extent in N-DLN of $Tramp_{OVA}$-4-1BBL treated mice compared to those having $Tramp_{OVA}$ cells.

These data indicate that OVA-specific $CD8^+$ T cells generated in response to $Tramp_{OVA}$-4-1BBL tumour cells consistently showed increased *in vivo* OVA-specific CTL responses, IFN-γ and IL-2 cytokine production both in the DLN and N-DLN but not in spleen, compared to animals seeded with $Tramp_{OVA}$ tumours. Therefore, the increased number of tumour-specific T cells, and their increased effector functions in LNs imply that 4-1BBL expression on tumour cells significantly enhances long-term antitumour immune responses.

A.

Group	Tumour cells
Control	--
$Tramp_{OVA}$	10x 10^6 $Tramp_{OVA}$
$Tramp_{OVA}$-4-1BBL	10x 10^6 $Tramp_{OVA}$-4-1BBL

B.



C.

Figure 5-8: Memory OVA-specific $CD8^+$ T cell response in C57BL/6 mice immunized with $Tramp_{OVA}$-4-1BBL tumour cells

A, tumour free mice inoculated with $Tramp_{OVA}$-4-1BBL tumour cells and $Tramp_{OVA}$ tumour bearing mice discussed in section 5.2.2.5 served as the experimental groups and naïve mice as a control group. After ≈3 month from tumour inoculation, a mixture of $5x10^6$ control splenocytes labelled with 2.5 µM CFSE ($CFSE^{Hi}$) and $5x10^6$ target splenocytes labelled with 1 µM CFSE ($CFSE^{Lo}$) and pulsed with 5 µg/ml SIINFEKL (total $1x10^7$ cells) was administered i.v. into all groups of mice to assess *in vivo* cytotoxicity. After 16 hr, lymphoid tissues were harvested and single-cell suspensions were prepared for flow cytometric analysis. **B**, frequency of OVA-specific $CD8^+$ T cells. Lymphocyte cell suspension from DLN, N-DLN and spleen isolated from different treatment groups was stained with H-$2K^b$/SIINFEKL pentamer and anti-CD3 and -CD8 antibody before analysis by flow cytometry. Data points represent the numbers of live $pentamer^+CD8^+CD3^+$ cells relative to total $CD8^+$ cells in individual animals.

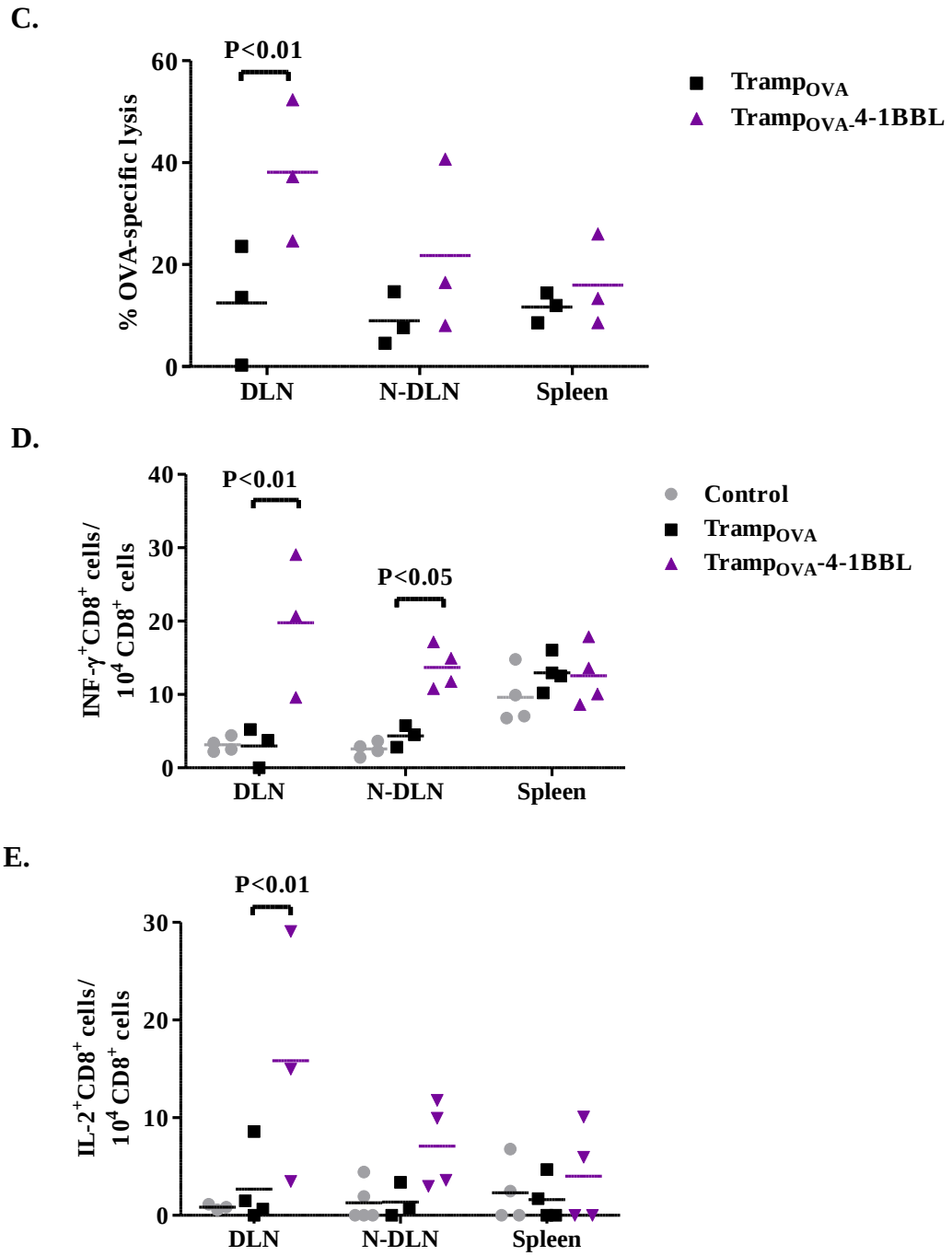


Figure 5-8 …cont. C, *in vivo* OVA-specific CTL response. Target cells labelled with $CFSE^{Hi}$ and $CFSE^{Lo}$ were analyzed by flow cytometry and the percentage of target cell killing was determined. Data points represent the percentage of lysed target cells in different animals. **D** and **E**, frequency of IFN-γ and IL-2 cytokine positive cells in $CD8^+$ T cells after *ex vivo* stimulation, respectively. $1x10^6$ lymphocytes were cultured in wells of a 96 well plate and stimulated with 1 µg/ ml SIINFEKL peptide overnight. Cells were surface stained with anti-CD3 and -CD8 antibodies followed by intracellular staining with anti-IFN-γ or -IL-2 antibodies before analysis by flow cytometry. Data points represent the numbers of cytokine$^+$CD8$^+$CD3$^+$ cells relative to total CD8$^+$ cells in different animals.
The horizontal line represents the group mean.
The P value indicates the statistical significance as analyzed by two-way ANOVA and post-hoc Bonferroni test.

5.2.4 Donor $CD8^+$ T cell response induced by combined NR/CB1954 and 4-1BBL treatment in a long-term *in vivo* model

Initially, we were interested in gaining insight into the kinetics of $CD8^+$ T cell expansion in response to NR/CB1954 and 4-1BBL combined therapy. To achieve this, a mixture of the indicated tumour cells (Fig 5-9B) were inoculated in wt C57Bl/6 mice. As the level of OVA-epitope presentation in $Tramp_{OVA}$-4-1BBL cells was nearly 1/5 that of $Tramp_{OVA}$ cells, $1x10^6$ $Tramp_{OVA}$ cells was used in tumour inoculation to ensure equal levels of OVA expression in tumour cells among different groups. After seeding tumour cells, OT-I T cells were adoptively transferred prior to vehicle/CB1954 treatment. Using this adoptive transfer experimental system, the frequency of OT-I $CD8^+$ T cells can be easily monitored in the peripheral blood via tail bleeds on a weekly basis for 5 weeks (Fig 5-9B). In addition, the frequency of OT-I $CD8^+$ T cells and antitumour CTL response were also assessed at the end of the experimental period.

As shown in Fig 5-9C, the percentage of OT-I $CD8^+$ T cells was 0.85% in control mice receiving Tramp-C1 tumours, this percentage was increased by 2.1 and 1.5 fold in response to CB1954 and 4-1BBL treatments, respectively. Combined treatment with CB1954 and 4-1BBL resulted in further expansion of OT-I $CD8^+$ T cells by 1.6 fold compared to mice receiving CB1954. These relative frequencies of OT-I T cells were maintained for the first 2 weeks. After 3weeks, the numbers of OT-I $CD8^+$ T cell started to decline in different groups and nearly reached the level of control group by week 5.

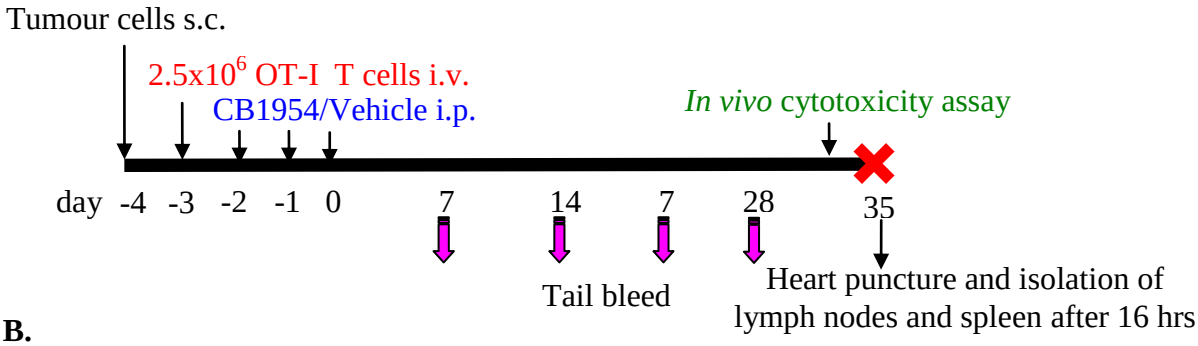
After 5 weeks, mice were culled and lymphoid tissues were isolated for assessing the frequency of OT-I T cells and OVA-specific lytic activity. Like control mice, the average percentage of OT-I $CD8^+$ T was $\approx$ 0.5% of total $CD8^+$ T cells in

lymphoid tissues from CB1954 treated groups. In the 4-1BBL treated group, a marginal increase (0.69%) was observed only in the DLN, whereas in the group of mice receiving combined CB1954 and 4-1BBL treatment the percentage of OT-I T cells was ≈ 0.72% in DLN and N-DLN and 0.56% in spleen (Fig. 5-9D and E).

As can be seen in (Fig. 5-9F and G), the mean OVA-specific cytotoxicity was ≈ 10% in lymphoid tissues and blood from control mice receiving only OT-I T cells, while there was a wide scatter in responses in each treatment group. However, the mean OVA-specific cytotoxicity was marginally increased in CB1954-treated mice compared to the 4-1BBL group suggesting a qualitative difference in the $CD8^+$ T cell response, perhaps reflecting different priming conditions. Combined treatment with CB1954 and 4-1BBL further enhanced OVA-specific CTL response in different lymphoid tissues and blood compared to CB1954 and 4-1BBL groups, respectively.

These results suggested that OT-I underwent initial expansion in response to the different treatments in the blood circulation, and that CB1954 and 4-1BBL combined treatment was more potent in stimulating $CD8^+$ T cell proliferation than CB1954 and 4-1BBL single treatments. This initial phase of $CD8^+$ T cell expansion was followed by a contraction phase that was clearly evident in the blood by week 4 post therapy. However, results for the percentages of OT-I T cells and CTL response in the lymphoid tissues from different treatment were difficult to interpret due to the high variability in responses within each treatment and the small group number.

A.



B.

Group	Tumour cells	OT-I T cells	Treatment
Control	10x 10^6 Tramp-C1	2.5x10^6 cells	CB1954
CB1954	5x 10^6 $Tramp_{OVA}$-NR+ 1x 10^6 $Tramp_{OVA}$	2.5x10^6 cells	CB1954
4-1BBL	5x 10^6 $Tramp_{OVA}$-NR+ 5x 10^6 $Tramp_{OVA}$-4-1BBL	2.5x10^6 cells	Vehicle
CB1954+4-1BBL	5x 10^6 $Tramp_{OVA}$-NR+ 5x 10^6 $Tramp_{OVA}$-4-1BBL	2.5x10^6 cells	CB1954

Figure 5-9: Donor OT-I $CD8^+$ T cell responses to NR/CB1954 combined therapy in wild-type C57BL/6 mice

Male C57BL/6 mice, 6-8 weeks old, were injected s.c. in the right flank with mixtures of the indicated tumour cells. One day later, mice received 2.5x10^6 CFSE labelled OT-I lymphocytes i.v. followed by 3 consecutive doses of 20 mg/kg CB1954 or vehicle for 3 days. After 5 weeks, a mixture of 5x10^6 control splenocytes labelled with 2.5 µM CFSE ($CFSE^{Hi}$) and 5x10^6 target splenocytes labelled with 1 µM CFSE ($CFSE^{Lo}$) and pulsed with 5 µg/ml SIINFEKL (total 1x10^7 cells) was administered i.v. into all groups of mice in an *in vivo* cytotoxicity assay. After 16 hr, lymphoid tissues were harvested and single-cell suspensions were prepared for flow cytometric analysis. **A** and **B**, schematic representation of the experimental design and the experimental groups, respectively.

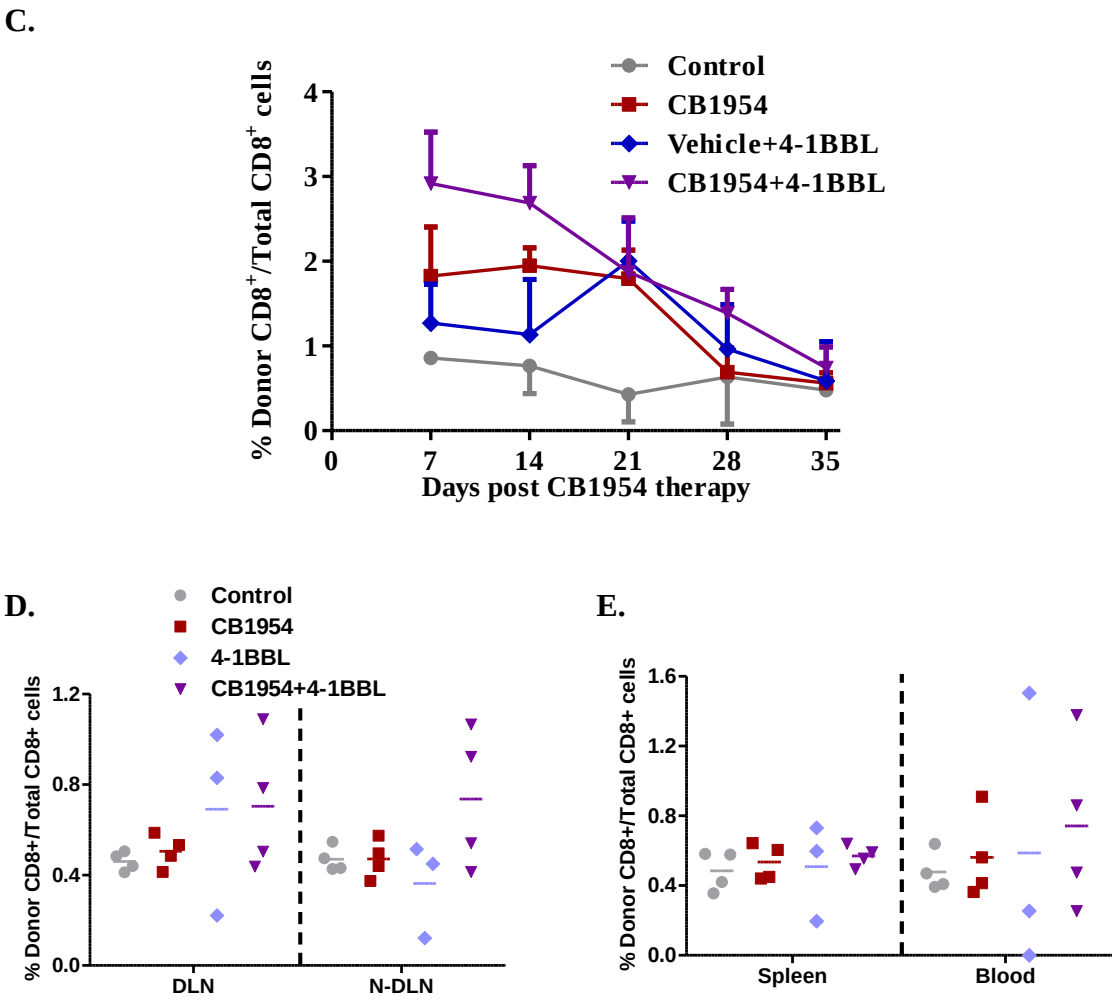


Figure 5-9 …cont. C, kinetics of OVA-specific $CD8^+$ T cells expansion in peripheral blood. Blood samples were collected via tail bleeds or terminal heart puncture and processed to remove RBCs. Lymphocytes were stained with anti-CD45.1, -CD3 and -CD8 antibodies before analysis by flow cytometry. Data points represent the percentages (±SEM) of live $CD45.1^+CD8^+CD3^+$ cells relative to total $CD8^+$ T cells.**D** and **E**, the frequency of OVA-specific $CD8^+$ T cells in different lymphoid tissues 5 weeks post treatment. Lymphocyte cell suspensions were stained with anti-CD45.1, -CD3 and -CD8 antibodies before analysis by flow cytometry. Data points represent percentages of live $CD45.1^+CD8^+CD3^+$ cells relative to total $CD8^+$ cells in different animals. n=3-4
The horizontal line represents the group mean.

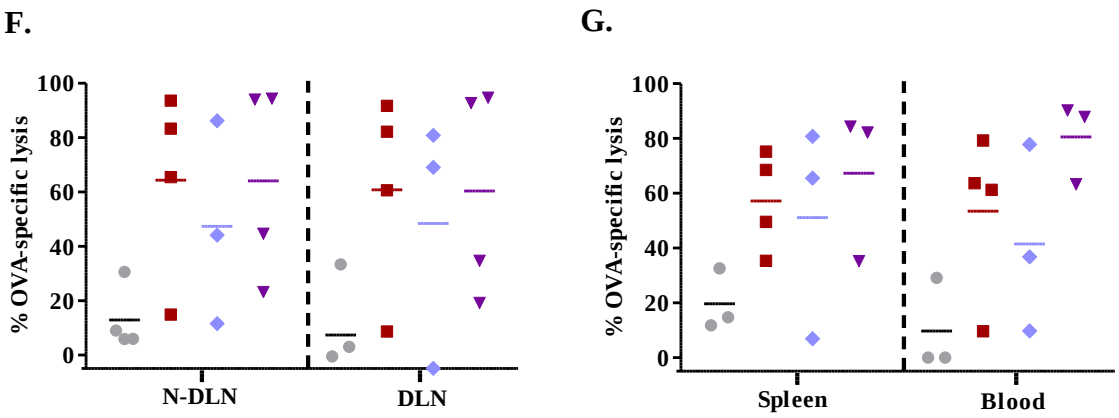


Figure 5-9 …cont. F and **G**, *in vivo* OVA-specific CTL response in different lymphoid tissues 5 weeks post treatment. Target cells labelled with $CFSE^{Hi}$ and $CFSE^{Lo}$ were analyzed by flow cytometry and the percentage of target cell killing was determined. Data points represent the percentage of lysed target cells in different animals. n=3-4
The horizontal line represents the group mean.

5.2.5 Endogenous $CD8^+$ T cell responses simulated by combined NR/CB1954 and 4-1BBL treatment in a long-term *in vivo* model

As the number of transgenic OT-I T cells used in the preceding adoptive transfer experiment does not reflect the low numbers of endogenous naïve T cell precursors specific for a single epitope found in a normal mouse, we were interested in evaluating the endogenous antitumour $CD8^+$ T cell responses to NR/CB1954 and 4-1BBL combined therapy in wt C57BL/6 mice after 4 weeks.

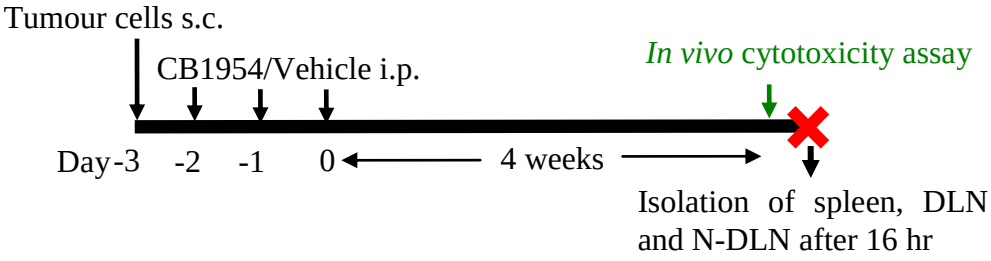
To test this, mixtures of tumour cell lines as indicated in Figure 5-10B were inoculated in C57BL/6 mice, followed by vehicle/CB1954 treatment as illustrated in Fig 5-10A. After 4 weeks, antitumour $CD8^+$ T cell responses were analysed in different lymphoid tissues by monitoring the frequency and CTL activity of OVA-specific $CD8^+$ T cells.

Analysis of OVA/K^b $pentamer^+$ $CD8^+$ $CD3^+$ T cells in the different experimental groups revealed that few OVA-specific $CD8^+$ T cells could be detected in lymphoid

organs of NR/CB1954 treated mice, comparable to the control group; while in 4-1BBL group these numbers were increased by 2 fold in DLN and to a lesser extent in N-DLN, but not in spleen. The combination of NR/CB1954 and 4-1BBL therapy clearly increased the frequency of OVA-specific $CD8^+$ T cells not only in DLN but also in spleen and N-DLN (although the latter did not achieve statistical significance) compared to NR/CB1954 treated group (Fig 5-10C).

Since CTL responses to tumour antigen are critical for the efficacy of $CD8^+$ T cell-dependent antitumour immunity, OVA-specific cytolytic activity was assessed in the different groups in an *in vivo* cytotoxicity assay. As shown in Fig 5-10D, NR/CB1954 treatment resulted in an average OVA-specific CTL-mediated cytotoxicity of 11.6% in different lymphoid organs, whereas 4-1BBL group showed increased levels of cytotoxicity in DLN (28%) and N-DLN (19%) and similar levels in spleen. Combining 4-1BBL with NR/CB1954 therapy increase OVA-specific cytotoxicity by 3.4 fold in DLN and 2.2 in both N-DLN and spleen compared to NR/CB1954 treatment. These results show that combining CB1954 with 4-1BBL treatment promotes expansion of OVA-specific $CD8^+$ T cells and tumour-specific CTL response compared to monotherapies.

A.



B.

Group	Tumour cells	Treatment
Control	10x 10^6 Tramp-C1	CB1954
CB1954	5x 10^6 $Tramp_{OVA}$-NR+ 1x 10^6 $Tramp_{OVA}$	CB1954
4-1BBL	5x 10^6 $Tramp_{OVA}$-NR+ 5x 10^6 $Tramp_{OVA}$-4-1BBL	Vehicle
CB1954+4-1BBL	5x 10^6 $Tramp_{OVA}$-NR+ 5x 10^6 $Tramp_{OVA}$-4-1BBL	CB1954

Figure 5-10: Endogenous OVA-specific CD8+ T cell responses to NR/CB1954 and 4-1BBL combined therapy in wild-type C57BL/6 mice
Male C57BL/6 mice, 6-8 weeks old, were injected s.c. in the right flank with mixtures of the indicated tumour cells. One day later, mice received 3 consecutive doses of 20 mg/kg CB1954 or vehicle for 3 days. After 4 weeks, a mixture of 5x10^6 control splenocytes labelled with 2.5 µM CFSE ($CFSE^{Hi}$) and 5x10^6 target splenocytes labelled with 1 µM CFSE ($CFSE^{Lo}$) and pulsed with 5 µg/ml SIINFEKL (total 1x10^7 cells) was administered i.v. into all groups of mice in an *in vivo* cytotoxicity assay. After 16 hr, lymphoid tissues were harvested and single-cell suspensions were prepared for flow cytometric analysis. **A** and **B**, schematic representation of the experimental design and experimental groups, respectively.

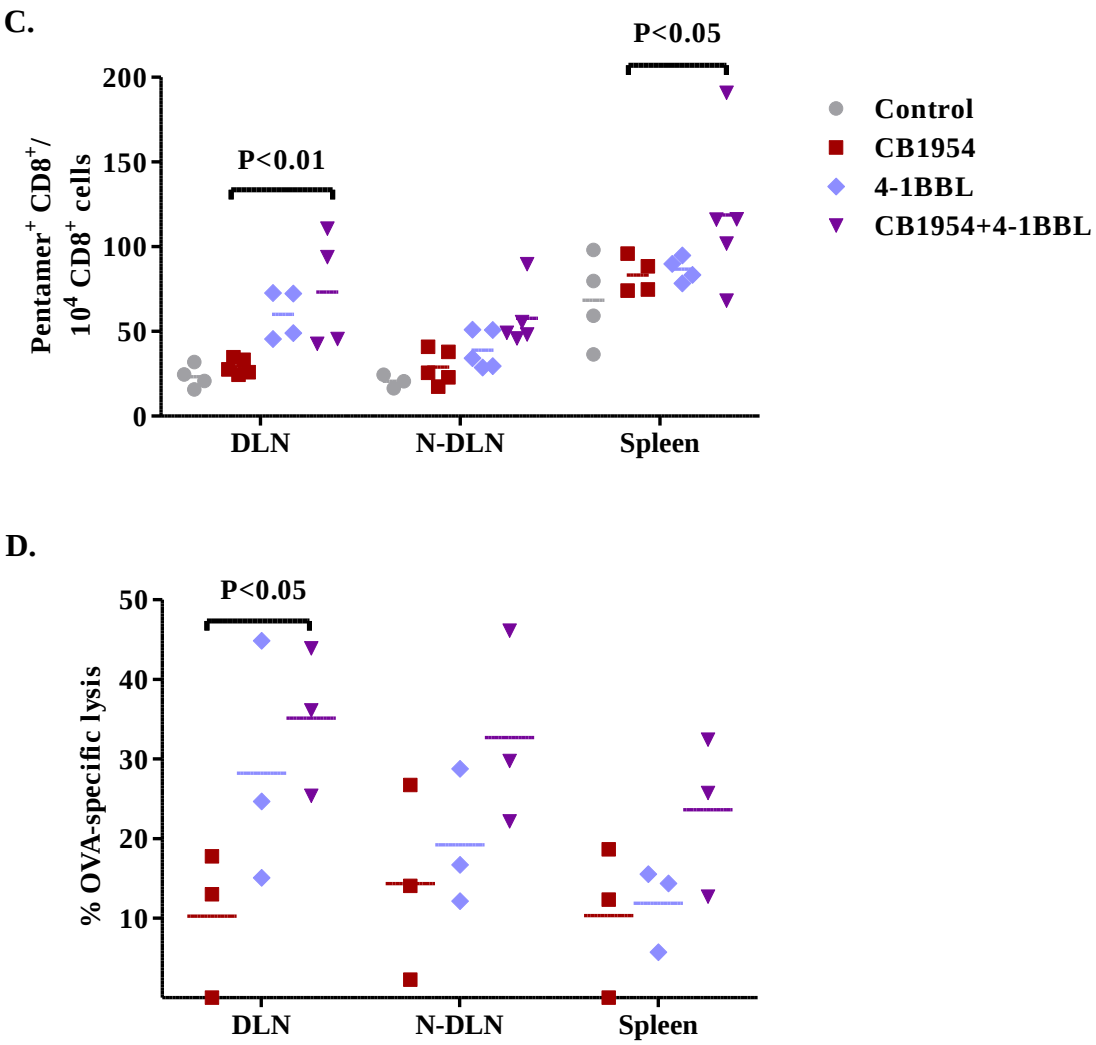


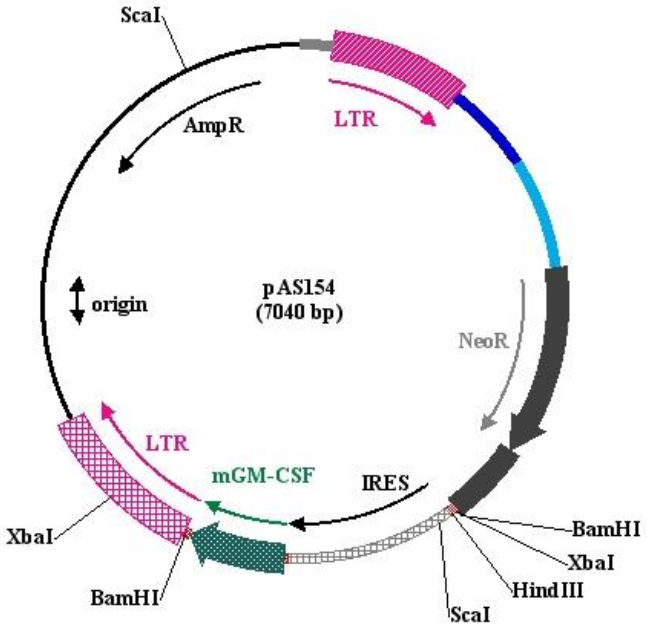
Figure 5-10 …cont. Single cell suspension from DLN, N-DLN and spleen isolated from different treatment groups was prepared. **C**, the frequency of OVA-specific $CD8^+$ T cells after 4 weeks from treatment. Lymphocyte cell suspension was stained for H-$2K^b$/SIINFEKL pentamer, CD3 and CD8 before analysis by flow cytometry. Data points represents the numbers of live pentamer$^+CD8^+CD3^+$ cells relative to total $CD8^+$ cells in different animals and the horizontal line represents the group mean. **D**, *in vivo* OVA-specific CTL response after 4 weeks from treatment. Target cells labelled with $CFSE^{Hi}$ and $CFSE^{Lo}$ were analyzed by flow cytometry and the percentage of target cell killing was determined. Data points represent the percentage of lysed target cells in different animals.
The horizontal line represents the group mean.
The P value indicates the statistical significance as analyzed by two-way ANOVA and post-hoc Bonferroni test.

5.3 $CD8^+$ T cell responses to combined therapy of NR/CB1954 and granulocyte macrophage-colony stimulating factor (GM-CSF)

5.3.1 Generation of pxLNI-murine GM-CSF retroviral vector

For construction of pxLNI-mGM-CSF plasmid, murine GM-CSF (mGM-CSF) cDNA was obtained from Ad.left.NR-mGM-CSF adenoviral vector by *Bam*HI enzyme digestion. The purified DNA fragment (1235bp) was inserted into *Bam*HI linearized pxLNIX retroviral vector downstream the LTR region. After ligation, the plasmid was transformed in competent *E. coli* XL2 bacteria to generate several plasmid preparations designated as pAS154A1 to pAS154A19. The correct plasmid constructs was verified by *Xba*I, and *Sca*I restriction enzyme digestion. Gel electrophoresis analysis of the plasmid digests showed 4 plasmid preparations gave the expected numbers and the approximate band sizes; two fragments of 5460, 1580 bp with *Xba*I enzyme and two fragments of 3637 and 3403 bp *Sca*I digest indicating the right orientation of the insert. DNA sequence analysis of pAS154 plasmids using m-GM-CSF-F1 forward: 5'- CTT TTC CTG GGC ATT GTG G-3' and m-GM-CSF-R1 reverse: 5'- ATG CGG ATA GGT AAC-3' primers also confirmed the correct coding sequence.

A.



B.

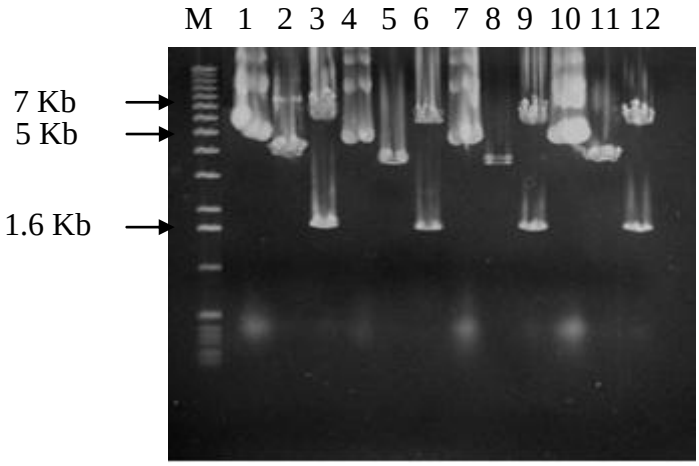


Figure 5-11: Map and restriction enzyme digests of the mGM-CSF expression vector pAS154

A, pAS154 (pxLNI-mGM-CSF) retroviral plasmid map showing the position of the neomycin resistance gene, followed by the poliovirus internal ribosome entry site (IRES) and mGM-CSF gene; expression of both genes is controlled by the 5' LTR region. The map also shows the location of restriction enzyme sites used in the cloning or characterization process. **B**, agarose gel electrophoresis of pAS154A2 (lane 1, 2, 3); pAS154A4 (lane 4, 5, 6); pAS154A9 (lane 7, 8, 9); pAS154A11 (lane 10, 11, 12) plasmid digests. M: size marker (1kb DNA ladder); lanes 1, 4, 7 and 10: uncut plasmids; lanes 2, 5, 8 and 11: *Sca*I digests; lanes 3, 6, 9 and 12: *Xba*I digests.

5.3.2 Generation of $Tramp_{OVA}$-GM-CSF cells

pAS154 was used to transduce $Tramp_{OVA}$ cells in a similar fashion to that used earlier with other gene constructs. The generated $Tramp_{OVA}$-GM-CSF cells were expanded as explained in section 3.4.4 to prepare cell surplus required to inoculate tumour cells in mice.

5.3.3 Characterization of $Tramp_{OVA}$-GM-CSF cells

5.3.3.1 Murine-GM-CSF production by $Tramp_{OVA}$-GM-CSF cells

To validate mGM-CSF production by $Tramp_{OVA}$-GM-CSF cells, the level of cytokine was analyzed in the supernatant of $Tramp_{OVA}$-GM-CSF cultures using ELISA. As shown in Figure 5-12, control $Tramp_{OVA}$ cells do not constitutively secrete m-GM-CSF cytokine; whereas the initial seed stock of $Tramp_{OVA}$-GM-CSF cells (passage1; P1) produced 520 pg/ml per 0.5 $x10^6$ cells per 48 hr, this dropped to 336 pg/ml in passage 4 (P4) that was usually used to seed tumour cells in mice.

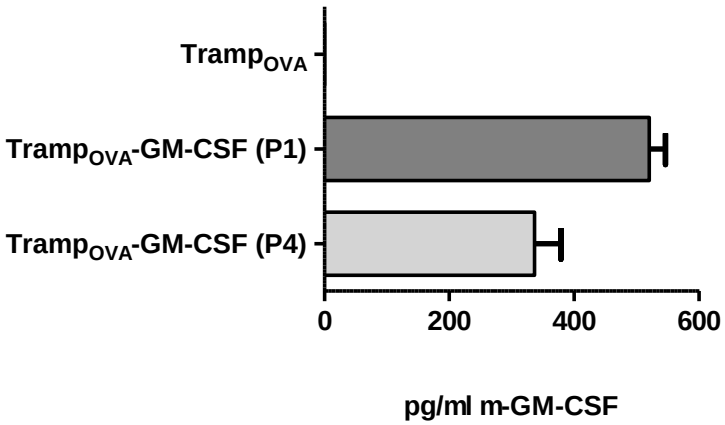


Figure 5-12: Murine GM-CSF cytokine production by $Tramp_{OVA}$-GM-CSF cells
A total of 0.5 $x10^6$ $Tramp_{OVA}$ or $Tramp_{OVA}$-GM-CSF cells were cultured in 6 cm plates for 48 hr. Supernatants were diluted and concentrations of mGM-CSF were determined by ELISA. Values shown represent the mean of quadruplicate cultures (±SEM).

5.3.3.2 Presentation of OVA-epitope by $Tramp_{OVA}$-GM-CSF cells

The ability of $Tramp_{OVA}$-GM-CSF cells to present the SIINFEKL epitope was examined using B3Z T cell activation assay. As shown in figure 5-13, control Tramp-C1 cells showed background enzyme activation; whereas $Tramp_{OVA}$ cells activated B3Z T cells by 3.2 fold compared to that of $Tramp_{OVA}$-GM-CSF cells indicating reduced levels of OVA epitope presentation in $Tramp_{OVA}$-GM-CSF.

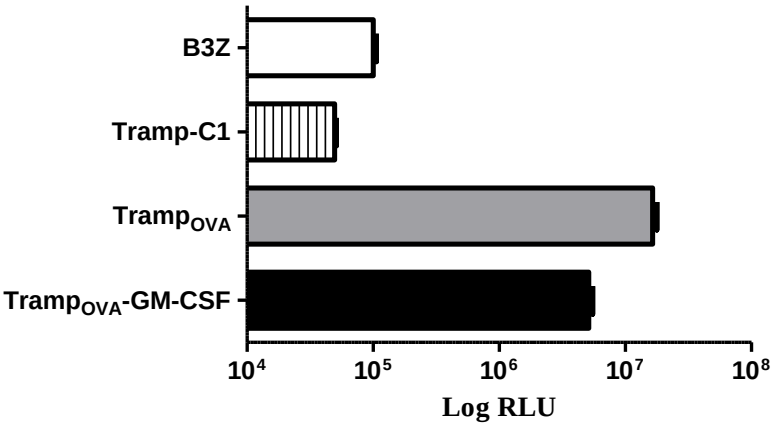


Figure 5-13: B3Z T cell activation by $Tramp_{OVA}$-GM-CSF cells
$Tramp_{OVA}$-GM-CSF or $Tramp_{OVA}$ cells were cocultured at $1x10^4$/well in a 96 well plate with $1x10^5$ B3Z T cell hybridomas for 16 hr. β-galactosidase activity in B3Z T cells was assayed in total culture lysates after incubation with β-galactosidase luminescent substrate for 1 hr at 37ºC. Data are presented as the mean relative luminescence (RLU) of quadruplicate wells (±SEM) on a log scale.

5.3.4 Endogenous $CD8^+$ T cell responses stimulated by combined NR/CB1954 and GM-CSF treatment in a long-term *in vivo* model

In the present experiment we investigated the effectiveness of combining GM-CSF with NR/CB1954 gene therapies in priming and activation of endogenous naïve T cells to boost the generation of long-term antitumour $CD8^+$ T cell responses in wt C57Bl/6 mice.

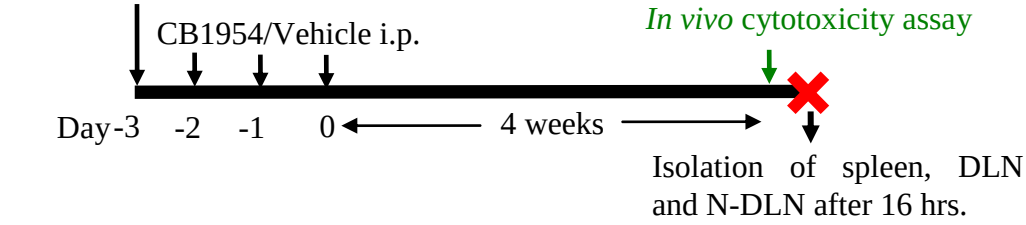
Similar experimental design and groups used in NR/CB1954 and 4-1BBL combined therapy were also used in the present experiment except for that the tumour cells produced GM-CSF instead of expressing 4-1BB Ligand.

Analysis of OVA/K^b pentamer positive $CD8^+$ T cells by flow cytometry showed a preferential increase in the numbers of OVA-specific $CD8^+$ T cells in response to different treatments in DLN but not in the N-DLN and spleen. In CB1954 group, the numbers of OVA-specific CD8 T cells was marginally increased compared to control mice, whereas greater numbers were observed in GM-CSF group relative to both CB1954 treated and control mice. Interestingly, combined treatment of CB1954 and GM-CSF secreting cells induced a significant increase in OVA-specific $CD8^+$ T cells compared to mice receiving either CB1954 or GM-CSF secreting cells alone (Fig 5-14A).

The effector function of OVA-specific $CD8^+$ T cells induced by different treatment was assessed in an *in vivo* cytotoxicity assay. Although, the numbers of OVA-specific $CD8^+$ T cells was predominantly evident in the DLN compared to N-DLN and spleen from different treatment groups; this was not translated into a preferential increase in OVA-specific cytotoxic activity in the DLN where relatively equal levels of cytotoxicity was observed across the lymphoid tissues of each group. As shown in Figure 5-14B, mice receiving CB1954 treatment showed an average

level of 23.5% OVA-specific lysis, while those receiving GM-CSF secreting cells induced higher CTL response (31.8%). As expected the combination of CB1954 and GM-CSF enhanced OVA-specific cytotoxicity by 1.4 fold and 2 fold relative to GM-CSF and CB1954 treatment alone, respectively. These results indicate that combined therapy of CB1954 and GM-CSF enhances the frequency and effector function of memory tumour-specific $CD8^+$ T cell response relevant to NR/CB1954 or GM-CSF single treatment.

A.



B.

Group	Tumour cells	Treatment
Control	10x 10^6 Tramp-C1	CB1954
CB1954	5x 10^6 $Tramp_{OVA}$-NR+ 1.56x 10^6 $Tramp_{OVA}$	CB1954
GM-CSF	5x 10^6 $Tramp_{OVA}$-NR+ 5x 10^6 $Tramp_{OVA}$-GMCSF	Vehicle
CB1954+GM-CSF	5x 10^6 $Tramp_{OVA}$-NR+ 5x 10^6 $Tramp_{OVA}$- GMCSF	CB1954

Figure 5-14: Endogenous OVA-specific $CD8^+$ T cell responses to NR/CB1954 and GM-CSF combined therapy in wild-type C57BL/6 mice

Male C57BL/6 mice, 6-8 weeks old, were injected s.c. in the right flank with mixtures of the indicated tumour cells. One day later, mice received 3 consecutive doses of 20 mg/kg CB1954 or vehicle for 3 days. After 4 weeks, a mixture of $5x10^6$ control splenocytes labelled with 2.5 µM CFSE ($CFSE^{Hi}$) and $5x10^6$ target splenocytes labelled with 1 µM CFSE ($CFSE^{Lo}$) and pulsed with 5 µg/ml SIINFEKL (total $1x10^7$ cells) was administered i.v. into all groups of mice in an *in vivo* cytotoxicity assay. After 16 hr, lymphoid tissues were harvested and single-cell suspensions were prepared for flow cytometric analysis. **A** and **B**, schematic representation of the experimental design and experimental groups, respectively. A and B, schematic representation of the experimental design and experimental groups, respectively.

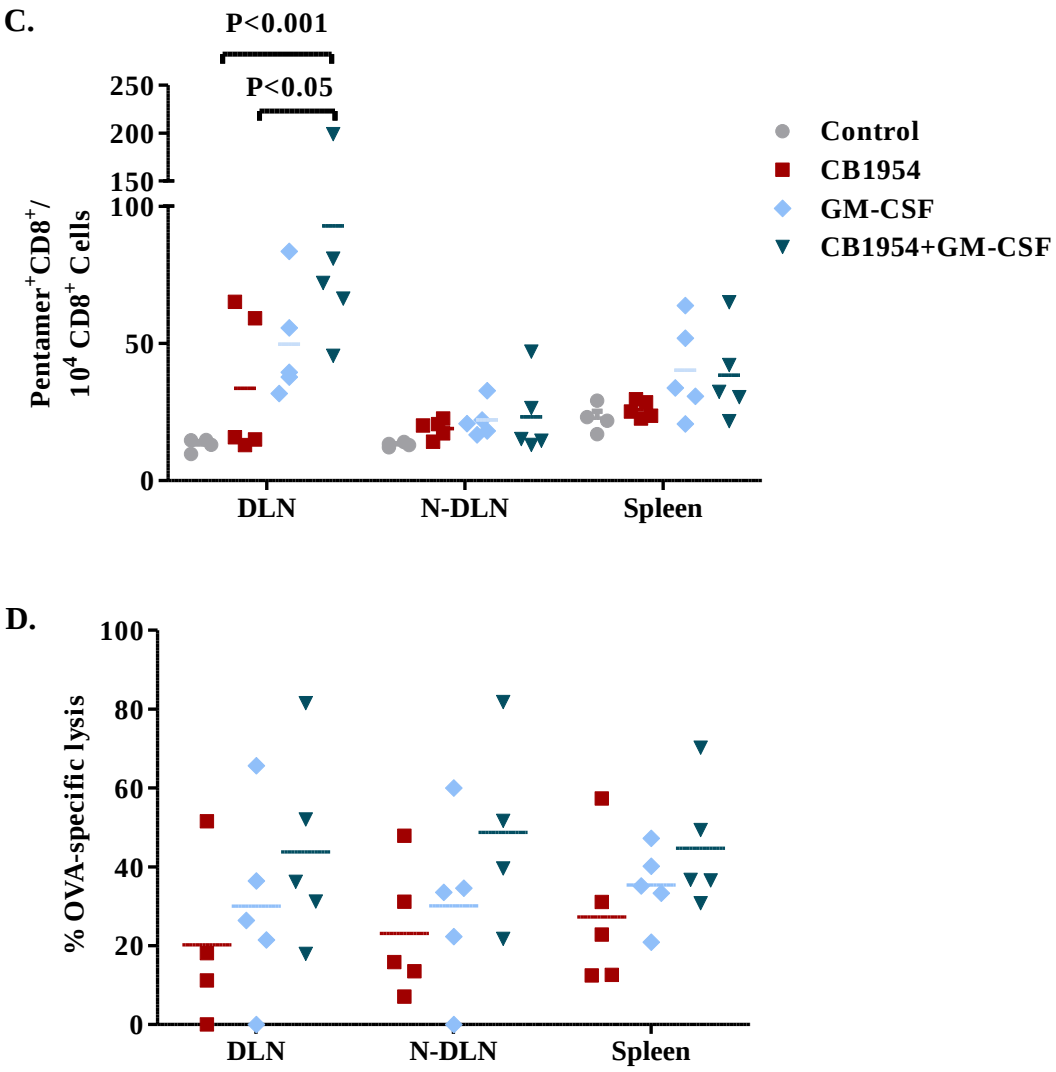


Figure 5-14 …cont. Single cell suspension from isolated DLN, N-DLN and spleen from different treatment groups was prepared. **C**, The frequency of OVA-specific $CD8^+$ T cells after 4 weeks from treatment. Lymphocyte cell suspension was stained for H-$2K^b$/SIINFEKL pentamer, CD3 and CD8 before analysis by flow cytometry. Data points represent the numbers (±SEM) of live pentamer$^+$$CD8^+$$CD3^+$ cells relative to total $CD8^+$ cells in individual animals. **D**, *in vivo* OVA-specific CTL response after 4 weeks from treatment. Target cells labelled with $CFSE^{Hi}$ and $CFSE^{Lo}$ were analyzed by flow cytometry and the percentage of target cell killing was determined. Data points represent the percentage of lysed target cells in individual animals.
The horizontal line represents the group mean.
The P value indicates the statistical significance as analyzed by two-way ANOVA and post-hoc Bonferroni test.

5.4 Discussion

Elimination of tumour cells using NR/CB1954 is likely to increase the amount of tumour antigens available for presentation to $CD8^+$ T cells and to modulate the immunological tumour microenvironment via the release of immunostimulatory molecules, hence favouring generation of antitumour immune responses. However, the initial immuno-adjuvant effect of NR/CB1954 was followed by minimal memory CTL response implying that long-term antitumour immunity and clinical benefit is expected to be limited. This suboptimal immune response could be further improved and amplified using immunotherapy to boost newly primed $CD8^+$ T cell responses and to promote differentiation of activated cells into memory $CD8^+$ T cells. Long-term antitumour immunity is essential for protection against tumour recurrence and metastasis.

Laderach et al. (2002) demonstrated that co-stimulation using 4-1BBL or agonistic anti-4-1BB antibodies is an effective adjuvant capable of enhancing the numbers, effector function and survival of primed $CD8^+$ T cells and suggested its potential use in tumour immunotherapy. Indeed, gene transfer of 4-1BBL molecule into the tumour cells increased the immunogenicity of tumour cells and stimulated tumour immunity (reviewed in Cheuk et al., 2004). In this context, $Tramp_{OVA}$ tumour cells were modified to express 4-1BBL in an attempt to provide a costimulatory signal for efficient priming of naïve T cells, and also to deliver a second costimulatory signal to promote effector functions and survival of T cells activated by APCs.

$Tramp_{OVA}$-4-1BBL tumour cells expressing high levels of 4-1BBL were highly immunogenic and inhibited tumour growth in wt C57BL/6 mice compared to parental $Tramp_{OVA}$ cells. Tumour rejection was associated with long-term survival that suggested generation of memory antitumour immunity (Fig 5-5B). This result

was consistent with Guinns' report that mice inoculated with tumour variants expressing moderate to high levels of 4-1BBL resisted tumour formation and showed increased survival rates. However, only those receiving high 4-1BBL expressing variants demonstrated long-term systemic antitumour immunity against parental challenge (Guinn et al., 2001). Also, other studies have reported that transduction of tumour cells from different origins with 4-1BBL resulted in reduced tumourigenicity and generation of systemic T cell-mediated antitumour immunity in syngeneic mice. Melero et al. and others using depletion experiments and cytokine analysis pointed that tumour rejection by 4-1BBL transduction was mainly $CD8^+$ T cell but not $CD4^+$ T cell dependent (Li et al., 2008; Martinet et al., 2000; Melero et al., 1998; Mogi et al., 2000; Xiang, 1999)

Our results demonstrated that 4-1BBL expression by tumour cells promoted the frequency of tumour-specific $CD8^+$ T cells that showed efficient CTL response (Fig. 5-7B and C). The cytokine signature of OVA-specific $CD8^+$ T cells indicated that they are of type I (Tc1 effector) phenotype as they can secret IL-2 and IFN-γ cytokine upon stimulation (Fig. 5-7D and E). Tc1 cells were reported to exhibit superior therapeutic antitumour effect than their counterparts Tc2 cells, which secret IL-4 and IL-5 cytokines due to their efficient perforin-mediated cytotoxicity to tumour cells and prolonged capacity for *in vivo* survival (Ye et al., 2007). Also, probably due to its increased capacity to localize in tumours (Huang et al., 2005) and to migrate and reside in inflamed tissues (Cerwenka et al., 1999).

Using adoptive transfer experimental system, NR/CB1954 treatment combined with tumoural 4-1BBL expression stimulated further expansion of circulating OT-I $CD8^+$ T cells for 2 weeks compared to either NR/CB1954 or 4-1BBL single treatments following vehicle/CB1954 treatment (Fig 5-9C). After 4 weeks, the frequency of

OT-I $CD8^+$ T cells was reduced in the blood of different treatment groups suggesting that the cells are migrating to non lymphoid tissues (Masopust et al., 2001) or undergoing AICD within the contraction phase. The proportion of OT-I $CD8^+$ T cells in the blood remained marginally elevated in the combined therapy relative to monotherapies by week 4; however the difference was negligible between different groups by week 5. Although, adoptive transfer of OT-I T cells readily allowed us to follow the kinetics of T cell responses to different treatments in the blood; however analysis of OT-I $CD8^+$ T cell accumulation and OVA-specific CTL response within secondary lymphoid tissues were inconclusive due to the small group number and variation in responses within each group.

On the other hand, endogenous $CD8^+$ T cell responses of mice receiving combined therapy of tumoural 4-1BBL and NR/CB1954 showed accumulation of OVA-specific $CD8^+$ T cells in DLN and spleen, however OVA-specific CTL response was significant only in DLN compared to monotherapies alone (5-10C and D). From these data we were able to conclude that tumoural 4-1BBL expression can promote memory immune responses generated by NR/CB1954-mediated tumour cell killing.

Cytokines secreted within the tumour microenvironment dictates the quality and the strength of the interaction between tumour cells and the host immune response. GM-CSF, in particular, is an essential cytokine for stimulating cross-presentation of tumour antigens and activation of antitumour immunity. Although many cytokine (IL-1, IL-2, IL-4, IL-5, IL-6, INFγ and TNFα) was used to transduce tumour cells, GM-CSF was proven to be the most potent inducer of specific and long-term tumour immunity in a preclinical model (Dranoff et al., 1993). This prompted the

use of GM-CSF secreting tumour cells in this study to enhance the suboptimal antitumour immune response of NR/CB1954 treatment.

The amount of GM-CSF secreted by $Tramp_{OVA}$-GM-CSF cells was 672 pg/ml (Fig 5-12), this level was comparably lower than the levels observed from other tumour vaccine models (13–200 ng/ml) (Dranoff et al., 1993; Dunussi-Joannopoulos et al., 1998). The reduced cytokine production by $Tramp_{OVA}$-GM-CSF seems to be due to the expression of GM-CSF as an internal ribosome entry site-dependent second gene under LTR retroviral promoter. Also, the producer cells are a pool of cells that produce different levels varying form negligible to high expression at a single cell levels, thus raising the possibility of reducing the total level of GM-CSF.

Combining local secretion of GM-CSF at the tumour site with NR/CB1954 treatment stimulated regional expansion of memory tumour-specific $CD8^+$ T cells only in the DLN (Fig 5-14C). A regional but not a systemic effect is likely due to reduced cytokine levels reaching the circulation thereby only recruiting APCs to the tumour site to process tumour antigens that subsequently migrate primarily to the draining lymph nodes for priming of tumour-specific T lymphocytes.

Although regional accumulation of tumour-specific $CD8^+$ T cells was induced by combined therapy of GM-CSF and NR/CB1954 treatment, a trend towards increased OVA-specific cytotoxicity was observed in different lymphoid tissues relative to either GM-CSF or NR/CB1954 treatment alone (Fig 5-14D). This systemic CTL response could be explained by the fact that the long 16 hr period following infusion of target cells in the *in vivo* cytotoxicity assay will allow circulation of target cells within the lymphatic system resulting in inaccurate correlation between the numbers of antigen-specific $CD8^+$ T cells and the CTL response within each lymphoid tissue. In support of this hypothesis, Regoes et al.

(2007) showed that target cell killing is detectable within the first hr following adoptive transfer of the target cells and reaches maximal target lysis within ≈ 4 hr (Regoes et al., 2007). Furthermore, this short *in vivo* cytotoxicity assay was shown to be relatively sensitive for detection of the CTL response of central memory (T_{CM}) cells that was shown to induce minimal CTL response compared to effector memory (T_{EM}) cells in a traditional ^{51}Cr release assay (Barber et al., 2003). Therefore a 4 hr *in vivo* cytotoxicity assay would permit ideal analysis of the functionality of T cells accumulated within tissues.

These studies showed that endogenous memory $CD8^+$ T cell responses to either 4-1BBL or GM-CSF treatment alone were comparably higher than NR/CB1954 treatment but this did not reach significance, thus suggesting that immunotherapy using 4-1BBL or GM-CSF alone are superior to NR/CB1954 treatment. Furthermore, tumoural expression of either 4-1BBL or GM-CSF further enhanced the generation of functional memory antitumour $CD8^+$ T cell responses following NR/CB1954 treatment.

6 Results: Effect of combined therapy of NR/CB1954 and 4-1BBL on anergic $CD8^+$ T cell responses *in vivo*

6.1 Introduction

The inability of the immune system to naturally control tumour is likely to be due to immune escape mechanisms employed by the tumours to suppress antitumour immune response. Tumour cells may evade detection by tumour intrinsic mechanisms including down-regulation of MHC class I and peptide transporter genes (TAP-1) and reduced expression of tumour associated-antigens at early phases of tumour growth (Khanna, 1998; Steer et al., 2010). Other tumour extrinsic factors contributing towards immune escape involve T-cell tolerance, production of immunosuppressive cytokines (e.g. TGF-β and IL-10) and immunosuppressive myeloid or regulatory T cells (Mapara and Sykes, 2004). The mechanisms underlying T cell tolerance mainly includes antigen-specific non-responsiveness (anergy), deletion of T cells by apoptosis, and non specific suppression of T cell functions (Lechler et al., 2001). Several studies have shown that anergic $CD8^+$ T cells arise when encountering antigen in the context of minimal costimulation and cytokine help thereby favouring T cells non-responsiveness to subsequent stimulation (Aichele et al., 1995; Boussiotis et al., 1994; Jenkins, 1994). This state of T cell anergy however has been reported to develop early during the course of tumour progression (Staveley-O'Carroll et al., 1998) and to limit protective $CD8^+$ T cell-based antitumour immunity (Kreuwel et al., 2002).

In this context, an envisaged therapeutic approach for cancer treatment would not only eradicate tumours but also reactivate anergic tumour-specific memory T cells and in addition help to activate naïve T cells. To this end, we aim to examine the capacity of NR/CB1954 and 4-1BBL combined therapy to restore the responsiveness of anergized T cells to tumour antigens.

Previous studies have shown that $CD8^+$ T cell anergy can be induced in TCR transgenic mice by multiple systemic exposures to cognate antigenic peptide (Dubois et al., 1998; Frauwirth et al., 2001; Kyburz et al., 1993), while single-injection protocols generate hyper-reactive $CD8^+$ T cells (Pihlgren et al., 1996). $CD8^+$ T cells recovered from anergized TCR transgenic mice display defective TCR signalling, increased sensitivity to cell death, impaired proliferative responses to both *in vitro* and *in vivo* antigenic stimulation, and fail to differentiate into cytotoxic effector cells when stimulated *in vitro* with their cognate peptide. However, exogenously added IL-2 can restore the cytolytic activity and to a lesser extent the proliferative capacity of anergized T cells.

This chapter describes induction of $CD8^+$ T cell anergy in OT-I mice by using the protocol developed by Dubois et al., 1998, to provide a highly reliable homogenous pool of anergized cells for usage in adoptive transfer experiments. The phenotypic and functional characteristics of *in vitro* and *in vivo* stimulated anergized OT-I T cell were demonstrated and ultimately the effect of NR/CB1954 and 4-1BBL combined gene therapy on the state of anergized $CD8^+$ T cells was examined.

6.2 Induction of $CD8^+$ T cell anergy in OT-I BoyJ mice using multiple OVA-peptide injections

6.2.1 Characterization of anergized OT-I $CD8^+$ T cells *in vitro*

Initially the effect of multiple peptide administrations on the phenotype and the proliferative and functional responsiveness of OT-I $CD8^+$ T cells were examined in comparison to naïve and activated T cells. To achieve this, mice were either non-immunized (naïve group), given a single dose of the OVA-peptide antigen to induce a

functional response in T cells (activated group), or treated with 3 doses of OVA-peptide antigen to induce a tolerogenic response in T cells (anergized group) (Fig. 6-1A). Lymph nodes and spleen were harvested and single-cell suspensions were prepared for flow cytometric analysis.

As shown in Figure 6-1B, the average total numbers of OT-I lymphocytes recovered from lymphoid tissues of activated and anergized mice (1.305×10^8 and 0.83×10^8, respectively) were significantly reduced to approximately 1/3 of the numbers recovered from naive animals (3.172×10^8). This was associated with a marked decrease in the proportion of CD3 cells compared to B cells in LNs of both activated and anergized mice relative to naïve mice (Fig. 6-1C), however the skewed proportion of CD3 in spleen of anergized mice was modest compared to activated and naïve animals (Fig. 6-1D).

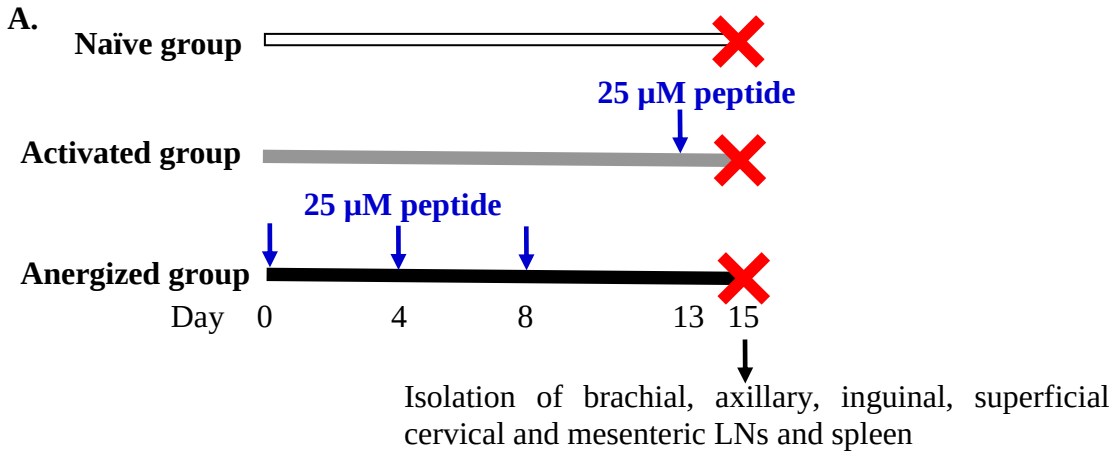


Figure 6-1: Effect of multiple SIINFEKL-peptide administrations on total lymphocytes numbers and CD 8^+ T cell to B cell proportion in lymphoid tissues of male OT-I BoyJ mice.
A, experimental design for inducing T cell activation and anergy. Male OT-I BoyJ mice (4-6 week old) were left untreated, or given single 25µM SIINFEKL peptide i.p 2 days prior analysis, or received 3 doses of 25µM SIINFEKL peptide i.p at 4 days interval followed by 7 days resting period. Lymphoid tissues (pool of brachial, axillary, inguinal, superficial cervical and mesenteric LNs and spleen) were harvested and single-cell suspensions were surface-stained with anti-CD3, -CD8, -B220 for flow cytometric analysis.

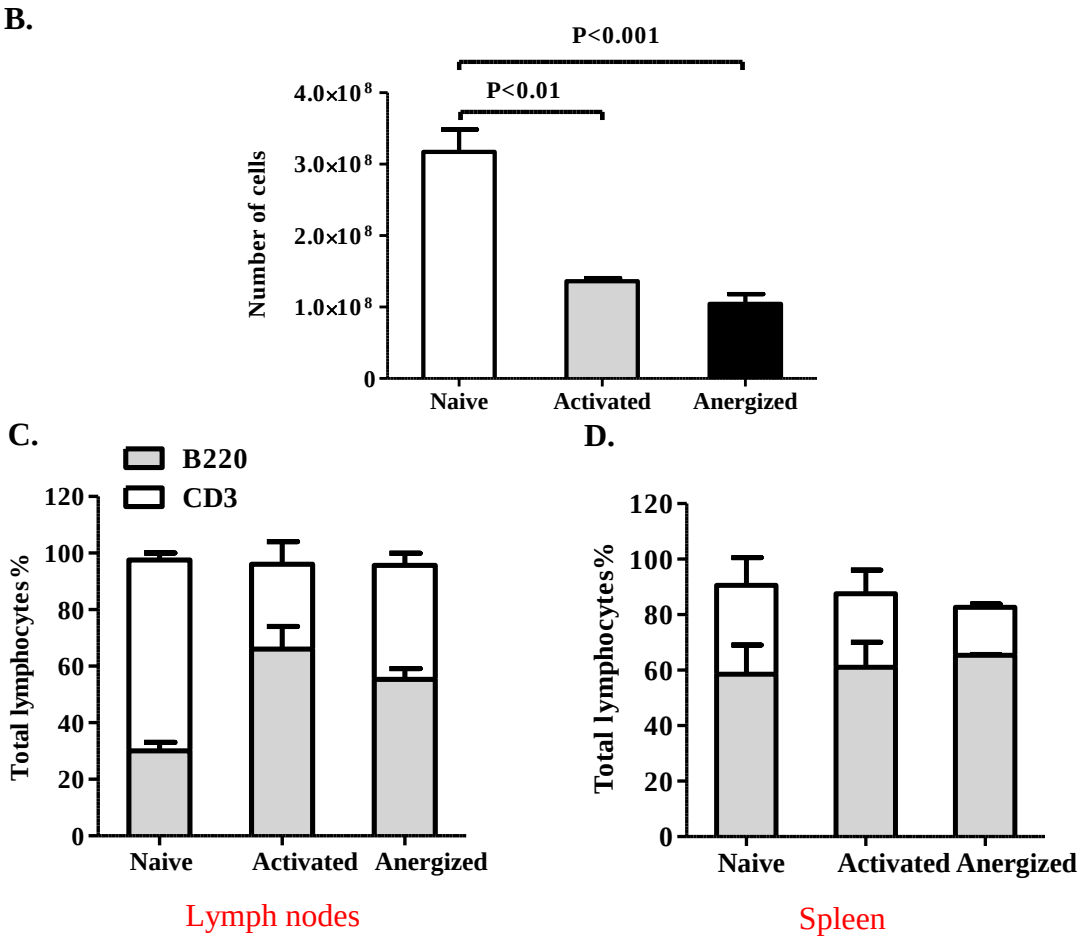


Figure 6-1 …cont. B, total numbers of lymphocytes (±SEM) in lymphoid tissues of naïve, activated and anergized mice (3 mice from two independent experiments). **C** and **D**, the percentages (±SEM) of B and CD3 cells, respectively, in lymph nodes and spleen of naïve, activated and anergized mice (data are derived from two independent experiments). The P value indicates the statistical significance as analyzed by one-way ANOVA and post-hoc Bonferroni test.

Because TCR down-regulation is one of the possible mechanisms underlying induction of T cell tolerance, the effect of multiple peptide administration on the TCR level was investigated. Surface expression of the OT-I TCR (Vα2/Vβ5) was examined by staining for the α-chain of the OT-I TCR using Vα2-specific antibodies. T cells from naïve and activated mice expressed similar levels of the transgenic TCR Vα2-chain in both spleen and LNs. However, the level of Vα2 was marginally reduced on the surface of anergized $CD8^+$ T cells from LNs but was significantly different in spleen (Fig 6-2A).

The level of the late activation markers CD44 and Ly-6 on the surface of T cells was also studied. These two markers were selected based on an earlier observation that they were the only up-regulated markers on the surface of $CD8^+$ T cells stimulated *in vivo* 7 days before analysis with either single or multiple antigenic peptide injections (Dubois et al., 1998). Flow cytometric analysis revealed that CD44 surface expression was highly up-regulated on $CD8^+$ T cells from LNs and spleen of activated mice relative to naive mice, while it was expressed at intermediary level on $CD8^+$ T cells from LNs and spleen of anergized mice (Fig 6-2B). In contrast, Ly-6 activation marker was markedly up-regulated by anergized T cells from LNs and to a lesser extent by activated cells compared to naïve cells. However similar levels of Ly-6 expression were observed in $CD8^+$ T cells from spleen of activated and anergized mice (Fig 6-2C).

These data demonstrate that multiple peptide administration results in reduction of the number of OT-I $CD8^+$ T cells and partially affect the level of TCR on $CD8^+$ T cells. Furthermore the phenotypic characteristics of anergized cells indicated antigen encounter and T cell differentiation into memory cells.

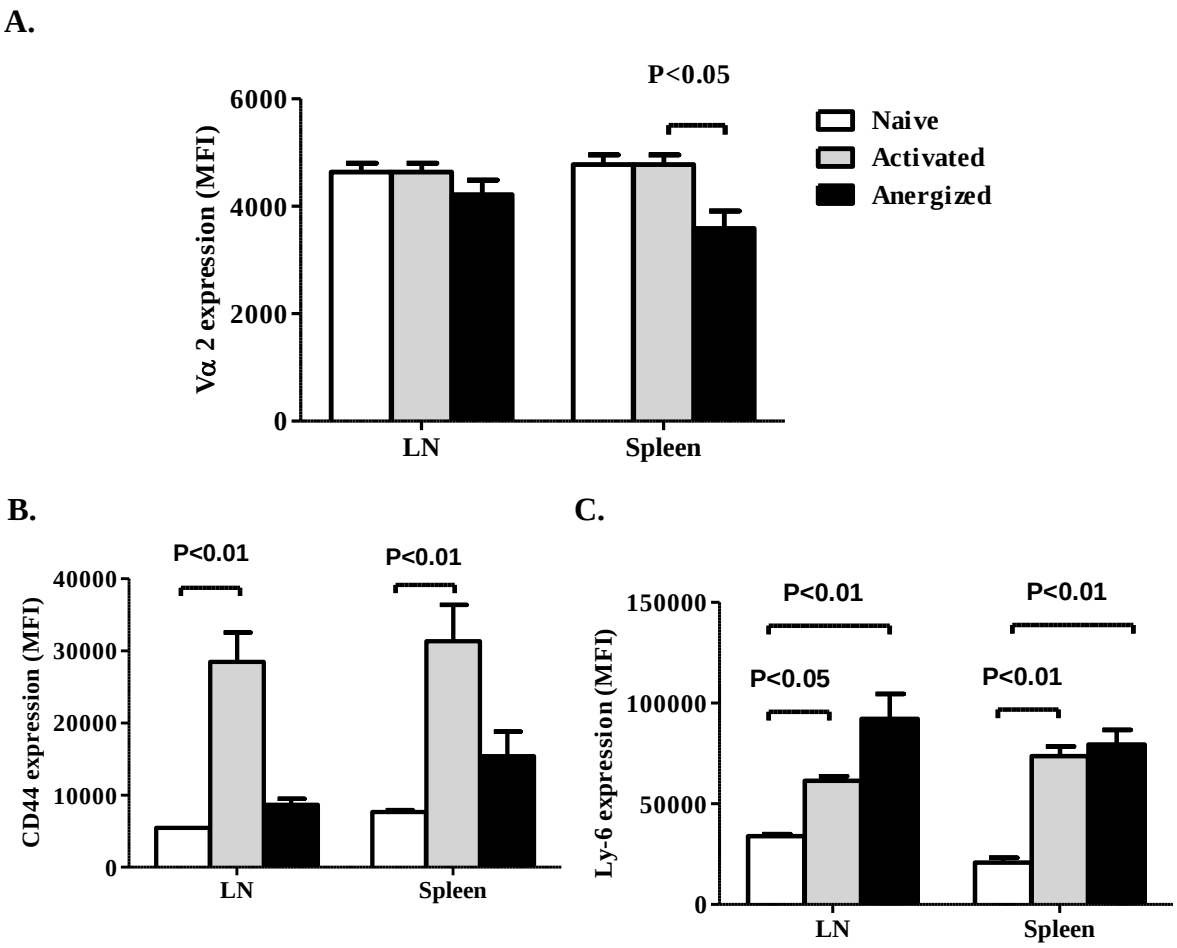


Figure 6-2: Surface phenotype of OT-I CD8$^+$ T cells following single or multiple SIINFEKL-peptide stimulations

Male OT-I BoyJ mice (4-6 week old) were treated as indicated in Figure 6-1A. Lymphoid tissues were harvested and single-cell suspensions were surface-stained with anti-CD3, -CD8, -Vα2, -CD44 and –Ly-6 antibodies for flow cytometric analysis. **A**, **B** and **C**, bar graph representation of the MFI (±SEM) of Vα2, CD44 and Ly-6 expression, respectively, in viable CD3$^+$CD8$^+$ cells from LNs and spleen of naïve, activated and anergized mice.

6.2.2 Functional and proliferative responses of anergized OT-I CD8$^+$ T cells *in vitro*

The main characteristics of anergized cells are: i) being non-responsive to antigenic stimulation and ii) having diminished ability to induce effector functions. Therefore the functional status of CD8$^+$ T cells recovered from anergized mice was assessed by examining, cytokine production (IFNγ), a key Th1 cytokine produced upon CD8$^+$ T cell activation; and CD107a an activation-induced degranulation marker associated with cytolytic function; using intracellular staining and flow cytometry.

After *in vitro* stimulation of OT-I cells with their cognate peptide, the percentage of IFNγ-secreting CD8$^+$ T cells was the highest in lymphocytes from LNs (9.3%) and spleen (3.9%) of activated animals while minimal in lymphoid tissues of anergized and naïve mice (Fig. 6-3A). Similarly, surface mobilization of CD107a was more evident and accounted for > 30% of total CD8$^+$ T cells from LNs and spleens of activated mice, whereas CD8$^+$ T cells from anergized mice showed a marginal increase in CD107a expression relative to naïve cells (Fig. 6-3B).

The proliferative response of anergized OT-I T cells following *in vitro* antigenic stimulation was then examined using thymidine incorporation into DNA of dividing cells as a marker for proliferation. Freshly isolated naïve or anergized OT-I T cells were cocultured with irradiated parental Tramp-C1 or Tramp$_{OVA}$ cells for 3 days. Unlike naïve OT-I cells, which proliferated vigorously upon stimulation with irradiated Tramp$_{OVA}$ cells, anergic OT-I cells showed a modest proliferative response in the presence of Tramp$_{OVA}$ cells (Fig. 6-3C). As expected parental Tramp-C1 cells induced neither naïve nor anergic OT-I to proliferate.

These results demonstrated that multiple SIINFEKL-peptide administrations rendered OT-I CD8$^+$ T cells anergic as confirmed by impaired proliferative response

and altered effector functions including IFN-γ production and up-regulation of CD107a.

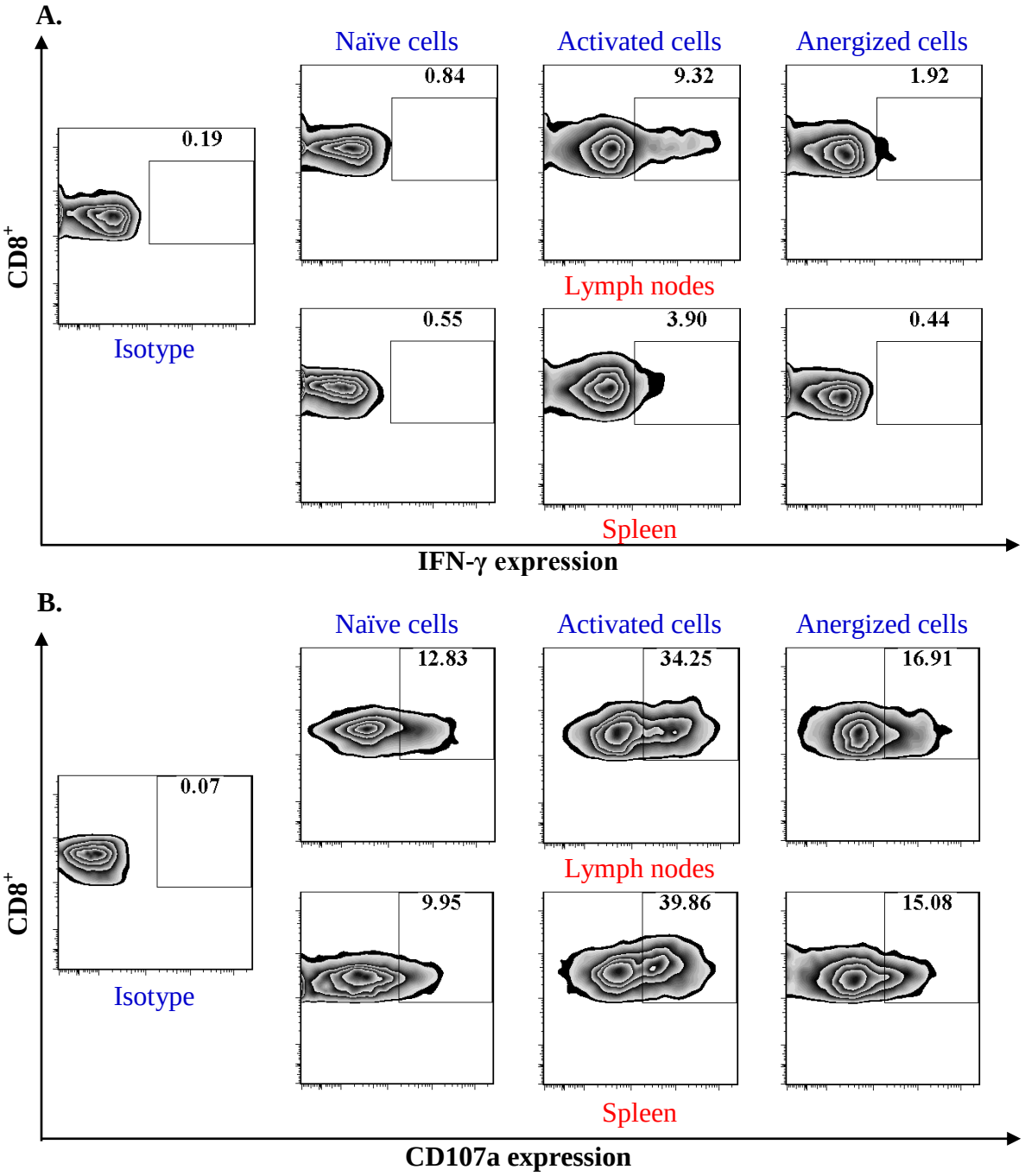


Figure 6-3: Functional and proliferative responses of OT-I CD8$^+$ T cells following single or multiple SIINFEKL-peptide stimulations *in vivo*.
Male OT-I BoyJ mice (4-6 week old) were treated as indicated in Figure 6-1A, then lymphoid tissues were recovered and single-cell suspensions were prepared. Lymphocytes were cultured at 1x10^6/well in 96-well plates and stimulated with 1μg/ml SIINFEKL peptide for 5 hr in the presence of 10 μg/ml monensin and CD107a antibody. After incubation for 5 hr, cells were surface stained with anti-CD3 and -CD8, followed by intracellular staining for IFN-γ and flow cytometric analysis. **A** and **B**, the frequency of IFN-γ$^+$ and CD107a$^+$ CD8$^+$ T cells following antigenic *in vitro* stimulation of OT-I lymphocytes from naïve, activated or anergized cells. FACS plots are gated on CD3$^+$CD8$^+$ cells and numbers indicate the percentage of cells within the illustrated gate. Representative results are shown from two experiments.

C.

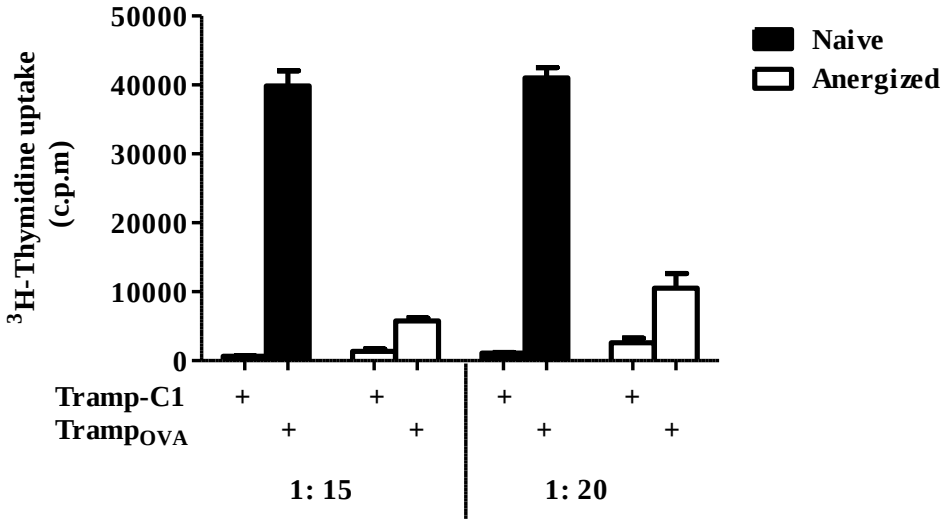


Figure 6-3 …cont. C, *in vitro* proliferation of OT-I lymphocytes from anergized or naïve mice following stimulation with Tramp-C1 or Tramp$_{OVA}$ cells. The number of CD8$^+$ T cells in different lymphocyte suspensions from anergized and naïve mice was adjusted to 1x10^6/ml based on flow cytometric analysis of CD3$^+$CD8$^+$ T cell percentage in each sample. Lymphocytes were cocultured with 1x10^4 120 Gy irradiated Tramp$_{OVA}$ or Tramp-C1 cells at a 15:1 or 20:1 ratio in wells of a 96-well plate. Thymidine was added 16 hr before the plate was harvested following 3 days of coculture. Results are expressed as mean (±SEM) counts per minute (c.p.m) of ^{3}H-thymidine uptake by duplicate lymphocyte cultures for 2 mice.

6.2.3 Characterization of anergized donor OT-I CD8$^+$ T cells in C57BL/6 mice bearing subcutaneous Tramp$_{OVA}$-NR cells

We next set up to examine whether anergic OT-I T cells would retain their hyporesponsive phenotype upon antigenic stimulation *in vivo*. To do so naïve, activated or anergic OT-I T cells were recovered from lymphoid tissues of mice treated as previously discussed in Figure 6-1A, then they were adoptively transferred to C57BL/6 mice bearing Tramp$_{OVA}$-NR tumour cells (Fig 6-4A).

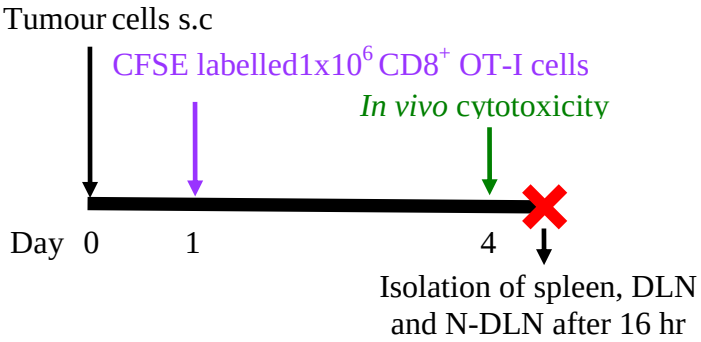
Initially, their ability to accumulate and proliferate upon encountering OVA-tumour antigen was determined. The transferred OT-I cells were pre-labelled with the CFSE to assess proliferation in recipient mice by CFSE dilution.

The frequency of naïve OT-I T cells recovered from the DLNs were significantly increased by ≈ 3 and 4.3 fold relative to activated or anergized cells, respectively (Fig. 6-4C). Consistent with this the frequencies of naïve OT-I cells in the spleen exceeded that of activated or anergized cells by 2.3 and 6.1 fold, respectively. By contrast, no significant difference was observed between naïve, activated and anergic OT-I cells in N-DLNs.

In DLNs, the impaired accumulation of anergic OT-I cells compared to naïve OT-I cells is attributable to a defect in cell proliferation in response to OVA antigen stimulation (Fig. 6-4D). While most of the naïve and activated OT-I cells have accomplished more than 7 rounds of divisions, anergic OT-I cells mainly remained non-divided or went through fewer than 4 rounds of divisions. The division profile of naïve, activated or anergized cells in both lymph node and spleen appears to be comparable suggesting that OT-I T cells were initially activated in the DLNs then they were released to the circulation and migrated to the spleen.

Finally, the ability of anergized OT-I cells to lyse OVA-loaded target cells was examined in an *in vivo* cytotoxicity assay. Because adoptively transferred CFSE labelled target cells were CD45.2 donor derived cells, they could be distinguished from CD45.1^{+} OT-I T cells. Consistent with the proliferation profile, anergized cells showed the least OVA-specific CTL response with an average of 16% specific lysis compared to activated (34.8%) and (47%) naïve T cells in different lymphoid tissues (Fig. 6-4E).

A.



B.

Group	Tumour cells	OT-I T cells
Naive	5x 10^6 $Tramp_{OVA}$-NR	Naïve 1x10^6 cells
Activated	5x 10^6 $Tramp_{OVA}$-NR	Activated 1x10^6 cells
Anergized	5x 10^6 $Tramp_{OVA}$-NR	Anergized 1x10^6 cells

Figure 6-4: Anergized OT-I $CD8^+$ T cell responses in C57BL/6 recipient mice inoculated with $Tramp_{OVA}$-NR tumour cells

Male OT-I BoyJ mice (4-6 week old) were treated as indicated in Figure 6-1A. Lymphoid tissues were harvested and single-cell suspensions were surface-stained with anti-CD3 and -CD8 for flow cytometric analysis. The number of $CD8^+$ T cells in different lymphocyte suspensions from anergized, activated and naïve mice was adjusted to 10x10^6/ml. **A** and **B**, schematic representation of the experimental design and experimental groups, respectively. Male wt C57BL/6 mice, 6-8 weeks old, were injected s.c in the right flank with 5x10^6 $Tramp_{OVA}$-NR cells one day prior the adoptive transfer of 1x10^6/200 µl CFSE labelled anergized, activated or naïve OT-I $CD8^+$ T cells i.v. After 3 days, a mixture of 5x10^6 control splenocytes labelled with 2.5 µM CFSE ($CFSE^{Hi}$) and 5x10^6 target splenocytes labelled with 1 µM CFSE ($CFSE^{Lo}$) and pulsed with 5 µg/ml SIINFEKL (total 1x10^7 cells) was administered i.v. into all groups of mice to assess *in vivo* cytotoxicity. After 16 hr, lymphoid tissues were harvested and single-cell suspensions were prepared for flow cytometric analysis.

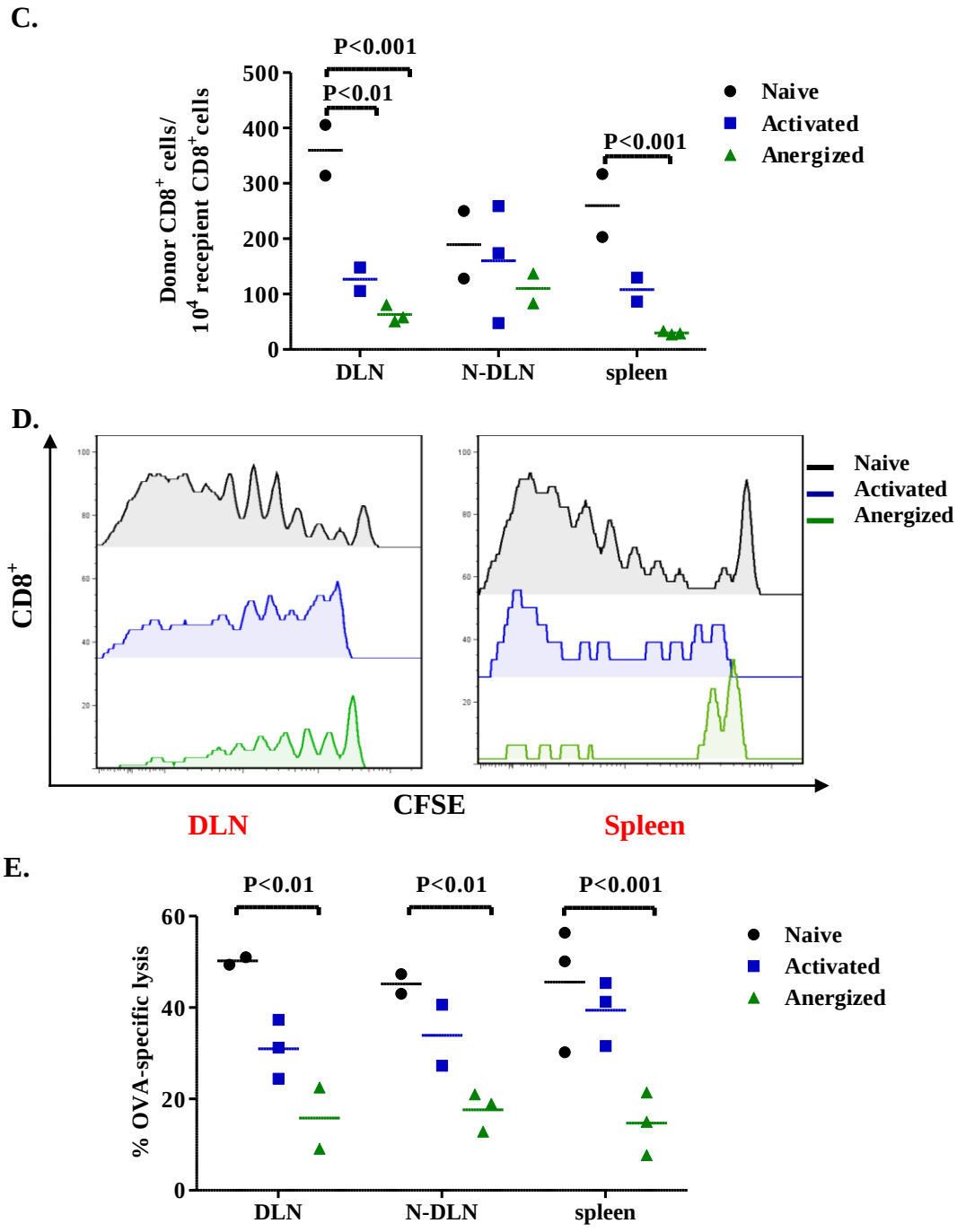


Figure 6-4 …cont. C, The frequency of OVA-specific $CD8^+$ T cells in lymphoid tissues from different groups. Lymphocyte cell suspensions were stained with anti-CD45.1, -CD3 and -CD8 antibodies before analysis by flow cytometry. Data points represent the numbers of live $CD45.1^+CD8^+CD3^+$ cells relative to total $CD8^+$ cells in individual animals. **D** histograms for CFSE dilution of viable $CD45.1^+$ $CD3^+CD8^+$ cells from pooled flow cytometric data from lymph nodes and spleen, recovered from different groups of mice as indicated. **E**, *in vivo* OVA-specific CTL response. Target cells labelled with $CFSE^{Hi}$ and $CFSE^{Lo}$ were analyzed by flow cytometry and the percentage of target cell killing was determined. Data points represent the percentage of lysed target cells in individual animals.
The horizontal line represents the group mean. The P value indicates the statistical significance as analyzed by two-way ANOVA and post-hoc Bonferroni test.

6.3 Examining reversal of OT-I $CD8^+$ T cell hyporesponsiveness by 4-1BBL

6.3.1 Anergized OT-I $CD8^+$ T cells response to $Tramp_{OVA}$-4-1BBL cells *in vitro*

Given that 4-1BB-ligation has been reported to enhance CD8 T cell proliferation, and to selectively rescue activated $CD8^+$ T cells from activation induced cell death (Hurtado et al., 1997), we asked whether the level of 4-1BBL expression by $Tramp_{OVA}$-4-1BBL could provide a sufficient signal to improve the number and survival of anergized $CD8^+$ T cells.

Anergized OT-I T cells were recovered from lymphoid tissues of mice treated as previously discussed in Figure 6-1A. After the percentage of $CD8^+$ T cells was determined by flow cytometry, OT-I T cells were CFSE-labelled and co-cultured with irradiated either Tramp-C1, $Tramp_{OVA}$ or $Tramp_{OVA}$-4-1BBL cells and proliferation was assessed on day 3 and day 5 of stimulation.

After 3 days, anergized OT-I T cells stimulated with Tramp-C1 cells remained substantially undivided; whereas OT-I cultured with $Tramp_{OVA}$ or $Tramp_{OVA}$-4-1BBL cells divided rapidly and underwent several rounds of divisions with comparable proliferation profiles (Fig. 6-5A). Although anergized cells cultured with $Tramp_{OVA}$ cells underwent more rounds of division compared to those cultured with $Tramp_{OVA}$-4-1BBL cells by day 5, they showed marked reduced numbers of viable cells in each round of division relative to anergized cells stimulated with 4-1BBL. This data suggested that 4-1BBL stimulation can enhance the survival of proliferated anergized OT-I T cells.

To confirm the previous result, the magnitude of anergized OT-I T cell proliferation in response to different stimulation was analyzed using ^{3}H-thymidine incorporation assay. As shown in figure 6-5C, significant proliferation of anergized OT-I T cells

was observed when stimulated for 3 days with either $Tramp_{OVA}$ or $Tramp_{OVA}$-4-1BBL, compared to untransduced tumour cells. The difference between $Tramp_{OVA}$ and $Tramp_{OVA}$-4-1BBL in their ability to induce or/and maintain anergic OT-I T cell proliferation was more pronounced at day 5.

In conclusion, although $Tramp_{OVA}$ cells express relatively higher levels of OVA than $Tramp_{OVA}$-4-1BBL cells (as shown in Fig 5-3), the main difference in proliferation is observed at day 5 but not day 3. This suggests that 4-1BBL costimulation provides survival signals that limits the induction of activation induced cell death, and consequently prolongs the proliferative response of anergized OT-I cells.

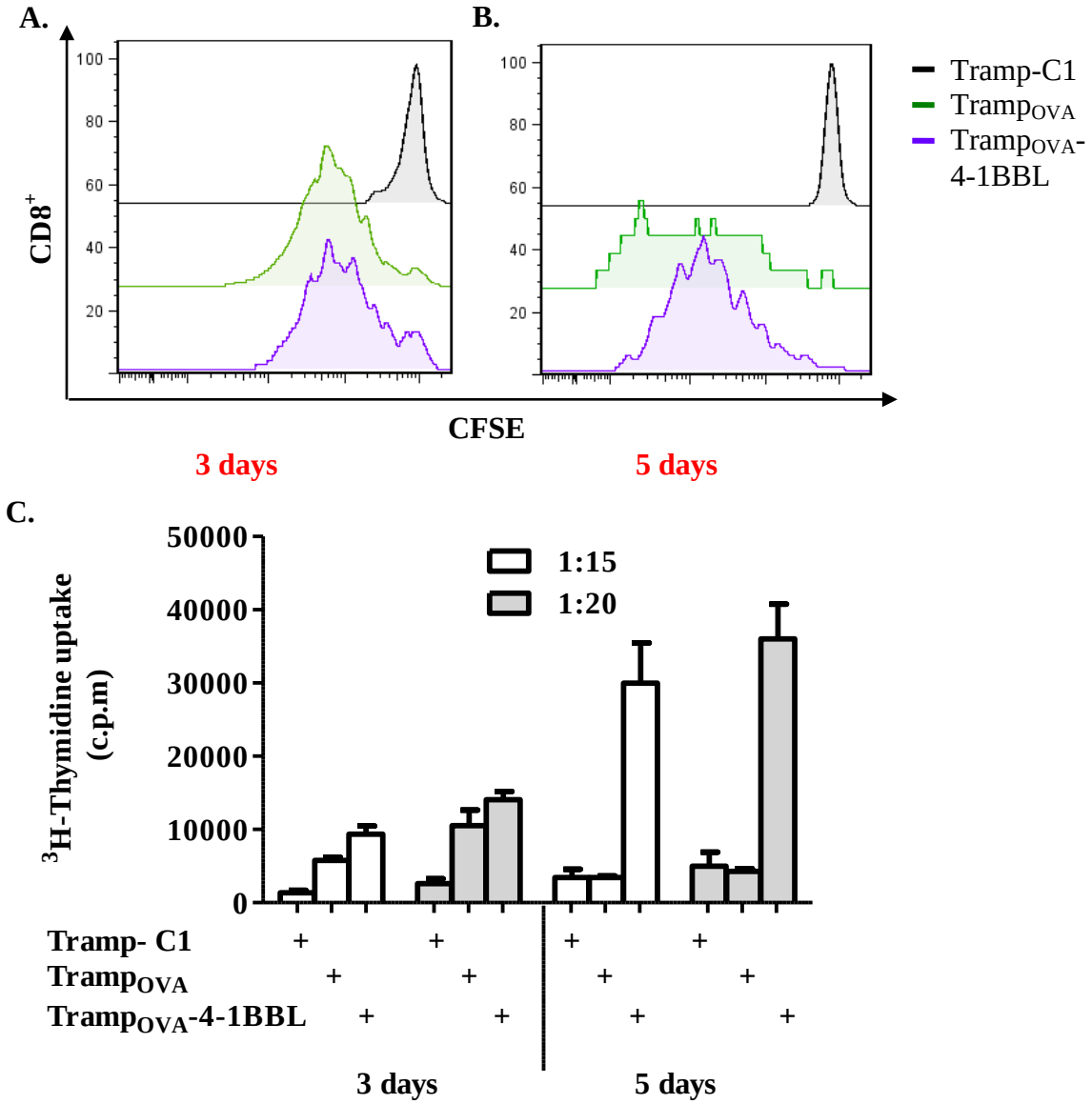


Figure 6-5: Activation of anergized OT-I CD8$^+$ T cells following stimulation with Tramp$_{OVA}$-4-1BBL cells *in vitro*.

Male OT-I BoyJ mice (4-6 week old) were treated as mentioned in Figure 6-1A, then lymphoid tissues were harvested and single-cell suspensions were surface-stained with anti-CD3, -CD8 for flow cytometric analysis. The number of CD8$^+$ T cells in different lymphocyte suspensions from anergized mice was adjusted to 10x10^6/ml. **A** and **B**, histograms of CFSE dilution of viable CD3$^+$CD8$^+$ cells stimulated with irradiated Tramp-C1, Tramp$_{OVA}$ or Tramp$_{OVA}$-4-1BBl cells, for 3 and 5 days respectively. Representative results are shown from 3 cultures. **C**, anergized lymphocytes were cocultured with 1x10^4 120 Gy irradiate Tramp-C1, Tramp$_{OVA}$ or Tramp$_{OVA}$-4-1BBl cells at a 15:1 or 20:1 ratio in wells of a 96-well plates. Tritiated thymidine was added 16 hr before the plate was harvested following 3 days or 5 days of coculture. Results are expressed as mean (±SEM) counts per minute (c.p.m) of ^{3}H-thymidine uptake by duplicate lymphocyte cultures for 2 mice.

6.3.2 Anergized OT-I $CD8^+$ T cell responses to combined NR/CB1954 and 4-1BBL therapy in C57BL/6 mice

As demonstrated in chapter 5, NR/CB1954 and 4-1BBL combined gene therapy stimulated the generation of functional memory tumour-specific $CD8^+$ T cells. Therefore the potential of this combination to restore the proliferative and functional response of anergized OT-I $CD8^+$ T cell was examined in an adoptive transfer setting *in vivo*.

C57BL/6 mice were implanted subcutaneously with different combinations of tumour cells according to their experimental group (Fig 6-6A and B), followed by adoptive transfer of anergized OT-I cells and 3 consecutive doses of CB1954 or vehicle. Two days later, *in vivo* cytotoxicity was assessed. Lymphoid tissues were isolated after 16 hr to determine the frequency of OT-I $CD8^+$ T cells and their distribution in the DLNs, and N-DLNs and spleen.

As shown in Figure 6-6C, the frequency of anergized OT-I T cells in DLNs of control mice implanted with untransduced Tramp-C1 was negligible (Mean=3.5 OT-I cells in 10^4 $CD8^+$ T cells), while the numbers were increased to 56.4 OT-I cells in 10^4 $CD8^+$ T cells in mice bearing $Tramp_{OVA}$-NR cells and receiving vehicle. This indicated that OVA expression by tumour cells induced proliferation of anergized cells compared to OT-I T cells in untransduced tumour bearing mice. The mean proportion of OT-I T cells was further increased by ≈2 fold (104 OT-I cell in 10^4 $CD8^+$ T cell) in mice treated with CB1954 compared to vehicle treated mice signifying that NR/CB1954-mediated cytotoxicity induced the release of OVA-tumour antigen thereby inducing increased accumulation of OT-I T cells within the DLNs. Interestingly, 4-1BBL and OVA expression induced proliferation of anergized OT-I T cells by ≈2.4 fold compared to those in vehicle group; whereas

combining 4-1BBL with CB1954 showed comparable numbers of OT-I T cells (93.5 OT-I cells in 10^4 $CD8^+$ T cells) to that of CB1954 group, however the average was reduced relative to the 4-1BBL group (135 OT-I cells in 10^4 $CD8^+$ T cells).

In N-DLNs, the frequency of OT-I T cells ranged from 6 to 7.7 OT-I cell in 10^4 $CD8^+$ T cell among controls, vehicle and CB1954 groups, whereas it was increased to 32.7 and 34.8 OT-I cell in 10^4 $CD8^+$ T cell in the 4-1BBL and CB1954+4-1BBL groups, respectively. This suggests that 4-1BBL transduced tumours, either alone or in combination with CB1954 treatment, can stimulate proliferation of anergized OT-I T cells in N-DLNs. This observation was also mirrored in the spleen (Fig. 6-6D), however mice implanted with 4-1BBL transduced tumours showed marked significant increase in the numbers of OT-I T cells (259.2 OT-I cells in 10^4 $CD8^+$ T cells) compared to vehicle (31.6 OT-I cells in 10^4 $CD8^+$ T cells) and CB1954 (32 OT-I cells in 10^4 $CD8^+$ T cells) groups. However marginal increase in the frequency of OT-I T cells (1.5 fold) could be seen in the spleen of CB1954+4-1BBL group relative to vehicle and CB1954 group.

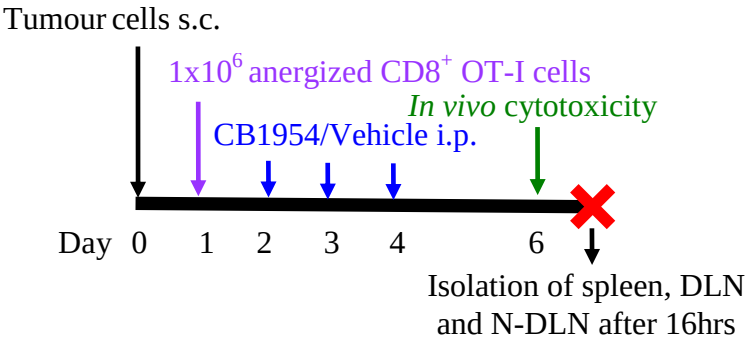
To determine whether in addition to proliferation, the anergized OT-I T cells exhibited CTL activity in response to different treatments, mice were assessed for OVA-specific lysis in an *in vivo* cytotoxicity assay. In this experiment, there was a technical problem in i.v. administration of target splenocytes to vehicle treated group; therefore this group was excluded from the present comparison.

In control mice receiving only anergized OT-I T cells, the background level of OVA-specific lysis was ≈ 13% in different lymphoid tissues (Fig. 6-6E). By contrast CB1954+4-1BBL group of mice showed 74% OVA-specific CTL activity versus 43.6% and 53% in CB1954 and 4-1BBL groups, respectively. Comparable results were also observed in N-DLNs; however in spleen the CTL response of

CB1954+4-1BBL group was increased by 31% compared to mice receiving CB1954 but marginally (5.3%) to 4-1BBL group. This suggests that treatment of mice with a combination of NR/CB1954 and 4-1BBL can slightly potentiate the cytotoxic function of previously anergized cells compared to single treatment with CB1954 but not 4-1BBL treatment.

Taken together, these data suggests that combined NR/CB1954 and 4-1BBL treatment may enhance the functional but not the proliferative responses of anergized $CD8^+$ T relative to NR/CB1954.

A.



B.

Group	Tumour cells	OT-I T cells	Treatment
Control	10x 10^6 Tramp-C1	1x10^6 cells	CB1954
Vehicle	5x 10^6 $Tramp_{OVA}$-NR+ 1x 10^6 $Tramp_{OVA}$	1x10^6 cells	Vehicle
CB1954	5x 10^6 $Tramp_{OVA}$-NR+ 1x 10^6 $Tramp_{OVA}$	1x10^6 cells	CB1954
4-1BBL	5x 10^6 $Tramp_{OVA}$-NR+ 5x 10^6 $Tramp_{OVA}$-4-1BBL	1x10^6 cells	Vehicle
CB1954+4-1BBL	5x 10^6 $Tramp_{OVA}$-NR+ 5x 10^6 $Tramp_{OVA}$-4-1BBL	1x10^6 cells	CB1954

Figure 6-6: Activation of anergized donor OT-I $CD8^+$ T cells following NR/CB1954 and 4-1BBL combined therapy in C57BL/6 mice

Male OT-I BoyJ mice (4-6 week old) were treated as indicated in Figure 6-1A, then lymphoid tissues were harvested and single-cell suspensions were surface-stained with anti-CD3, -CD8 for flow cytometric analysis. The number of $CD8^+$ T cells in lymphocyte suspensions from anergized mice was adjusted to 10x10^6/ml. **A** and **B**, schematic representation of the experimental design and experimental groups, respectively. At day 0, male wt C57BL/6 mice, 6-8 weeks old, were injected s.c in the right flank with tumour cells as indicated for the different experimental groups. One day later, 1x10^6 $CD8^+$ anergic T cells were adoptively transferred in 200 µl PBS i.v. At day 2, mice were given 3 consecutive daily doses of 20 mg/kg CB1954 or vehicle for 3 days. After 2 more days, a mixture of 5x10^6 control splenocytes labelled with 2.5 µM CFSE ($CFSE^{Hi}$) and 5x10^6 target splenocytes labelled with 1 µM CFSE ($CFSE^{Lo}$) and pulsed with 5 µg/ml SIINFEKL (total 1x10^7 cells) was administered i.v. into all groups of mice to assess *in vivo* cytotoxicity. After 16 hr, lymphoid tissues were harvested and single-cell suspensions were prepared for flow cytometric analysis.

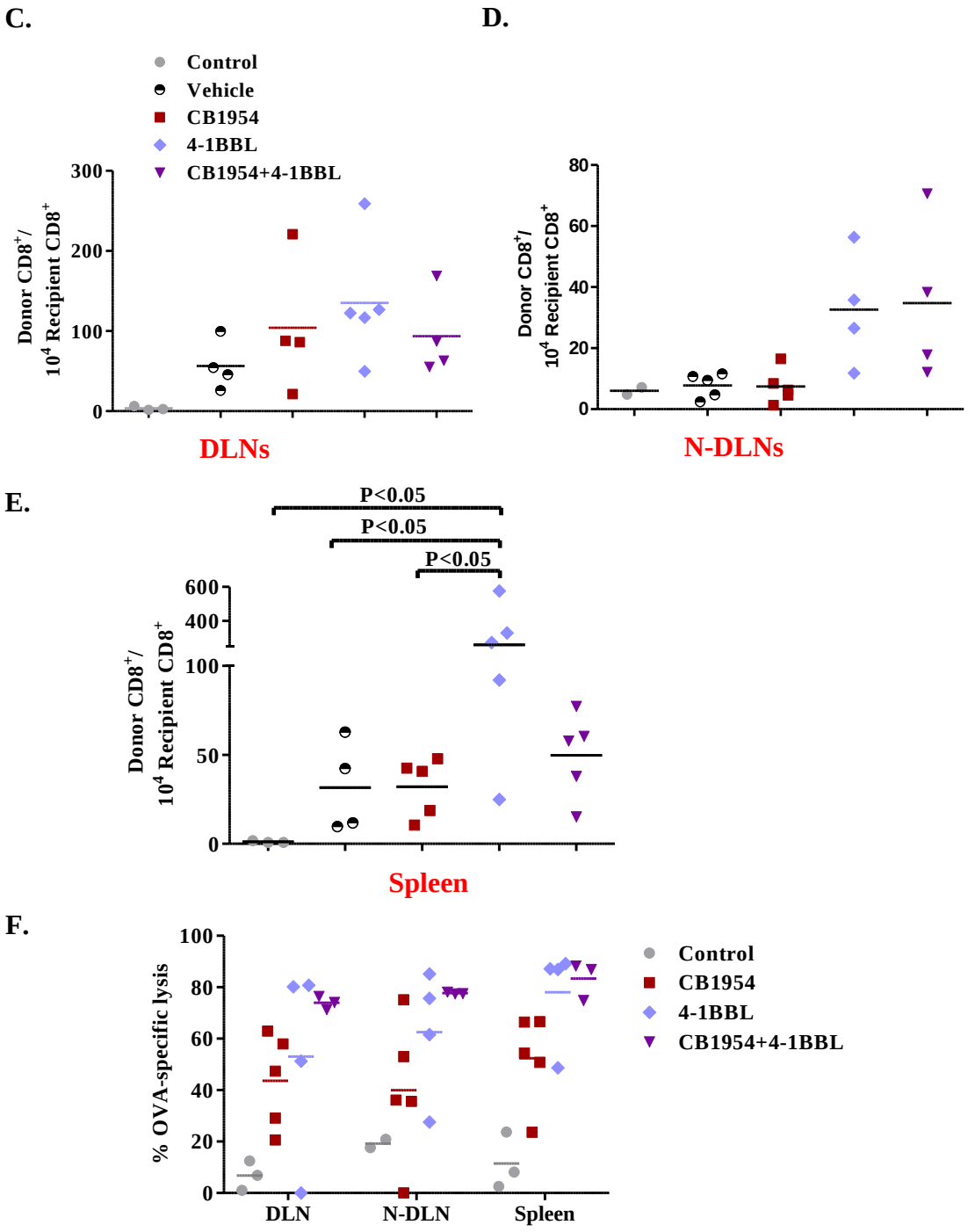


Figure 6-6 …cont. C, **D** and **E**, the frequency of donor anergized OVA-specific $CD8^+$ T cells after 3 days from treatment. Single-cell suspensions were stained with anti-CD45.1, -CD3 and -CD8 antibodies before analysis by flow cytometry. Data points represent the numbers of live $CD45.1^+CD8^+CD3^+$ cells relative to total $CD8^+$ cells in individual mice. **F**, *in vivo* OVA-specific CTL response after 3 days from treatment. Target cells labelled with $CFSE^{Hi}$ and $CFSE^{Lo}$ target cells were analyzed by flow cytometry and the percentage of target cell killing was determined. Data points represent the percentage of lysed target cells in individual animals.
The horizontal line represents the group mean**.** The P value indicates the statistical significance as analyzed by two-way ANOVA and post-hoc Bonferroni test.

6.4 Discussion

Tumours typically express two types of antigens tumour-specific antigens (TSA) and tumour-associated antigens (TAA). The latter represents the majority of known tumour antigens, which are normal self-proteins, but expressed in abnormal quantities or locations. During T cell development, T cells specific for TAAs have been tolerized either centrally or peripherally resulting in deletion or selection of low affinity tumour reactive T cells. This makes self tolerance, although essential for preventing autoimmunity, a substantial obstacle to the generation of immune responses against tumour antigens (Goodnow et al., 2005; Novellino et al., 2005). However, some tumour antigens are encoded by mutant protein or differ in their expression level on tumour cells, which may allow selective targeting of tumour cells. Nevertheless, the development of tolerized antitumour CTLs following some peptide-based vaccines and T cell therapies indicates that T cell tolerance represents a significant challenge for efficient tumour immunotherapy (Mapara and Sykes, 2004; Shafer-Weaver et al., 2009; Toes et al., 1996; Toes et al., 1998). Among the promising approaches adopted to bypass tolerance to endogenous tumour antigens is the provision of additional T cell costimulation. Recently, the TNFR family member 4-1BB has been shown to play a key role in the survival effector and memory T cells. In addition, 4-1BBL appears capable of overcoming T cell tolerance which may contribute to its enhancement of tumour immunity (Mittler et al., 2004; Wilcox et al., 2002; Zhang et al., 2003).

To study reversal of T cell anergy by immuno-gene therapy, OT-I $CD8^+$ T cell tolerance to OVA-tumour antigen was induced using a well documented protocol used with other transgenic TCR mice to induce peripheral T cell anergy (Aichele et al., 1995; Frauwirth et al., 2001; Kyburz et al., 1993; Mamalaki et al., 1993).

In the present study, multiple peptide administrations resulted in a significant decrease within the total frequency of OT-I lymphocyte and 40% reduction in $CD8^+$ T cells in the OT-I mice (Fig 6-1). The remaining $CD8^+$ T cells expressed low CD44 and high Ly-6c levels, a pattern associated with antigen-experienced memory cells suggesting that activated OT-I $CD8^+$ T cells underwent activation induced cell death (AICD) (Fig 6-2B and C). This is consistent with establishment of T cell anergy in TCR transgenic mice in which high doses of peptide led to apoptosis of many of the activated $CD8^+$ T cells (deletional tolerance) and induction of hyporesponsiveness in many of the remaining antigen-specific T cells (CTL tolerance) (Miethke et al., 1994; Ohlen et al., 2002).

One of the mechanisms proposed to explain establishment of $CD8^+$ T cell anergy in the surviving antigen-specific T cells is down-regulation of the TCR (Schonrich et al., 1991). However, flow cytometric analysis of the surface levels of TCR Vα2-chain of OT-I T cells recovered from anergized mice revealed that peptide administration has modest effect on the TCR level and that other mechanisms are likely to be responsible for $CD8^+$ T cell anergy in the present model (Fig 6-2A). Also, by staining against the Vα2-chain of the TCR we ruled out that the anergized $CD8^+$ T cells resulted from selection of cell populations expressing a rearranged second TCR α-chain CD8 as reported in other studies (Heath et al., 1995; Heath and Miller, 1993).

Furthermore, anergic $CD8^+$ T cells showed a state of hyporesponsive phenotype with suppressed proliferative response, reduced IFN-γ production and reduced direct cytolytic activity following *in vitro* antigenic stimulation. Also the results further suggest that there is slight regional difference in the response of cells from spleen or lymph nodes. Interestingly, single-peptide injection stimulated rapid

AICD within 2 days following immunization with the peptide similar to that seen with multiple-peptide injections, nevertheless they showed increased IFN-γ production and CTL degranulation compared to naïve and anergized (Fig 6-3).

The state of anergy observed in the present model is similar to that reported with anergic CD4 or CD8 T cells from other TCR transgenic mice receiving either oral or systemic soluble antigen that is characterized by impaired proliferative response and IL-2 production and inhibition in IL-4 and IFN-γ production (Bercovici et al., 2000; Burkhart et al., 1999; Mueller et al., 1991). This state was also reported to result from a combination of both clonal anergy and cytokine-mediated immunosuppression that involve IL-10 and TGF-β production (Miller et al., 1999; Sundstedt et al., 1997).

The protocol that we used to induce tolerance in OT-I T cells in the present study was shown to completely abrogate the *ex-vivo* cytotoxic effector function in F5 transgenic $CD8^+$ T cells (that are specific for a peptide from the influenza nucleoprotein in the context of $H\text{-}2D^b$) (Dubois et al., 1998). Surprisingly, when applied to OT-I transgenic $CD8^+$ T cells, the same protocol significantly inhibited but not completely abolished the CTL response, as measured by CD107a degranulation marker. This is likely to be due to the difference in the condition of T cell stimulation *ex-vivo*, F5 TCR cells were stimulated with the peptide for 3 days allowing for further functional exhaustion of T cells compared to a 5 hr stimulation period of OT-I T cells in the present study. In agreement with this, Koniaras et al., (1998) reported the CTL activity of OT-I transgenic T cells activated *in vivo* by single peptide injection was completely lost when cocultured with OVA-expressing stimulator cells for 5 days while showing increased cytotoxic activity when examined directly without second peptide stimulation (Koniaras et al., 1998).

The hyporesponsive phenotype of tolerant OT-I $CD8^+$ T cells described in the present study is closely similar to one described by Geramin and coworkers as "split anergy" in an *in vitro* model. In this form of tolerance, T cells lose their capacity to proliferate and produce IL-2 in response to antigen while maintaining their lytic ability. Importantly, anergic $CD8^+$ T cells developed using multiple peptide administrations or due to split anergy can be reversed by IL-2, provided either therapeutically or by activated $CD4^+$ T helper cells (Mescher et al., 2007).

In the different *in vitro* systems used in the present study, OT-I $CD8^+$ T cells were stimulated with either soluble peptide or OVA-expressing tumour cells in the presence of APCs and in absence of $CD4^+$ T helper cells as the OT-I lymphocyte suspension used consisted mainly of $CD8^+$ T cells and APCs. Therefore it was of interest to confirm that the hyporesponsive phenotype of OT-I $CD8^+$ T cells to OVA antigen can be successfully maintained in an *in vivo* tumour setting where the $CD4^+$ T cell helper arm is intact. Upon adoptive transfer of CFSE labelled OT-I $CD8^+$ T cells to mice inoculated with $Tramp_{OVA}$ tumour cells it was apparent that anergized but not activated or naïve OT-I T cells failed to proliferate or differentiate into cytotoxic effectors (Fig 6-4). This suggested that the anergic state of OT-I $CD8^+$ T cell was not altered in the present short term *in vivo* transfer model. In addition, OT-I T cells activated using single-peptide administration were slightly hyporesponsive to s.c. $Tramp_{OVA}$ tumour cells compared to naïve T cells suggesting that single immunization of OT-I mice with OVA-peptide can induce a level of T cell tolerance.

More recently, the role of 4-1BBL costimulation in reversal of the tumour-mediated $CD8^+$ T cell anergy has attracted the interest of Sharma et al. (2009). Their studies demonstrated that the use of peptide-based vaccine containing a novel soluble form

of 4-1BB ligand (SA-4-1BBL) restored the killing response of anergic antigen-specific $CD8^+$ T cell developed as a consequence of tumour growth in a TC-1 tumour model (Sharma et al., 2009). Moreover, results from the same group showed SA-4-1BBL could license $CD4^+CD25^-$ T effector cells (Teff) cells to overcome the suppressive effect of $CD4^+CD25^+FoxP3^+$ T regulatory (Treg) cells (Elpek et al., 2007).

In the present study, CFSE-labelling of anergic OT-I $CD8^+$ T cells stimulated with either $Tramp_{OVA}$ or $Tramp_{OVA}$-4-1BBL expressing cells, although the latter express lower level of OVA antigen, showed that both can promote equivalent *in vitro* expansion of $CD8^+$ T cells by 3 day; while only 4-1BBL expressing cells can increase the rate of proliferation over a longer time (Fig 6-5A and B). In parallel, the rate of proliferation was examined using [3H] thymidine incorporation assay at similar time points confirming that 4-1BBL expressing $Tramp_{OVA}$ cells significantly increased proliferation of anergized $CD8^+$ T cells by day 5 (Fig 6-5C). This suggests that 4-1BBL was able to break the unresponsive state of anergized $CD8^+$ T cell as well as increase the rate of proliferation of these reactivated cells *in vitro*. Similarly, human $CD8^+$ T cells that had become non-responsive either to anti-CD3 antibody alone or to anti-CD3 antibody combined with CD80/CD86 costimulation, and continued to show progressive AICD, were rescued from the anergic state and become reactivated evidenced by further proliferation when costimulated with 4-1BBL *in vitro* (Habib-Agahi et al., 2007). Using an adoptive transfer system, agonistic 4-1BB mAb was also reported to restore the proliferative capacity and cytotoxic function of anergized OT-I $CD8^+$ T cells that were tolerized in C57BL/6 mice by administration of a single dose of OVA peptide i.v. (Wilcox et al., 2004).

One of the aims of this study was to assess the effects of combined NR/CB1954 and 4-1BBL gene therapy on anergized $CD8^+$ T cells responses. To achieve this OT-I $CD8^+$ T cells were anergized *in vivo*, then adoptively transferred into normal C57BL/6 groups of mice previously inoculated with tumour cells expressing various combination of gene therapy and followed by treatment with CB1954 or vehicle. Although combined NR/CB1954 and 4-1BBL treatment did not enhance expansion of anergized $CD8^+$ T cells, it induced a marginal increase in antitumour CTL response compared to NR/CB1954 or 4-1BBL alone (Fig 6-6). Thus, suggesting that combined therapy may restore the functional responsiveness of anergized cells. However, further functional analysis of anergized cells (e.g. cytokine production and CTL CD107a degranulation) is required to preclude interference of endogenous immune response to treatment in assessment of OVA-specific lysis *in vivo*.

The apparent inability of combined NR/CB1954 and 4-1BBL treatment to restore the proliferative response of anergized cells could be for a number of reasons a) increased sensitivity of anergized cells compared to naïve cells to the cytotoxic metabolites released in the tumour microenvironment, b) Limited benefit of the intratumoural 4-1BBL costimulation compared to the generalized effect of agonistic 4-1BB mAb used in experimental tumour model demonstrating the efficacy of 4-BB costimulation in breaking T cell tolerance (Sun et al., 2004). In addition, treatment–mediated activation of anergized $CD8^+$ T cells may have stimulated trafficking of effector $CD8^+$ T cells to non-lymphoid tissues such as liver, lung, intestine or skin, therefore studying enhanced response to combined therapy in our short-term model in non lymphoid tissues rather than in LN could be more informative. Therefore, further studies are required to investigate the unexpected little benefit of the

combined therapy of NR/CB1954 and 4-1BBL in stimulating the proliferation of anergized T cells *in vivo*.

7 Summary, future work and conclusions

7.1 Summary

The effectiveness of NR/CB1954 gene therapy to induce tumour regression has been demonstrated in a variety of animal tumour models (Portsmouth et al., 2007). This system also showed promising results in a phase I studies and preliminary biological efficacy in a phase I/II clinical trial in prostate cancer patients (Palmer et al., 2004; Patel et al., 2009). Although NR/CB1954 acts directly by selectively ablating tumour cells expressing the therapeutic gene, tumour cell death may create a favorable condition for stimulation of the host's own immune response against the tumour. The development of tailored specific-antitumour immune responses may potentially eradicate untransduced tumour cells thereby overcoming limitations due to inefficient gene delivery of the therapeutic gene to tumour cells, and could also help in treatment of metastatic cancers. Ideally, generation of long-term antitumour immunity would be beneficial in preventing tumour recurrence and in increasing the patient's survival. However, the capacity of NR/CB1954-mediated cell death to stimulate specific antitumour immune response is still unclear and requires further detailed studies. Thus, work presented in this study aimed to demonstrate the effect of NR/CB1954-cytotoxicity on activation of tumour-specific $CD8^+$ T cell response, and to evaluate the capacity of this therapy to induce long-term antitumour immunity using an *in vivo* model tumour system.

The first part of the study focused on establishing the model system which involved the initial generation of a model prostate Tramp-C1 cell line that expresses OVA as a neo-tumour antigen. OVA expression was restricted to the cytoplasm using a truncated-cytoplasmic form of the gene to mimic expression of tumour associated antigen. A single cell-derived $Tramp_{OVA}$ clone was chosen based on its capacity to activate OVA-specific T cell responses *in vitro* (section 3.1.2.4). The therapeutic

NR enzyme was introduced to the selected $Tramp_{OVA}$ clone, generating $Tramp_{OVA}$-NR clones. Surprisingly, the relative sensitivity of the clones was not directly correlated with the level of NR expression detected by western blot (Fig 3-9 and 10). The $Tramp_{OVA}$-NR clone showing the highest sensitivity to CB1954 was further selected for *in vivo* studies. Nevertheless, it was observed that the sensitivity of $Tramp_{OVA}$-NR clones was reduced with continuous cell culture. Thus, to minimize variability between experiments due to differences in the level of OVA or NR expression, a large cell surplus was established by expansion of early passaged cells, so that all *in vivo* experiments could use cells at the same passage number (Fig 3-12). This strategy was also applied to other single cell-derived clones expressing therapeutic genes that were further generated in the course of the present thesis.

Down-regulation of transfected or virally transduced genes upon continued growth in culture is a common problem, and could be due to methylation of CpG dinucleotides in the introduced DNA and/or epigenetic effects. Possible approaches that could be adopted to avoid this problem in future include inserting ubiquitously acting chromatin opening elements (UCOEs) upstream of the promoter-driven cassettes within the viral vector to prevent transgene silencing and provide high levels of protein expression (Zhang et al., 2007). Also, flanking the transgenes with chromatin insulators can protect the transgene from repressive position effect (Kuhn and Geyer, 2003).

The next step in establishing the model system was verifying the tumourigenicity of the generated $Tramp_{OVA}$ and $Tramp_{OVA}$-NR single cell derived clones in the presence of a sub-therapeutic dose of OVA-specific OT-I lymphocytes in athymic C57BL6 mice. Although both $Tramp_{OVA}$ and $Tramp_{OVA}$-NR cells stimulated

reasonable tumour growth in a satisfactory duration post tumour inoculation, the size of the tumours was relatively smaller than those achieved by the parental tumour growth (Fig 3-13). In adoptive transfer experiments, a high dose of $10x10^6$ naïve OT-I lymphocytes affected tumour growth and resulted in complete tumour rejection of established tumours in some of the mice, demonstrating that such a dose is highly therapeutic and would obscure the effect of NR/CB1954 on tumour growth (Fig 3-14). The use of a reduced number of OT-I lymphocytes ($2.5x10^6$) was found not to affect tumour progression and thus was more suitable for studying $CD8^+$ T cell response to therapy (Fig 3-16).

In the second part of the study, the initial experiments showed that CB1954 induced complete tumour regression of established $Tramp_{OVA}$-NR tumours in athymic C57BL/6 mice receiving OT-I lymphocytes prior to CB1954 administration (Fig 4-1). Generation of antitumour OVA-specific CTL response in long-term tumour-free survivors was assessed using *in vivo* cytotoxicity assay. Unexpectedly, mere inoculation of the $Tramp_{OVA}$-NR cells in nude mice resulted in activation of adoptively transferred OT-I T cells and stimulation of high OVA-specific cytotoxicity. Administration of CB1954 however slightly increased the antitumour CTL response in lymph nodes but not in the spleen (Fig 4-2). In another experimental setting, marginal expansion of OT-I T cells was also observed in response to NR/CB1954 treatment (Fig 4-6).

Furthermore, studies examining the generation of protective immunity to the enzyme/prodrug therapy showed that lymphocytes from vehicle-treated or CB1954-cured mice adoptively transferred into secondary naïve recipients failed to prevent tumour outgrowth and there was minimal difference in tumour growth rate between the treatment groups (Fig 4-3). However, it was difficult to interpret the results due

to: a) loss of some of the mice early in the course; b) high tumour cell load relative to the effector T cells (50:1); c) absence of $CD4^+$ T cells to provide help during priming of $CD8^+$ T cells for efficient memory CTL generation; d) loss of OVA antigen expression as suggested by loss of the ability to activate B3Z hybridoma (Fig 4-5)

Taken together these results suggested that NR/CB1954 appears to have no obvious effect on the generation of antitumour immunity using the present adoptive transfer tumour model system.

Because of the several limitations with using athymic C57BL/6 mice including: a) health problems and b) high CTL response of adoptively transferred OT-I T cells to $Tramp_{OVA}$-NR cells without further treatment and the possible homeostatic expansion of OT-I T cells in the lymphopenic host, it was decided to evaluate immunocompetent mice as alternative recipients for the adoptive transfer experiments.

Initial establishment of $Tramp_{OVA}$ or $Tramp_{OVA}$-NR tumour growth in wild-type C57BL/6 mice was unsuccessful using the same dose of $Tramp_{OVA}$ cells that was a tumourigenic in athymic animals. However, doubling the cell dose allowed tumour growth by 6 weeks (Fig 4-7). Attempting to improve tumour establishment in the wt C57BL/6 mice, mixing matrigel with the injected tumour cell inoculum did not facilitate tumour development and was associated with a fluctuating phase of growth and regression (Fig 4-8). Another approach explored to enhance tumour development was prior sublethal irradiation of mice, to transiently suppress the immune response; however irregular pattern of tumour growth was evident in 1 and 2.5 Gy irradiated mice (Fig 4-9A and B). Conversely, tumour formation was initiated in a shorter time and progressively developed in 5 Gy irradiated mice (Fig

4-9C). However, $Tramp_{OVA}$-NR tumour cells failed to induce tumour growth in 5 Gy irradiated mice probably due to the additional viral transduction and single cell cloning processes (Fig 4-9D). This further manipulation may have contributed to increased immunogenicity, possibly due to development of an immune response against the transduced therapeutic and antibiotic transgenes or else reduced tumourigenicity due to genetic or epigenetic changes in the different cell clones. Another possibility to explain the inability of $Tramp_{OVA}$-NR cells relative to $Tramp_{OVA}$ cells to induce tumour formation could be attributed to differences in the proliferation rates between the two single cell clones, however this was not explored *in vitro*.

Since stable growth of NR expressing tumours was found difficult to accomplish in the present study, it was decided to investigate the immune response to NR/CB1954 treatment of fresh tumour cell implants rather than established solid tumours. In an adoptive transfer setting, NR/CB1954-mediated cytotoxicity stimulated a modest increase in proliferation and the frequency of donor OT-I T cells only in the DLN four days post CB1954 treatment (Fig 4-10C and D). Furthermore, marginal OVA-specific CTL response was recorded in different lymphoid tissues seven days post therapy; however this experiment was done with limited number of animals per group and the difference was not statistically significant.

Although this system was sensitive to detect slight differences between vehicle and CB1954 treated group, inoculation of OVA-expressing tumour cells alone stimulated an increase in the numbers of OT-I T cells and lysis of OVA-target cells. These results together with earlier observations indicated that s.c. injection of $Tramp_{OVA}$-NR cells can inherently stimulate a significant immune response. Such a response might have been amplified by the presence of high input numbers of high

affinity OVA-specific transgenic OT-I T cells in our model system. In future studies, this system may be further improved by downward titration of the numbers of transgenic OT-I T cells to attain a cell dose that may better reveal any differences in the amount of antigen released from tumours in response to treatment. Selection of other $Tramp_{OVA}$ clones e.g. $Tramp_{OVA}$ clone 1 (Fig 3-6) that express lower level of OVA during the initial screening process may also reduce treatment-unrelated activation of OT-I T cells and improve the sensitivity of the present *in vivo* model system.

As an alternative approach, that was adopted here, *ex-vivo* identification of the endogenous antigen-specific CD8 T cells through the use of MHC class I tetramers or pentamers could give insight into the normal physiological immune responses that may be distorted in adoptive transfer systems.

Examining the endogenous $CD8^+$ T cell responses to NR/CB1954-mediated tumour cell death revealed that the frequency of OVA-specific $CD8^+$ T cells was significantly increased compared to the vehicle-treated group during the expansion phase (7 days) (Fig 4-12C). However, high OVA-specific lysis was again observed just by inoculation of $Tramp_{OVA}$-NR cells without treatment, yet NR/CB1954 treatment stimulated further marginal increase in the OVA-specific CTL response (4-13C). Four weeks post therapy, the numbers of OVA-specific $CD8^+$ T cells in NR/CB1954 was relatively higher than vehicle treated group but did not reach significance. Nevertheless, similar level of OVA-specific lysis was induced in vehicle and CB1954 treated groups.

These results together with earlier observation using short-term adoptive transfer experiments in immunocompetent mice demonstrated that NR/CB1954-mediated tumour cell death stimulates short-lived tumour-specific $CD8^+$ T cell responses and

does not support the generation of long-term $CD8^+$ T cell-mediated immunity. Thus, the third part of the study focussed on adopting additional approaches to enhance generation of possible memory $CD8^+$ T cells.

Other studies have shown that 4-1BB ligation expression on tumour cells can promote antitumour immune response in mice, involving both T and NK cells (Cheuk et al., 2009; Wilcox et al., 2002). However, no previous studies have investigated the combination with NR/CB1954 treatment. Potentially, prodrug administration might reduce the immune response induced by intratumoural 4-1BBL costimulation due to premature ablation of the tumour cells. Nevertheless, the combined effect of prodrug-mediated tumour cells death releasing tumour antigens with the local costimulation might increase immune responses. To test our hypothesis, several stable $Tramp_{OVA}$-4-1BBL single cell clones were generated following 4-1BBL retrorviral-mediated delivery to parental $Tramp_{OVA}$ cells (Fig 5-2). A highly 4-1BBL expressing single cell clone was selected for further studies. Although the level of OVA expression by these cells was markedly reduced relative to that of parental $Tramp_{OVA}$ cells (Fig 5-3), the level of 4-1BBL was sufficient to provide enhanced proliferative and effector function of OT-I cells in *in vitro* comparative studies with parental $Tramp_{OVA}$ cells (Fig 5-4 and 5-5). Furthermore, inoculation of $10x10^6$ $Tramp_{OVA}$-4-1BBL cells failed to establish tumours in immunocompetent C57BL/6 hosts and enhanced survival of mice compared to mice injected with $Tramp_{OVA}$ cells (Fig 5-6A). Further investigation of the generation of memory antitumour $CD8^+$ T cell responses revealed that animals receiving $Tramp_{OVA}$-4-1BBL cells showed significant higher numbers of OVA-specific $CD8^+$ T cells and CTL response primarily in the DLN compared to $Tramp_{OVA}$ bearing mice after ≈ 3 month from tumour inoculation (Fig 5-8B and C). Also, *ex vivo*

stimulation of lymphocytes from different lymphoid tissues of these mice with OVA-epitope induced Tc1 cytokines (IFN-γ and IL-2) production in DLN in mice inoculated with $Tramp_{OVA}$-4-1BBL but not in those bearing parental $Tramp_{OVA}$ tumours (Fig 5-8D and E). A Tc1 response is correlated with a more effective antitumour immunity in the majority of tumour models (Dobrzanski et al., 2000; Kemp and Ronchese, 2001; Sato et al., 2003). These results indicated that immunization with $Tramp_{OVA}$-4-1BBL cells can enhance the development of antitumour $CD8^+$ T cell immunity.

Combining NR/CB1954 treatment with intratumoural 4-1BBL resulted in increased numbers of endogenous OVA-specific $CD8^+$ T cells in DLN and spleen that was associated with OVA-specific CTL response in the DLN but did not reach statistical significance in other lymphoid tissues, when compared to NR/CB1954 or 4-1BBL alone (Fig 5-10C and D). Thus, offering intratumoural 4-1BBL costimulation with NR/CB1954 treatment can promote the development of possible functional memory $CD8^+$ T cells.

The second approach adopted to improve long-term immune response was the use of intratumoural GM-CSF, aiming at in situ recruitment and activation of APCs for optimal antigen presentation and enhancement of T cell activation. Previous studies reported that combining NR/CB1954 treatment with GM-CSF therapy enhance tumour regression and stimulate generation of tumour immunity in a vaccination model (Djeha et al., 2005; Green et al., 2003). However, there has been no direct investigation of cellular component of the immune response. Our results showed that, in comparison with single treatments, immunization with $Tramp_{OVA}$-GM-CSF cells mixed $Tramp_{OVA}$-NR cells combined with CB1954 treatment induced significant expansion of OVA-specific $CD8^+$ T cells and high cytolytic activity

against OVA-target cells in the DLN (Fig 5-14C and D). These results indicated that GM-CSF- secreting tumour cells synergize with NR/CB1954-mediated cytotoxicity to boost long-term antitumour $CD8^+$ T cell responses. These data also support the rationale for a planned phase I clinical trial of adenoviral gene transfer of NR-GMCSF and CB1954 treatment in prostate cancer patients.

Phenotypic characterization of memory $CD8^+$ T cells observed with combined therapies would have been very useful in verifying whether these are central memory cells (TCM) or effector memory cells (TEM). TCM express CD62L and CCR7 receptors necessary for homing to lymph nodes and have limited effector function whereas TEM down-regulated this markers, home to the periphery and can rapidly induce effector functions in response to antigen (Lanzavecchia and Sallusto, 2005). However, regional localization of OVA-specific $CD8^+$ T cells in tumoural DLN may suggest that these cells are of TCM phenotype.

The findings in this part of the study support our earlier hypothesis that costimulation with 4-1BBL or provision of GM-CSF at the site of the tumour together with NR/CB1954 treatment enhances and prolongs $CD8^+$ T cell-mediated antitumour immunity.

One of the reasons of failure of antitumour T cells in eradicating naturally occurring tumours is the development of T cell anergy following persistent exposure to tumour antigens in absence of adequate costimulation. T cell anergy can also occur at the peak of the effector T cell phase due to up-regulation of coinhibitory receptors or by virtue of the immunosuppressive tumour microenvironment. It was therefore of interest to investigate the effects of intratumoural 4-1BB costimulatory ligand combined with NR/CB1954 treatment on anergized tumour-specific $CD8^+$ T cell responses. $CD8^+$ T cell anergy was induced in OT-I mice by multiple systemic

exposure to OVA-peptide according to a well documented protocol (Dubois et al., 1998). Since this was the first study to use this protocol in OT-I transgenic mice, initial experiments were performed to characterize the non-responsive state of OT-I T cells following multiple peptide administrations. In these experiments the anergic state of these cells was compared to naïve cells from non-immunized mice and activated cells generated in OT I mice following a single dose of OVA-peptide. The results showed that administration of either multiple or single dose of OVA-peptide to OT-I mice resulted in a marked reduction of the ratio of CD3 cell/B cells in the lymphocytes recovered from lymph nodes of activated and anergized mice relative to those found in non-immunized mice, however this effect was less pronounced in the spleens from different groups. Unlike naïve and activated cells, anergized $CD8^+$ T cells recovered from the lymph nodes showed a subtle reduction in TCR level, yet a significant down-regulation of TCR was observed in spleen. These results suggested that peptide administration triggered deletional tolerance in activated and anergized mice, while multiple doses of the peptide additionally induced modest down-regulation of TCR levels in the remaining $CD8^+$ T cells. Functional analysis of OT-I T cells from naïve, activated or anergized mice, also demonstrated reduced ability of anergized $CD8^+$ T cells to secret IFN-γ or to induce degranulation and cytotoxicity upon *ex vivo* antigenic stimulation compared to activated $CD8^+$ T cells. This was also associated with reduced capacity of anergized $CD8^+$ T cells to proliferate in response to antigen compared to naïve cells. These results demonstrated that multiple OVA-peptide administration to OT-I transgenic mice induces a state of $CD8^+$ T cell hyporesponsiveness. Consistent with *in vitro* data, adoptively transferred anergized $CD8^+$ T cells showed reduced proliferative and OVA-specific lytic activity in response to s.c $Tramp_{OVA}$ tumours compared to naïve

and activated $CD8^+$ T cells. Thus, confirming the hyporesponsiveness phenotype of anergized $CD8^+$ T cells in our model tumour system.

Interestingly, coculturing of anergized $CD8^+$ T cells with $Tramp_{OVA}$-4-1BBL cells enhanced proliferation of $CD8^+$ T cells compared to those cultured with $Tramp_{OVA}$ alone. Subsequent *in vivo* experiments examining anergized $CD8^+$ T cell responses to combined NR/CB1954 and 4-1BBL treatment demonstrated that similar level of OVA-specific $CD8^+$ T cell expansion was induced with NR/CB1954 single treatment or combined with 4-1BBL. However, the greater level of CTL activity observed in combined therapy was inconclusive due to the possibility of endogenous OVA-specific CTL being generated in response to treatment that may interfere in assessing the CTL response of anergized cells. Therefore, in future studies characterization of the anergized OT-I T cells functional response using the CD107a or perforin expression as markers of CTL activity would be more informative.

7.2 Future work

The main focus of the present study was studying $CD8^+$ T cell responses to NR/CB1954-mediated tumour cell killing however examining the effect of the therapy on $CD4^+$ T cell and NK cell responses will be also important.

The present work showed that combined therapy of NR/CB1954 and 4-1BBL or GM-CSF can enhance generation of long-term antitumour $CD8^+$ T cell responses; therefore it would be of a key interest to examine development of protective antitumour immunity following combined therapy in tumour rechallenge experiments. Furthermore, T cells or NK cells depletion studies will contribute to further delineating which cellular components of the immune system are involved in mediating antitumour immunity.

The main limitation in using the present experimental model system was the inherent immunogenicity of the $Tramp_{OVA}$ cells resulting in difficulty in establishment of tumour growth and assessment of the CTL responses. To overcome this problem a system where OVA expression can be tightly regulated in tumour cells using a tetracycline-inducible system for conditional expression would likely be beneficial (Ryding et al., 2001; Yu et al., 2009). This way, tumours could be implanted and established without any possibility of inducing immune response against OVA. Transgene expression is then induced only during treatment administration thereby allowing analysis of the immune response associated with NR/CB1954 treatment.

Another possible model is the use of B16 melanoma tumour cell line derived from C57BL/6. B16 is a poorly immunogenic and aggressive tumour. It expresses very low levels of major histocompatability complex (MHC) class I molecules, making it difficult for $CD8^+$ T cells to recognize. These cells normally express Tyrosinase-related protein-2 (TRP-2) and gp100 proteins that contain immunogenic MHC class I–presented epitopes (Bloom et al., 1997; Overwijk et al., 1998). Also, TRP-1 is a cell surface protein that can be the target of antibodies against B16 (Overwijk et al., 1999). The availabilities of tetramers for TRP-2 epitopes and TRP 1-specific $CD4^+$ TCR transgenic (Tg) mouse would allow direct analysis of immune responses to therapy without having to introduce model tumour antigens (Cho et al., 2011; Muranski et al., 2008). Another advantages in using this tumour model system, is that the growth of B16 melanoma tumours stimulates expansion of a small proportion of short-lived hyporesponsive TRP-2-specific $CD8^+$ T cells (McWilliams et al., 2006). This endogenous induction of peripheral T cell tolerance during tumour development would potentially eliminate the need for prior induction of non

physiological anergy of adoptively transferred cells to study reversal of T cell anergy.

7.3 Conclusions

The present study demonstrated that NR/CB1954-mediated cytotoxicity increased the numbers of antitumour-specific $CD8^+$ T cell in lymphoid tissues during the expansion phase, indicating generation of a systemic antitumour immune response. However, the primary $CD8^+$ T cell response initiated by the immunogenic NR/CB1954-induced cell death did not support the generation of long-term antitumour $CD8^+$ T cells. Combining intratumoural GM-CSF or 4-1BBL with NR/CB1954 significantly improved the regional antitumour $CD8^+$ T cell responses 4 weeks post therapy. Therefore these data support the particular use of GM-CSF or 4-1BBL in combination with NR/CB1954 therapy as a promising strategy to increase the likelihood of a systemic long lasting antitumour response in cancer patients.

The present study also reports establishment of $CD8^+$ T cell anergy in OT-I TCR transgenic mice, which could be useful for further studies on the ability of gene/immunotherapeutic strategies to overcome tumour-specific $CD8^+$ T cell anergy.

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