Characterising the role of dendritic cells in TCR gene therapy

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I, Alastair Charles Hotblack confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

Adoptive transfer of T cell receptor (TCR) gene-modified T cells is a promising field of tumour immunology. However whilst these cells can potently control tumour growth, this occurs in only a subset of patients. In order to devise new strategies to improve the anti-tumour response we need a clear understanding of the mechanisms by which transferred T cells are activated to kill tumours. Whilst dendritic cells (DC) are required to activate and control T cell function in adaptive immune responses it is not known whether control of tumours by adoptively transferred T cells depends on similar interactions with endogenous DC.

To address this the CD11c.DTR model was used to transiently deplete DC in mice with established B16.F10 sub-cutaneous tumours after having been treated with TCR-transduced T cells. Unexpectedly we found that depletion of $CD11c^+$ cells facilitates enhanced expansion and effector-phenotype differentiation of the transferred T cells. This appears to be mediated by depletion of a DC population with regulatory capabilities. However depletion of DC in the closely related CD11c.DOG model fails to promote accumulation of TCR-transduced T cells. Indeed, in this model DC depletion leads to less T cell infiltration into tumours. These contrasting results following depletion likely reflect the heterogeneous $CD11c^+$/DC compartment in the tumour, where different populations contribute pro- or anti-inflammatory roles. Nonetheless these data suggest that TCR-transduced T cells interact with the endogenous immune system, although the exact nature of this interaction requires further investigation.

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'look on my works ye mighty and despair!'
Nothing beside remains. Round the decay
of that colossal wreck, boundless and bare
the lone and level sands stretch far away.

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-/-	knock out
ACT	adoptive cell therapy
AICD	activation induced cell death
AP-1	activator protein 1
APC	antigen presenting cell
Batf3	basic leucine zipper ATF-like transcription factor 3
BID	BH3 interacting-domain death agonist
BM-DC	bone marrow-derived dendritic cell
CAR	chimeric antigen receptor
CASM	ConA-activated splenocyte media
CCR6,7..	CC-chemokine receptor 6,7..
CD4,8..	cluster of differentiation 4,8..
cDC	classical DC
CDP	common DC progenitor
CDR	complementarity-determining region
CEA	carcinoembryonic antigen
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
ConA	concanavalin A
CSF1	macrophage colony-stimulating factor
cTEC	cortical thymic epithelial cells
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte associated antigen 4
DAG	diacylglycerol
DAMPs	damage associated molecular patterns
DC	dendritic cell
DC1	CD8/CD103$^+$ cDC population
DC2	CD11b$^+$ cDC population

DISC	death-inducing signaling complex
DNA	deoxyribonucleic acid
DT	diptheria toxin
DTR	diptheria toxin receptor
E2-2	E-box protein transcription factor/Tcf4
ER	endoplasmic reticulum
FasL	Fas ligand
FLT3	fms-like tyrosine kinase 3
FSC	forward scatter
GFP	green fluorescent protein
Gy	gray
HLA	human leukocyte antigen
HSC	haematopoetic stem cell
i.p.	intra-peritoneal
i.v.	intra-venous
ID2	inhibitor of DNA binding 2
IFNα or γ	interferon alpha or gamma
IL-2,4..	interleukin 2,4..
Iono	ionomycin
IP3	inositol trisphosphate
IRES	internal ribosome entry site
IRF4,8	interferon regulatory factor 4,8
ITAM	immunoreceptor tyrosine-based activation motif
Lck	lymphocyte-specific protein tyrosine kinase
LCMV	lymphocytic choriomeningitis virus
LN	lymph node
LTR	long terminal repeat
LV	lentivirus
MAGE A-3	melanoma associated antigen 3
MAPK	mitogen-activated protein kinase
MART1	melanoma antigen recognised by T cells 1
MDP	macrophage DC progenitor

MDSC	myeloid-derived suppressor cell
MHCI,II	major histocompatibility complex class I,II
MoDC	monocyte-derived DC
mRNA	messenger RNA
mTEC	medullary thymic epithelial cells
NFAT	nuclear factor of activated T cells
NK cell	natural killer cell
NP	nucleoprotein
ns	not significant
OVA	ovalbumin
PAMPs	pathogen associated molecular patterns
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD-1	programmed cell death protein 1
PD-L1	programmed death-ligand 1
pDC	plasmacytoid DC
PI	propidium iodide
PLCγ1	phospholipase C, gamma 1
PMA	phorbol 12-myristate 13-acetate
pMHC	peptide-major histocompatibility complex
Pre-DC	DC precursor
PRR	pattern recognition receptor
RAG	recombination activating gene
s.c.	sub-cutaneous
SIRPα	signal regulatory protein alpha/CD172a
SLP-76	SH2 domain containing leukocyte protein of 76kDa
SSC	side scatter
T_{CM}	central memory T cells
T_{EM}	effector memory T cells
TAA	tumour associated antigen
TAP	transporter associated with antigen processing
TCR	T cell receptor

TGF-β	transforming growth factor beta
Th1,2..	type 1,2.. T helper cell
TIL	tumour infiltrating lymphocyte
TLR	toll like receptor
Treg	regulatory T cell
TRP1,2	tyrosinase-related protein 1,2
TSA	tumour specific antigen
Vα1,2..	TCR variable alpha chain 1,2..
Vβ1,2..	TCR variable beta chain 1,2..
VEGF	vascular endothelial growth factor
WT	wild type
ZAP-70	Zeta-chain-associated protein kinase of 70kDa

Chapter 1 Introduction

1.1 Immune surveillance of cancer

The immune system has evolved as an intricately detailed cellular system to destroy pathogens. However the concept that the immune system could control and eradicate transformed, cancerous cells that arise throughout our lives was, until relatively recently, widely disputed.

First postulated by Paul Ehrlich in 1909, tumour immune surveillance proposes that tumour cells can be distinguished from healthy cells and that they will be eliminated by the immune system before they are clinically detected (Ehrlich 1909). However lack of convincing evidence meant this theory fell from popular thought and wasn't rekindled until the latter half of the 20th century. Evidence then began to emerge that the immune system was able to control tumour growth and this was formalised as the theory of 'cancer immune surveillance' by Sir MacFarlane Burnet and Lewis Thomas in 1957, who stated:

> *"It is by no means inconceivable that small accumulations of tumour cells may develop and because of their possession of new antigenic potentialities provoke an effective immunological reaction with regression of the tumour and no clinical hint of its existence"*

Burnet argued that it was an *"evolutionary necessity"* that there be a system in place for dealing with mutated, cancerous cells. Central to this idea was the concept that tumour cells would express antigens that would allow the immune system to differentiate them from healthy cells. However at this time the field of cellular immunology was relatively new and there was conflicting evidence over the ability of the immune system to destroy cancerous cells. What was known was that lymphocytes caused the rejection of transplanted tissue-graphs with incredible precision. However the mechanism that caused this and perhaps as importantly, the evolutionary reason for this mechanism, were not understood.

As Thomas stated in 1982:

> *"Surely this intricate and powerful apparatus was not selected in evolution in order to provide experimental surgeons and pathologists with something to do for a living"*.

They believed that the reason foreign tissue-graphs were able to be rejected was as a result of an immune system designed to recognise subtle differences in cells and eradicate those that it deemed 'foreign'. However despite this conviction, the data generated in animal models largely refuted the tumour immune surveillance hypothesis. As Thomas astutely noted:

> *"The greatest trouble with the idea of immunosurveillance is that it cannot be shown to exist in experimental animals."*

This changed in the 1990s when our understanding of cellular immunity improved, partly through the availability of better animal models. It was then that evidence of immunosurveillance began to emerge and the theory eventually became well accepted.

1.1.1 Evidence of immune surveillance

Key to the initial misgivings was work from two labs which showed that athymic, nude mice (which should lack mature T cells) were no more susceptible to spontaneous or induced tumours than wild-type (WT) mice (Povlsen 1974; Stutman 1974). However the flaws in these animal models were unknown to researchers at the time. It was later discovered that these mice were not completely immunocompromised as they did contain a small population of T cells. Secondly these mice had an increased abundance in natural killer (NK) cells (Ikehara et al. 1984; Maleckar and Sherman 1987) which themselves have potent abilities to control tumour growth (Cerwenka and Lanier 2001).

The first conclusive evidence supporting a role for the immune system in tumour control came through experiments in mice lacking interferon gamma (IFN-γ). These experiments showed that in mice injected with an IFN-γ neutralising

monoclonal antibody, transplanted sarcomas grew faster than in control mice. Likewise fibrosarcomas which over-expressed the truncated dominant-negative IFN-γ receptor α were insensitive to IFN-γ and showed more tumorigenicity and less immunogenicity when transplanted into syngeneic mice (Dighe et al. 1994). This IFN-γ mediated control was shown not only in transplantable tumours but also in tumours induced by the chemical carcinogen, methylcholanthrene (MCA) (Kaplan et al. 1998). As well as IFN-γ, later experiments showed that mice lacking perforin (a key element of cellular cytotoxicity) were similarly more susceptible to a variety of tumours than perforin-sufficient mice (Broek et al. 1996; Smyth et al. 2000; Street et al. 2001).

These data showed a clear role for the immune system, particularly lymphocytes, in tumour control. This was built upon in subsequent work, primarily using knockout mice, which aimed to identify that cellular component of immune control. Experiments in mice lacking recombinase activating gene 1 or 2 (RAG-1 or RAG-2) were central to this. These are DNA repair enzymes that are expressed solely in lymphoid cells and are essential for lymphocytes to rearrange their antigen receptors. Mice deficient in RAG-1 or RAG-2 therefore completely lack T cells, B cells and NKT cells (Shinkai et al. 1992). Injection of MCA into these mice was found to induce tumour formation at a greater rate and frequency than WT mice. RAG-2 knockout (-/-) mice also spontaneously formed significantly more neoplastic lesions than WT mice (Shankaran et al. 2001). Interestingly when RAG-$2^{-/-}$ mice were crossed with mice which can't respond to IFN-γ (Stat-$1^{-/-}$), there was no different in the formation of induced tumours compared to RAG-$2^{-/-}$ or Stat-$1^{-/-}$ mice alone. This suggested there was a heavy overlap between these two mechanisms of tumour control. The prospect of T cell mediated control of tumours was given further credence by the identification of a human melanoma-specific antigen able to be presented to cytotoxic T cells (Bruggen et al. 1991). These works were a fundamental step in determining how the immune system controls cancer. However this raised a pertinent question; if the immune system is able to differentiate healthy cells from tumour cells and control tumour growth, why do immunocompetent hosts still get tumours?

1.1.2 Immunoediting

Whilst the theory of cancer immune surveillance postulated that a developing tumour could be controlled by the immune response, this did not fully describe the range of interactions a tumour had with the immune system. Instead Shankaran and Schreiber suggested that the term 'cancer immunoediting' be used. This alteration was based on the observation that cancers do develop in the presence of an intact immune system and that these tumours are less immunogenic than tumours which develop in immunodeficient mice. This lead them to suggest that a developing tumour is constantly sculpted by the immune response to it, and that those tumours that do develop will, by default, be less able to be controlled by the immune system (Shankaran et al. 2001). This immunoediting concept can be separated into three different processes: elimination, equilibrium and escape (Fig 1.1)

'Elimination' refers to what was originally described in the tumour immune surveillance hypothesis; that the immune system will destroy nascent tumour cells before they become clinically relevant. This has been extensively reviewed by Dunn et al. 2002, in brief: The immune response to a tumour begins when innate cells such as NK cells and NKT cells respond to local inflammation and produce IFN-γ. These have direct anti-proliferative and apoptotic effects on the tumour, or can indirectly prevent angiogenesis, which both cause tumour cell death. Tumour antigens are acquired by DC which migrate to the LN and present antigen to T cells. At the same time the escalating immune response recruits NK cells and macrophages which themselves can induce tumour cell death. Lastly tumour antigen-specific T cells migrate from the LN to the tumour and mediate tumour regression. The specific roles of T cells and DC in this anti-tumour response are discussed in more detail later. However although effective, this immune response is not always sufficient to prevent tumour development.

'Equilibrium' therefore refers to when the immune system and the tumour reach a state of dynamic equilibrium, in which further tumour growth is controlled but the tumour is not eliminated. Evidence for this process arose from experiments

in which mice were injected with MCA to induce tumorigenesis. However those mice that did not develop tumours were then injected with antibodies to deplete CD8 or CD4 T cells or neutralise IFN-γ . More than half of mice with these compromised immune systems subsequently developed sarcomas, suggesting that microscopic tumours were being controlled by the immune response and were only able to grow when this control was removed (Koebel et al. 2007). Yet whilst most cancer cells will be eliminated by the immune response in the equilibrium phase, proliferation of the genetically unstable tumour mass will constantly produce new tumour cell variants. During this process the immune system is paradoxically selecting for variants which can escape selective immune pressure. This process has been demonstrated in an MCA-induced tumour from a RAG-2$^{-/-}$ mouse. This expressed a highly immunogenic antigen which, when transplanted into WT mice, was presented by MHCI to CD8 T cells. However subsequent tumours that grew in these mice lacked expression of this antigen due to this immune pressure (Matsushita et al. 2012).

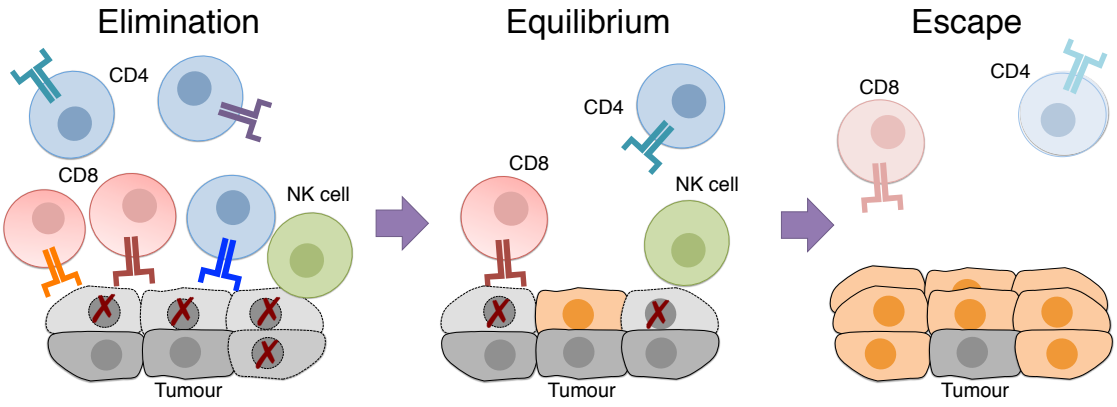


Figure 1.1: *Cancer immunoediting.*

Elimination: The immune response against the tumour is able to kill tumour cells and reject the tumour.
Equilibrium: Incomplete elimination of the tumour leads to tumour cells being present but controlled by the immune response.
Escape: Accumulation of mutations that prevent the tumour being recognised by the immune system, or active mechanisms to induce immune system dysfunction allow the tumour to evade the immune response.

'Escape' therefore represents the final process of immunoediting in which new mutations accumulated in tumour variants prevent their recognition or elimination by cells of the immune system. This leads to uncontrolled growth of the tumour and clinical presentation as a malignant disease. One primary mechanism by which tumour cells can lose immunogenicity is though reduced expression of the tumour antigen, or of the proteins involved in antigen presentation. For example tumours have been shown to limit antigen presentation though reducing expression either of human leukocyte antigen (HLA) directly (Algarra et al. 2000) or of the components of the antigen processing pathways (Seliger et al. 2000). Alternatively, immune pressure can also silence expression of tumour antigens themselves. This is particularly prevalent in immunotherapies that focus on a particular tumour antigen, which is often lost on relapsed/growing tumours (Jäger et al. 1997; Maude et al. 2014; Verdegaal et al. 2016; Yee et al. 2002). Alternatively, tumours can lessen the effect of immune recognition by acquiring mutations that evade apoptosis. For example human tumours have been shown to down-regulate proteins involved in apoptosis signalling such as the TRAIL receptors or caspase proteins. Alternatively tumours can over-express proteins that inhibit apoptosis such as the 'inhibitor of apoptosis proteins' (IAB), FLIP or Bcl-2 (Fulda 2009).

An alternative and perhaps complimentary explanation for tumour immune escape is not through reduced immunogenicity of the tumour itself, but by promotion of immune dysfunction (Willimsky and Blankenstein 2005). Tumours can over-express immunosuppressive cytokines such as interleukin 10 (IL-10) and transforming growth factor beta (TGF-β) (Khong and Restifo 2002). Alternatively tumours can also recruit immunosuppressive cells such as regulatory T cells (Tregs) and NKT cells (Terabe and Berzofsky 2004). More recently the importance of tumour-resident myeloid derived suppressor cells (MDSC) (Gabrilovich and Nagaraj 2009) and dysfunctional dendritic cells (DC) (Pinzon-Charry et al. 2005) in promoting immune suppression have been recognised. These mechanisms all contribute to suppress T cell responses and promote tumour escape.

1.1.3 Evidence in humans

Despite the emergence of convincing data in pre-clinical mouse models, evidence of immunoediting in humans was less forthcoming. However longitudinal studies of patients who were under long-term immunosuppression, such as after organ transplants, did begin to reveal a propensity for cancer formation. This was most prevalent in tumours thought to be of a viral etiology, which had been previously accepted to heavily rely on the immune system for prevention. However tumorigenesis of a plethora of tumours not thought to be induced by viral infection, including but not limited to melanomas and lung cancer, were also shown to be more frequent in immunosuppressed patients. (Vesely et al. 2011). These data therefore replicated the results seen in immune suppressed mice which showed that a suppressed immune system allows tumour growth, or conversely that a functional immune system can prevent tumour growth.

These data from transplant patients suggested that the immune system could mediate tumour elimination in humans as well as mice. Evidence of the equilibrium phase of tumour immunoediting arose from a similar source; numerous incidences of tumours being passed from a seemingly disease free host to an organ transplant recipient have been recorded (MacKie et al. 2003; Penn 1996). These suggest that a clinically undetectable tumour was being controlled by the host which then outgrew when transferred to an immunosuppressed recipient.

Perhaps the most convincing data for the involvement of the immune system in controlling or shaping human tumours is from whether immune cell infiltration is associated with improved prognosis. A wide variety of immune cells have been shown to infiltrate tumours including myeloid cells and different T cell subsets (Bindea et al. 2013) and the composition of immune cells in the tumour can affect patient survival. Infiltration of $CD8^+$ T cells is a positive prognostic factor in colorectal cancer (Galon et al. 2006), melanoma (Zhang et al. 2003) and breast cancer (Mahmoud et al. 2011). In contrast, in line with their predicted role in

promoting tumour escape, infiltration of Tregs has been associated with a poor prognosis (DeLeeuw et al. 2012; Shang et al. 2015). These data suggest that the immune cell composition of the tumour can be an important prognostic factor.

The importance of immune suppression in or by the tumour can also be seen in the striking recent data from patients treated with immune checkpoint inhibitors. Expression of the inhibitory receptor cytotoxic T lymphocyte associated antigen 4 (CTLA-4) on Tregs is a critical regulator of T cell activation (Schwartz 1992). Likewise dysfunction of tumour-infiltrating T cells can be promoted by over-expression of the immuno-modulatory receptor programmed death-ligand 1 (PD-L1), which is a consistent feature of malignant tumours (Chen et al. 2012). Recent attempts to prevent this immunosuppression via monoclonal antibodies that block CTLA-4, PD-L1 or its receptor on T cells, PD-1, have led to promising clinical outcomes (Korman et al. 2006; Pardoll 2012). These data further show that, as in the pre-clinical setting, human tumours subvert immune responses to escape immune control but therapeutic measures to diminish this suppression can enhance pre-existing anti-tumour immunity.

From a position of widespread debate, cancer immune surveillance and later cancer immunoediting has now arisen as a fundamental aspect of tumour growth. Indeed the strength of the data supporting these hypotheses led Hanahan and Weinberg to include ‘avoiding immune destruction’ as one of their updated ‘hallmarks of cancer’ in 2011 (Hanahan et al. 2011). This research has led to great improvements in our knowledge of how the immune system functions and how it is able to control tumour growth. However work still remains to identify the best way to exploit these advances to provide better therapeutic options for cancer patients.

1.2 T cell biology

As discussed above, T cells are one of the principle components of anti-tumour immunity. Along with B cells, T cells form the adaptive immune response that is utilised when innate immune cells such as macrophages, NK cells and neutrophils are insufficient to deal with a pathogen. Whereas innate immunity is characterised by rapid responses which recognise a broad set of pathogen associated signals, adaptive immunity is slower but cells recognise a more defined, specific signal. For T cells this recognition is determined by the specificity of a unique T cell receptor which defines antigen recognition by that cell.

1.2.1 The T cell receptor

T cell receptors (TCRs) are expressed on the surface of all T cells and specify which antigens that T cell is able to respond to. The TCR is a heterodimeric, membrane anchored protein consisting of a disulphide-linked alpha (α) and beta (β) chain (Fig 1.2). Each chain consists of a constant region that spans the cell membrane and a variable region that projects outward and is responsible for antigen binding (Davis and Bjorkman 1988). This variable region recognises short antigen fragments (peptides) when presented by major histocompatibility complex (MHC) molecules, and antigen binding leads to T cell activation via TCR-mediated signalling. However as the cytoplasmic domain of the TCR constant region does not itself confer signalling properties, TCR signalling is dependent on the association with the CD3 molecules; ε, δ, γ and ζ. Phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) on intracellular CD3 leads to the signalling cascade that activates the T cell (Smith-Garvin et al. 2009). This activation pathway is discussed in more detail later (section 1.2.3).

The crucial aspect of the TCR, which itself characterises the T cell, is the highly variable regions that confer antigen specificity. This specificity is partly generated by the assembly of the two different TCR chains, which promotes variation in

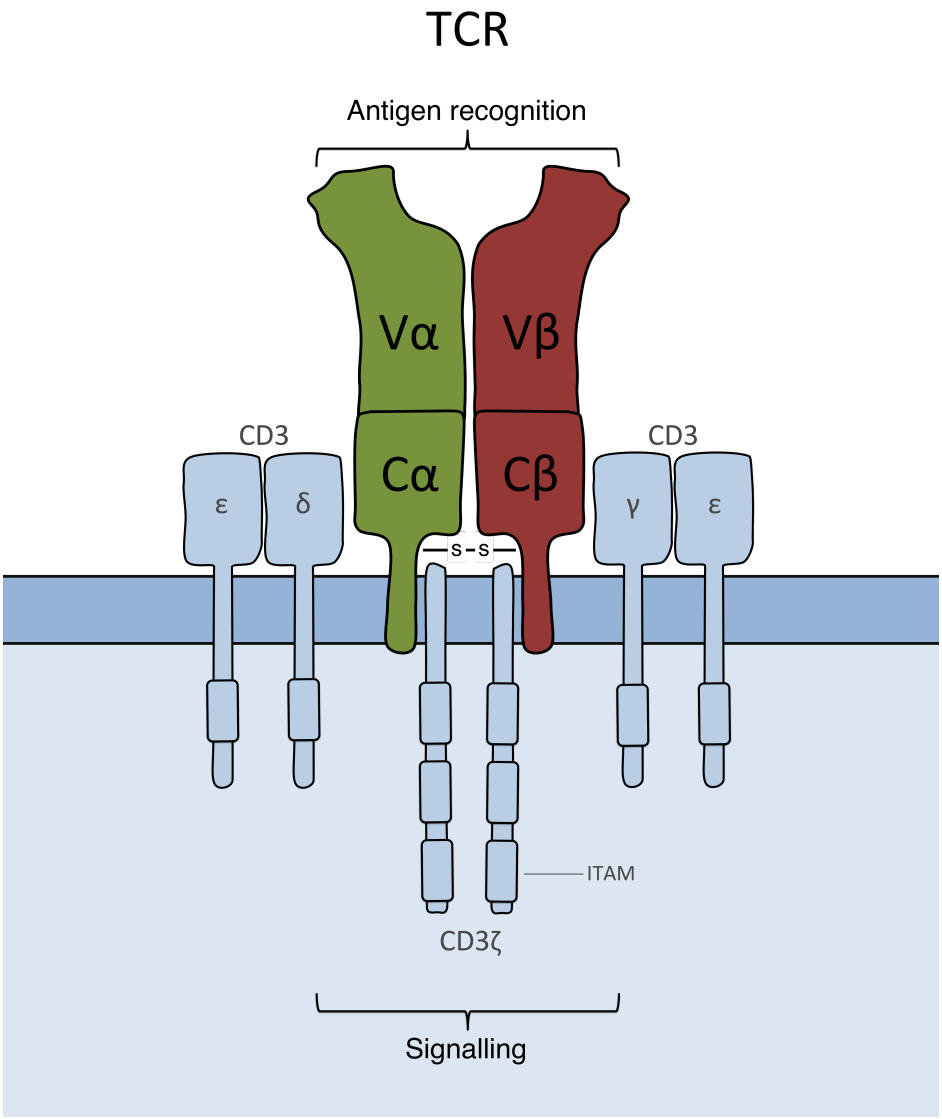


Figure 1.2: *T cell receptor structure.*

antigen binding. However in humans whilst there are numerous Vα and Vβ genes, this combination alone can not possibly confer the antigen specificity necessary to respond to the myriad pathogens encountered by the immune system. Additional variation is added to the TCR via a process known as V(D)J recombination.

As T cells mature in the thymus, they undergo a process of somatic recombination which rearranges the variable (V), joining (J) and, in the β-chain, diversity (D) gene segments that comprise the variable region of the TCR. During these recombination events additional nucleotides are also inserted or deleted at the junctions between these rearranged regions (Schatz et al. 1992). The V(D)J genes that get recombined are found in the complementarity-

determining regions (CDR) of the TCR chains, particularly CDR3 which is responsible for antigen recognition. Therefore this novel CDR3 amino acid sequence in the TCR can theoretically generate an enormous number of TCRs, each with different antigen specificity. It was initially thought that each TCR and therefore each individual T cell was specific for a unique antigen. However there are $>10^{15}$ potential foreign peptides that an immune system would need to be able to respond to (Mason 1998; Sewell 2012). To put that in context there are only 10^{12} T cells in the human body and in order to mount an effective immune response, many of those cells will be clones expressing the same TCR. It has been estimated that of the naive T cells there are only 10^{8} unique TCRs (Arstila et al. 1999). Therefore in order to generate immunity to the breadth of possible foreign peptides, TCRs are thought to be degenerate: a specific TCR will actually recognise a variety of different peptides, and conversely many TCRs will recognise the same peptide (Mason 1998; Sewell 2012).

1.2.2 Central tolerance

Although V(D)J recombination is capable of generating a TCR repertoire that can recognise a vast array of peptides, not all newly generated TCRs will be functional and some may be potentially self-reactive. Therefore during T cell maturation in the thymus, developing T cells undergo a process known as central tolerance in which only TCRs capable of binding to pMHC complexes without self-reactivity are maintained. There are three broad outcomes for T cell progenitors (thymocytes) in the thymus; if the TCR has no affinity for MHC these cells do not receive further TCR signalling and will die by neglect. TCRs with low-medium affinity for self-peptide-MHC will proliferate and mature into functional T cells in what is known as ‘positive selection’. This occurs in the thymic cortex and is controlled by MHC expression on epithelial cells (cTECs). This process also determines whether the CD4/CD8 double positive thymocytes mature into CD4 or CD8 T cells, depending on whether they bind to MHCII or MHCI respectively. However thymocytes also migrate to the thymic medulla where those cells with high self-peptide-MHC affinity will be selected against.

'Negative selection' depends on a population of medullary thymic epithelial cells (mTECs) which, through the expression of the autoimmune regulator (AIRE) protein, can present tissue specific antigens not normally found in the thymus. Alternatively cross-presenting migratory DC can also present tissue antigens in the thymus. Negative selection of these self-reactive T cells can occur through a number of mechanisms, although most commonly self-reactive T cells are deleted from the repertoire. Alternatively the TCR may be further edited to reduce self-reactivity. Lastly to exploit TCRs with high self-reactivity without deleting them, these T cells can be converted into regulatory T cells (Tregs) (reviewed in Derbinski and Kyewski 2010; Hogquist et al. 2005 and Xing and Hogquist 2012).

Theoretically the mature T cells that leave the thymus will either be naive T cells that have the potential to recognise foreign antigen or self-reactive Tregs. However the occurrence of autoimmunity shows that some self-reactive T cell clones do escape central tolerance and migrate into the periphery. To prevent these T cells from mediating damaging autoimmunity however these cells are subject to peripheral tolerance. This includes T cell suppression through regulatory T cells (Tregs) which produce immuno-suppressive cytokines such as IL-10 and express inhibitory receptors such as CTLA-4. These concepts will be discussed in the context of Treg function later. In additional DC can also mediate peripheral tolerance by presenting antigen to self-reactive T cells and induing T cell hypo-responsiveness. The role of DC in peripheral tolerance will be discussed in chapter 1.3.

For the T cells not under the control of central or peripheral tolerance, in order to assume effector functions, naive T cells need to bind to peptide as presented by MHC. A naive T cell will then undergo a sequence of signalling reactions that lead to T cell activation and proliferation. This process governing T cell activation is summarised in Fig 1.3 and discussed in more detail below.

1.2.3 T cell activation

Upon binding of the TCR to a peptide-MHC complex (pMHC) a series of phosphorylation events drives transcription factor mediated gene expression. An important facet of this TCR-pMHC binding is the CD8 and CD4 co-receptors. These are expressed on the cell surface of CD8 or CD4 T cells and are able to bind to either MHCI or MHCII molecules respectively. Whilst these may act to help stabilise the TCR-pMHC interaction, their more important function is to bring the lymphocyte-specific protein tyrosine kinase (Lck) into contact with the TCR complex (Artyomov et al. 2010). Lck is bound to the cytoplasmic domain of the CD8/CD4 co-receptor and upon co-receptor binding to MHC, Lck is able to phosphorylate ITAMs on the CD3 chains. Phosphorylation of ITAMs then recruits the cytosolic zeta-chain-associated protein kinase of 70kDa (ZAP-70) which itself is phosphorylated by Lck. The activated ZAP-70 can then phosphorylate two adaptor molecules; linker of activated T cells (LAT) and SH2 domain containing leukocyte protein of 76kDa (SLP-76). The phosphorylated form of these molecules then form a complex which activates phospholipase C gamma 1 (PLCγ1). Finally, this produces inositol trisphosphate (IP3) and diacylglycerol (DAG). These two substrates induce T cell activation by three separate mechanisms. IP3 causes the release of calcium from the endoplasmic reticulum into the cytosol and activates calcineurin, which dephosphorylates and activates a transcription factor; nuclear factor of activated T cells (NFAT). Alternatively DAG can de-inhibit the transcription factor NFκB or activate the mitogen-activated protein (MAP) kinase cascade and activate another transcription factor; Activator protein 1 (AP-1). These three transcription factors (NFAT, NFκB and AP-1) then translocate to the nucleus and induce the transcription of a variety of genes necessary for T cell proliferation, differentiation and cytokine production (Malissen and Bongrand 2015; Smith-Garvin et al. 2009).

Whilst the molecular mechanisms behind T cell activation are now well understood there remains uncertainty over the initial triggering event for TCR signalling. It was initially supposed that upon TCR-pMHC engagement,

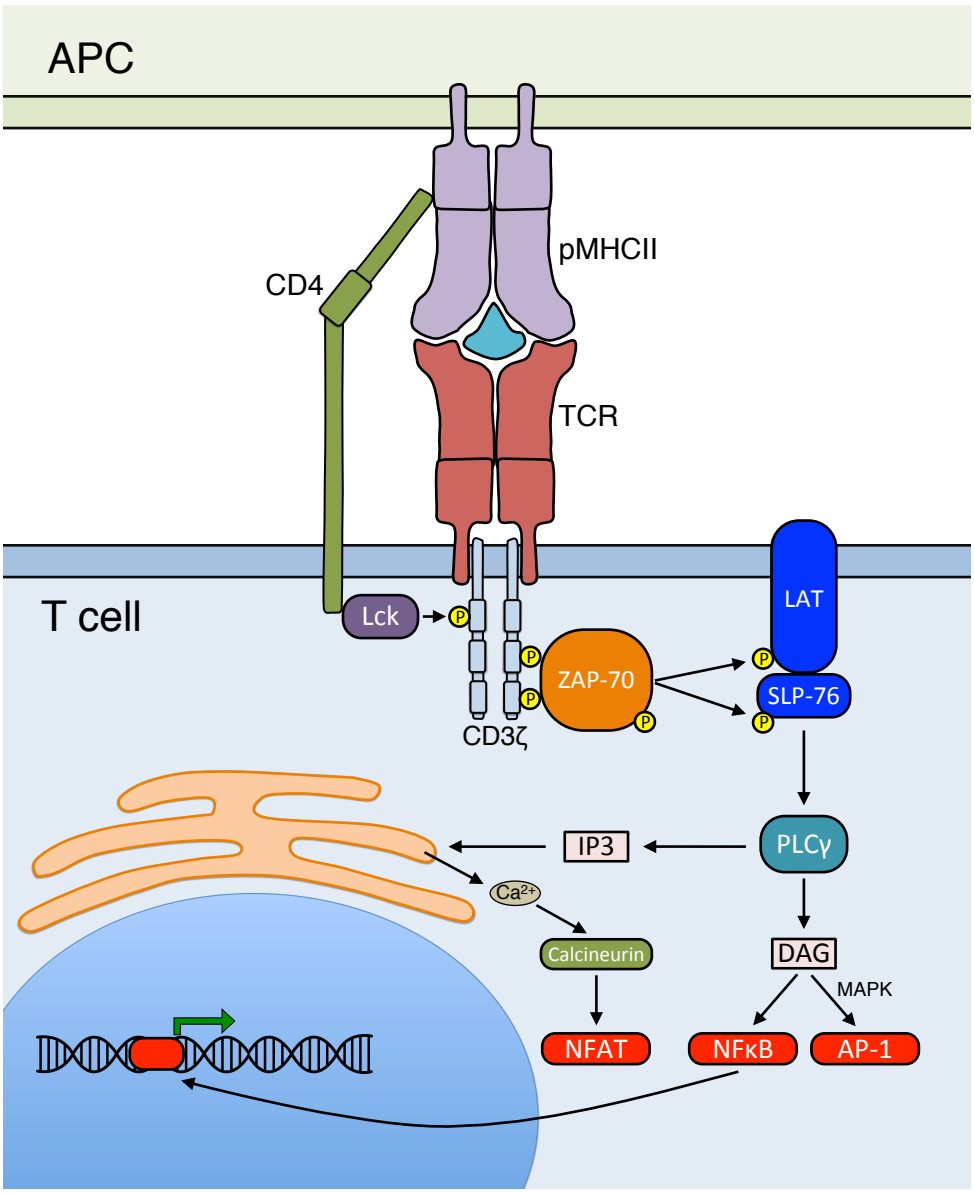


Figure 1.3: *T cell receptor signalling.*

conformational changes in the intracellular TCR regions allowed phosphorylation of ITAMs and the signal was thusly propagated. Whereas this was true of other transmembrane receptors such as G protein-coupled receptors, the relevance of conformational changes to the TCR-CD3 complex has been questioned (Malissen and Bongrand 2015). Indeed, chimeric antigen receptors (CARs) have utilised CD3-ζ signalling to induce T cell activation in a setting where changes to TCR conformation are irrelevant (Sadelain et al. 2013). An alternative explanation is one based on the spatial arrangement of proteins at the site of TCR-pMHC engagement. The 'kinetic segregation model' supposes that the

TCR/CD3 complex is continuously being simultaneously phosphorylated by Lck and subsequently dephosphorylated by the phosphatases CD45 and CD148. However upon the binding of the TCR with pMHC, the surfaces of the T cell and antigen presenting cell (APC) are brought close together and this is sufficient to exclude proteins with large ectodomains (such as CD45 and CD148) from this zone. This steric exclusion of the phosphatases prevents ITAM dephosphorylation, allows recruitment of ZAP-70 and the TCR signal is propagated as described above (Davis and Merwe 2006; Merwe et al. 2000).

However whilst it is a requirement for T cell activation, TCR signalling via TCR-pMHC binding alone is not sufficient to fully activate T cells. Full T cell activation and differentiation requires three signals, of which TCR signalling is one. The two further signals are both provided by the APC which provides co-stimulation via binding of CD80/86 to CD28 on the T cell (signal 2) (Chen and Flies 2013) and will secrete inflammatory cytokines such as IL-12 (signal 3) (Curtsinger and Mescher 2010). TCR signalling in the absence of these additional signals generates a T cell response unable to optimally expand, develop effector functions or generate memory (Chen and Flies 2013; Curtsinger and Mescher 2010). The conditions under which APC provide signals 2 and 3 are discussed in section 1.3.

1.2.4 T cell effector function

Upon activation through TCR signalling and co-stimulation, a naive T cell will proliferate, differentiate and begin to assume effector functions. Despite TCR promiscuity there are relatively few T cells specific for an antigen in the naive population. However once primed, these cells have a remarkable capacity for proliferation; for example in mice a population of naive T cells specific for a lymphocytic choriomeningitis virus (LCMV) epitope expanded 10^5-fold (from ~100 to 10^7 cells) upon LCMV infection (Blattman 2002). The acquisition of effector function is also tightly linked to this proliferation (Kaech and Ahmed 2001). Priming of naive T cells can therefore generate a large population of antigen-specific, effector T cells.

However there are differences in the proliferative response between CD4 and CD8 T cells. $CD8^+$ T cells will rapidly and extensively proliferate and differentiate into cytotoxic T lymphocytes (CTLs). In comparison, $CD4^+$ T cells begin proliferation after a slight delay and have a relatively lesser ability to divide (Foulds et al. 2002; Kaech et al. 2002). CD4 T cells also have a more diverse differentiation repertoire including type 1 T helper (Th1), Th2, Th17, Treg and follicular B helper T cells (Tfh) (Wan and Flavell 2009). Some of the differences between CD8 and CD4 T cell functions are described below.

$CD4^+$ T cell effector function

$CD4^+$ T cells were initially thought to differentiate into either Th1 or Th2 cells (Mosmann et al. 1986), however this has now been expanded to include Th17, Tfh, Th9, Th22 and Treg cells (O'Shea and Paul 2010). These cells are all functionally distinct and can be distinguished by the different cytokine profile that defines each population and contributes to their effector function. As well as this distinctive cytokine production, differentiated CD4 T cells also express a defined transcription factor. The transcription factors and cytokines associated with the main CD4 populations are summarised in Fig 1.4.

Th1 cells produce pro-inflammatory cytokines such as IFN-γ, TNF-α and TNF-β. These are thought primarily to stimulate cellular immunity by activating innate cells such as macrophages, by promoting cellular cytolytic function of T cells or by inducing IgG2a production by B cells (Wan and Flavell 2009).

Th2 cells however produce cytokines including IL-4, IL-5, IL-9, IL-10 and IL-13. These are responsible for promoting B cell proliferation and antibody class-switching to IgG1 and IgE antibodies (Mosmann et al. 1986).

Th17 cells are named due to their signature production of IL-17, although they also produce IL-21 and IL-22. Th17 cells have important roles in promoting inflammation, however whereas Th1 cells protect against intracellular pathogens, Th17 responses are directed against extracellular pathogens/fungi, and have also been implicated in auto-immunity (Zambrano-Zaragoza et al. 2014).

In contrast to Th1, Th2 and Th17 cells, the 'effector' functions of Tregs are broadly to suppress immune responses. Tregs can either develop from self-reactive T cells in the thymus during central tolerance or can differentiate from naive CD4 T cells in the periphery. Induced Tregs (iTregs) and natural Tregs have similar although non-redundant immunosuppressive functions *in vivo* (Haribhai et al. 2011). These are achieved through production of the regulatory cytokines IL-10 and TGF-β (Stassen et al. 2004) which help Tregs maintain self-tolerance and immune homeostasis. Treg dysfunction or depletion can lead to a wide variety of autoimmune and inflammatory diseases (Sakaguchi et al. 1995). Along with production of these cytokines, Tregs also suppress immune responses by expressing high levels of CTLA-4 (Schwartz 1992). This binds to CD80/86 on APCs with a higher affinity that CD28, and so limits T cell co-stimulation, either by out-competing CD28 or by physically removing CD80/86 from the APC surface (Krummel and Allison 1995; Qureshi et al. 2011; Sansom 2000).

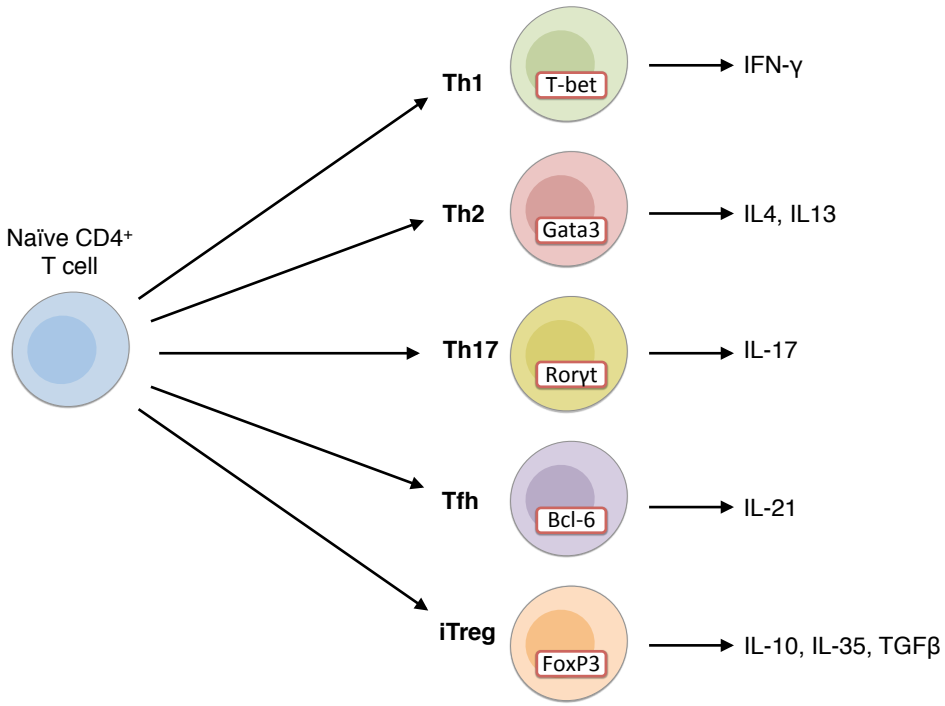


Figure 1.4: *CD4 T cell differentiation.*
Adapted from O'Shea and Paul 2010.

Lastly Tfh express the chemokine receptor CXCR5 and so are found predominantly in the LN B cell zones where they promote memory B cells and the production of high affinity antibodies (Wan and Flavell 2009).

However whether these CD4 subtypes are truly distinct lineages has recently been questioned as cytokine production may not be as rigidly determined by differentiation as first thought (O'Shea and Paul 2010). Nonetheless despite questions remaining over the plasticity of differentiated CD4 T cells, they clearly have a diverse but important role in the immune system.

$CD8^+$ T cell effector function

Cytotoxic T lymphocytes (CTLs) are so named as they are able to induce cytotoxicity of target cells by inducing two separate apoptosis pathways. Firstly, upon TCR engagement with pMHC, the CTL and target cell form a tight 'immunological synapse' (Stinchcombe et al. 2001). TCR signalling leads to influx of calcium, which causes polarisation of the microtubule cytoskeleton towards the synapse (Pores-Fernando and Zweifach 2009). This initiates degranulation of the CTL and exocytosis of lytic granules containing perforin and granzymes across the synapse where they contact the target cell (Jenkins and Griffiths 2010). Perforin molecules form a pore in the target cell membrane (but not the CTL membrane) into which the granzymes can enter (Lopez et al. 2013). Once inside the cell, granzymes induce target cell death by cleaving the BH3 interacting-domain death agonist (BID) and activating the 'intrinsic' apoptosis signalling pathway (Sutton et al. 2000).

Alternatively CTLs can also induce cytotoxicity via an calcium independent mechanism based upon the interaction of FAS with FAS ligand (FasL). FasL is a transmembrane protein that belongs to the tumour necrosis factor family and is expressed on activated CTLs (Berke 1995). The target cell expresses the Fas receptor which, upon FasL binding, trimerises to form the death-inducing signalling complex (DISC). DISC formation then leads to target cell death by activating the 'extrinsic' apoptosis signalling pathway (Kischkel et al. 1995).

The overall purpose of CD8 and CD4 T cell effector activity is the resolution of the infection or pathogen. As mentioned, whereas the innate immune response is very rapid it lacks the specific antigen recognition capabilities of adaptive immunity. The other distinguishing feature is that the adaptive immune system can generate memory and so respond more rapidly to a repeated infection. The mechanisms underlying this memory formation are discussed below.

1.2.5 Memory formation

A complete T cell response to a pathogen will consist of 4 phases. As discussed above, upon antigen exposure naive T cells will proliferate and begin to develop effector functions. During this 'expansion' phase CTLs traffic to the site of infection, secrete inflammatory cytokines and kill infected cells. The number of antigen-specific CTLs peak at around day 7 after infection and after that T cell numbers begin to reduce as cells undergo apoptosis. During this 'contraction' phase around 90-95% of expanded CTLs will die. However there is a population of antigen-specific memory cells that remain and will be stably maintained for years in the 'memory' phase. Lastly, if the same pathogen was to re-infect the host at a later date, this memory population is able to rapidly respond and provide better protection than antigen-inexperienced T cells (Williams and Bevan 2007).

The T cell memory compartment is comprised of two main populations; central memory (T_{CM}) and effector memory (T_{EM}), which can be separated by CCR7 expression. T_{CM} are $CCR7^+$ so will traffic to secondary lymphoid organs. T_{CM} are less dependent on co-stimulation than naive T cells and after TCR signalling will mainly produce IL-2 (Sallusto et al. 2004). Whilst they display limited effector functions, T_{CM} have a greater potential to proliferate and differentiate into effector cells able to produce large amounts of IFN-γ or IL-4. Conversely, T_{EM} are $CCR7^-$ and express chemokine receptors that promote migration to inflamed tissues. These have less proliferative capacity but are better able to rapidly produce effector cytokines and lyse target cells (Sallusto et al. 1999; Sallusto et al. 2004).

Naive T cell expansion to form multiple effector cells followed by contraction and subsequent memory formation has been well established, however the differentiation pathway that generates these memory cells is less well understood. The expression of the transcription factors T-bet and Eomes determines the differentiation of T cells into either central or effector memory subsets. High expression of T-bet is associated with formation of T_{EM} whilst expression of Eomes is linked to maintainance of T_{CM} (Intlekofer et al. 2005; Joshi et al. 2007). It was initially assumed that a sub-population of effector T cells, possibly those with the greatest effector function, go on to form memory cells, whilst the remaining cells undergo apoptosis (Youngblood et al. 2013). However this model (as shown in Fig 1.5A) proposes that the cytolytic effector cells 'de-differentiate' into a more naive-like memory cell.

An alternative model (Fig 1.5B) suggests instead that T cells follow a more linear differentiation pathway. In this model, memory cells do not form from effector cells, but directly from naive cells. This would still occur in primed T cells following TCR signalling, but these cells won't have reached the full cytotoxic effector stage yet. T cells progress through this pathway, losing their proliferative capacity but gaining effector functions associated with effector T cells. In contrast to the 'de-differentiation' model, here effector T cells eventually become terminally differentiated, lose effector function and become senescent or die (Restifo and Gattinoni 2013). Progression along this pathway is predicted to be set by the strength of the T cell activation signal, which could include the amount of antigen presented, the frequency of pMHC-TCR engagement, or the level of co-stimulation. To support this it has been shown that when T cells are injected into mice later in an infection (where they may receive a weaker activation signals), these cells show signs of increased memory potential (D'Souza and Hedrick 2006).

This linear differentiation model has been given further credence by recent work utilising *in vivo* fate mapping of murine T cells. These have shown that increased proliferation tends to generate short-lived effector cells, whereas reduced proliferation favours longer-lived memory cells. Likewise, in contrast to the

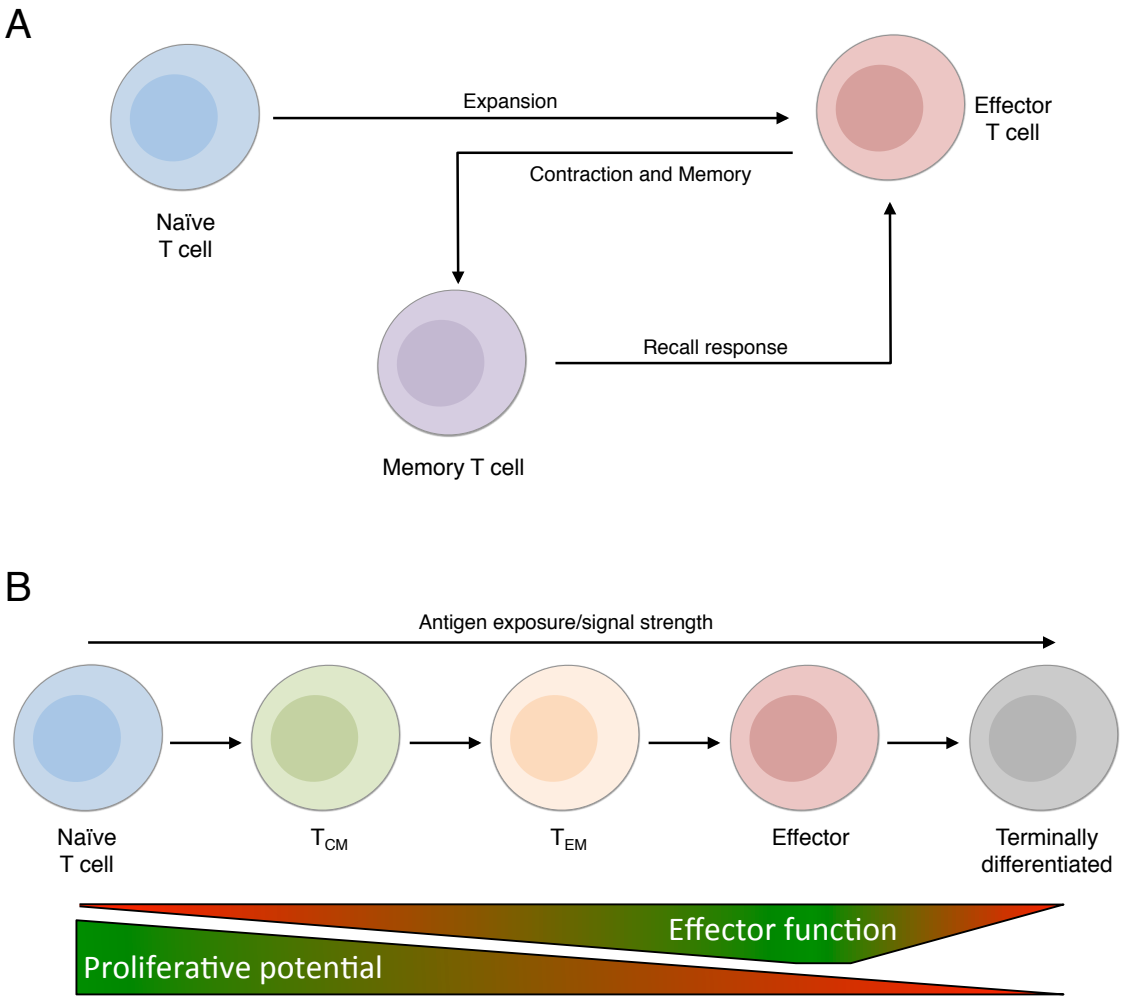


Figure 1.5: *T cell memory differentiation.*

original model, this data suggested that the T cell populations that comprise the effector response are less likely to be involved in a later re-call response (Buchholz et al. 2013; Gerlach et al. 2013).

Memory T cells are clearly a fundamental aspect of the adaptive immune response, however more work is required to elucidate the exact model of differentiation that drives their formation. This may have important consequences as the differentiation status of therapeutic T cells may play a significant role in their functional ability. This will be discussed in more detail in the context of tumour immunotherapy in section 1.4.

These sections have shown that T cells are able to recognise tumour cells through their unique TCR and expand and acquire effector functions that allow the specific cytolysis of that tumour cell. However an important facet of this response, which controls T cell priming, is the presentation of antigen to the T cell. The biology of one of the key antigen presenting cells, the dendritic cell, is discussed in more detail in the next section.

1.3 Dendritic cell biology

Since their discovery by Ralph Steinman in the 1970s (Steinman and Cohn 1973), Dendritic cells (DC) have arisen as a crucial cell population necessary to control and regulate the adaptive immune response. Broadly speaking the role of DC are to act as sentinels; tissue resident DC sample their environment and engulf any pathogens. DC then migrate to the LNs and present antigen to the T and B cells of the adaptive immune system. As shown earlier, this antigen presentation primes T cells and allows them to travel to the site of infection and kill the pathogen. DC therefore are the link between the innate and adaptive immune systems. However DC orchestrate adaptive immunity not only through priming antigen-specific T cells but they also have roles in preserving peripheral tolerance to self-antigens (Banchereau and Steinman 1998) and directly controlling effector T cell responses in tissues (Bennett and Chakraverty 2012). The various features that DC exhibit in order to efficiently carry out these functions are described in more detail below.

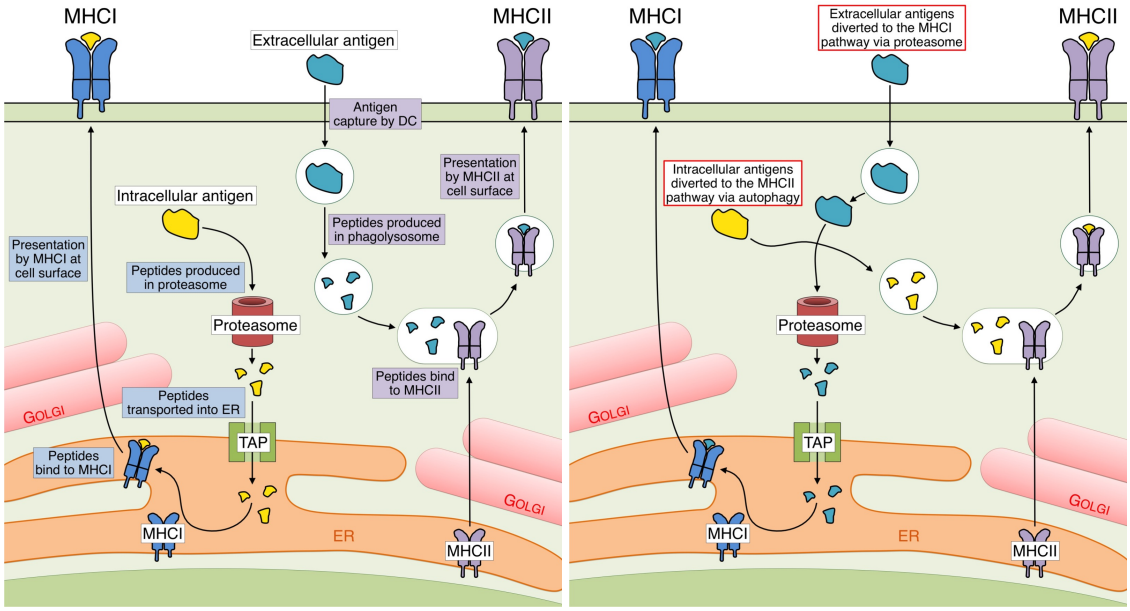
1.3.1 Antigen processing and presentation

The primary role of DC in the immune system is to capture, process and present antigens to lymphocytes in the context of MHCI and II. Whereas other cell populations such as macrophages and B cells can express MHCII and are able to present antigens, DC are professional antigen presenting cells (APCs) and as such are uniquely able to activate naive T cells (Croft et al. 1992; Ronchese and Hausmann 1993). This superior ability as APC is dependent on various functional characteristics that DC possess. Firstly, DC were initially defined and named due to their unusual morphology. Unlike other haematopoetic cells, DC are stellate and have numerous processes or ‘dendrites’ that extent outwards into their surroundings (Banchereau and Steinman 1998). This greatly increases the DC surface area and allows contact with multiple surrounding cells. Immature, tissue-resident DC utilise this morphology to capture antigens and are proficient at doing so via three mechanisms. Firstly, DC are able to phagocytose

particles and pathogens from their surroundings (Inaba et al. 1993). Alternatively they express receptors that mediate endocytosis. These include the Fcγ and Fcε receptors that endocytose immune complexes (Sallusto and Lanzavecchia 1994) or the macrophage mannose receptor and DEC-205 which bind to carbohydrates (Jiang et al. 1995). Lastly DC are also able to take up large amounts of extracellular fluid into pinocytic vesicles from which antigens can be sampled in a process called macropinocytosis (Norbury et al. 1997).

DC then have different mechanisms for processing and presenting either exogenously acquired antigens or endogenously synthesised antigens. DC process endogenous antigen, either from the cells own proteins or from viral infections, and present this via MHCI molecules. As shown in Fig 1.6A, intracellular antigens are degraded into multiple oligopeptides by the cytosolic proteasome, a complex of multiple proteolytic and regulatory subunits (Rock and Goldberg 1999). Peptides are then transported into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP) and loaded onto nascent MHCI molecules (Purcell 2000). pMHCI complexes are then transported to the cell surface by the Golgi apparatus where they can present the peptide to CD8 T cells. DC also utilise a separate mechanism to process and present extracellular antigens. After antigen capture, internalised antigens reside in endosomal and lysosomal compartments. These contain various proteases known as cathepsins, that degrade endocytosed proteins (Honey and Rudensky 2003). Peptides are then loaded onto MHCII molecules and transported to the cell surface where they are presented to CD4 T cells.

However these two mechanisms alone would severely restrict the repertoire of peptides able to be presented to T cells. Therefore DC can also direct endogenous antigens to be presented via the MHCII pathway by transfer of antigen into endosomal compartments during autophagy (Strawbridge and Blum 2007). DC can additionally use a separate mechanism that allows exogenous antigen (usually presented on MHCII) to be presented on MHCI to CD8 T cells. This process, known as cross-presentation, can occur by two mechanisms, the first, which relies on the proteasome, is summarised in Fig 1.6B. Internalised



(a) Direct presentation **(b)** Cross-presentation

Figure 1.6: Dendritic cell antigen presentation.

antigens are transferred from the phagosome to the cytosol, where they are able to be degraded by the proteasome (Kovacsovics-Bankowski and Rock 1995). As before, newly generated peptides enter the ER through TAP and bind to MHCI molecules. Whilst proteasome inhibition prevents this cytosolic cross-presentation, a separate proteasome-independent mechanism is also available to DC. This process is sensitive to lysosome proteolysis inhibition which suggests that MHCI molecules can access the phagosome and be loaded with peptide directly (Shen et al. 2004). However cross-presentation is not yet fully understood and the exact mechanisms used to cross-present antigens are unclear. For example the role of the ER has recently been questioned as ER-resident proteins that mediate MHCI loading have been found on phagosomes (Guermonprez et al. 2003). The recruitment of these proteins to the phagosome depends on the ER protein, Sec22b and cells deficient in this protein are unable to cross-present antigens (Cebrian et al. 2011). Likewise whilst TAP mediated import into the ER/phagosome was thought to be required for peptide loading onto MHCI, this may also occur via a TAP-independent mechanism (Lawand et al. 2016).

The machinery that enables cross-presentation via these pathways appears to be specific to DC as other APC such as macrophages are unable to cross-present antigen *in vivo* (Jung et al. 2002). DC may be able to preserve antigens so that they are available for cross-presentation as lysosomal proteolysis is reduced in DC compared to macrophages (Delamarre et al. 2005). DC that are adept at cross-presentation also express low levels of the lectin Siglec-G, which has recently been described to inhibit cross-presentation by increasing phagosomal hydrolysis of internalised antigens (Ding et al. 2016). Using these mechanisms DC are therefore capable of processing and presenting a broad range of peptides via both MHCI and II to activate CD8 and CD4 T cells respectively.

1.3.2 DC activation

However presentation of peptide alone is insufficient to fully prime effector T cells and can lead to T cell anergy (Steinman et al. 2003). Therefore DC require activation, from an immature to a mature state, where they are able to provide co-stimulation and inflammatory cytokines. DC can acquire activation signals at the same time as they acquire antigen and these signals are usually from pathogen associated molecular patterns (PAMPs). DC are able to recognise PAMPs through the expression of pattern recognition receptors (PRR), of which the most studied are toll-like receptors (TLR). Unlike receptors of the adaptive immune response such as TCRs, TLR are germ-line encoded receptors that are much less specific and recognise structurally similar, but widely distributed molecules. Cell surface TLR are involved in recognition of a series of conserved molecular patterns, specific to bacterial, fungal or protozoan pathogens while the intracellular TLR recognise viral nucleic acids (Kanzler et al. 2007). However in a cancer setting, DC can also promote immune responses to antigens in the absence of pathogen associated signals. DC express PRR, including some TLR, which can also bind to molecular structures not associated with pathogens but which are released from cells upon tissue damage (damage associated molecular patterns or DAMPs) (Gallo and Gallucci 2013). These have included

intracellular proteins such as the heat shock proteins (HSP70, HSP90, and gp96), the S100 calcium-binding protein family and HMGB1. In additon intracellular molecules such as ATP, uric acid and dsDNA have also been shown to induce DC maturation (Zitvogel et al. 2010). These intracellular 'danger signals' are upregulated after cellular stress and can be released upon cell apoptosis or necrosis. DAMPs can then bind to receptors on the DC surface, principally TLR4 and induce DC maturation (Apetoh et al. 2007; Fang et al. 2014).

TLR signalling induces this shift from 'immature' to 'mature' DC which are better able to prime T cells (Hemmi and Akira 2005). Mature DC express higher levels of co-stimulation molecules such as CD40, CD80 and CD86 and produce inflammatory cytokines such as IFN-α, IFN-β, TNF, IL-1 and IL-12 (Reis E Sousa 2004). These not only provide signal 2 and signal 3 to T cells, but also the cytokines act in an autocrine fashion to promote DC maturation. Cytokine and PRR-mediated activation appear to occur in combination though as DC activation by inflammatory cytokines in the absence of PRR signalling is insufficient to fully activate DC (Sporri and Reis E Sousa 2005).

A key aspect of DC maturation is the migration of DC from where they reside in peripheral tissues to draining LN. DC migration is controlled by the expression of chemokine receptors on the DC surface. Immature DC express chemokine receptors involved in migration to non-lymphoid tissue, such as CC-chemokine receptor 6 (CCR6), however as DC mature they down-regulate expression of these receptors and up-regulate expression of CCR7, a receptor that causes DC migration to draining LNs (Dieu et al. 1998). Therefore via these processes DC are able to sample their environment and internalise antigen, mature in response to pathogen associated signals, traffic to LN and present acquired antigen to naive T cells. However whilst this outlines their broad function in priming naive T cells in response to infection, DC have also been shown to control the effector function of T cells in tissues.

1.3.3 DC control of T cells *in situ*

Although DC prime T cell responses in the LN, these effector T cells need to be able to adapt to a changing environment in the tissue to be able to induce clearance of infected cells whilst minimising damage to healthy tissue. DC are thought to have a central role in controlling T cell effector function *in situ*. Although DC migrate to LN following infection, not all tissue resident DC migrate and those that remain are further bolstered by recruitment of DC from blood precursors (Eidsmo et al. 2009; León et al. 2007). A large proportion of these DC that migrate to the site of infection are 'inflammatory DC' that differentiate from monocytes in response to blood-borne TLR agonists (Shi et al. 2011).

These tissue-resident DC have been shown to have important roles in the control of effector T cells. After T cell priming in the LN, depletion of lung DC results in enhanced viral titres and mortality in influenza infected mice. This was as a result of impaired CD8 T cell responses which were dependant on presentation of influenza peptides by lung APC. (McGill et al. 2008). These data suggest that the recruitment of DC into inflamed tissue may promote subsequent T cell effector function. This inflammatory response is also noted in the intestine and skin of patients with autoimmune diseases, which show an accumulation of DC at these sites (Rescigno and Di Sabatino 2009; Zaba et al. 2008).

However the role of tissue-resident DC is not always to promote T cell responses. As infections are cleared, DC are able to restrict the function of effector T cells to prevent unwanted damage. For example whilst antigen presentation in the skin drives production of inflammatory cytokines such as IFN-γ, this also directly promotes IL-10 production by Tregs (McLachlan et al. 2009). Effector T cells have also been shown to interact with tissue-resident DC upon recognition of antigen. This induces upregulation of PD-1 on the T cell, which then loses effector function (Honda et al. 2014). The ability of DC to limit the effector function of T cells is also shown in their ability to enforce 'peripheral tolerance' in the absence of infection.

1.3.4 DC-mediated peripheral tolerance

As mentioned previously (section 1.2.2) central tolerance is crucial to preventing self-reactive T cells from developing, although a small amount of potentially self-reactive T cells will escape this process. DC therefore mediate peripheral tolerance which is crucial in preventing these self-reactive T cells from mounting responses to healthy tissues (Xing and Hogquist 2012). In the steady-state DC are able to promote T cell hypo-responsiveness ('anergy') or delete T cells to prevent unwanted immune reactions. This is primarily achieved through presentation of self-antigen to T cells in the absence of co-stimulation, which is required for full T cell activation (Steinman et al. 2003). Alternatively the absence of co-stimulation can be coupled with expression of inhibitory receptors such as PD-L1. This can interfere with TCR signalling in a antigen-specific manner and induce T cell anergy (Probst et al. 2005b). Although there exists a lymphocyte counterpart in the regulatory T cell, a specific 'regulatory DC' that would be required for peripheral tolerance has not been identified. Instead it has been suggested that these functions are carried out by immature DC of different subsets (Reis E Sousa 2006; Steinman and Nussenzweig 2002). However although a regulatory DC subset has not been identified, DC are a heterogenous population which do have different functions in the immune system. These DC subsets and their roles in various immune reactions are described in more detail below.

1.3.5 Murine DC subsets and development

DC can be broadly split into three groups, classical or conventional DC (cDC), plasmacytoid DC (pDC) and monocyte-derived DC (moDC). Despite some early scepticism, these DC populations have been shown to originate from a distinct cellular precursor that doesn't produce other lymphoid cells (Satpathy et al. 2012a). The exact expression of the various transcription factors and cytokines that regulate DC differentiation are not yet fully understood however a summary of DC ontology is shown in Fig 1.7.

Common myeloid progenitors (CMP) originate in the bone marrow and gradually differentiate into the macrophage DC progenitor (MDP) when exposed to the growth factor fms-like tyrosine kinase 3 ligand (FLT3L) (Karsunky et al. 2003). This population will bifurcate, of which one arm can give rise to monocytes when exposed to macrophage colony-stimulating factor (CSF1). Generation of monocytes was originally thought to be upstream of DC development as they are able to differentiate into a DC population that exhibit many features associated with what we now know as cDC (Merad et al. 2013). Differentiation of monocyte derived DC (MoDC) occurs during inflammatory conditions (Naik et al. 2006) so they are therefore often referred to as 'inflammatory DC'. They have very similar roles in antigen presentation as cDC and express similar cell surface molecules such as CD11c, CD11b and MHCII. However due to their monocytic lineage they also express the Fc-γ receptor 1 (CD64) not found on cDC (Mildner and Jung 2014). We now know that MoDC and cDC are separate DC populations and as mentioned earlier, it has been suggested that MoDC migrate from the blood to inflamed tissue to boost the cDC response to infections (Hespel and Moser 2012).

However without CSF1 and in the continued presence of FLT3L, MDP lose the potential to generate monocytes and will become common DC progenitors (CDP). These are committed to generating either of the remaining cells of the DC lineage, pDCs or cDCs (Merad et al. 2013). Development of pDCs is positively controlled by expression of the E-box protein transcription factor *E2-2* (Cisse et al. 2008), although they can arise from either CDP or common lymphoid progenitors (CLP) (Manz et al. 2001; Onai et al. 2013). pDCs are terminally differentiated in the bone marrow and migrate to the blood. Here they exist as a specialised subset of cells that express TLRs which bind to viral nucleic acids and are able to rapidly produce large amounts of type I IFNs in response to viral infections (Asselin-Paturel and Trinchieri 2005). However in addition, pDC are also able to act as APC and present antigen to naive T cells, the hallmark of a DC response, although this is dependent on TLR activation. (Mouriès et al. 2008; Sapoznikov et al. 2007).

However the most well-studied DC population are the cDC, which are the major

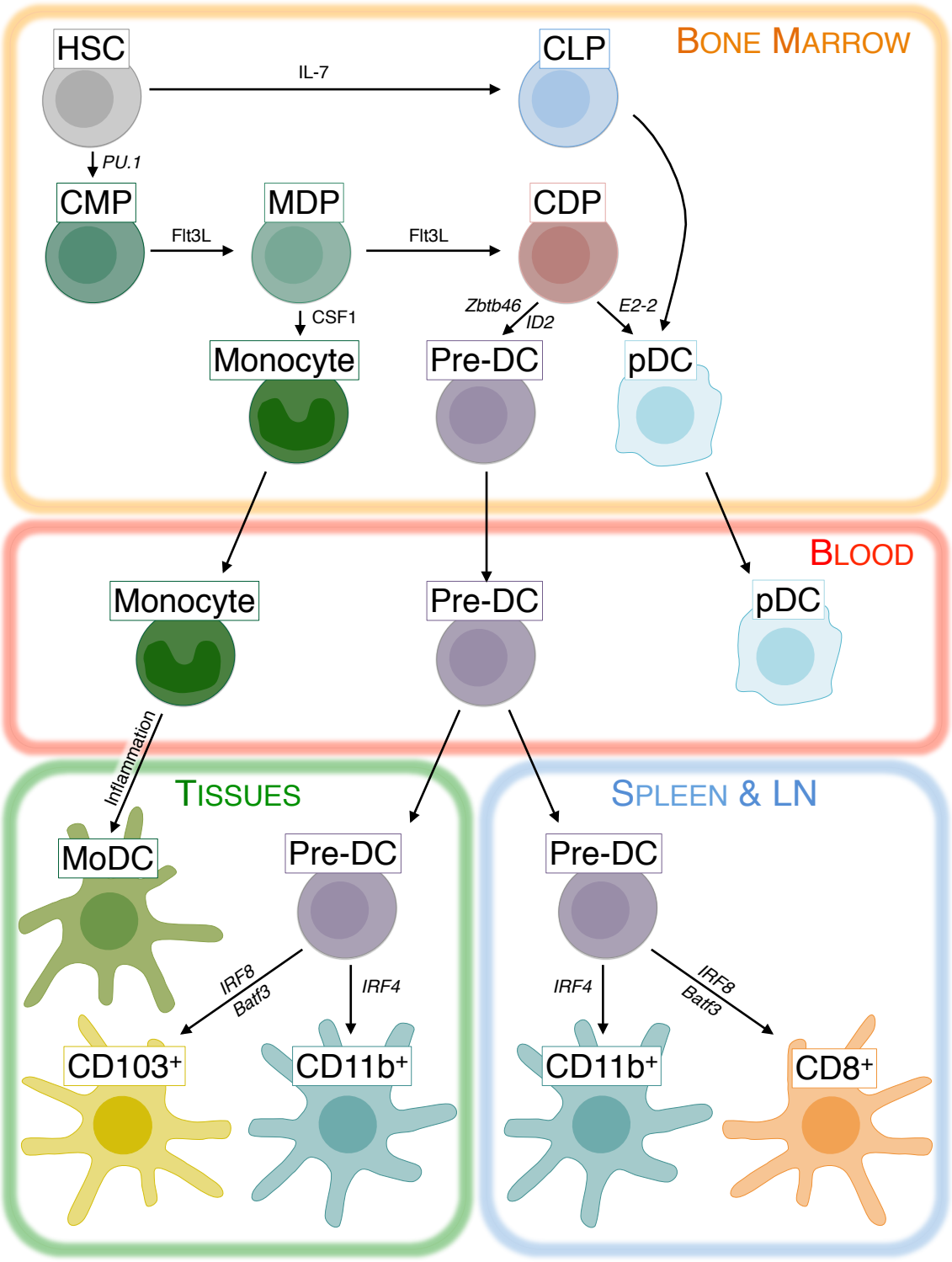


Figure 1.7: *Dendritic cell ontology.*

Adapted from Merad et al. 2013, with key cytokines or transcription factors (italics) noted. (HSC = hematopoietic stem cell; CLP = common lymphoid progenitor; CMP = common myeloid progenitor; MDP = macrophage DC progenitor; CDP = common DC progenitor).

antigen presenting population of DC. Expression of the DNA-binding protein inhibitor *ID-2* inhibits *E2-2* transcription factor mediated pDC differentiation and is thought to drive differentiation of CDP into pre-DCs and later cDCs (Jackson et al. 2011). Alongside this, pre-DCs also exclusively express the transcription factor *Zbtb46*, although this appears not to be strictly required for development of pre-DC or cDC (Satpathy et al. 2012b). Pre-DCs migrate from the bone marrow to the blood where where they populate both lymphoid and non-lymphoid tissues. There, pre-DCs will differentiate into one of two types of cDC; CD103/CD8$^+$ cDC1 or CD11b$^+$ cDC2 (Merad et al. 2013). Differentiation between these two subsets is controlled by the expression of the transcription factors interferon regulatory factor 8 (*IRF8*) and basic leucine zipper ATF-like transcription factor 3 (*Batf3*) on cDC1 (Grajales-Reyes et al. 2015), or *IRF4* on cDC2 (Suzuki et al. 2004). This lineage decision is made early in pre-DC development, when they are still in the bone marrow although it doesn't manifest until pre-DCs migrate to the periphery (Schlitzer et al. 2015)

The discover of CD8$^+$ cDC1 in the lymphoid organs was one of the first examples in DC heterogeneity (Crowley et al. 1989; Vremec et al. 1992) and was followed by the identification of their tissue-resident counterparts, CD103$^+$ cDC1 (Bursch et al. 2007; Rio et al. 2007). Despite these differences in marker expression, both CD8 and CD103$^+$ DCs belong to the same lineage and can be collectively identified by the expression of the chemokine receptor XCR1 (lymphotactin receptor/G-protein-coupled receptor 5) (Guilliams et al. 2016). cDC1 are the quintessential antigen presenting cells of the immune system and are specialised in cross-presentation. Both splenic CD8$^+$ (Haan et al. 2000) and tissue-resident CD103$^+$ (Bedoui et al. 2009; Broz et al. 2014) cDC have been shown to be able to cross-present antigen *in vivo*. Additionally, TLR signalling on cDC1 induces significant IL-12 production, which has important roles in promoting T cell and DC maturation (Reis e Sousa et al. 1997).

CD11b$^+$ cDC2 however are less well characterised than cDC1, partly because they represent a more heterogenous population. These can be distiguished from cDC1 as they are XCR1$^-$ and express the signal regulatory protein alpha (SIRPα

or CD172a) (Guilliams et al. 2016). cDC2 development is controlled by the expression of IRF4 and this appears to influence their functional role *in vivo*. IRF4 expression in $CD11b^{+}$ DC has been shown to induce high levels of MHCII expression (Vander Lugt et al. 2014) and therefore these cells are adept at presenting antigen to CD4 T cells (Dudziak et al. 2007). However this appears to limit their ability to cross-present antigen to CD8 T cells (Mildner and Jung 2014).

Whilst these DC subsets are functionally conserved in humans, their expression of some surface markers can vary (Satpathy et al. 2012a). However recent attempts to identify these populations have revealed a subset phenotype that is conserved across species. cDC1s can therefore be identified as $CADM1^{hi}$ $CD172a^{lo}$ $CD11c^{int\text{-}hi}$ $CD26^{hi}$ $IRF8^{hi}$ $IRF4^{lo}$ and cDC2s can be identified as $CADM1^{lo}$ $CD172a^{hi}$ $CD1c^{hi}$ $CD11c^{hi}$ $IRF4^{hi}$ $IRF8^{lo}$ cells (Guilliams et al. 2016).

1.3.6 DC in tumour immunology

DC have numerous potential roles in tumour immunology which reflect the heterogeneous nature of these cells and their functions. As discussed earlier, T cell responses to tumours have been associated with improved prognoses and patient survival (Clark et al. 1989; Clemente et al. 1996; Fridman et al. 2012; Galon et al. 2006). The logical assumption therefore is that, as T cells require antigen presentation to assume effector function, the DC that orchestrate this response would have similar prognostic value. The ‘cancer-immunity cycle’ has recently been proposed in which DC acquire tumour antigen then mature and migrate to draining LN. Antigen presentation primes naive T cells which migrate back to the tumour and recognise the specific p-MHC expressed on tumour cells. Effector T cells can then induce tumour cell death, which releases further antigen and the cycle can be repeated with increased depth and breadth of response (Chen and Mellman 2013). Indeed infiltration of DC into tumours has been associated with improved survival and reduced metastases (Ananiev et al. 2011; Iwamoto et al. 2003; Tsujitani et al. 1993; Zeid and Muller 1993). However

separate studies have conversely linked tumour infiltrating DC with poorer overall survival (Dadabayev et al. 2004; Sandel et al. 2005). This discrepancy is likely due to differences in the localisation and maturation of DC in different tumours. Likewise the DC that infiltrate the tumour are as phenotypically and developmentally varied as those found in steady state tissues and lymphoid organs (Broz et al. 2014; Salmon et al. 2016). In addition the stage of cancer progression in which the DC are found can also have a profound impact on their function. Scarlett et al recently showed in a inducible ovarian cancer model that during the initial phases of tumour equilibrium, tumour infiltrating DC were capable of stimulating T cell responses and expressed high levels of MHCII and CD40. However the phenotype of these DC later switched as they became immunosuppressive. Late-tumour infiltrating DC expressed lower levels of co-stimulatory molecules, whilst up-regulating expression of PD-L1. This phenotypic switch coincided with a phase of rapid tumour growth. In line with this data, depletion of early-DC induced rapid tumour growth, whereas depletion of late-DC prevented tumour escape (Scarlett et al. 2012). These data show that DC can have various functions in the tumour that either promote or control tumour growth.

One of the functions of DC that may have an important effect on this ability to promote tumour escape is not the priming of effector T cells but the maintenance of self-tolerance. This is relevant in a cancer setting as tumours clearly have mechanisms that allow them to subvert peripheral tolerance to disrupt the cancer-immunity cycle and evade immune responses. One mechanism that tumours use to manipulate immunosurveillance is through preventing DC maturation or recruiting immature DC. Tumour resident DC are known to have low levels of CD86, MHCII and produce few pro-inflammatory cytokines (Drake et al. 2006). Tumours are thought to directly inhibit DC maturation by over-expressing inhibitory cytokines such as VEGF, M-CSF, IL-10 and IL-6 (Drake et al. 2006; Gabrilovich 2004). Therefore tumour-resident DC and DC which migrate to tumour draining LN are able to acquire and present tumour antigen to naive T cells but can only provide poor co-stimulation. In addition, DC in tumour draining LNs have also been shown to express higher levels of PD-L1

than those in non-draining LNs (Salmon et al. 2016). These factors contribute to promoting anergic T cell responses that tolerise adaptive T cells to tumour antigens and promote tumour escape.

However not all tumour resident DC are in this immature state which promote T cell anergy. Recently $CD103^+$ cDC1 have been described as a DC population that infiltrate tumours and are crucial for inducing T cell immunity to tumour antigens (Broz et al. 2014; Salmon et al. 2016). This cDC1 population acquire tumour antigen and migrate to draining LN by expressing CCR7 (Roberts et al. 2015). In the draining LN, $CD103^+$ DC are essential for cross-presentation of tumour antigen to naive T cells and the subsequent priming of T cell responses against the tumour antigens (Broz et al. 2014; Roberts et al. 2015; Salmon et al. 2016). However although effective, $CD103^+$ DC are only present in small numbers and so cannot overcome tumour immune evasion. This has been shown in experiments with *Batf3*$^{-/-}$ mice, which lack cDC1 but in which transplanted B16 tumours grow as quickly as WT controls (Salmon et al. 2016). In contrast, injection of FLT3L promotes differentiation of CD103/cDC1 and results in accumulation of $CD103^+$ DC in the tumour. This promotes greater T cell infiltration and IFN-γ production in tumours (Salmon et al. 2016). As well as priming T cell responses in the LN, $CD103^+$ DC are suggested to have roles in controlling T cell function in the tumour. Depletion of $CD103^+$ DC limits adoptively transferred, tumour specific T cell-mediated tumour control, independent of LN priming (Broz et al. 2014). The importance of these cells is also evident in melanoma patients where high levels of $CD103^+$ DC or CCR7 expression correspond to a better prognosis and survival (Broz et al. 2014; Roberts et al. 2015). The importance of $CD103^+$ DC in the efficacy of various immunotherapies still needs to be fully assessed. However pre-clinical studies have suggested that enhanced tumour control after PD-L1 checkpoint blockade requires $CD103^+$ DC to be effective (Salmon et al. 2016). Likewise vaccines expressing FLT3L have been shown to synergise with CTLA-4 blockade (Curran and Allison 2009). The various approaches to developing immune responses against tumours including use of DC vaccines are discussed in more detail in the section 1.4.

Combined, these data show that whilst the $CD103^+$ subset are clearly important in mediating T cell control of tumour growth, overall DC have mixed role in tumour control or progression. This is reflected in the fact that they are a heterogenous population of cells in which each subset can also exist in a different state of maturation. Future studies will therefore need to carefully examine the different roles of these DC subsets and the importance of their maturation status. The next section will therefore explain the current murine models that have allowed researchers to elucidate DC function and in particular, the diphtheria toxin receptor depletion model.

1.3.7 Mouse models to study DC: DTR models

The availability of transgenic mice has had a dramatic impact on the study of various aspects of immunology including DC biology. Transgenic mice in which specific transcription factors are knocked out have been crucial in developing our understanding of the various DC subsets and how they develop. Likewise mice in which the Cre recombinase is expressed in DC has allowed for specific gene deletion and the elucidation of various DC functions. However of recent abundance and of particular importance in this study are the models of conditional DC ablation using the diphtheria toxin receptor (DTR).

The DTR system exploits a series of advantageous aspects of cell biology to allow specific and efficient depletion of target cells. Firstly, diphtheria toxin (DT), the toxin produced by *Corynebacterium diphtheriae*, is incredibly potent and able to efficiently block protein synthesis and induce cell apoptosis. However the activity of DT is strictly dependent on receptor-mediated endocytosis. The DT receptor (DTR) is a membrane-anchored form of the heparin-binding epidermal growth factor receptor. However the murine and human/simian versions of the DTR vary by three amino acids in the region that bind DT. This results in murine cells being 10^5 times less susceptible to DT mediated killing than human cells. These circumstances have been exploited to genetically engineer mice to express the high-affinity, human DTR in specific cell types, making these cells acutely susceptible to depletion upon DT injection (Saito et al. 2001).

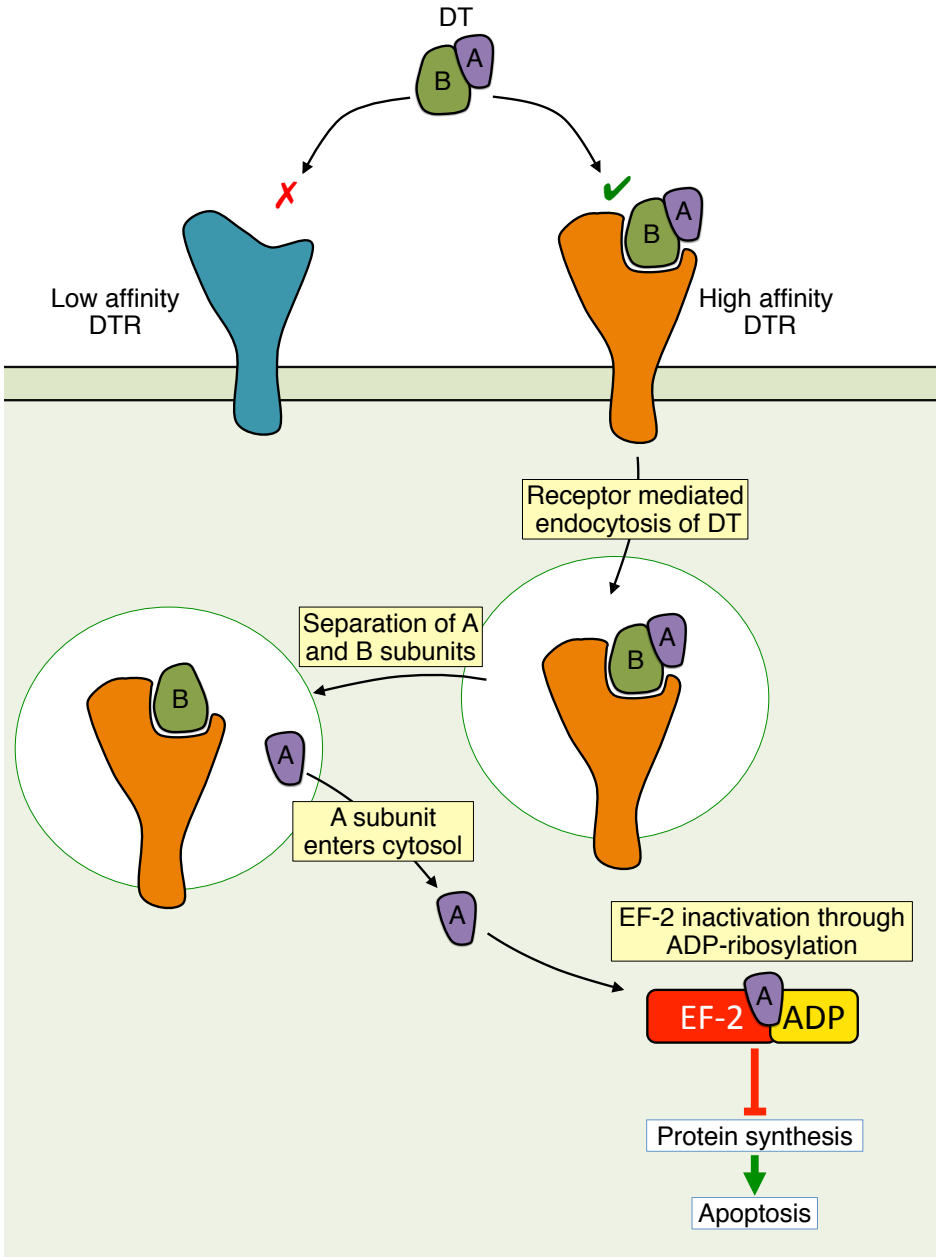


Figure 1.8: *DT mediated toxicity.*

1. DT is composed of two subunits. The B subunit binds to the human (high affinity) DTR but not the murine (low affinity) DTR.
2. DT entry into the cell is dependant on receptor-mediated endocytosis.
3. Increased acidification in the endosome results in the separation of the A and B subunits.
4. The A subunits enters to cytosol where it ADP-ribosylates elongation factor-2 (EF-2).
5. The inactive EF-2 prevents protein synthesis in the cell, which rapidly begins to undergo apoptosis.

The expression of the high-affinity DTR and hence the susceptibility to depletion can be controlled by placing DTR expression under the control of cell specific promotors. Previously, cell ablation had been achieved by constitutively

expressing DT-A under the control of these promotors (Saito et al. 2001). However conditional depletion of DC offers an attractive alternative to constitutive ablation as it has numerous advantages. Injection of a depleting agent offers a level of temporal and possibly spatial control over cell depletion not possible in constitutive depletion models. Likewise, conditional depletion avoids both developmental concerns and the appearance of compensatory mechanisms that are both associated with mice permanently depleted of cell subsets (Sapoznikov and Jung 2008).

Previous attempts at conditional DC depletion however had been fraught with difficulties, in part due to the heterogenous nature of these cells. Use of depleting antibodies was limited due to the lack of unique markers on DC (Bennett and Clausen 2007). Whilst some level of DC depletion could be achieved using chlodronate lipsomes, these will deplete all phagocytotic cells and are more routinely used to deplete macrophages (Rooijen and Hendrikx 2010). Therefore the DTR system was first utilised to deplete DC by Jung et al in 2002, by placing the high-affinity DTR under the control of the CD11c (*Itgax*) promotor. This system avoided the downsides associated with constitutive depletion whilst also offering improved specificity and efficiency compared to previous conditional depletion models. High efficiency of depletion takes on a more pronounced importance in DC depletion models due to the remarkable ability of few DC to promote T cell responses (Bennett and Clausen 2007). The DTR model is capable of this efficiency because of the high toxicity of DT; as little as one DT molecule in the cytosol may be sufficient to induce cell death (Yamaizumi et al. 1978). Lastly, the DTR model is also well suited to depletion in an immunological context as DT-mediated killing of target cells occurs through programmed apoptosis (Thorburn et al. 2003) which, in a similar depletion model, did not cause inflammation (Bennett et al. 2005).

In CD11c.DTR mice a single i.p. injection of DT (4ng/g) will deplete 85-90% of splenic DC within 24 hours and this is maintained for 3 days, after which time DC repopulate and return to homeostatic levels (Jung et al. 2002). However *cd11c* is expressed at varying levels in DC and depletion favours $CD11c^{hi}$ DC. It may be

that the *Itgax* promoter fragment is insufficient to fully control CD11c expression in all cells. For example $CD11c^{int}$ pDC and NK cells transcribe their endogenous *Itgax* allele and express CD11c but are not susceptible to depletion (Lucas et al. 2007; Sapoznikov et al. 2007). Whereas the *Itgax* promotor is also active in some activated T cells (Jung et al. 2002) and B cell plasma blasts (Hebel et al. 2006), however these cells are susceptible to depletion. Lastly detailed histological analysis has revealed that DT injection completely depletes marginal zone and metallophilic macrophages from the spleen and their sinusoidal counterparts from the lymph nodes (Probst et al. 2005a). Therefore careful consideration must be taken when interpreting data from this model due to the potential depletion of other $CD11c^{+}$ cells. CD11c.DTR mice also suffer from an unexpected phenotype where DTR expression causes a lack of migrating DC in the LN, and an overall LN hypocellularity (Blijswijk et al. 2015). The reason behind this is unclear but appears to be dependant on DTR expression on DC subsets as it is also found in other DC depletion models that use DTR expression.

However the biggest disadvantage of the CD11c.DTR model is the lethality in mice that are repeatedly injected with DT. Studies are therefore limited to one DT injection and hence DC can only be depleted for 3 days. This appears to be caused by CD11c expression on non-haematopoetic tissue, possibly in the gut or brain. CD11c.DTR mice reconstituted with WT bone marrow (BM) are still killed by repeated DT injection (Zaft et al. 2005). However this does allow for BM chimeras to be generated in which CD11c.DTR BM is used to reconstitute WT recipients, which circumvents the toxicity of repeated DT injections. The downside of this approach however is that radio-resistant dermal DC (Bogunovic et al. 2006) will be maintained from host origin and therefore not susceptible to depletion.

An alternative to the CD11c.DTR model therefore are the CD11c.DOG mice. These mice were generated by transferring a bacterial artificial chromosome (BAC) rather than the usual transgenesis used to created the CD11c.DTR mice. This means that in the CD11c.DTR mice, the DTR transgene is randomly

inserted into the genome and expression is only controlled by the minimal CD11c promotor. Whereas a BAC allows for the transfer of a much larger amount of genetic information, which means that in CD11c.DOG mice the expression of the DTR transgene is controlled by the whole CD11c gene locus, including the promotor and related gene enhancers and silencers (Hochweller et al. 2008). Due to this tighter restriction of DTR expression, CD11c.DOG mice are able to be injected with DT multiple times without toxicity. Moreover, the LN hypocellularity associated with other DTR models is not evident in the CD11c.DOG mice (Blijswijk et al. 2015).

In addition to these DTR models that broadly target $CD11c^+$ DC, recent attempts have been made to deplete specific DC subsets. These mouse models are shown in Table 1.1 and include targetting depletion to cDC (Meredith et al. 2012), pDC (Swiecki et al. 2010; Takagi et al. 2011) and $CD8^+$ DC (Fukaya et al. 2012; Piva et al. 2012). As with previous depletion models however, these models have various limitations that restrict their usage.

However one last limitation with DC depletion models not mentioned in Table 1.1 is the influx of neutrophils and monocytes after DC depletion (Hochweller et al. 2008; Sivakumaran et al. 2016; Tittel et al. 2012). This has been observed in both the CD11c.DTR and CD11c.DOG models but it is unclear whether other DC depletion models also exhibit these phenotpyes. Monocytosis and neutrophilia can have a confounding effect on the study of DC depletion, for example Tittel et al showed that despite DC depletion, DT injection promoted bacterial clearance as a result of this neutrophil recruitment. Similarly, the depletion-induced monocytosis occurs through an expansion of a unique population of monocytes primed to produce TNFα (Sivakumaran et al. 2016). These studies show that caution that must be used when interpreting data generated through the use of these DC depletion models. However despite these caveats, the CD11c.DTR and CD11c.DOG models are still powerful tools to use in the investigation of DC biology.

Table 1.1: Murine DTR models of DC depletion

Mouse model	Cell depletion	Limitations	Ref.
CD11c.DTR (Transgenic)	$CD11c^+$ DCs and some macrophages	Possible depletion of other cells: pDCs, NK cells, activated T and B cells. Toxicity in CD11c.DTR mice.	Jung et al. 2002
CD11c.DOG (BAC transfer)	$CD11c^+$ DCs and some macrophages	Similar possible depletion to CD11c.DTR model, although less well defined.	Hochweller et al. 2008
zDC.DTR (Knockin)	*Zbtb46*$^+$ cDC, but also activated monocytes	*Zbtb46* expression in erythroid progenitors and epithelial cells leads to lethality unless BM chimeras are made.	Meredith et al. 2012
BDCA2.DTR (Transgenic)	pDCs	BDCA2 expression varies so off-target DTR expression possible.	Swiecki et al. 2010
SiglecH.DTR (BAC transfer)	pDCs	Homozygous SiglecH.DTR mice are deficient in endogenous SiglecH expression.	Takagi et al. 2011
Clec9a.DTR (BAC transfer)	$CD8^+$ (and likely $CD103^+$) cDCs	Partial depletion of $Clec9^{lo}$ pDC.	Piva et al. 2012
CD205.DTR (Knockin)	$CD8^+$ DC, dermal DC, and LC	DT mediated lethality; requires generation of BM chimeras	Fukaya et al. 2012

Adapted from Van Blijswijk et al. 2013

1.4 Cancer immunotherapy

The formalisation of the theory of immunoediting (as discussed earlier) has led to many attempts to utilise the potential of the immune system to control tumours in patients. This is because, despite recent advances, cancer still remains a leading cause of mortality in the developed world. Key to this anti-tumour immune response is the response generated by the adaptive T cells, which are able to recognise tumour antigens and induce cytotoxic effects. The added attractiveness of cancer immunotherapies is that tumour recognition can occur systemically in sites distal to the tumour, potentially allowing T cells to cure tumour metastases. Likewise the addition of immunological memory might generate long lived, persistent protection. The majority of recent cancer immunotherapies therefore have focused on generating a population of cytotoxic T cells that are able to destroy the tumour. There are three main approaches to this that have been attempted:

- Vaccination against tumour antigens to promote an endogenous T cell response
- Expansion and adoptive transfer of tumour infiltrating lymphocytes
- Generation of a new anti-tumour T cell population by gene therapy

This section will discuss the foundations, as well as recent developments of these three approaches.

1.4.1 Vaccines for tumour immunotherapy

DC are responsible for orchestrating adaptive immunity and so are attractive targets for controlling anti-tumour immunity. Particularly, considerable interest has been generated in utilising DC through vaccinations. However whilst vaccines for infectious diseases are incredibly potent at inducing durable immune responses, this has proved to be more challenging in a tumour setting. For use in tumour immunotherapy, vaccination refers to therapeutic use, rather

than prophylactic. This means patients are already burdened with an established tumour that has likely evolved to avoid recognition or elimination by the immune system. DC in these patients are therefore often dysfunctional and unable to generate effector T cell responses (Pinzon-Charry et al. 2005). Provision of additional tumour antigen in this setting is therefore not sufficient to generate CD8 T cell responses able to effectively control tumours. The challenge that tumour vaccines face is how to overcome the myriad immunosuppressive mechanisms that tumours use to blunt T cell immunity. To achieve this the DC used for vaccination must not have been subverted by the tumour microenvironment and must be able to present antigen alongside relevant co-stimulation. The vaccination strategies that have been used to achieve this essentially fall into two categories; either DC are generated *ex vivo* and therefore outside the influence of the tumour or endogenous DC are targeted *in vivo* alongside an approach to ensure DC maturation.

Vaccination with *ex vivo* generated DC

The majority of initial work with tumour vaccines involved use of DC *ex vivo*, such as pulsing DC with a tumour-specific peptide *in vitro* before transferring them back into the patient. Whilst this technique has been shown to induce antigen-specific T cell responses, its effect on tumour regression, particularly when used therapeutically has been limited (Celluzzi et al. 1996; Inaba et al. 1990; Zitvogel et al. 1996). A number of different factors could be limiting the potency of T cell responses to antigen-loaded DC. Several studies have suggested that presentation of antigen via MHCI by peptide-loaded DC is fairly short-lived (Amoscato et al. 1998; Kukutsch et al. 2000; Zehn et al. 2004). Likewise radiolabelled DC injected intra-dermally into patients did not migrate away from the injection site, which could limit their ability to present antigen to T cells in the LN (Lesterhuis et al. 2011). Most importantly however, the importance of providing adequate co-stimulation and inflammatory cytokines alongside antigen presentation were not fully realised. However the generation of DC *ex vivo* offer some approaches to improve this, namely that the maturation

of the DC population can be directly controlled.

Subsequent attempts to generate DC cultures were done so with the addition of various cytokines such as IL-1β, TNF-α , IFN-α and IL-6, or with the addition of TLR agonists. Generation of DC in these conditions produced mature DC that expressed higher levels of co-stimulatory molecules and cytokines such as IL-12. These advancements in pre-clinical work (as reviewed in Kalinski et al. 2013) led to numerous clinical trials utilising *ex vivo* generated DC. Of particular note is the treatment of prostate cancer patients with *sipuleucel-T*, a product derived from blood APC loaded with a prostate cancer antigen. This led to improved patient survival and as a result was one of the first cellular immunotherapies licensed for use by the FDA (Higano et al. 2009). However *ex vivo* generation and manipulation of DC is time-consuming and costly, so other methods to generate DC-mediated T cell responses have been designed which directly target DC *in vivo*.

Vaccinations targeting DC *in vivo*

One such technique to target *in vivo* DC is through the use of an relevant antigen, fused to a monoclonal antibody that recognises a DC-specific receptor. In a series of papers, Ralph Steinman and colleagues demonstrated the feasibility of this approach and showed it could be used to efficiently generate effector CD8 and CD4 T cell responses against tumour antigens (Bonifaz et al. 2002; Bonifaz et al. 2004; Hawiger et al. 2001). These studies also highlighted the importance of co-transfer of an agent to induce DC maturation, and ensure antigen presentation does not result in T cell tolerance. Targeting of antigen to specific DC subsets has also allowed the examination of the different roles these DC have in regulating immunity. For example by targeting $CD8^+$ or $CD8^-$ DC, Dudziak et al. 2007 showed the different ability of these cells to process and present antigen via MHCI and II.

However a separate technique to target endogenous DC is to transduce them directly *in vivo* using a viral vector that expresses a tumour-specific antigen.

Transduced DC would then present this stable, endogenously synthesised protein antigen through MHCI and II pathways and induce potent and sustained T cell responses. This was shown to be a viable approach as, after injection of a GFP-containing lentivirus (LV), transduced DC can be detected in the skin draining LN (Arce et al. 2009). Antigen expression can be targeted specifically to DC though use of DC specific promotors (Lopes et al. 2008), or through the use of pseudotyped vectors which bind to specific targets (Yang et al. 2008). This is an attractive approach as DC are required to prime the response to antigen encoded by LV *in vivo* (Goold et al. 2011). However although they are necessary to induce T cell immunity, DC do not necessarily require direct LV transduction as they can acquire antigen from other transduced cells and cross-present this to naive T cells *in vivo* (Hotblack et al. 2017). However the benefit of direct DC transduction is that the DC can be further genetically engineered to improve the immune response. For example DC transduced by LV that encode constitutively active components of DC maturation signalling pathways prime enhanced T cell responses (Escors et al. 2008).

Although vaccination via these various strategies can be effective at inducing CTL responses *in vivo*, translation of this work into clinical trials has been largely disappointing (Rosenberg et al. 2004). However recent improvements to these techniques may be used to improve the T cell response and provide better clinical efficacy. For example vaccination in combination with checkpoint inhibitors (antibodies that block negative immune receptors), may be able to boost T cell responses induced by vaccination (Kleponis et al. 2015). This was shown in a recent clinical trial utilising a NY-ESO antigen fused to a DEC-205 antibody to target DC *in vivo*. 6 of the 8 patients treated with this vaccine in combination with checkpoint blockade showed objective tumour regressions (Dhodapkar et al. 2014). Likewise the choice of antigen used to vaccinate is important in generating a functional immune response. Vaccinations with 'cancer neoantigens' (tumour specific antigens generated by mutations in the tumour) have recently been described and could potentially be used enhance T cell reactivity (Schumacher and Schreiber 2015). Neoantigen-specific tumour vaccines have been shown to induce pronounced immune responses and

tumour regression comparable to checkpoint blockade (Gubin et al. 2014). The choice of tumour antigen to use is also important in other tumour immunotherapies and will be discussed in more detail in the context of adoptively transferred T cells.

1.4.2 Adoptive T cell therapy

Rather than priming an endogenous T cell response to a vaccine, an alternative approach to generating tumour specific T cells is to isolate autologous tumour infiltrating lymphocytes (TILs). These are then expanded *ex vivo* using tumour specific peptides and re-infused back into the patient where they can assume effector functions and mediate tumour control (Fig 1.9). Pre-clinical models of murine tumours, mainly melanoma, treated using adoptive cell therapy (ACT) solidified the idea of cellular immunotherapy as a viable treatment for cancer. Of note, these models formed many of the tenets of ACT that are required for effective T cell responses.

The initial studies of ACT in murine models showed that adoptive transfer of *ex vivo* cultured T cells caused regression of established tumours (Eberlein et al. 1982), which can be improved by the co-administration of exogenous IL-2 (Donohue et al. 1984). Soon after, tumour specific T cells isolated directly from surgically removed tumours (TILs) were also shown to be effective, although only when given in combination with lymphodepletive chemotherapy (Rosenberg et al. 1986). It was initially thought that this aided transferred T cells as the removal of the endogenous pool of T cells and NK cells allowed for transferred T cells to homeostatically expand *in vivo* (Dummer et al. 2002). However it has since been shown that whilst the removal of 'cytokine sinks' does favour expansion of transferred T cells, the presence of excess IL-7 and IL-15 also directly improved their anti-tumour function. (Gattinoni et al. 2005). In addition, the removal of endogenous Tregs through host pre-conditioning is important in reducing Treg-mediated immune suppression of transferred T cells (Antony et al. 2005). These promising pre-clinical results led to the design of clinical trials to treat patients with metastatic melanoma. In these landmark studies, adoptive

transfer of autologous TILs, in combination with host lymphodepletion and exogenous IL-2, resulted in objective response rates in 50-70% of patients with refractory, metastatic melanoma (Dudley et al. 2002; Dudley et al. 2008; Morgan et al. 2006).

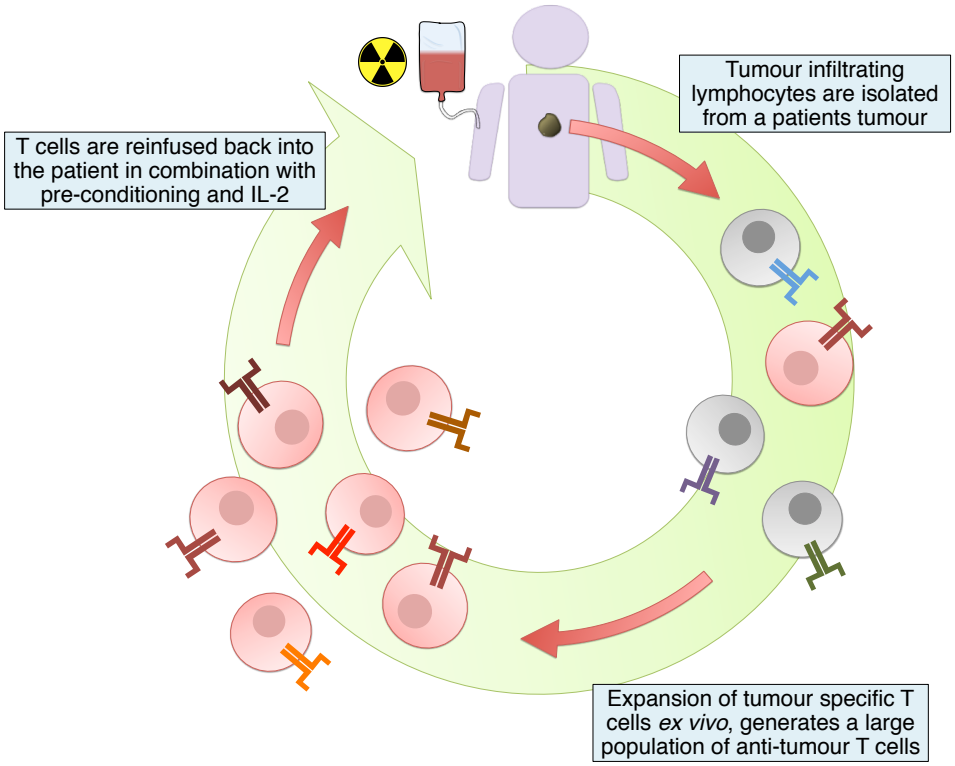


Figure 1.9: *Adoptive cell therapy.*

One of the benefits to producing tumour-specific T cells by isolating TILs is that the population of T cells that is generated are polyclonal and contain T cells with reactivity to a range of tumour antigens (Kvistborg et al. 2012; Lu et al. 2014). However whilst successful, isolating TILs isn't always viable for all patients. TIL extraction may not be possible in patients with tumours in locations that make surgical resection difficult or in patients with non-solid tumours. Likewise isolating sufficient numbers of TILs from less immunogenic tumours may be difficult. Lastly, exposure to immunosuppression in the tumour microenvironment, coupled with lengthy *ex vivo* culture may limit the efficacy of TILs once re-infused back into the patient (Restifo et al. 2012). Therefore alternative ways of generating tumour-

specific T cells, particularly ones that allow for a broader range of tumours to be treated, have been developed. Principal amongst these is the re-direction of polyclonal T cells to tumour-specificity through retroviral gene transfer.

1.4.3 TCR gene therapy

The antigen specificity of a T cell is defined solely by the TCR it expresses. Therefore tumour-specific T cells can be generated by introducing anti-tumour TCRs into peripheral blood lymphocytes. The concept of using viral transduction to introduce new TCR α and β chains into polyclonal T cells was shown by various groups. Retrovirally transduced T cells exhibited the same antigen-specificity as the T cell clone from which the TCR genes were isolated and these cells were functional *in vitro* (Clay et al. 1999; Cooper et al. 2000; Kessels et al. 2000; Stanislawski et al. 2001) and *in vivo* (Fujio et al. 2000). The feasibility of using this approach to generate tumour-specific T cells was subsequently demonstrated by Kessels et al. 2001. Here, T cells transduced with TCRs that recognise a model antigen were able to reject antigen-expressing tumours *in vivo*. These results paved the way for numerous further pre-clinical studies and clinical trials using TCR gene therapy for tumour immunotherapy.

Clinical trials of TCR gene therapy

The successful treatment of melanoma patients with ACT led investigators to attempt a similar approach with TCR-modified T cells. TCR genes were isolated from one of the responding T cell clones from a patient on this original ACT trial (Dudley et al. 2002). This TCR recognises the antigen 'melanoma antigen recognised by T cells 1' (MART1). After lymphodepletion, melanoma patients were treated with their own lymphocytes transduced with the HLA-A2 restricted MART1 TCR. Unfortunately only 13% of patients treated with these cells showed signs of a clinical response (Morgan et al. 2006). However this trial was shortly followed-up by another trial using a higher avidity MART1 TCR which led to an

improved clinical response rate of 30% (Johnson et al. 2009). This trial also tested a different TCR that recognises another melanoma antigen 'gp100', and reported clinical response rates of 19%. A similar trial using a TCR which recognises 'NY-ESO-1', a cancer/testis antigen expressed on some melanomas was also conducted. This resulted in clinical responses in 5 out of 11 patients tested (45%), including 2 complete responses (Robbins et al. 2011). Subsequent clinical trials have also been undertaken which have targeted p53, CEA (carcinoembryonic antigen) and MAGE A-3 (melanoma associated antigen 3), (reviewed in Duong et al. 2015). Results from these trials have suggested that TCR transduced T cells can induce clinical responses and control tumour growth, but only in a subset of patients. Therefore work is still needed, both pre-clinically and in clinical trials, to improve the reach of transduced T cells to non-responsive patients and improve overall efficacy.

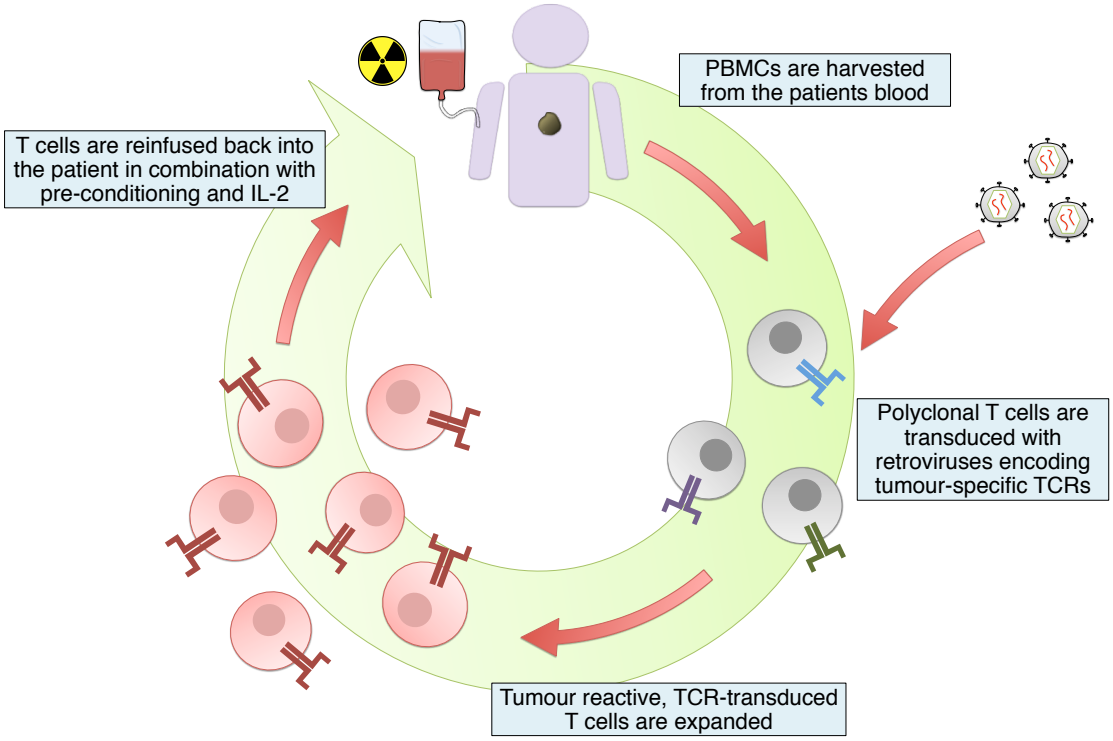


Figure 1.10: *TCR gene therapy.*

B16 melanoma models

One of the most well studied pre-clinical tumour models is the B16 melanoma. This is a spontaneous melanoma derived from a C57BL/6 mouse which has been used extensively for immunotherapy studies. T cells transduced with TCRs which recognise an antigen expressed on B16 cells were shown to be able to delay tumour growth in mice with already established B16 tumours (Abad et al. 2008). The TCR genes used in this study were designated 'Pmel-1' as they recognised the protein antigen Pmel-17, the mouse homologue of the human gp100 protein. These proteins, like MART1, are melanocyte differentiation antigens that are expressed in melanocytes but also in B16 melanoma and the majority of human melanomas (Kawakami et al. 1992; Overwijk et al. 2003). Subsequent identification of further self/melanoma antigens that are commonly shared between melanomas led to further pre-clinical models with TCRs targeting these antigens. TCR gene therapy with TCRs that recognise tyrosinase-related protein 1 (TRP1) (Kerkar et al. 2011) and TRP2 (Chinnasamy et al. 2013) have also been shown to control B16 progression *in vivo*. However in the majority of these studies, anti-melanoma TCR expressing T cells were only able to induce transient tumour control. Generating a durable T cell response that can mediate complete tumour elimination remains a challenge in the field.

One of the factors that could be limiting these T cells is that the introduced TCRs recognise self antigens. The benefits of this approach is that these self antigens are often broadly applicable to many patients, as is the case with melanoma antigens such as MART1 and gp100. However the downside is that the T cell response to a self antigen is often limited. The choice between using self- or foreign-reactive TCRs is discussed in more detail below.

Choice of antigen for TCR gene therapy

The choice of a suitable antigen to target is an important issue for both TCR gene therapy and tumour vaccines. Tumour antigens can be split into two

groups; tumour associated antigens (TAAs) or tumour specific antigens (TSAs). TAAs are non-mutated self antigens that are usually only expressed on certain cell types or in specific tissues. These include the cancer testis antigens (such as NY-ESO-1), which are antigens usually only expressed on male germline cells, but are also often expressed in cancers. The second class of TAA are tissue differentiation antigens. These are usually only expressed in specific tissues but will often be upregulated in tumours arising from those tissues. These include the melanocyte differentiation antigens, MART1, TRP2 and gp100 which are expressed in melanocytes and melanomas. Lastly, other TAAs can derive from ubiquitously expressed proteins that are overexpressed in tumours, including Her-2/Neu and PRAME antigens (Heemskerk et al. 2012). The benefit of TAAs is that many are shared between tumours and patients (Kawakami et al. 1992), which increases the practicality of targeting these antigens with TCR gene therapy. However TAA-specific T cells that are generated in patients have been regulated by central and peripheral tolerance mechanisms designed to prevent autoimmunity and the TCRs generated are often of a low avidity to TAAs. For example thymic expression of gp100 and TRP1 limits the generation of T cells reactive to those self antigens (Träger et al. 2012; Zhu et al. 2013). The induction of peripheral tolerance to these antigens may also limit the efficacy of adoptively transferred T cells. For example adoptively transfered, gp100 specific T cells were functionally tolerant to a B16 tumour unless mice were also given an antigen-specific vaccination (Overwijk et al. 2003). Likewise, CD8 T cells transduced with a TCR for the TAA 'MDM2' became tolerant *in vivo* unless provided with antigen specific helper CD4 T cells (Ghorashian et al. 2014). In addition, whilst targeting TAAs can be effective, this can be coupled with 'off-tumour, on-target' effects where the T cells attack healthy tissue which expresses the target antigen. This has been shown in clinical trials where gp100 and MART1 specific T cells caused adverse effects due to TAA expression in the skin, inner ear and retina (Johnson et al. 2009).

Therefore as an alternative, tumour specific antigens (TSAs) are an attractive target for TCR gene therapy. TSAs or 'neoantigens' are formed through DNA mutations that gather in the tumour genome and produce novel protein

sequences. Alternatively tumours that arise as a result of oncogenic viruses can also express TSAs from the viral open reading frames. Theoretically TSAs make ideal targets for immunotherapy as expression of neoantigens are entirely absent from normal tissues and TCRs with neoantigen specificity are devoid of control by central tolerance (Schumacher and Schreiber 2015). Neoantigen-specific T cells are therefore expected to be more tumour reactive than TAA-specific T cells (Heemskerk et al. 2012). Indeed tumour-responding T cells from a melanoma patients blood were predominantly reactive against patient specific neoantigens rather than the shared TAAs (Lennerz et al. 2005). Therefore generation of neoantigen specific T cells by providing patients with a vaccine containing neoantigen peptides has been proposed (Vonderheide and Nathanson 2013). Alternatively the feasibility of producing neoantigen-specific TCR transduced T cells has been described. TCRs specific for a patients melanoma neoantigens have been identified and isolated from the blood of healthy controls and T cells transduced with these TCRs were able to recognise patient tumour samples *in vitro* (Stronen et al. 2016). A potential downside to these neoantigen specific responses however is that the infiltration of neoantigen-specific T cells into the tumour has been associated with the subsequent loss of that neoantigen expression from the tumour (Verdegaal et al. 2016). This shows that neoantigen specific responses are prone to immunoediting by the tumour. However identification of so called 'trunk' mutations that are universally expressed in the heterogenous tumour population may help prevent immune escape (Gerlinger et al. 2012; Mcgranahan et al. 2016). However the major downsides to targeting neoantigens is that the vast majority are patient specific (Heemskerk et al. 2012). As a result, the current TCR gene therapy strategies for patients all focus on TAA and the feasibility of targeting neoantigens in a patient specific manner will need to be addressed if therapies targeting these antigens are to realise their potential.

Chimeric antigen receptors

An alternative to transducing T cells with tumour reactive TCRs, is to use a novel class of engineered receptor called the chimeric antigen receptor (CAR). The basic structure of a CAR is an extracellular antigen binding domain that is linked by a transmembrane domain to intracellular signalling provided by CD3-ζ chain (Fig 1.11). Antigen specificity is typically provided by a single-chain antibody variable fragment (scFv), although other receptor-ligand interactions can also be used such as cytokine receptors (Kahlon et al. 2004; Kong et al. 2012). Initially CAR-expressing T cells would proliferate poorly as antigen presentation and CD3-ζ signalling alone was insufficient to fully activate the T cell (Brocker and Karjalainen 1995). However subsequent generations of CARs had co-stimulatory domains such as CD28, 4-1BB and OX-40 added to their signalling domains, which has improved T cell activation (Finney et al. 2004). One of the major benefits to using CAR gene therapy is, unlike TCR gene therapy, recognition of antigen is not dependant on presentation by MHC. This

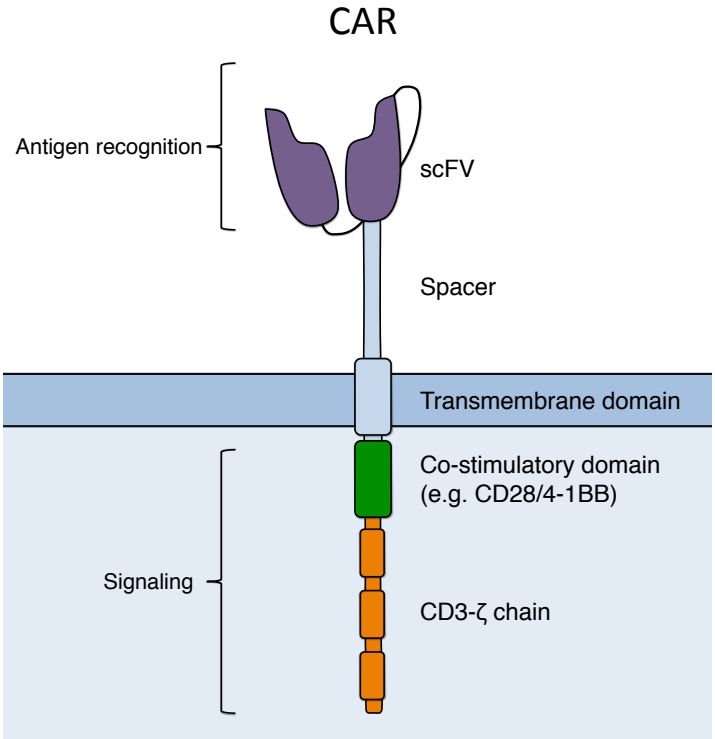


Figure 1.11: *Chimeric antigen receptor.*

vastly improves the scope of treatments using CARs as the transduced cells are not restricted by HLA type. A recent clinical trial using T cells transduced with a CD19-specific CAR to treat patients with relapsed acute lymphoblastic leukaemia has shown the efficacy of CAR therapy. Complete remissions were achieved in 27 out of 30 patients treated with these cells (90%) (Maude et al. 2014). However the disadvantage with CAR therapy is that the range of antigens able to be targeted is less than for TCR modified T cells. For example while TCRs can recognise intracellular antigens including TAAs and TSAs, CARs are restricted to antigens expressed on the cell surface.

Maximising introduced TCR avidity

Although TAA specific TCRs are often of low affinity, responses from T cells transduced with these TCRs can be improved by enhancing the TCR avidity. One of the primary mechanisms to achieve this is by enhancing TCR expression on the cell surface. The TCR is formed when the TCR α and β chain heterodimer complexes with the CD3 molecules, so these components need to be optimally expressed in TCR transduced T cells to enhance expression. One potential problem associated with TCR gene therapy is that the introduced α and β chains can pair, not with each other, but with the endogenous α and β chains. This not only reduces the availability of CD3 for the introduced TCR but these 'mispaired' TCRs can also generate unknown and potentially autoreactive specificities (Bendle et al. 2010). To help promote specific pairing of the introduced TCRs, additional cysteine residues have been added to the TCR constant regions (Cohen et al. 2007; Kuball et al. 2007). This promotes formation of an additional disulphide bond between the introduced α and β chains and was shown to reduce mispairing-associated toxicity (Bendle et al. 2010). Similar approaches have altered the configuration of the constant regions of introduced α chains to favour binding of the reciprocally altered β chain (Voss et al. 2007). Alternatively the constant regions of human TCRs have been replaced with murine constant regions, which preferentially pair with themselves, rather than the fully human endogenous TCR chains. This has been shown to

increase introduced TCR cell surface expression and improved T cell function (Cohen et al. 2006; Thomas et al. 2007).

Mispairing can also be reduced by ensuring that there are equal amounts of introduced TCR α and β chains available. Use of a viral self-cleaving 2A peptide sequence to separate the α and β chains in the viral vector results in the co-translational cleavage of one mRNA strand containing both TCR chains. This has been proposed to improve specific pairing as both chains are expressed equally (Furler et al. 2001; Szymczak et al. 2004). This approach differs from use of an internal ribosome entry site (IRES) which requires two transcription events to produce the α and β chains.

Lastly, even in the absence of mispairing, to be expressed on the cell surface introduced TCRs must compete with endogenous TCRs for CD3 (Call and Wucherpfennig 2005; Thomas et al. 2010). Codon optimisation of the introduced TCR sequences can improve gene expression and allow introduced TCRs to outcompete endogenous TCRs. This has been shown to be crucial for high levels of TCR expression on the cell surface (De Witte et al. 2008a; Kerkar et al. 2011). Alternatively as CD3 availability can be limiting, provision of additional CD3 by co-transduction of the TCR with CD3 γ, δ, ε and ζ chains can enhance introduced TCR expression on the cell surface and improve anti-tumour function *in vivo* (Ahmadi et al. 2011).

Optimising TCR gene therapy - DC interaction

The potential role of T cell - DC interactions have been shown in ACT models, where effective responses from adoptively transferred transgenic T cells require the provision of tumour-antigen expressing vaccines (Overwijk et al. 2003). This effect may be as a result of tumour-vaccines being required to break tolerance to tumour antigen in TCR transgenic mice (Schreurs et al. 2000), however in another study whilst vaccination enhanced the proliferation of TRP2 transgenic T cells *in vivo*, they still became tolerised and were unable to control B16 growth (Singh et al. 2009). In addition, the use of vaccines to supplement *in vitro*

activated, TCR transduced T cells is less clear. One study has found that vaccination was essential for tumour control by TCR transduced T cells in a prostate cancer model (De Witte et al. 2008b), however the immunological mechanism behind this synergy was not investigated. Importantly, the role of endogenous DC rather than DC in the context of a vaccine, has not been investigated in these models, and could have an important impact on the efficacy of TCR-transduced T cells.

The role of DC in the adoptive transfer of T cells may vary when TCR-transduced T cells are transferred, rather than transgenic T cells. In order to transduce T cells with retroviruses expressing TCR genes, the T cells must be activated *in vitro*. This could have numerous effects on the T cell population used to treat tumours and affect their interaction with endogenous immune cells. For example T cell activation and transduction with retrovirus *in vitro* has been shown to impair the function of adoptively transferred T cells compared to naive T cells (Sauce et al. 2002). This could be as a result of enhanced effector differentiation; in pre-clinical tumour models transfer of T_{CM} cells resulted in greater expansion and anti-tumour efficacy than transfer of T_{EM} cells (Klebanoff et al. 2005). In contrast to T_{CM} populations, T_{EM} are able to rapidly differentiate into effector T cells but have limited proliferative potential (Sallusto et al. 1999). Effector cells that differentiate from adoptively transferred naive populations, rather than T_{CM} have also been shown mediate enhanced tumour responses *in vivo* (Hinrichs et al. 2009). Interestingly these data suggest that *in vitro* activated, transduced T cells can interact with DC *in vivo* to differentiate and acquire effector function. In line with this, the greater efficacy of tumour control mediated by these less-differentiated T cells corresponds to enhanced trafficking to secondary lymphoid organs (Klebanoff et al. 2005). Indeed, following adoptive transfer, TCR transduced T cells have also been shown to accumulate in tumour draining, but not in non-tumour draining LN (Koya et al. 2010). This suggests a role for DC mediated antigen presentation to transferred T cells in the lymphoid organs. However the importance of these potential interactions in mediating tumour control by transferred T cells is not known. In addition to potentially controlling transferred T cell responses in the lymphoid organs, DC may also

have a role in controlling T cell effector function in the tumour. Recently, depletion of $CD103^+$ DC during the transfer of activated, antigen-specific T cells was shown to limit, but not prevent tumour control. This was thought to be dependant on tumour resident DC as the importance of priming CTLs in the LN was avoided by blocking lymph node egress with FTY-720 (Broz et al. 2014). Transferred T cells therefore have numerous opportunities to interact with endogenous DC, however the extent/importance of these potential interactions are unclear.

1.5 Research aims and hypothesis

Considering the numerous roles of DC in promoting and controlling endogenous T cell responses, it is likely that DC are also involved in regulating or promoting transferred T cell responses. However DC have varied functions that can either enhance or diminish tumour control. These appear to be dependant on a number of factors, such as the specific DC subset, their maturation state and the phase of immunoediting that the tumour is exposed to. A greater understanding of the roles these DC have in the anti-tumour immune response is important for improving tumour immunotherapies. In particular, the context in which DC interact with TCR-transduced T cells will effect their ability to control tumour growth.

This project therefore was designed to investigate the interactions between endogenous DC and TCR-transduced T cells. We hypothesised that endogenous DC were required for the effective clearance of tumours by TCR transduced T cells. To address this we chose to use the CD11c.DTR and CD11c.DOG depletion models. This would allow us to interrogate numerous aspects of transferred T cells efficacy, including *in vivo* expansion and tumour control, in the absence of DC.

Chapter 2 Materials and Methods

2.1 Cloning of TCRs into pMP71 vector

2.1.1 PCR reactions

DNA for the TRP2 and Pmel-1 TCRs were a kind gift from Prof Ton Schumacher (Netherlands Cancer Institute). These were originally in the pMX plasmid, but were cloned into the pMP71 IRES CD19 vector. To clone the TRP2 TCR sequence into the pMP71 vector, primers were designed to be flanked by the restriction sites Not1 and Sal1 (highlighted in bold). These were designed using the DNA analysis program SnapGene and ordered from Invitrogen:

Not1 TRP2 β fw:
5'- ATTT**GCGGCCGC**ATGGCCCCGCGGCTGCTGTG -3'
TRP2 α Sal1 rv:
5'- CGCG**GTCGAC**TCAGCTGCTCCACAGCCTCAGGGT -3'

A polymerase chain reaction (PCR) was carried out with 1µg DNA from the pMX plasmid containing the TRP2 TCR. This was added to 10µl 2xPhusion PCR master mix (Invitrogen), 10mM forward primer, 10mM reverse primer and made up to 20µl with nuclease free water. The PCR reaction was then carried out as follows: 3min initial denaturation at 94℃, then 30 cycles of: 30s denaturation at 94℃, 1min annealing at 58℃ and 1min elongation at 72℃, lastly there was a final 10min elongation at 72℃.

To clone the Pmel-1 TCR and replace the IRES sequence with a 2A sequence an overlap extension PCR was performed using the pMX Pmel-1 DNA. The first PCR amplified the α chain and a separate PCR reaction amplified the β chain. These products omitted the IRES sequence which was replaced with overlapping sections containing the 2A sequence (underlined):

Not1 Pmel-1 α fw:
5'- ATTT**GCGGCCGC**ATGAAGAGCCTGAGCGTGAGCCTGGTGGTGCTG -3'
Pmel-1 α P2A rv:
5'-CCGGCCTGCTTCAGCAGGCTGAAGTTGGTGGCGCCGCTGCCGCTGCTG
CTCCACAGCCTCAGGGTCATCA -3'

This PCR was done alongside another PCR to amplify the β chain:

P2A Pmel-1 β fw:
5'- CACCAACTTCAGCCTGCTGAAGCAGGCCGGCGACGTGGAGGAAAACCC
TGGCCATGGCCACAACCATGGCCCC -3'
Pmel-1 β Sal1 rv:
5'- GAC**GTCGAC**TCAGCTGTTCTTCTTCTTCACCATGGCCATC -3'

These two PCR reactions were carried out as described above for the TRP2 TCR, although the annealing temperature was reduced from 58 to 55℃. Finally the fusion PCR was performed using the products from these two PCR reactions (0.5µg each) and the primers that bind either side of the whole sequence (Not1 Pmel-1 α fw and Pmel-1 β Sal1 rv). This produced a final PCR product that contained the Pmel-1 α and β chains, separated by the 2A sequence and flanked by Not1 and Sal1.

PCR products were extracted by gel electrophoresis (2% Agarose, 0.5x TBE + 0.5x water, Ethidium Bromide 1:1000) after addition of Gel Loading Solution (Sigma G2526) in a 1:5 dilution with 5µl of Hyper Ladder 1 (Bioline 33025) to determine the fragment size. Gels were analysed using Ultrospec 1100 pro (Amersham Biosciences) and the specific band cut and purified with QIA Quick Gel extraction kit (Qiagen 28704).

2.1.2 TRP1 TCR sequence design and synthesis

The TRP1 TCR sequence (Kerkar et al. 2011; Muranski et al. 2008) was synthesised by GeneArt (Invitrogen) with Not1 and Sal1 sites flanking the TCR, so a PCR reaction was not required. In addition the TCR was codon-optimised

and an extra cysteine was added by amino acid substitution in both chains: α-T179C and β-S186C.

2.1.3 Plasmid digestion and ligation

The TRP2, Pmel-1 and TRP1 TCRs were cloned into the pMP71 IRES CD19 vector backbone by excision of the F5 TCR and replacing it with the relevant TCR. The PCR products (TRP2 and Pmel-1 TCRs), 1µg of the pMX TRP1 vector (from GeneArt) and 1µg of the pMP71 F5 CD19 vector (from laboratory stocks, see appendix for details) were separately digested with 1µl of Not1 (NEB R0189L) and 1µl of Sal1 (NEB R0138L) restriction enzymes, with 2µl of 10x buffer 3 (NEB B7003S) and made up to 20µl with nuclease free water. Digestion reactions took place at 37℃ for 1 hour. After which, the digested pMX TRP1 and pMP71 F5 CD19 vectors were analysed by gel electrophoresis as described above. The TRP1 TCR (1800bp) and the pMP71 CD19 backbone (6301bp) from which the F5 TCR had been excised were extracted with QIAquick Gel Extraction Kit and eluted in 50µl of nuclease free water. The digested PCR products containing the TRP2 (1801bp) or Pmel-1 (1813bp) TCRs were purified with QIA Quick PCR purification kit (Qiagen 28106) and DNA was eluted in 30µl of nuclease free water.

A ligation reaction was carried out to ligate the TRP2, Pmel-1 or TRP1 TCRs into the empty pMP71 CD19 vector. This was performed in an Eppendorf tube at room temperature for 10 minutes containing the following reagents: 10µl 2x Quick Ligase Buffer (NEB B2200S), 1.5µl of the digested vector backbone, 3µl digested TCR inserts, 1µl Quick T4 DNA Ligase (NEB M2200L) and 4.5µl nuclease free water.

2µl of the ligation product was added to 25µl of Max Efficiency DH5α bacteria (NEB C2987H) in a 1.5ml eppendorf and tubes left on ice for 30min. Tubes were heat-shocked at 42℃ for 45s followed by a 2min incubation on ice. Transformed bacteria were plated onto LB agar plates (0.1 mg/ml, Sigma-Aldrich) containing Ampicillin and left in the incubator overnight at 37℃. Colonies were inoculated into 5ml of Ampicillin containing LB Broth medium (0.1 mg/ml, Sigma-Aldrich)

and incubated overnight. DNA was extracted using QIAPrep Spin Miniprep kit (Qiagen 27106) according to manufacturers instructions and sent to Eurofins MWG Operon for sequencing. For larger DNA preparations, correctly transformed colonies were inoculated into 100ml of LB broth overnight and DNA extracted using the Qiagen Plasmid Midi Kit (Qiagen 12143).

2.1.4 Final TCR retroviral constructs

The plasmid maps and DNA sequences for these TCRs can be found in the appendix. The retroviral vector pMP71 was used for all TCR constructs with a 2A sequence separating the α and β chains, followed by an IRES CD19 sequence. The F5 TCR recognises the influenza A virus nucleoprotein ($NP_{366-379}$) in the context of H-$2D^b$. The Pmel-1 TCR recognises the gp100 peptide ($gp100_{25-33}$) in the context of H-$2D^b$. The TRP2 TCR recognises Tyrosinase related protein 2 ($TRP2_{181-188}$) in the context of H-$2K^b$. The TRP1 TCR recognises Tyrosinase related protein 1 ($TRP1_{113-127}$) on MHCII. All TCRs were codon optimised and the F5, TRP1 and TRP2 also contain an extra cysteine residue in the constant chain.

2.2 Cell culture

2.2.1 Tissue culture and cell counting

Tissue culture work was performed in Biohit Biological Safety Cabinet Class 2 hoods. Cells were counted on a haemocytometer (Abcam). 10µl of cells were mixed with 10µl Trypan Blue (Life Technologies, 15250-061) and live cells were counted under a light microscope as cells that had not taken up the dye.

For counting cells from FACS samples, CountBright Absolute Counting Beads (Life Technologies, C36950) were used. A set volume (10µl) of beads were added per sample, which relates to a certain amount of beads. From this the total number of cells per sample can be calculated.

2.2.2 Phoenix eco and ampho cells

The human 293T cell line (a human embryonic kidney line transformed with adenovirus E1a) had been stably transfected with DNA encoding for the gag-pol proteins as well as the ecotropic or amphotropic virus envelope (Nolan laboratories). These packaging cells are easily transfected with DNA for the production of retroviruses. Cells were grown in tissue culture flasks 75 qcm (TPP 90076) with Isocove's Modified Dulbecco medium (IMDM) (Lonza BE12722F), supplemented with 10% Fetal Calf Serum (FCS) (Biosera), 1% L-glutamine 200mM (GIBCO 25030) (2 mM) and 1% Penicillin/Streptomycin (GIBCO 15070) (100 U/ml). Cells were detached by treating them with 3ml of 0.05% Trypsin-EDTA (GIBCO 25300) for 1 minute before neutralisation with normal medium. Cells were split 1/8 every 2 days as the cells became over 90% confluent.

2.2.3 B16.F10

B16.F10 is a murine melanoma cell line derived from a C57BL/6 mouse. Cells were cultured in tissue culture flasks 75 qcm (TPP 90076) in RPMI 1640 medium (Lonza BE12-167F), supplemented with 10% FCS (Biosera), 1% L-glutamine (2 mM) and 1% Penicillin/Streptomycin (100 U/ml). Cells were detached by treating them with 3ml of 0.05% Trypsin-EDTA (GIBCO 25300) for 3 mins in a 37℃ incubator, before neutralisation with normal medium. Cells were split 1/10 every 2 days as the cells became over 90% confluent.

2.2.4 EL4-NP

EL4 and EL4-NP are murine lymphoma cell lines derived from a C57BL/6 mouse. EL4-NP was made to stably express the influenza A virus nucleoprotein (NP), and will express this peptide in the context of H-2D^b. These were a kind gift from Prof B. Stockinger (Francis Crick Institute, London). EL4-NP cells were maintained in RPMI 1640 medium (Lonza BE12-167F), supplemented with 10% FCS (Biosera), 1% L-Glutamine (2mM) and 1% Penicillin/Streptomycin (100 U/ml). Cells were split 1/10 every 2 days. Transfected EL4-NP cells are also resistant to the antibiotic Geneticin (Sigma G418 disulfate salt solution, A1720). 1mg/ml of Geneticin was added to culture media once a week to select for transfected cells.

2.2.5 Murine T cell culture

C57BL/6 splenocytes were mashed through a 40µM cell strainer (BD Falcon 352340) into a 50ml Falcon centrifuge tube (TPP) and washed with PBS. Red blood cells were lysed by resuspending the cell pellet in 2ml ammonium-chloride-potassium (ACK) lysing buffer (Lonza 10-548E) for 2mins. Cells were then washed with 20ml PBS. Cells were MACS sorted (see below) or not, and cultured in RPMI 1640 medium (Lonza BE12-167F), supplemented with 10% Fetal Calf Serum (FCS) (Biosera), 1% L-glutamine (2 mM), 1%

Penicillin/Streptomycin (100 U/ml) and 2-Beta-Mercaptoethanol (50µM).

2.2.6 T cell selection: MACS sorting

Miltenyi CD8a (Ly-2) MicroBeads (130-049-401) or CD4 (L3T4) MicroBeads (130-049-201) were used to sort CD8 or CD4 T cells respectively. Splenocytes were prepared as described above and resuspended in 630µl MACS buffer (0.5% bovine serum albumin (BSA) and 2mM Ethylendiamintetraacetat (EDTA)) per spleen. 70µl of microbeads were added per spleen and incubated on ice for 20min. LS magnetic separation columns (Miltenyi 130-042-401) were rinsed with 3ml of MACS buffer. Cells were washed with 30ml of MACS buffer and resuspended in 500µl MACS buffer per 10^8 cells/per spleen. Cells were loaded into columns through a pre-separation filter (Miltenyi, 130-101-812) and washed 3 times with 3ml MACS buffer. Cells were eluted twice with 4ml MACS buffer into a 15ml Falcon tube.

2.2.7 RMA/S cells

RMA-S cells are a TAP deficient derivative of the RMA cell line, which can efficiently present exogenously loaded peptide (De Bruijn et al. 1991). RMA/S cells were cultured in RPMI 1640 medium (Lonza BE12-167F), supplemented with 10% FCS (Biosera), 1% L-Glutamine (2mM) and 1% Penicillin/Streptomycin (100 U/ml). Cells were split 1/10 every 2 days.

2.2.8 Generation of BM-DC

Bone marrow derived DC (BM-DC) were generated by flushing the tibia and femur of C57BL/6 mice. Bone marrow was flushed with 5ml RPMI using a 2ml syringe (BD, 300185) and 27G needle (BD, 300635). Cells were pipetted to generate a single cell suspension and passed through a 40µM cell strainer (BD Falcon 352340) into a 50ml Falcon centrifuge tubes (TPP). Red blood cells were lysed by resuspending the cell pellet in 2ml ACK lysing buffer (Lonza 10-548E)

for 2mins. Cells were then washed with 20ml PBS, then again in complete RPMI media (10% FCS (Biosera), 1% L- glutamine (2 mM), 1% Penicillin/Streptomycin (100 U/ml) and 2-Beta-Mercaptoethanol (50µM)). Cells were counted and resuspended at $2x10^6$/ml. 1ml of cells were plated in a 24 well plate (TPP) with 40ng/ml recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF) (Peprotech, 315-03). 48 hours laters, non-adherent cells were washed off by rinsing around the well with 1ml RPMI media which was discarded and replaced with fresh media and GM-CSF. Media was replaced every 2-3 days, and BM-DC were mature by day 7.

2.3 Retroviral production and transduction

2.3.1 Virus production

1.5-2.0x10^6 Phoenix eco packaging cells were plated on a 60.1 qcm tissue culture dish (TPP 93100) in 8ml of complete IMDM medium. The next day medium was replaced with 5ml of fresh IMDM medium. The transfection mix was set up as follows; 10µl Fugene HD Transfection Reagent (Roche 04709713001) was added to 75 µl OPTIMEM media (GIBCO 31985) in a 1.5ml Eppendorf tube. The DNA mix was prepared separately; 2.6µg of vector DNA and 1.5µg pCl-Eco DNA were added to a total volume of 50µl water. DNA mix was carefully added to the Fugene mix and incubated at room temperature for 15-20 minutes before dripping it onto the Phoenix eco plates. The next morning, medium was replaced with 5ml complete T cell medium and cells were incubated during the day and overnight. On day 4, virus supernatant was collected from Phoenix eco plates and spun down to remove cell debris. Virus supernatant could either be used for transduction or stored at -80℃.

2.3.2 T cell transduction

Bulk or CD4/CD8 sorted splenocytes were resuspended at 1.5x10^6 cells/ml and activated with Concanavalin A (ConA) (Sigma, L7647) (2 µg/ml) and IL-7 (R&D Systems, 207-IL) (1ng/ml) for 24 hours before transduction. The next day cells were counted and 4-6x10^6 T cells were resuspended in 1.5ml of viral supernatant. Non-tissue culture plates were prepared by coating with 2ml RetroNectin (Takara T100B) for 3 hours at room temperature, then blocked with filter sterilised 2% BSA/PBS for 30 minutes and washed twice with PBS. Cells were added to these prepared plates and spun at 2,000 rpm for 90 minutes at 32℃. 2.5ml of T cell media containing IL-2 (Roche, 11011456001) (total volume of 100 U/ml) was added and cells were cultured in a 37℃ incubator. Fresh media and IL-2 was added every 2-3 days.

2.3.3 B16.F10 transduction

Retroviral supernatant was generated as above, although Phoenix ampho cells and pCl-Ampho DNA were used. pMP71 NP IRES GFP had previously been generated by a PhD student in the laboratory, Bernado Alvares. $1x10^5$ B16.F10 cells were plated onto a 6 well plate (TPP) and 1.5ml of viral supernatant was added overnight. The next day, cells were detached with 500µl 0.05% Trypsin-EDTA (GIBCO 25300) for 3 mins in a 37℃ incubator, before neutralisation with normal medium. Cells were transferred to a T75 tissue culture flask (TPP 90076) for continued culture. Transduction was measured by GFP expression by FACS. Transduced cells were FACS sorted by gating on GFP^+ cells on a BD FACSAria.

2.4 Flow cytometry

2.4.1 Surface staining

<$2x10^6$ cells (typically 0.5-$1x10^6$ cells) were routinely stained for FACS analysis. Cells were washed once with PBS and resuspended in 50µl of FACS buffer (1% FCS/PBS) containing the monoclonal antibodies of interest and Fc block in the appropriate dilutions (see table 2.1 for details). Cells were incubated on ice in the dark for 20-30 minutes before being washed in FACS buffer and analysed on the FACS machines. FACS data was acquired on a BD Fortessa or LSR 2 FACS machine.

2.4.2 Intracellular staining

BD cytofix/cytoperm kit (BD Biosciences 554714) was used for intracellular cytokine staining. Cells were firstly stained for surface molecules as above, and fixed with 100µl of 2% paraformaldehyde for 10mins on ice. Cells were washed with 200µl FACS buffer and left in the fridge overnight. The next day cells were fixed with 50µl of the fixation solution and incubated on ice for 15 mins. Cells were washed with 150µl of perm/wash solution, and incubated for 30mins with the intracellular antibodies (IFN-γ APC) diluted in perm/wash buffer. Cells were finally washed once in perm/wash and again in FACS buffer before the cells were analysed.

Ki67 staining was performed using the FoxP3 staining kit (eBioscience, 00-5523-00). Cells were surface stained as above and fixed with 100µl of FoxP3 Fix/Perm solution for 20-30mins on ice. Cells were then washed in Perm wash and stained with Ki67 eFluor660 diluted in Perm wash for 30mins. Cells were finally washed in Perm wash, and again in FACS buffer before being analysed.

Table 2.1: FACS antibodies

Specificity	Fluorochrome	Manufacturer	Dilution
CD3	FITC	BD (553062)	1/100
CD4	APC-H7	BD (580181)	1/400
CD4	FITC	BD (553047)	1/400
CD8	APC	BD (5530350)	1/400
CD8	v450	BD (560469)	1/400
CD11b	v450	eBioscience (48-0112-82)	1/400
CD11c	APC	BD (550261)	1/200
CD11c	FITC	BD (553801)	1/200
CD19	PerCP-Cy5.5	eBioscience (45-0193)	1/200
CD24	BV711	BD (563450)	1/1000
CD45.2	v500	BD (562129)	1/200
CD62L	PE	BD (55315)	1/400
CD62L	Alexa700	BD (560517)	1/400
CD64	PE	Biologend (139303)	1/200
CD103	Biotin	BD (557493)	1/100
CD127	eFluor660	eBioscience (50-1271-80)	1/100
F4/80	APC-eFluor780	eBioscience (47-4801-82)	1/400
H2-Kb	FITC	BD (553569)	1/100
I-Ab	PE	BD (553552)	1/800
IFN-γ	APC	BD (554413)	1/200
Ki-67	eFluor660	eBioscience (50-5698)	1/200
Ly6C	PeCy7	BD (560593)	1/400
NK1.1	APC-eFluor780	eBioscience (47-5941-80)	1/200
PD-L1	APC	BD (564715)	1/400
Streptavadin	PerCP-Cy5.5	BD (551419)	1/100
Thy1.1	PeCy7	eBioscience (25-0900-82)	1/10,000
Vβ3	PE	BD (553209)	1/400
Vβ11	PE	BD (553198)	1/200
Vβ13	PE	BD (561541)	1/800
Vβ14	FITC	BD (553258)	1/400
Fc-block (CD16/CD32)	*N/A*	BD (14-0161-86)	1/100

2.5 Functional assays

2.5.1 T cell co-culture with peptide-loaded cells

T cells transduced with TCRs were left for 5 days post-transduction then stimulated with peptide loaded cells. $1x10^6$ RMA/S cells were cultured overnight at 20℃ to stabilise empty MHC molecules. The next day RMA/S cells were loaded with 10μM - 100pM of relevant or irrelevant peptide. Alternatively, on day 7 of BM-DC culture, cells were loaded with peptide as above. Peptides used were $TRP2_{181\text{-}188}$: VYDFFVWL
$gp100_{25\text{-}33}$: EGSRNQDWL
$TRP1_{113\text{-}127}$: CRPGWRGAACNQKI
Cells were incubated with peptide in an 37℃ incubator for 2-3 hours. RMA/S cells were then irradiated with 80Gy. Cells were then washed in complete RPMI and $1x10^5$ RMA/S cells or $1x10^4$ BM-DC were plated in a 96-well plate. To this, $1x10^5$ mock or TCR transduced T cells were added, and cells were co-cultured overnight (at ratios of 1:1 or 10:1 respectively). The next morning, cells were spun and 100-150μl of supernatant was harvested for ELISA, if necessary. Cells were resuspended in media to which 5μg/μl brefeldin A had been added. Brefeldin A blocks the transport of proteins in the Golgi apparatus, so prevents secretion of cytokines to allow intracellular staining. After 4 hours, cells were washed in FACS buffer and stained for intracellular IFN-γ as described above.

2.5.2 Upregulation of MHC on B16.F10

MHCI is upregulated by B16.F10 in the presence of IFN-γ (Seliger et al. 2001). To generate media containing murine IFN-γ, splenocytes were activated with ConA and IL-7 as described previously. After 24 hours, cells were centrifuged and supernatant was collected and frozen at -20℃. B16.F10 were cultured overnight in this defrosted ConA activated splenocyte media (CASM) to

upregulate expression of MHC. Cells were trypsinised the next day and stained with H-2Kb, I-Ab and PD-L1 antibodies.

2.5.3 T cell co-culture with tumour cells

CASM was added to B16.F10 cells overnight and the next day, B16.F10 were washed with complete RPMI. B16.F10 and EL4-NP cells were then irradiated with 80Gy and $1x10^5$ tumour cells were washed and plated in 96-well plates. $1x10^5$ mock or TCR transduced T cells were then added overnight. Alternatively for shorter incubations, tumour cells weren't irradiated and were cultured with T cells for 4 hours. The next morning (or 4 hours later) cells were spun and 100-150µl of supernatant was harvested for ELISA, if necessary. Cells were resuspended in media to which 5µg/µl brefeldin A had been added. After 4 hours, cells were washed in FACS buffer and stained for intracellular IFN-γ as described above.

2.5.4 ELISA

Cytokine-containing supernatants were generated as above and IFN-γ was measured using the BD OptEIA kit (555138), according to manufacturers instructions. Supernatants were diluted 1/4 in RPMI media before use in the assay.

2.6 *in vivo* experiments

2.6.1 Mice

Animal protocols were approved by local institutional research committees and in accordance with U.K. Home Office guidelines. C57BL/6 mice aged between 8 and 15 weeks were obtained from the in-house animal facility at the Royal Free Hospital. Similarly aged Thy1.1 C57BL/6 mice from the animal facility were also used as donors in multiple experiments. Homozygous CD11c.DTR/GFP (B6.FVB-Tg(Itgax-DTR/ EGFP)57Lan/J) mice were bred in-house and used experimentally as heterozygotes. Homozygous CD11c.DOG mice (Tg(Itgax-DTR/OVA/EGFP)1Garbi) were a kind gift from Prof Andrew MacDonald (University of Manchester) and breeders were established at the Royal Free Hospital. Heterozygous mice were used for experiments. RAG-1$^{(-/-)}$ mice (B6.129S7-Rag1tm1Mom/J) were ordered from Charles River and bred with CD11c.DTR mice in-house to generate CD11c.DTRxRAG-1$^{(-/-)}$ mice. All experiments were carried out under a home office license (project license number 70/7475).

2.6.2 Generation of bone marrow chimeras

C57BL/6 were irradiated with a total of 11Gy split over 2 days. On the second day donor bone marrow was harvested by flushing tibia and femur with 5ml RMPI. Cells were washed, counted and resuspended at 25x10^6/ml in PBS. Irradiated mice were reconstituted with 200µl (5x10^6) bone marrow cells by tail vein injection and were allowed to reconstitute for a minimum of 8 weeks before mice were used for experiments.

2.6.3 Genotyping

CD11c.DTR mice were genotyped by PCR with primers that bind to the DTR/GFP transgene, to give a 630bp band:
DTR fw primer: 5'-GCCACCATGAAGCTGCTGCCG-3'
DTR rv primer: 5'-TCAGTGGGAATTAGTCATGCC-3'
CD11c.DOG mice were genotyped by a PCR with primers that bind to the ovalbumin sequence in the DTR/OVA/GFP transgene, to give a 500bp band:
DOG fw primer: 5'-AACCTGTGCAGATGATGTACCA-3'
DOG rv primer: 5'-GCGATGTGCTTGATACAGAAGA-3'.
PCR reactions were carried out as follows; 4min at 95℃, then 35 cycles of; 30sec at 94℃, 30sec at 58℃ and 30sec at 72℃. Followed by a final extension phase of 30mins at 72℃.

CD11c.DTRxRAG-$1^{(-/-)}$ mice were genotyped as above to check expression of the DTR transgene. RAG-$1^{(-/-)}$ was assessed by phenotype, mice were tail bled and red blood cells were lysed from 100µl of blood by transferring into 9ml water for 30secs. 1ml of 10xHBSS was then added to prevent further lysis. Cells were then stained for expression of CD11b, CD8, CD4 and CD19.

2.6.4 Tumour protection experiments

B16.F10 or B16 NP GFP cells were trypsinised when ~50% confluent after between 5 and 10 days of culture. Cells were washed twice in PBS and resuspended in fresh PBS at $5x10^6$/ml. Mice were shaved on the flank and injected sub-cutaneously with 100µl ($5x10^5$) tumour cells. For B16 NP GFP injections, mice were sub-lethally irradiated with 4Gy before tumour inoculation to enhance tumour engraftment. Tumours became established and visible 7-10 days after injection. Without treatment tumours would continue growing and mice would have to be sacrificed by day ~20 if tumours grew to >15mm in any direction, as per home office regulations. Tumour size was measured using callipers every 2-3 days. Width (w) and length (l) of the tumours were measured and the tumour size was calculated according to the following formula:

$w \times l \times \pi/4$. Mice were also sacrificed if they lost more than 20% of their body weight, although this was rare.

For treatment with TCR transduced T cells, CD8 or CD4 sorted splenocytes were transduced as described above. On day 1-3 after transduction, cells were counted, washed and resuspended in PBS. 200µl of T cells were injected into mice by tail vein injection. For B16.F10 experiments, on day 10 mice were irradiated with 4Gy in the morning then injected with T cells >4 hours later. For B16 NP GFP mice were treated with T cells on day 7.

2.6.5 DC depletion

DC were depleted in CD11c.DTR mice/bone marrow chimeras by injecting 100ng of DT (Sigma, D0564) in 200ul PBS intra-peritoneally (i.p.). This corresponds to ~5ng/g body weight. CD11c.DOG mice were similarly injected i.p. with DT that ranged from 100ng to 1.2µg (5 to 64 ng/g). $CD11c^+$ cells were depleted for up to 3 days and depletion was measured in the spleen and tumour as appropriate. Non-depleted mice were injected i.p. with 200µl PBS as a control.

2.6.6 T cell expansion after DC depletion

Mice were injected with B16.F10 or B16 NP GFP tumours as described above. As described for each experiment, DC were depleted by i.p. injection of DT or PBS, typically on day 6, 9 and 12. Transduced T cells were injected on day 7 (1 day post-transduction) into mice and tumour size was measured as described above.

2.6.7 Lymphocyte isolation from tumours

Tumours were harvested from tumour-bearing mice and weighed in Bijoux tubes. Tumour samples were mechanically digested using dissection scissors before culturing in 1ml of RMPI containing 320µg of Liberase TL (Roche, 05401020001)

and 200µg of DNase 1 grade 2 (Roche 10104159001). These were incubated at 37℃ in a shaking incubator for 30mins. Liberase contains a mixture of collagenase I and II which break down the extracellular matrix and release the cells. DNase degrades DNA released from dying cells to decrease the viscosity of the sample and improve cell yield. Tumours were then transferred to ice and mashed through a 70µM cell strainer (BD Falcon, 352350) with 5mM EDTA/PBS. Cells were spun and resuspended in 3ml complete RPMI at 37℃ in a 15ml Falcon tube. Cells were underlaid with 3ml of Histopaque-1077 (Sigma-aldrich, 10771). Cells were then centrifuged at room temperature for 10mins at 700g. The upper layer containing live cells were then carefully removed and transferred to a new 15ml Falcon tube and centrifuged again with 10ml fresh complete RPMI for 10mins at 1500rpm to remove any remaining histopaque. Cells were then resuspended in FACS buffer for staining.

2.6.8 *ex vivo* isolated tumours

Tumours were harvested and mechanically digested with dissection scissors and washed/mashed through 70µM cell strainer (BD Falcon, 352350) with 5mM EDTA/PBS. Live tumour cells were isolated by centrifugation with Histopaque-1077 as above. $1x10^6$ tumour cells were plated in a T75 flask (TPP) and incubated for 3-7 days. Cells were trypsinised and split 1/10 when necessary to establish an stable *ex vivo* tumour line.

2.6.9 MACS sorting $CD11c^+$ cells from the tumour

Lymphocytes were isolated from the tumour as described above. $CD11c^+$ cells were then isolated using murine CD11c MicroBeads (Miltenyi, 130-097-059). Cells were resuspended in 400µl MACS buffer (0.5% bovine serum albumin (BSA) and 2mM Ethylendiamintetraacetat (EDTA)). 10µl of microbeads were added per sample with 5µl Fc block and incubated at 4℃ for 20 minutes. MS magnetic separation columns (Miltenyi 130- 042-201) were rinsed with 500µl MACS buffer. Cells were washed with 30ml of MACS buffer and resuspended in

500µl MACS buffer. Cells were loaded into columns through a pre-separation filter (Miltenyi, 130-101-812) and washed 3 times with 500µl MACS buffer. Cells were eluted twice with 500µl MACS buffer into a 15ml Falcon tube, then passed through another MS column to increase purity. Cells were washed twice and eluted again with 500µl MACS buffer.

$CD11c^+$ cells were peptide loaded with 1µM relevant peptide for 2 hours, then washed and plated in a 96-well plate. As before $1x10^4$ $CD11c^+$ cells were co-cultured overnight with $1x10^5$ transduced T cells. The next morning cells were incubated with brefeldin A and stained for intracellular IFN-γ.

2.7 Analysis and statistical tests

FCS flow cytometry files were analysed using FlowJo v10 software. Data was further analysed in GraphPad Prism 6, which was also used to generate graphs including means, medians, standard deviations and to perform statistical analysis. Paired and unpaired students t-tests were calculated for data sets, Kaplan-Meier survival curves and Log Rank tests were performed for survival data. Differences were considered statistically significant when p values were <0.05 (significance was represented by *: <0.05, **: <0.01, ***: <0.001, ****: <0.0001).

Chapter 3 Testing the specificity of anti-B16 TCR transduced T cells *in vitro*

To examine the role of DC in the context of tumour immunotherapy by adoptively transferred, TCR-modified T cells, we established a clinically relevant murine model in which T cells transduced with self-reactive TCRs controlled tumour growth. The B16.F10 melanoma model was chosen because a variety of TCRs have been identified that confer specificity for melanocyte differentiation antigens, such as tyrosinase, MART-1, gp100, tyrosinase-related protein 1 (TRP1) and TRP2 (Rosenberg et al. 1999). These antigens are expressed on normal melanocytes but also often over-expressed in melanomas. T cells specific for these antigens have been identified from tumour infiltrating lymphocytes (Boon and Bruggen 1996) and T cells transduced with TCRs which recognise these antigens provide tumour regression *in vivo*. However this control is only temporary and insufficient to clear the tumour burden as tumours eventually evade the immune response and regrow (Abad et al. 2008; Chinnasamy et al. 2013; Kerkar et al. 2011). We reasoned that use of a model in which T cells only mediated partial control of tumour growth would allow us to dissect the role of DC in the absence of an over-whelming T cell response.

Alongside the standard B16.F10 melanoma model we also established a model in which the TCR-transduced T cells recognise a tumour specific antigen (TSA). However despite the T cell response to melanoma in patients being pre-dominated by T cells responding to neo-antigens (Lennerz et al. 2005), there are no suitable TCRs for TSAs in the pre-clinical B16.F10 model. However our group and others have previously used the F5 TCR to control tumours expressing the influenza nucleoprotein (NP). This foreign antigen can be used in lieu of a TSA generated by a mutation in the tumour. EL4 thymoma tumour cells that have been engineered to stably express NP are recognised by the F5 TCR which potently controls tumour growth (Ahmadi et al. 2011; Morris et al. 2005). We chose to use a similar approach in the B16.F10 model, by transducing

B16.F10 melanocytes with an retrovirus encoding the NP protein. This would allow us to compare immune responses that had been tolerised or not to tumour antigen when expressed on the same tumour cells.

Whether T cells are transduced with TCRs which recognise tumour associated/self antigens or tumour specific/foreign antigens may have an important bearing on their functional ability. As discussed in chapter 1.2.2, the TCR affinity for self, but not foreign antigens is limited by central tolerance. Self-reactive TCRs are therefore typified by reduced TCR affinity compared to foreign antigen specific TCRs (Stone et al. 2015). Lower TCR avidity also translates into lower cytotoxic ability in CTLs (Alexander-Miller et al. 1996).

Although T cell responses to tumour associated antigens in the B16.F10 melanoma model have been well studied by others, the response to tumour specific antigens is less clear. In addition, neither model had been used by our group previously. Therefore the aims of the following experiments were to:

1. Clone melanoma specific TCRs into retroviral expression vectors
2. Show that TCR transduced T cells recognise tumour associated antigen as expressed on peptide loaded target cells or directly on B16.F10 tumour cells
3. Generate a NP-expressing B16.F10 cell line and show it is recognised by F5-TCR transduced T cells

3.1 Cloning of anti-B16.F10 TCRs into retroviral expression vectors

To generate retrovirus that encoded the genes for melanoma specific TCRs we were kindly provided with two MHC class I restricted TCRs by Ton Schumacher (Netherlands Cancer Institute). These recognise melanocyte differentiation antigens expressed by the B16.F10 melanoma (Fig 3.1A). The TRP2 TCR recognises tyrosinase related protein 2 ($TRP2_{181\text{-}188}$ on $H\text{-}2K^b$) and the Pmel-1 TCR recognises $gp100_{25\text{-}33}$ on $H\text{-}2D^b$. The α and β chains of the TRP2 TCR are separated by a 2A sequence which induces a co-translational cleavage of the polypeptide chain (Holst et al. 2006). This differs from the IRES separating the Pmel-1 α and β chains because it uses a single mRNA strand to produce two equimolar proteins. This stoichiometric expression is particularly important in reducing the chance of mispairing of the introduced α/β chains with endogenous α/β chains which can create TCRs of unknown specificity/toxicity (Bendle et al. 2010). To replace the Pmel-1 IRES sequence with a 2A sequence an overlapping/fusion PCR was performed as demonstrated in Fig 3.1A. As well as the 2A sequence the TRP2 TCR also had extra modifications to improve expression not found in the Pmel-1 TCR. The DNA sequences of both TCRs had been codon optimised however an additional cysteine residue had been added to the TRP2 constant chains, to promote pairing between the two introduced chains (Cohen et al. 2007; Kuball et al. 2007).

Alongside these class I restricted TCRs, the sequence for an MHC class II restricted TCR, which recognises $TRP1_{113\text{-}127}$ (Muranski et al. 2008; Quezada et al. 2010) was synthesised by GeneArt (Life Technologies) (Fig 3.1B). As in the TRP2 TCR, to improve TCR expression we codon optimised the TRP1 TCR and added the extra cysteine residues by substituting one amino acid in both chains (α-T179C and β-S186C). To further improve expression the DNA for all three TCRs was cloned into pMP71, a retroviral expression vector optimised for expression in lymphocytes (Fig 3.1C). This plasmid contains a truncated CD19 epitope to allow easy staining by FACS as a marker of transduction (Tey et al.

2007). In the same vector backbone we also already had the F5 TCR, a class I restricted TCR which recognises the influenza nucleoprotein ($NP_{366-379}$) as presented by H-$2D^b$ (Townsend et al. 1985).

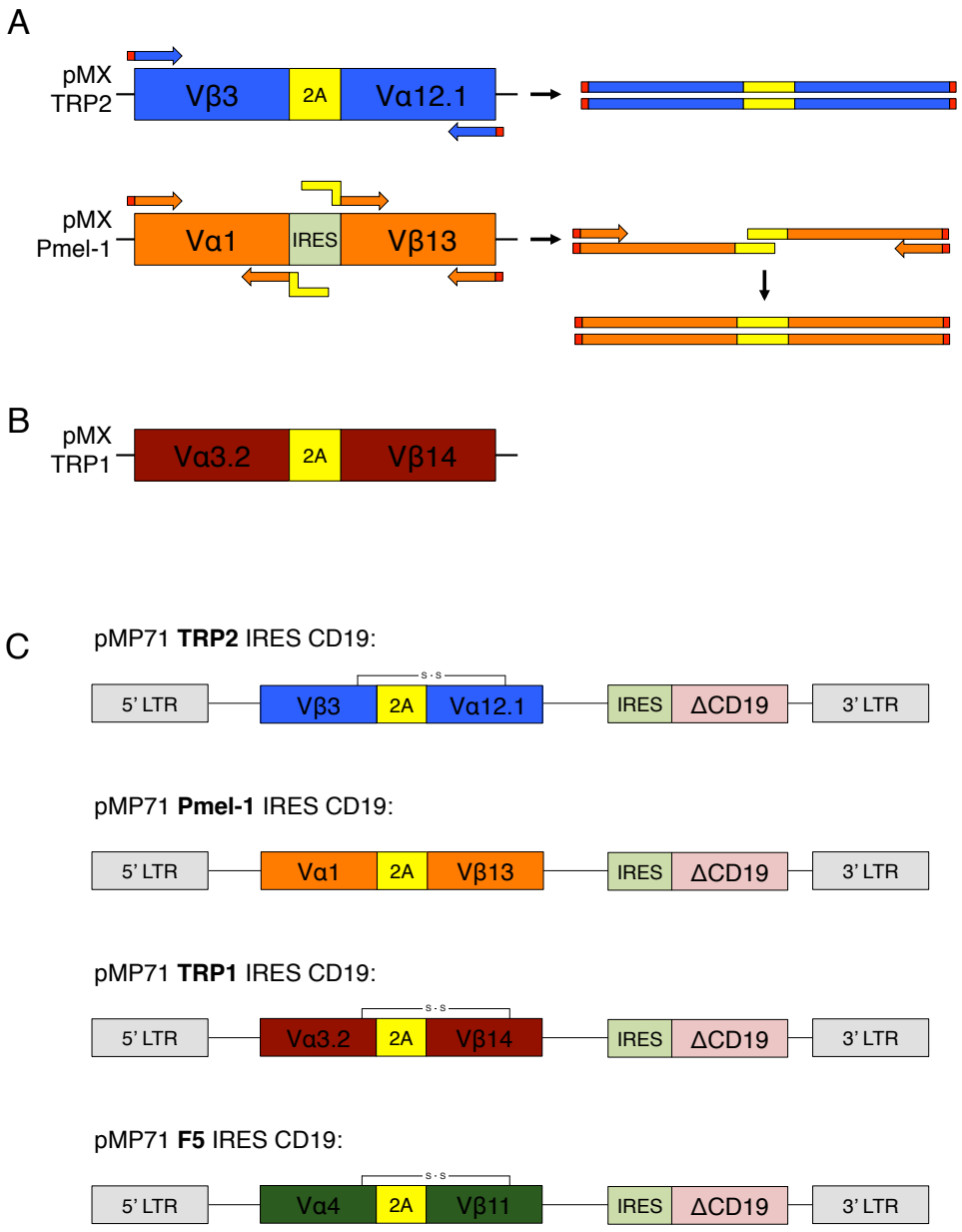


Figure 3.1: *Schematic representation of the retroviral expression vectors.*
(A) Retroviral vectors in a pMX backbone were donated by Ton Schumacher (Netherlands cancer institute). PCR reactions were performed to replace the IRES sequence in the Pmel-1 vector with a 2A sequence.
(B) TRP1 TCR as ordered from GeneArt.
(C) TRP2, Pmel-1 and TRP1 TCRs were cloned into the pMP71 vector. This includes a truncated CD19 epitope as a transduction marker, alongside the TCR.
The 2A sequence is from the porcine teschovirus-1 and allows co-translational cleavage of the two proteins. IRES denotes an internal ribosome entry site. —S-S— denotes the presence of an extra cysteine in the α and β chains.

3.2 TCR transduced T cells recognise cognate antigen

To test whether the melanoma-specific TCRs were functional in transduced T cells, C57BL/6 splenocytes were transduced with retroviral vectors encoding the TRP2, Pmel-1, TRP1 and F5 TCRs or mock transduced without retrovirus. Expression of both the specific variable β chain and the CD19 marker were detectable by FACS 3 days after transduction (Fig 3.2A). Summary data for these transductions (Fig 3.2B) show broadly similar transduction efficiencies between the different TCR constructs (TRP2 mean 60±14%, Pmel-1 mean 59±12%, TRP1 mean 63±14% and F5 mean 59±15%).

To ensure that T cells transduced with the Pmel-1 and TRP2 TCRs could recognise their cognate antigen, RMA/S cells were loaded with relevant or irrelevant peptide and co-cultured with TRP2 and Pmel-1 transduced T cells at decreasing concentrations. After 24 hours of co-culture the $CD19^+$ transduced CD8 cells were stained for intracellular IFN-γ production (Fig 3.3A and B). Whilst both transduced populations produced minimal IFN-γ when cultured with RMA/S cells loaded with irrelevant peptide, the relevant peptide induced a clear IFN-γ response in both TRP2 and Pmel-1 transduced cells, comparable to PMA and ionomycin activated T cells. At high peptide concentrations of 10μM - 100nM there is a trend for higher IFN-γ responses in TRP2 than Pmel-1 transduced cells, although this is not significant. However as the T cell response starts to decrease with lower peptide concentrations (10nM - 100pM) this difference is lost.

Whilst RMA/S are suitable to present class I restricted peptides they lack MHCII expression so will not present the class II restricted TRP1 peptide. To test the antigen specificity of TRP1 transduced T cells we generated bone marrow derived DC (BM-DC) which express high levels of MHCI and II. These were loaded with relevant or irrelevant peptide and co-cultured with transduced T cells as in Fig 3.3A. Both TRP2 and Pmel-1 transduced cells produced a similar level

of IFN-γ when cultured with peptide loaded BM-DC as compared to peptide loaded RMA/S cells. However with peptide loaded BM-DC they appear to maintain IFN-γ production at lower peptide concentrations (Fig 3.3C). At the highest peptide concentration (10μM), TRP1 transduced cells produced IFN-γ at the same level as TRP2 and Pmel-1 cells. However this rapidly decreased with lower peptide concentrations. IFN-γ production became undetectable at 100-fold lower concentration in TRP2 or Pmel-1 transduced cells than TRP1. The likely explanation for this is that the IFN-γ production in this experiment has been measured in the transduced $CD8^+$ population. Whilst gene transfer of the same TCR has been shown to confer antigen specificity to both CD8 and CD4 cells (Morris et al. 2005; Roszkowski et al. 2005), it is reasonable to suggest that in this case the T cell response following TRP1 TCR-pMHCII engagement is inferior in the absence of the CD4 co-receptor. Whilst this is not limiting at saturating peptide concentrations, when the peptide concentration decreases the lack of the CD4 co-receptor in $CD8^+$ TRP1 transduced cells limits their activation. Surprisingly there was no IFN-γ detectable after 24 hours in the $CD4^+$ TRP1 transduced cells. This may be a fault in the experiment design as IL-2 may be a preferable read-out for $CD4^+$ T cell activation. Likewise the cytokine production kinetics may differ between CD4 and CD8 T cells. Checking for cytokine production in the supernatants by ELISA rather than intracellular cytokine staining may offer a more sensitive approach. However this was not pursued as these experiments had succeeded in their aim of showing that the recently constructed TCR retroviral vectors conferred antigen specificity for the relevant B16.F10-associated peptides.

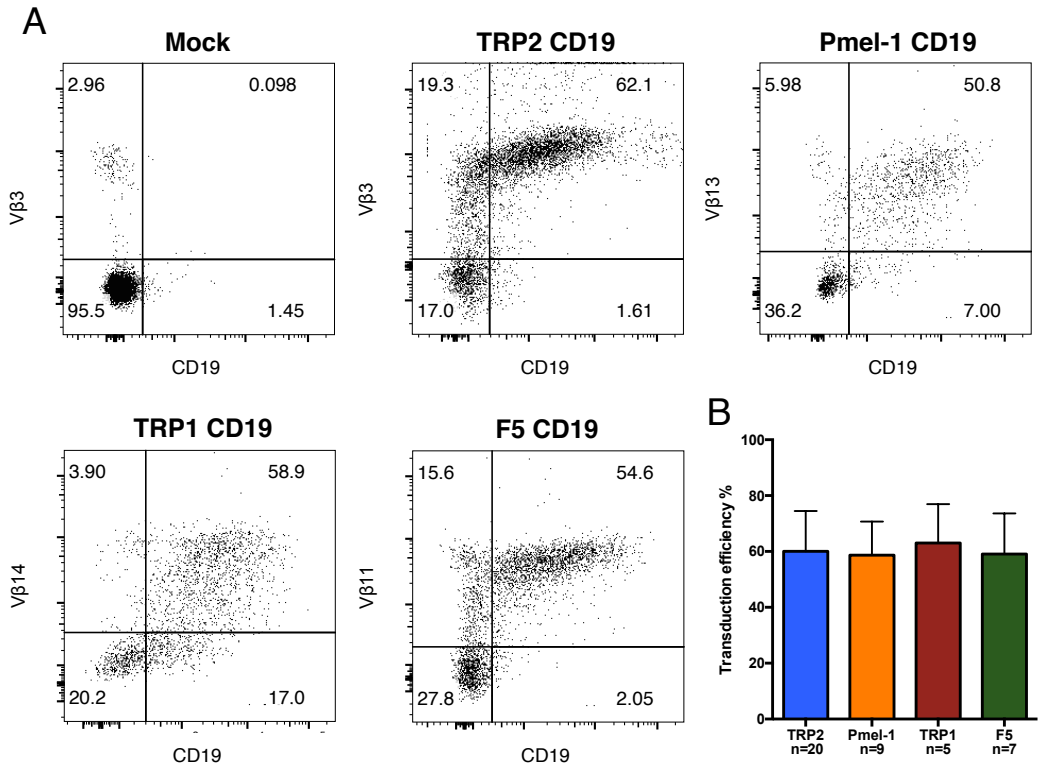


Figure 3.2: *T cell receptor transduction of murine splenocytes.*
(A) C57BL/6 splenocytes were transduced with retroviruses encoding TRP2, Pmel-1, TRP1 or F5 TCRs or without retrovirus (mock transduced). After 3 days, cells were stained for CD4, CD8, CD19 and the Vβ chain specific for that TCR. (TRP2 = Vβ3, Pmel-1 = Vβ13, TRP1 = Vβ14 and F5 = Vβ11). Cells are pre-gated on live lymphocytes (FSC v SSC) then CD8 (for mock, TRP2, Pmel-1 or F5) or CD4 for TRP1. Representative FACS plots are taken from different experiments.
(B) Summary transduction efficiencies across multiple experiments are plotted as the mean frequency of $CD19^+$ $V\beta^+$ in $CD8^+$ (TRP2, Pmel-1, F5) or $CD4^+$ (TRP1) live splenocytes ($\pm$SD).

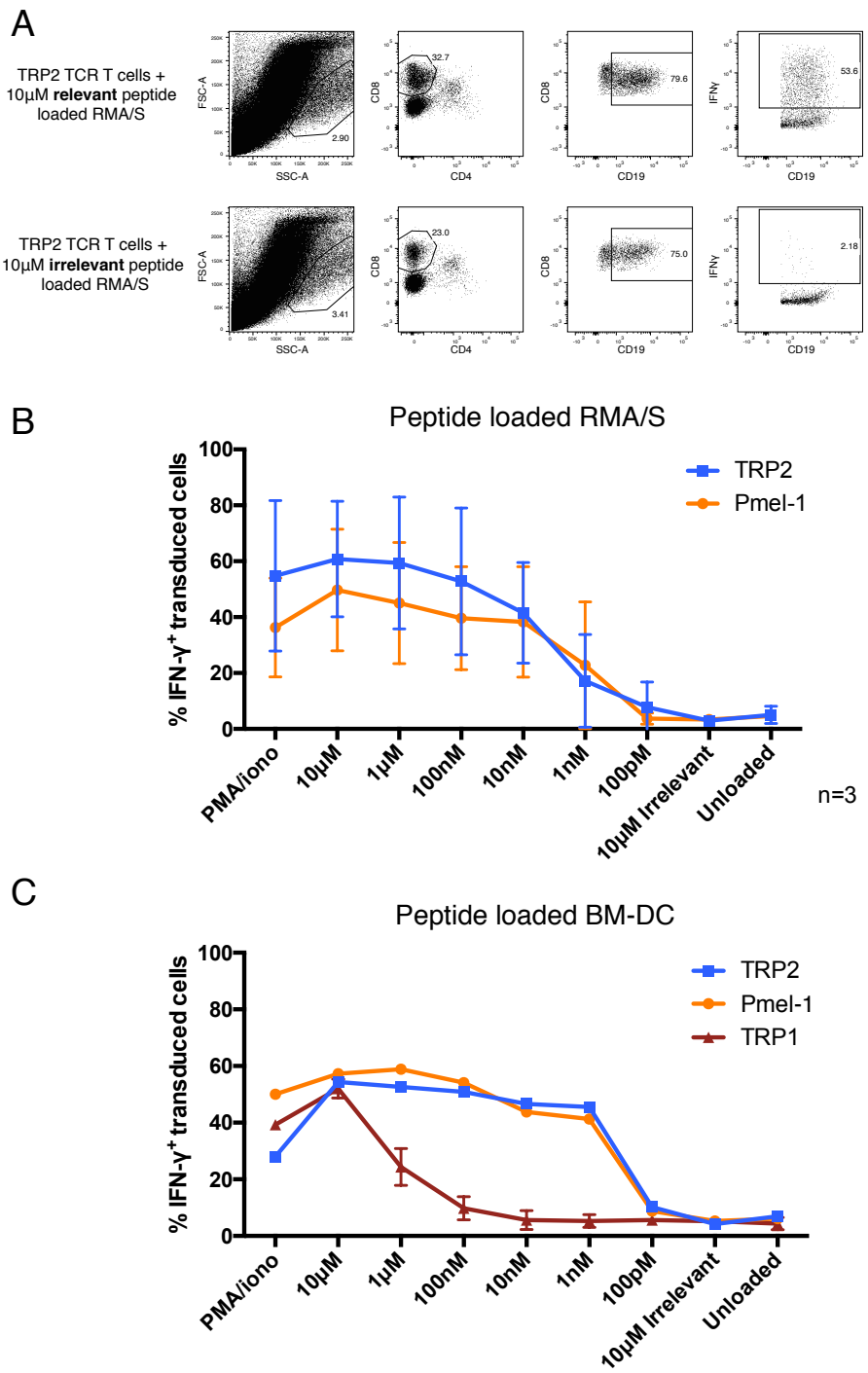


Figure 3.3: *TCR transduced T cells recognise cognate antigen in vitro.*
Bulk splenocytes were transduced with anti-TRP2, Pmel-1 or TRP1 TCRs and 5 days later were co-cultured overnight with RMA/S cells (A,B) or BM-DC (C). These were loaded with decreasing concentration of specific peptide, irrelevant peptide or unloaded. Cells were stained for intracellular IFN-γ in $CD8^+$, $CD19^+$ lymphocytes.
(A) Gating strategy shown in representative FACS plots.
(B) Mean IFN-γ^+ transduced T cells from 3 independent experiments ±SD.
(C) Mean IFN-γ^+ transduced T cells from 1 experiment for TRP2 and Pmel-1, or 2 experiments for TRP1 ±SD.

3.3 Transduced T cells recognise antigen expressed on tumour cells *in vitro*

As well as recognising peptide-loaded cells, TCR transduced T cells must be capable of recognising peptide when processed and presented directly by tumour cells. However B16.F10 melanoma is a poorly immunogenic tumour and has an almost complete lack of MHCI expression *in vitro*. Previous studies have shown that B16.F10 upregulates MHCI expression in response to IFN-γ (Seliger et al. 2001) so we generated media containing murine IFN-γ by harvesting the supernatant from ConA activated splenocytes. This ConA activated splenocyte media (CASM) contained 8.5ng of IFN-γ/ml, as quantified by ELISA (Fig 3.4A). B16.F10 cells cultured for 24 hours in CASM upregulated their expression of MHCI to a similar level as the highly immunogenic EL4-NP thymoma line (Fig 3.4B). Interestingly, incubation with CASM also upregulated MHCII on B16.F10 cells, although this depreciated more rapidly with decreasing IFN-γ concentration than MHCI expression. Based on these experiments CASM was diluted 1/2 (4.2ng/ml) for use in subsequent assays. However IFN-γ has been shown to upregulate PD-L1 expression on ovarian tumours (Abiko et al. 2015) and incubation with CASM upregulated expression of PD-L1 on B16.F10 melanoma cells (Fig 3.4C).

To test whether CASM-mediated, MHC upregulation renders B16.F10 capable of presenting the relevant peptides to transduced T cells, B16.F10 cells were incubated overnight in CASM or normal media, washed, then co-cultured in fresh media with mock or TRP2 transduced T cells (Fig 3.5A). Supernatant was collected after 4 hours and tested for T cell production of IFN-γ by ELISA. As expected, without prior activation of tumour cells with CASM, neither mock nor TRP2 transduced cells produced IFN-γ. However after activation of B16.F10 with CASM, TRP2 but not mock transduced cells produced high levels of IFN-γ. In this setting, CASM-mediated upregulation of PD-L1 on tumour cells was therefore insufficient to prevent IFN-γ production by TRP2 transduced T cells.

To examine the tumour specific response of TCR transduced T cells further, overnight co-culture experiments were set up with TRP2, Pmel-1 or TRP1 transduced cells (Fig 3.5B and C). Production of IFN-γ was measured by FACS staining of transduced cells for intracellular IFN-γ. The frequency of IFN-γ^+ TRP2 transduced cells was significantly higher when cultured with the antigen positive B16.F10 melanoma compared to the antigen negative EL4 thymoma. However in overnight cultures, unlike after 4 hours, there was no difference between the response to B16.F10 that had been pre-activated with CASM or not. This suggests that the minimal IFN-γ produced by resting T cells overnight is sufficient to induce MHC upregulation, which then enables antigen presentation to the TRP2 transduced T cells. Indeed, in Fig 3.4 we saw MHCI upregulation in ~50% of B16.F10 cells after culture with CASM diluted 1/100. This relates to an IFN-γ concentration of 80pg/ml which is broadly equivalent to the background level of IFN-γ produced after 4 hours by transduced cells in Fig 3.5A.

Although in Fig 3.3 both TRP2 and Pmel-1 transduced cells responded to peptide-loaded cells to a similar extent, Pmel-1 transduced cells responded more poorly when cultured with tumour cells. The frequency of IFN-γ^+ Pmel-1 cells was only detectable above background after pre-incubation with CASM, but this was still substantially lower (10%) than with TRP2 transduced cells (56%). Whilst the frequency of IFN-γ^+ TRP1 cells (41%) was higher than Pmel-1 cells this too was dependent on activation of the tumour with CASM. As shown in Fig 3.4, MHCII upregulation (and therefore presentation to TRP1 cells) requires higher IFN-γ concentrations than for MHCI upregulation. In this setting therefore it appears that background IFN-γ is not enough to induce sufficient MHCII upregulation to fully present to TRP1 cells.

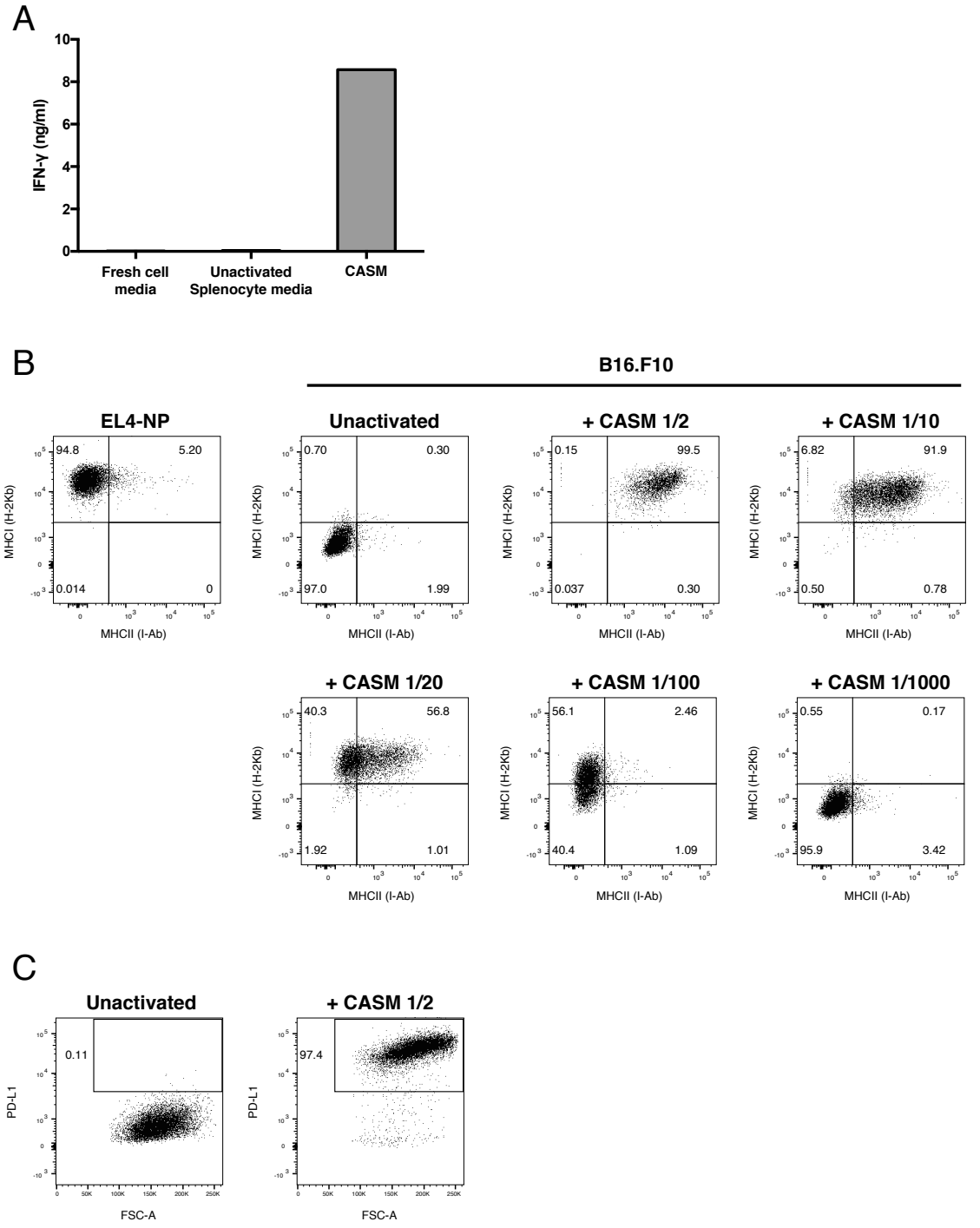


Figure 3.4: *Upregulation of MHC class I and II on B16.F10 melanoma in vitro.*
(A) Concentration of IFN-γ in cell-free ConA activated splenocyte media (CASM) as measured by ELISA.
(B) MHCI and II upregulation on B16.F10 after incubation wit CASM. CASM was added to B16.F10 melanoma at various dilutions, after 24 hours cells were harvested and stained for MHC class I (H-2K^b) and MHC class II (I-Ab).
(C) PD-L1 expression on B16.F10 after addition of CASM (diluted 1/2) for 24 hours.

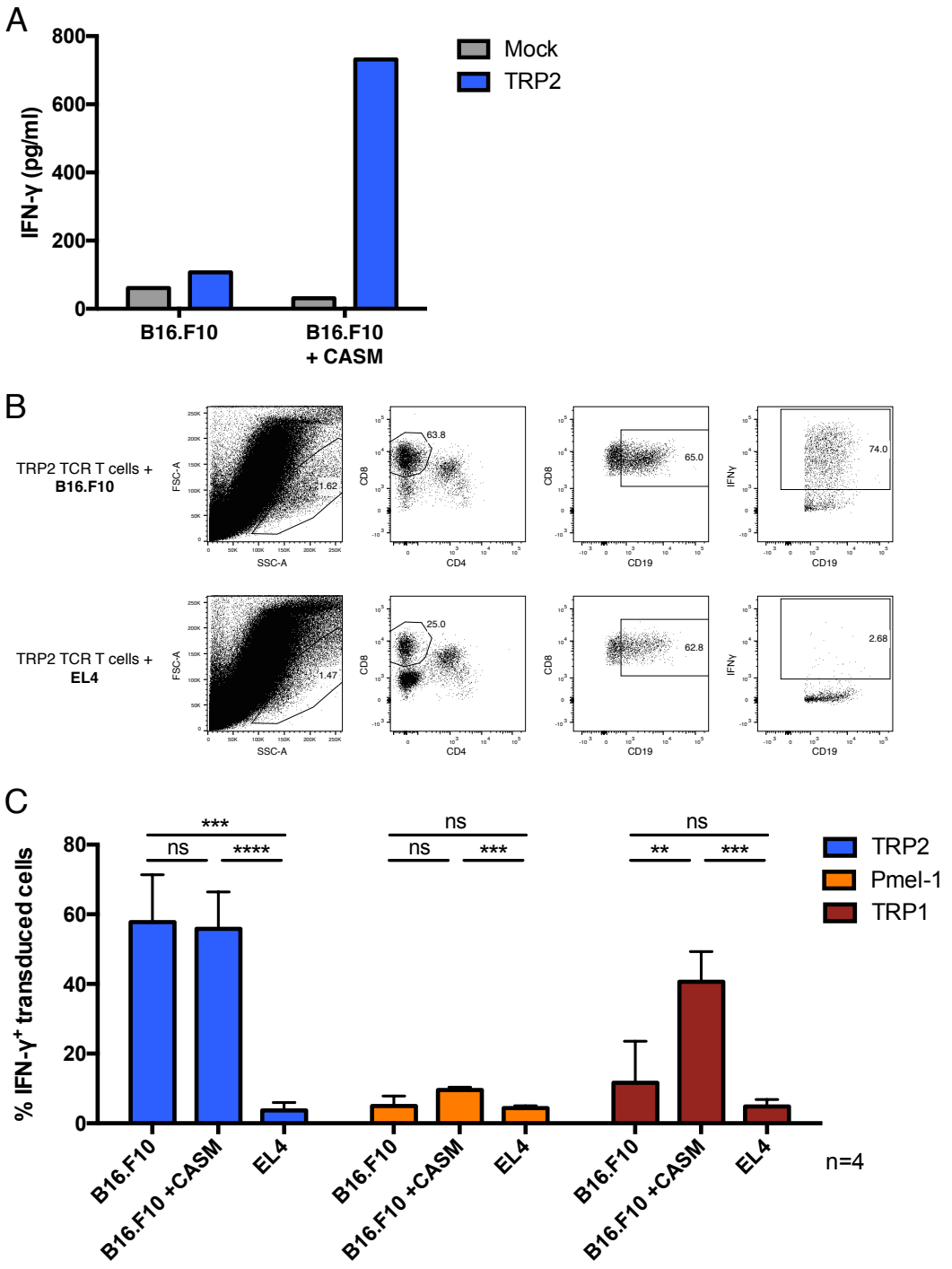


Figure 3.5: *Transduced T cells recognise antigen expressed on B16.F10 in vitro.*
(A) IFN-γ production by mock or TRP2 transduced splenocytes after co-culture with B16.F10 that had been activated with CASM or left unactivated for 24 hours previously. Supernatants were harvested after 4 hours and IFN-γ concentration was measured by ELISA.
(B,C) B16.F10 tumours were irradiated and co-cultured with transduced splenocytes overnight. Cells were then stained for intracellular IFN-γ in $CD8^+$, $CD19^+$ lymphocytes. Gating strategy shown in representative FACS plots (B). Results in (C) are from 4 independent experiments and show mean frequency of IFN-γ^+ cells in the $CD8^+$ $CD19^+$ population ±SD. Statistical test: unpaired T test. ns: non significant, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$

3.4 Recognition of B16 NP GFP by F5 TCR transduced T cells

As a comparison to these TCRs which recognise self-antigens presented by the B16.F10 tumour we established the F5 TCR model in which the tumour cells present the foreign NP peptide to TCR transduced T cells. To ensure stable, long-term expression of NP we transduced B16.F10 with a retrovirus that encoded the NP protein (Fig 3.6A). This vector also contained GFP as a transduction marker and had been generated by a previous PhD student, Bernado Alvares. Although transduction with this vector gave a high transduction efficiency (93% GFP^+) to prevent potential outgrowth of an NP-negative population we attempted to FACS sort a 100% GFP^+ population. However although this increased the purity of the transduced population there remained a small GFP^- population (Fig 3.6B). Therefore an alternative approach was attempted where single cells of the transduced B16 NP GFP population were plated in 96 well plates. Two clones were isolated by this approach that were 100% GFP^+, with either medium or high GFP expression (Fig 3.6C).

To ensure that these GFP^+ cells expressed and presented NP, the B16 NP GFP cell lines were activated with CASM and co-cultured overnight with F5 or TRP2 transduced T cells. The frequency of IFN-γ^+ F5 transduced cells was comparable when cultured with either B16 NP GFP or EL4-NP cells. This was specifically in response to tumours expressing NP as the standard EL4 tumour only induced backround IFN-γ (Fig 3.6D). As with the TRP2 transduced cells in Fig 3.5C, there was no difference when F5 transduced T cells were cultured with tumours that had been pre-activated with CASM or not. There was also no notable difference in the frequency of IFN-γ^+ F5 transduced cells when cultured with GFP medium or GFP high cell lines, suggesting expression of the NP protein is not a limiting factor. Nonetheless the high GFP expressing clone (B16 NP GFP clone 2) was selected for future experiments. Interestingly, despite the F5 TCR recognising a foreign antigen, the frequency of IFN-γ^+ F5 transduced cells was comparable to TRP2 transduced cells.

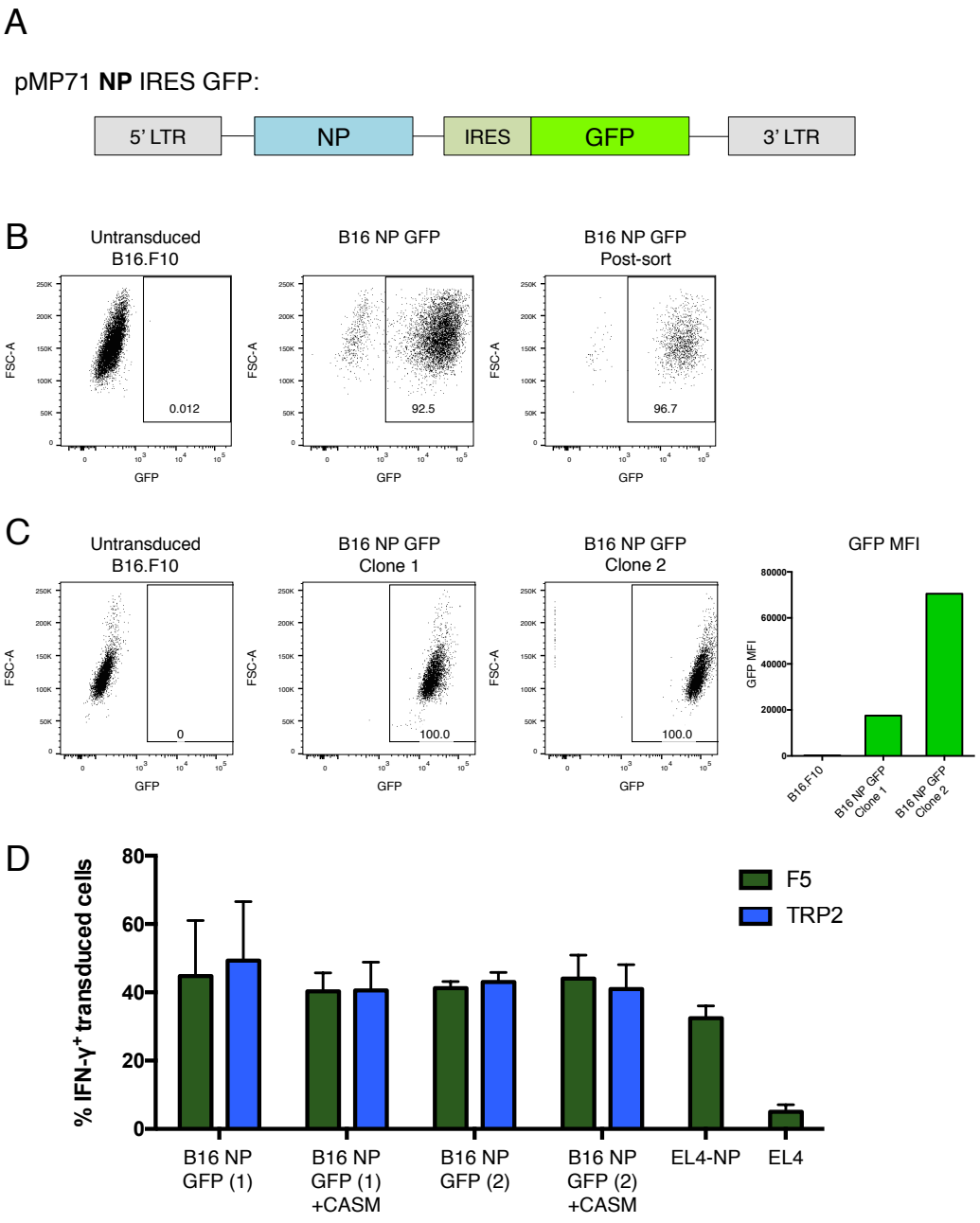


Figure 3.6: *Expression of NP in B16.F10 and recognition by F5 TCR transduced T cells.*
(A) The retroviral expression vector used to transduce B16.F10 with a construct expressing the influenza nucleoprotein (NP) and GFP.
(B) FACS profile of transduced B16.F10 cells and after FACS sorting.
(C) FACS profile and median GFP intensity of single cell clones of the B16 NP GFP.
(D) As in Fig 3.5C, tumours were irradiated and co-cultured overnight with TRP2 or F5 transduced T cells. Graph shows frequency of IFN-γ^+ cells in the $CD8^+$, $CD19^+$ population. Results are from 2 independent experiments $\pm$SD.

3.5 Summary and conclusion

The genes encoding the α and β chains of TCRs specific for the melanocyte differentiation antigens TRP2, Pmel-1 or TRP1 were cloned into the pMP71 retroviral expression vector. These were used to transduce splenocytes and these TCR engineered T cells were able to recognise their cognate antigens when loaded onto target cells or presented directly on the B16.F10 melanoma cell line *in vitro*. A number of approaches had been utilised in these introduced TCRs to ensure that transduced T cells were capable of responding to their cognate antigen. These modifications included codon optimisation, separation of α and β chains with 2A sequences (Holst et al. 2006) and the addition of extra cysteine residues to promote TCR expression on the cell surface (Cohen et al. 2007; Kuball et al. 2007). TCR expression can be further enhanced by providing transduced cells with surplus CD3 molecules, either by co-transduction with extra CD3 (Ahmadi et al. 2011) or by down-regulation of endogenous TCR chains (Berdien et al. 2014; Ochi et al. 2011; Okamoto et al. 2009; Provasi et al. 2012). However as transduced T cells exhibited good effector function *in vitro* these other modifications were not pursued.

TRP2 and Pmel-1 transduced cells displayed similar IFN-γ production in response to peptide-loaded target cells, suggesting the ability of each TCR to recognise their cognate antigen is similar. However Pmel-1 transduced cells were only able to induce a low level of IFN-γ in response to the tumour cells directly, relative to TRP2 transduced cells. It is possible this could be explained by the Pmel-1 TCR lacking the extra cysteine modification found in TRP2 TCR. However as both transduced T cells respond equally to peptide-loaded cells this is unlikely. Instead this difference in IFN-γ production may be due to differences in expression of the relevant protein in the B16.F10 cell line. Melanomas including the B16.F10 melanoma are known to be relatively 'unstable' tumours and although analysis of protein levels were not performed here they have been found to vary between different laboratories and samples (Overwijk and Restifo 2001; Riker et al. 2000). Therefore although Pmel-1 transduced T cells can recognise peptide loaded cells,

B16.F10 cells themselves may limit recognition by presenting low levels of the Pmel-1 peptide.

As well as these class I restricted TCRs, a class II restricted TCR (TRP1) was also generated. As previously discussed, although T cells transduced with this TCR responded to peptide loaded cells this was sub-optimal, possibly due to the experimental design being optimised for CD8 not CD4 T cells. Nonetheless these cells were able to respond to peptide presented directly by the tumour cell. However this was largely dependent on B16.F10 upregulation of MHCII.

The F5 TCR model, which recognises the foreign NP peptide, was also established to allow comparison to these self-reactive anti-tumour TCRs. B16.F10 cells were engineered to express NP and presented antigen to F5 TCR transduced T cells. This resulted in the induction of a IFN-γ response comparable to that of the well established EL4-NP cell line. Surprisingly the IFN-γ response from F5 transduced T cells was the same as from TRP2 transduced T cells. This suggests that either these TCRs have similar affinities, that the higher affinity of the F5 TCR does not correlate with an improved effector function, or that the detection of the effector response by this *in vitro* assay is saturated. Indeed higher TCR affinities do not necessarily relate to better effector function and have been shown not to improve T cell responses above a certain threshold (Zhong et al. 2013). However the control of tumour growth *in vivo* would be a more physiological assessment of the ability of these TCR transduced T cells to respond to tumour antigens, compared to *in vitro* assays.

Together the data in this chapter demonstrate that although the exact production of IFN-γ by T cells transduced with these TCRs varied, all four TCR constructs showed they were capable of redirecting the antigen specificity of a polyclonal T cell population towards a tumour associated antigen *in vitro*. The next chapter will therefore focus on the ability of these TCR transduced T cells to control tumour growth *in vivo*.

Chapter 4 Control of B16.F10 tumour growth by TCR transduced T cells *in vivo*

The previous chapter showed that T cells transduced with the anti-melanoma or anti-NP TCRs were capable of recognising their cognate antigen and producing IFN-γ *in vitro*. Here we tested the *in vivo* efficacy of T cells transduced with these TCRs. Previous studies have shown that TRP2 or Pmel-1 transduced CD8 T cells induce a transient delay in B16.F10 tumour growth for approximately 10 days after T cell transfer (Abad et al. 2008; Chinnasamy et al. 2013; Kerkar et al. 2011). However this is insufficient to completely control the tumour, which then quickly regrows. As discussed earlier it is possible that T cells transduced with these TCRs may only be able to induce partial tumour control as the TCRs only recognise tumour associated antigens with low affinity.

We would therefore predict that foreign-antigen specific and therefore high affinity TCRs would mediate enhanced tumour clearance. However T cells transduced with the ovalbumin (OVA) specific TCR, OT-1, induced significant but not complete tumour regression of a B16-OVA tumour (De Witte et al. 2006). We and others have also shown that T cells transduced with the foreign-antigen specific F5 TCR can completely eliminate the EL4-NP thymoma (Ahmadi et al. 2011; Morris et al. 2005). However the EL4 thymoma is thought to be a more immunogenic tumour than B16.F10, so the efficacy of elimination of a B16 NP tumour by F5 transduced T cells is not yet know.

However despite expressing a 'self-reactive' TCR, CD4 T cells transduced with the TRP1 TCR have been shown to mediate potent tumour control *in vivo* (Kerkar et al. 2011). These cells, unlike the class I restricted TCRs, were able to eliminate B16.F10 tumours without subsequent regrowth. Comparison between these models however is complicated as different cell types are used for the response, either CD8 or CD4 T cells. It may be that intrinsic differences between

these cell types, rather than differences in their TCR affinity is responsible for these different efficacies.

As mentioned earlier, above a certain threshold, increasing TCR affinity does not increase T cell effector function (Zhong et al. 2013). However enhancing TCR avidity, rather than affinity, of transduced T cells is an attractive strategy to improve T cell responses. The structural modifications to the TCR that promote TCR expression on the cell surface have been discussed earlier (chapter 1.4). In addition to these structural modifications to the introduced TCRs, other approaches to enhance the therapeutic effect of T cells consist of maximising the ability of transferred T cells to expand *in vivo*.

Proliferation of transferred T cells can be induced either through vaccination or through host pre-conditioning. However T cell proliferation through homeostatic expansion after lymphodepletive pre-conditioing, rather than vaccine-induced expansion has recently been preferred. Nonmyeloablative chemotherapy or irradiation has been shown to promote better expansion of introduced T cells than vaccination alone (De Witte et al. 2008a). Host pre-conditioning is thought to provide improved T cell expansion through a number of mechanisms; firstly expansion is driven by depletion of endogenous NK and T cells, which results in surplus IL-7 and IL-15. Unlike vaccination, these cytokines promote proliferation independent of antigen-recognition. This promotes long-term survival of expanded cells rather than the rapid expansion and contraction phases mediated by antigen-driven proliferation (De Witte et al. 2008a). Moreover depletion of endogenous lymphocytes has the additional benefit of depleting endogenous Tregs, which reduces potential immune suppression of the transferred T cells. Lastly the conditioning regimen itself can induce tumour cell destruction directly. This can also aid latter T cell expansion by release of tumour antigen (Zitvogel et al. 2008).

However whilst essential for optimal T cell responses, pre-conditioning irradiation also has downsides. Primarily we have noticed that mice often do not tolerate high levels (5 Gy) of irradiation well which can induce substantial weigh loss, requiring mice to be sacrificed regardless of tumour burden. Therefore we chose

to irradiate mice with a slightly lower 4 Gy of irradiation and test:

1. the control of B16.F10 by:
    - TRP2 or Pmel-1 transduced $CD8^+$ T cells
    - TRP1 transduced $CD4^+$ T cells
2. the control of B16 NP GFP by F5 transduced $CD8^+$ T cells

The purpose of these experiments was to establish a B16.F10 tumour model in which we could achieve partial tumour control by transferred T cells. This would allow later experiments to address the interactions these cells have with DC.

4.1 Control of B16.F10 by TRP2 or Pmel-1 transduced T cells *in vivo*

To test whether T cells transduced with anti-melanoma TCRs would control the growth of B16.F10, tumour-bearing mice were sub-lethally irradiated with 4 Gy before receiving TRP2, Pmel-1 or mock transduced T cells (Fig 4.1A). As both TCRs are class I restricted, $CD8^+$ cells were sorted prior to activation and transduction. By day 3 post-transduction the cells to be injected were $>$97% $CD8^+$ with transduction efficiencies of 61% $TRP2^+$ and 45% Pmel-1^+ (Fig 4.1B). Mice received 2x10^6 transduced T cells, but as the Pmel-1 transduced cells were less well transduced, these mice received a slightly higher total number of T cells as the population contained more untransduced cells. This number of total T cells was matched in mice treated with mock transduced T cells. Following T cell injection the size of the subcutaneous B16.F10 tumour was monitored and mice were sacrificed when tumours exceeded 15mm in any direction, as per home office regulations.

Both TRP2 and Pmel-1 transduced T cells showed a modest delay in tumour growth as compared to mice treated with mock transduced T cells (Fig 4.1C). For $\sim$10 days after transfer of TRP2 or Pmel-1 transduced T cells the tumour growth plateaued. However as in previous studies this control was transient and tumours then began to regrow. This eventual failure to control tumour growth suggests either the tumour stopped presenting the relevant antigen, that the transduced T cells became anergised/exhausted or that they failed to persist for longer than 10 days. However although the tumour control mediated by the transduced T cells was only partial it did result in a significant improvement in overall survival (Fig 4.1D), as mice developed tumours large enough to necessitate sacrifice at later time points. Interestingly despite Pmel-1 transduced T cells responding poorly to B16.F10 cells *in vitro* (Fig 3.5C), they were able to control tumour growth *in vivo* to a similar degree as TRP2 transduced T cells.

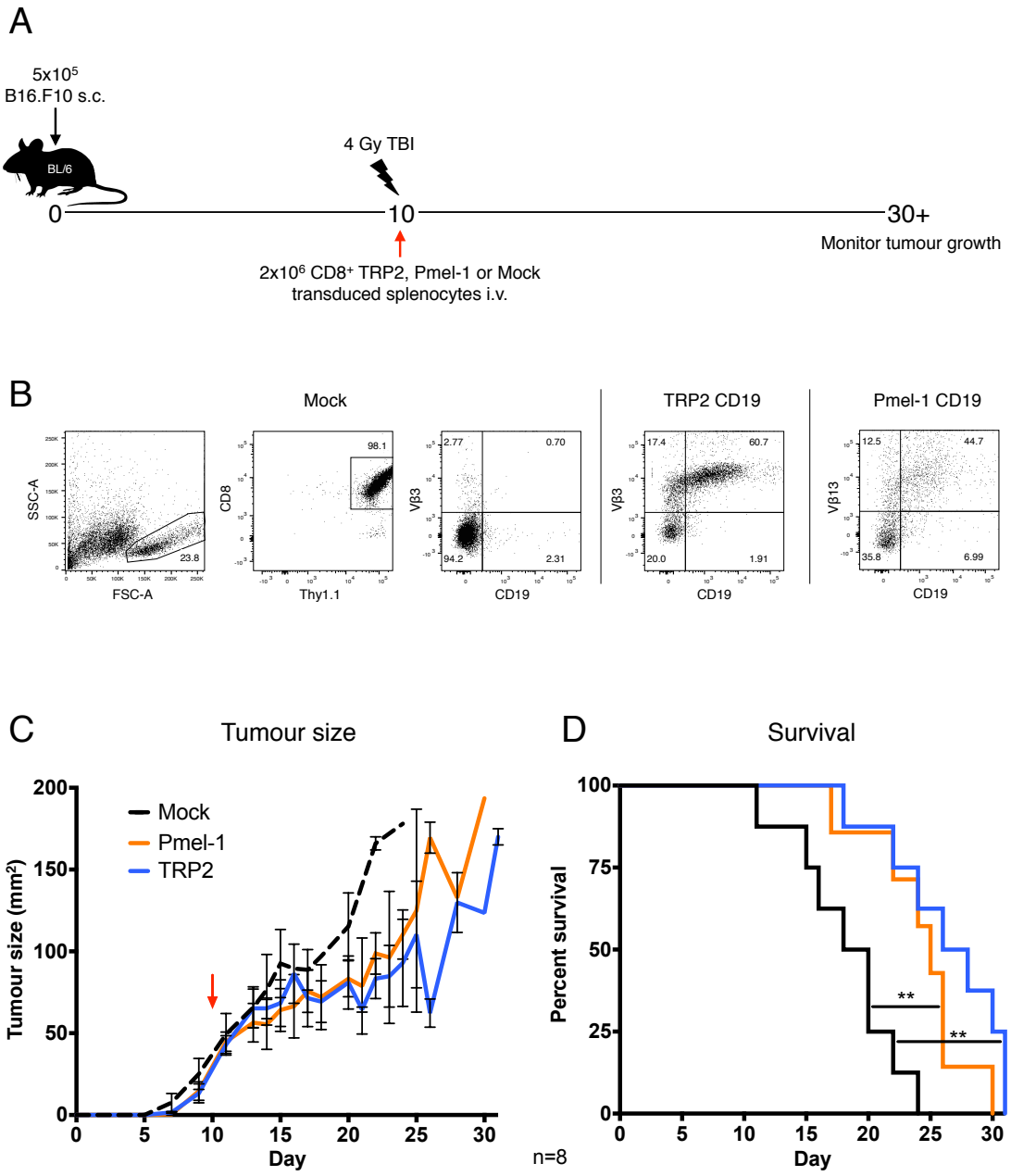


Figure 4.1: *Transient control of B16.F10 growth by TCR transduced CD8⁺ T cells in vivo.*
(A) Experiment outline. 5x10^5 B16.F10 cells were injected sub-cutaneously into the flank of C57/BL6 mice. On day 10 post-injection mice were irradiated with a sub-lethal dose of irradiation (4 Gy) and injected i.v. with 2x10^6 CD8$^+$ splenocytes that had been transduced with the TRP2 or Pmel-1 TCR or mock transduced.
(B) FACS profile of transduced T cells on day 3 post-transduction. Transduced cells are identified as CD19$^+$ and specific Vβ$^+$ lymphocytes. TRP2 = Vβ3, Pmel-1 = Vβ13.
(C) Tumour protection by transduced T cells was assessed by measuring tumour size. Results are the mean from 8 mice per group from 2 independent experiments ±SEM.
(D) Kaplan Meier survival graph. Significance measured by Log Rank test. **: p≤0.01.

4.2 Control of B16.F10 by TRP1 transduced T cells *in vivo*

To test the ability of TRP1 transduced T cells to control growth of B16.F10, the experiment as described in Fig 4.1 was repeated with TRP1 transduced T cells (Fig 4.2A). As the TRP1 TCR is class II restricted we sorted $CD4^+$ cells before transduction and by day 3 post-transduction the cells were 95% $CD4^+$ and 59% $TRP1^+$ (Fig 4.2B). As before, following T cell injection tumour size was monitored and mice were sacrificed when necessary.

In contrast to the transient control mediated by TRP2 and Pmel-1 transduced T cells, TRP1 transduced cells mediated profound tumour control. All mice treated with TRP1 TCR transduced T cells completely cleared the tumour and remained tumour-free until day 43 when the experiment was ended (Fig 4.2C). As no mice treated with TRP1 cells had to be sacrificed due to tumour size and cleared the tumour without any toxicity, 100% of mice survived until day 43 (Fig 4.2D).

To test whether the tumour control offered by TRP1 transduced T cells would lessen when fewer transduced T cells were transferred, we repeated the experiment in Fig 4.2 but transferred $1x10^5$, $2x10^5$, $1x10^6$ or $2x10^6$ TRP1 T cells (Fig 4.3A). As in Fig 4.2, $2x10^6$ TRP1 T cells mediated marked tumour control, however in this experiment only 1/3 mice completely cleared the tumour. The other 2 mice appeared to be clearing the tumour until $\sim$day 45 when the tumour rapidly regrew and the mice had to be sacrificed. A similar pattern of tumour regression before eventual rapid outgrowth at $\sim$day 40 was also observed in mice treated with $1x10^6$ and $2x10^5$ TRP1 T cells. Surprisingly, despite tumour control appearing to be the most effective at a dose of $2x10^6$, mice treated with 10-fold fewer cells still controlled the tumour to a broadly similar extent. However at a dose $1x10^5$, TRP1 T cells conferred suboptimal tumour control as tumour growth only plateaued for $\sim$15 days before outgrowth, similar to the control seen by TRP2 or Pmel-1 transduced T cells.

However in addition to this lasting tumour control and elimination, TRP1

transduced T cells also mediated off-tumour, on-target destruction of endogenous TRP1 expressing melanocytes. Mice treated with $2x10^6$ and $1x10^6$ but not $2x10^5$ TRP1 transduced T cells showed visible signs of vitiligo (skin depigmentation) (Fig 4.4). Whilst this was most prominent and systemic at $2x10^6$, mice treated with $1x10^6$ TRP1 cells still showed some signs of vitiligo, albeit limited to the skin at the tumour site. This is not unsuspected as extensive vitiligo has been reported in mice treated with TRP1 transgenic T cells (Quezada et al. 2010) and has been reported in some patients receiving adoptive cell therapy for melanoma (Dudley and Rosenberg 2003).

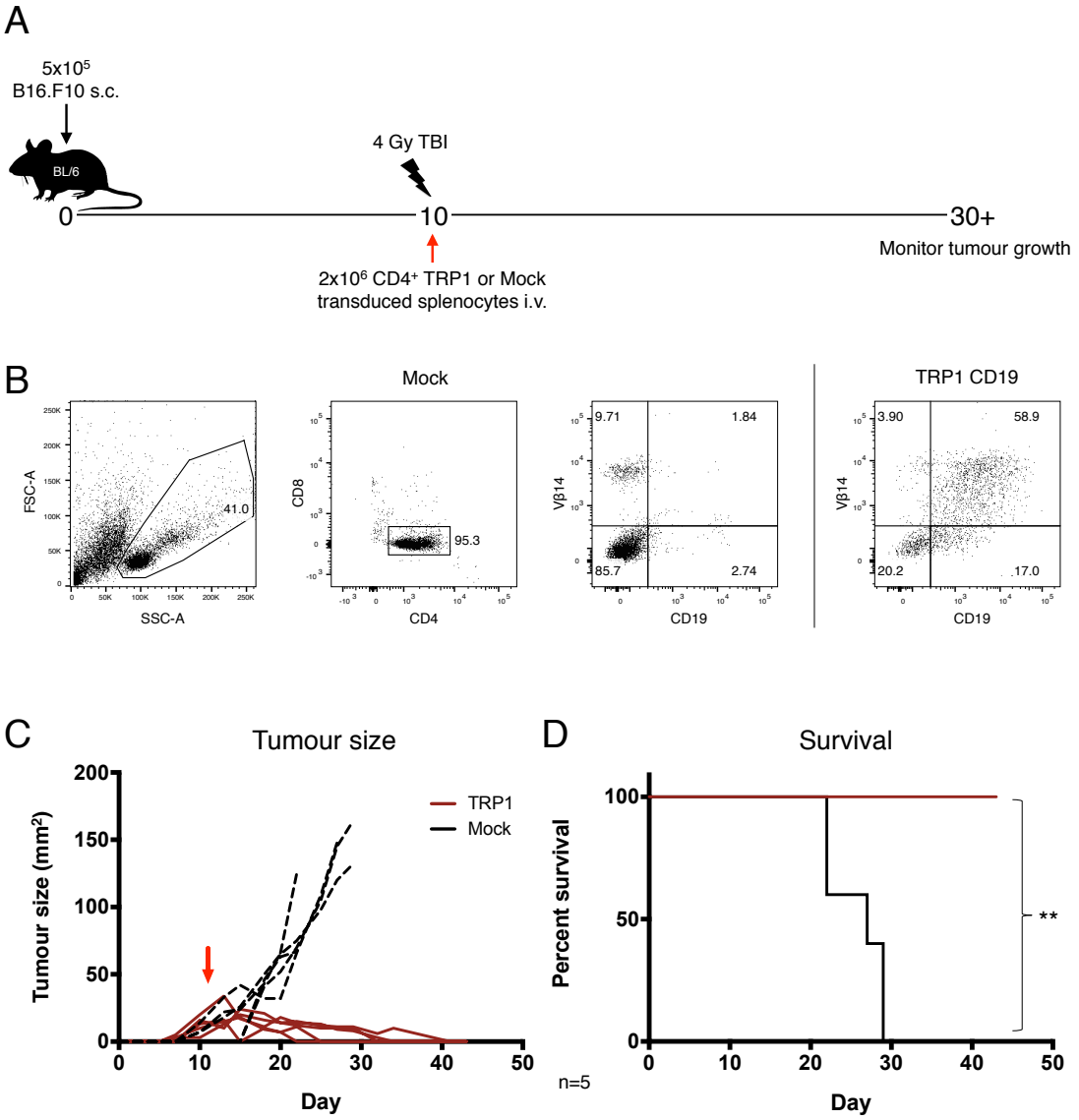


Figure 4.2: *Control of B16.F10 growth by TRP1 transduced CD4⁺ T cells in vivo.*
(A) Experiment outline. 5x10^5 B16.F10 cells were injected sub-cutaneously into the flank of C57/BL6 mice. On day 10 post-injection mice were irradiated with a sub-lethal dose of irradiation (4 Gy) and injected i.v. with 2x10^6 CD4$^+$ splenocytes that had been transduced with the TRP1 TCR or mock transduced.
(B) FACS profile of transduced T cells on day 3 post-transduction. Transduced CD4 T cells are identified as CD19$^+$ and Vβ14$^+$ lymphocytes.
(C) Tumour protection by transduced T cells was assessed by measuring tumour size.
(D) Kaplan Meier survival graph. Significance measured by Log Rank test. **: $p \leq 0.01$. Results are from 5 mice per group from 1 experiment

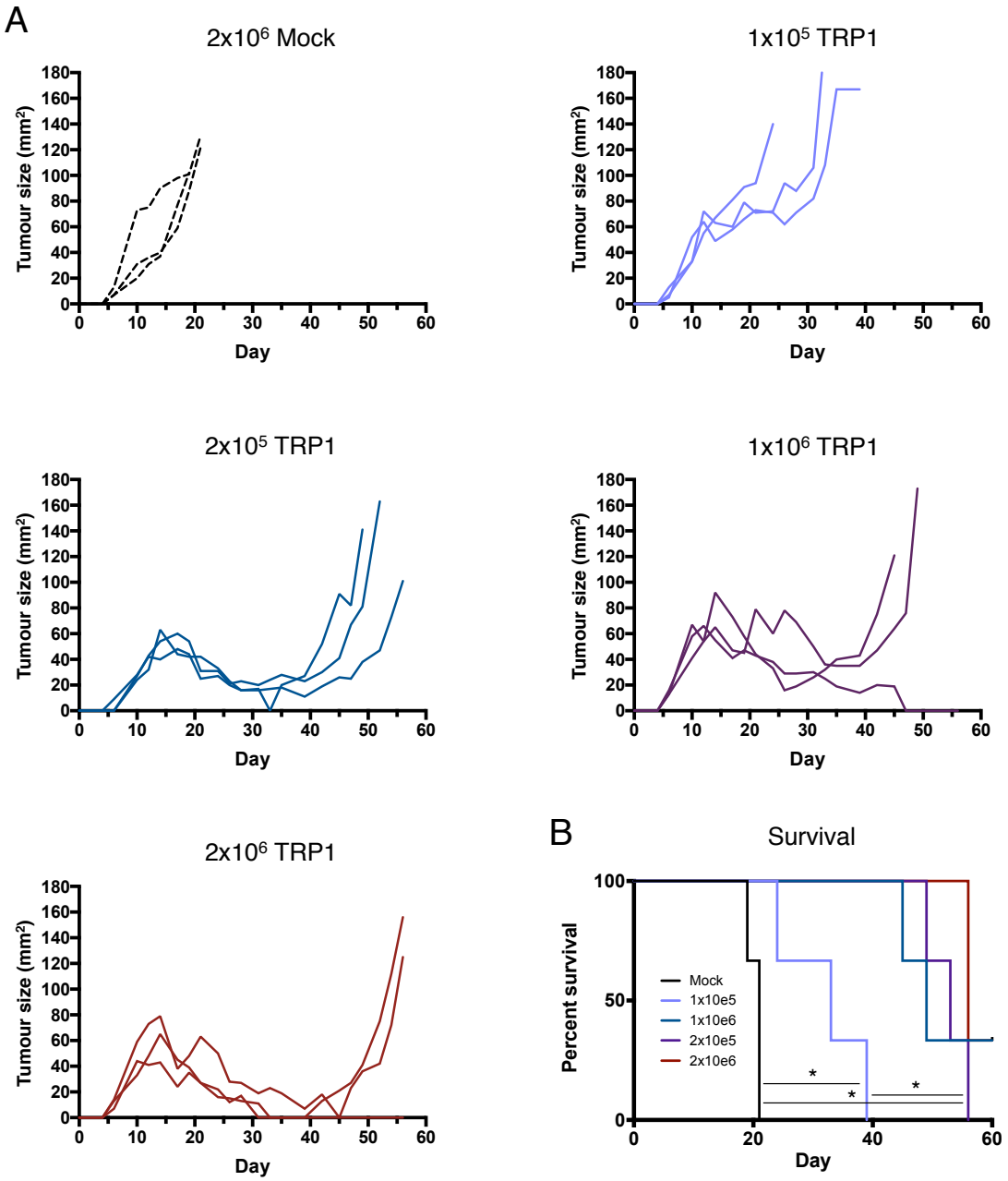


Figure 4.3: *Control of B16.F10 growth by TRP1 T cells depends on cell dose.*
(A) Tumour control by TRP1 transduced T cells. As before $5x10^5$ B16.F10 cells were injected sub-cutaneously into the flank of C57/BL6 mice. On day 10 post-injection mice were irradiated 4 Gy and injected i.v. with $1x10^5$, $2x10^5$, $1x10^6$ or $2x10^6$ $CD4^+$ TRP1 TCR transduced T cells. Tumour protection by transduced T cells was assessed by measuring tumour size. Results are from 3 mice per group from 1 experiment.
(B) Kaplan Meier survival graph. Significance measured by Log Rank test. *: $p \leq 0.05$.

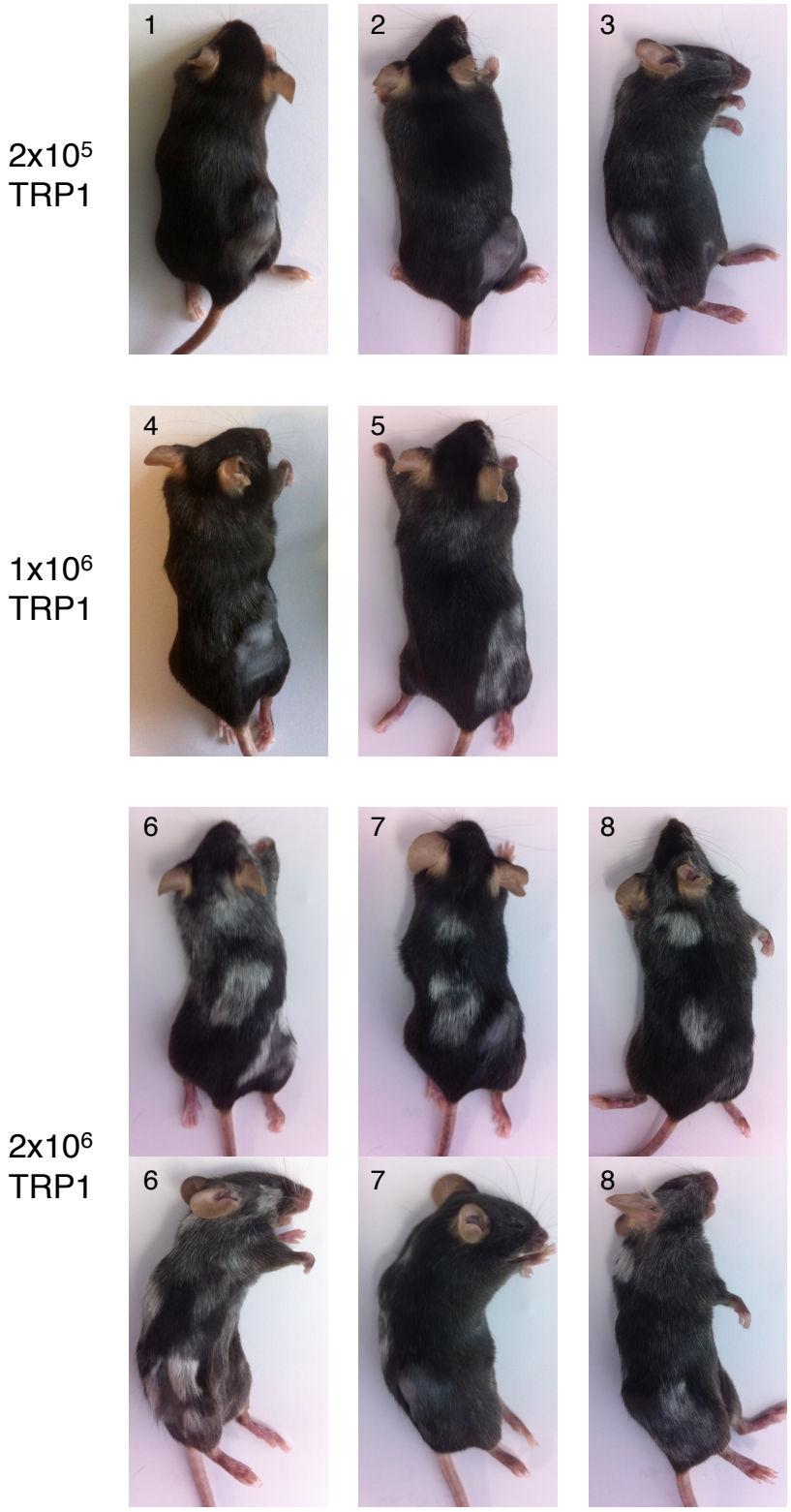


Figure 4.4: *Self-reactive TRP1 transduced T cells mediate autoimmune vitiligo.* Vitiligo in mice bearing B16.F10 tumours after treatment with high numbers of TRP1 transduced T cells. Photographs show mice upon sacrifice (day 49-56) in groups treated with $2x10^5$, $1x10^6$ or $2x10^6$ $CD4^+$ TRP1 TCR transduced T cells.

4.3 Control of B16 NP GFP by F5 transduced T cells *in vivo*

To test whether F5 TCR transduced T cells were able to control the growth of B16 NP GFP *in vivo*, the B16.F10 model as described previously had to be adapted. Mice were irradiated with 4 Gy on the day of tumour injection, rather than T cell transfer as done previously. This was done to remove endogenous NP-specific T cells that would not be tolerised to the foreign antigen. By day 7, tumours were well established so mice were then treated with $2x10^6$ F5 CD19 or mock transduced T cells (Fig 4.5A). One group of mice also received $1x10^6$ F5 TCR transgenic T cells as a positive control.

As for the previous experiments purified CD8 T cells were transduced, however in these experiments transduced T cells were injected 1 day, rather than 3 days, after transduction. This reduces the length of time cells are cultured for and improves their survival *in vitro*. However it makes an accurate calculation of their transduction efficiency more difficult. Whilst F5 TCR transduced T cells expressed the CD19 transduction marker after 1 day, TCR expression was not yet visible (Fig 4.5B). This may be because to be expressed on the cell surface, the TCR α and β chains must dimerise, then form a complex with the CD3 γ , δ , ε and ζ chains. This is inherently more complicated than expression of the smaller, truncated CD19 protein and so takes longer to be expressed on the cell surface. For this reason a small number of cells were cultured until day 4 to check transduction efficiency. As expected, by day 4 the $CD19^+$ transduced cells had become $V\beta11^+$.

The ability of F5 TCR transduced or transgenic T cells to mediate tumour control was monitored by measuring tumour size over the course of the experiment. Fig 4.6A demonstrates that F5 CD19 transduced T cells controlled tumour growth in half the mice tested. Of the 7 mice treated, 3 showed pronounced tumour regression until day $\sim$35 when tumour control was lost and tumours rapidly outgrew. 1 further mouse showed mild tumour control, but the remaining 3

showed no evidence of tumour control. The reason why only half of the F5 CD19 treated mice responded to treatment is unclear. The mice that controlled the tumour were spread between the two experiments so is not due to a fault in one experiment. In contrast 5 out of 6 mice treated with F5 transgenic T cells controlled tumour growth until day 30-35 when control was lost. 1 mouse in this group completely cleared and remained tumour free until day 50 when the experiment was ended. As in the B16.F10 model, treatment with mock transduced T cells had no effect on the growth of the B16 NP GFP tumour. Tumour control by F5 CD19 transduced or F5 transgenic T cells caused a significant improvement in survival, although this was more significant in F5 transgenic treated mice (Fig 4.6B).

Although mice treated with either F5 TCR T cell population were able to control tumours, the majority eventually lost this tumour control. To investigate whether this loss of control was due to the B16 NP GFP tumours losing NP expression *in vivo*, tumours from mice treated with F5 or mock transduced T cells were cultured *ex vivo*. As the B16 NP GFP tumour line is 100% GFP^+ (Fig 3.6C), outgrowth of a untransduced, NP negative tumour is unlikely. Fig 4.7A shows that tumours treated with either F5 CD19 or mock transduced T cells remain 100% GFP^+. However as GFP expression is controlled by an IRES sequence and not linked to NP expression by a 2A sequence, it is possible that GFP^+ cells have lost or down-regulated NP expression. To test this, *ex vivo* tumours were co-cultured with F5, TRP2 or mock transduced splenocytes *in vitro*. IFN-γ expression was measured by FACS in live, $CD8^+$, $CD19^+$ F5 or TRP2 transduced T cells or live, $CD8^+$ mock transduced T cells (Fig 4.7B). Regardless of whether tumours had been partially controlled by F5 transduced T cells or not, *ex vivo* B16 NP GFP tumours were capable of presenting NP to F5 transduced T cells to the same extent as the *in vitro* cell line (Fig 4.7C). The frequency of IFN-γ^+ cells was also similar between F5 and TRP2 TCR transduced T cells. This suggests that the loss of tumour control exhibited by F5 CD19 *in vivo* is not due to loss of NP expression on the tumour. Moreover *ex vivo* tumours taken from F5 treated mice completely failed to control the tumour were still able to present NP *in vitro*.

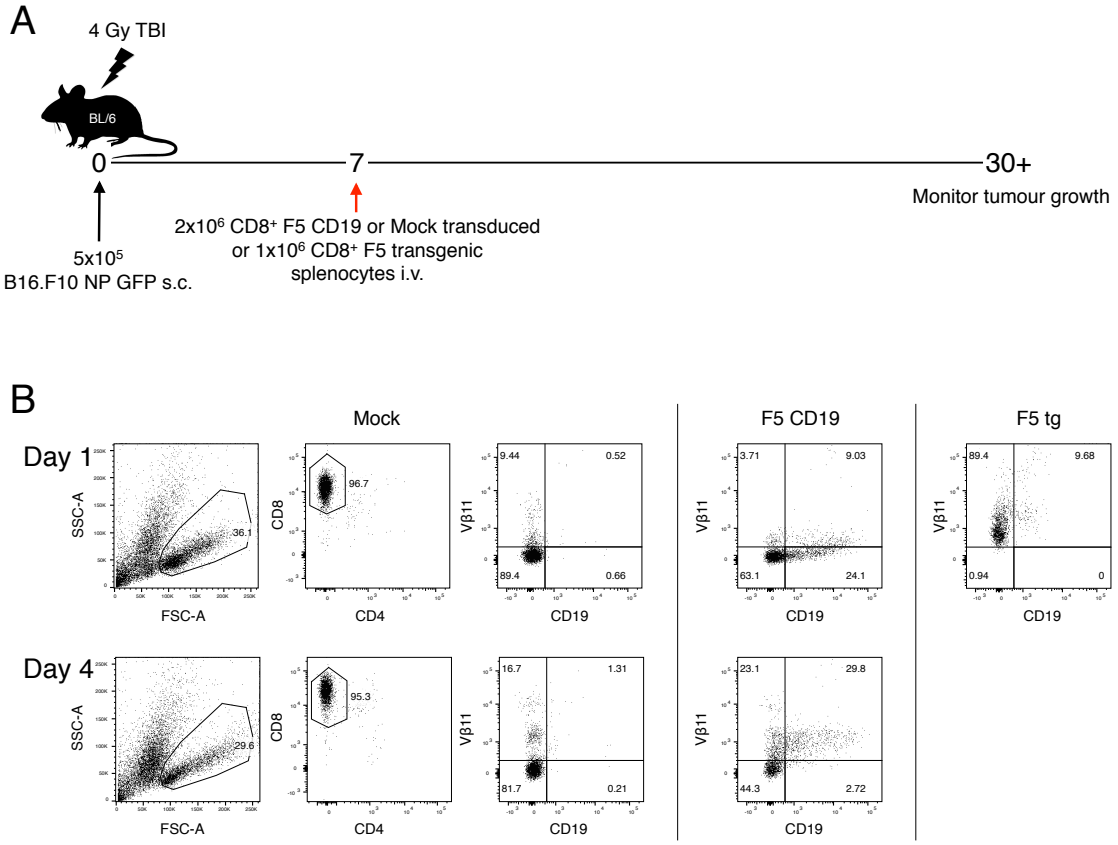


Figure 4.5: *Establishing the B16 NP GFP in vivo model.*
(A) Experiment outline. C57/BL6 mice were irradiated with 4 Gy and injected subcutaneously in the flank with 5x10^5 B16 NP GFP cells. On day 7 mice were injected with 2x10^6 CD8$^+$ splenocytes that had been transduced with the F5 TCR or mock transduced. A separate group was injected with 1x10^6 unactivated CD8$^+$ splenocytes from a transgenic F5 TCR mouse.
(B) FACS profile of transduced and transgenic T cells on day 1 and day 4 post-transduction. Transduced cells are identified as CD19$^+$ and Vβ11$^+$ lymphocytes.
F5 CD19 denotes transduced T cells, F5 tg denotes transgenic T cells.

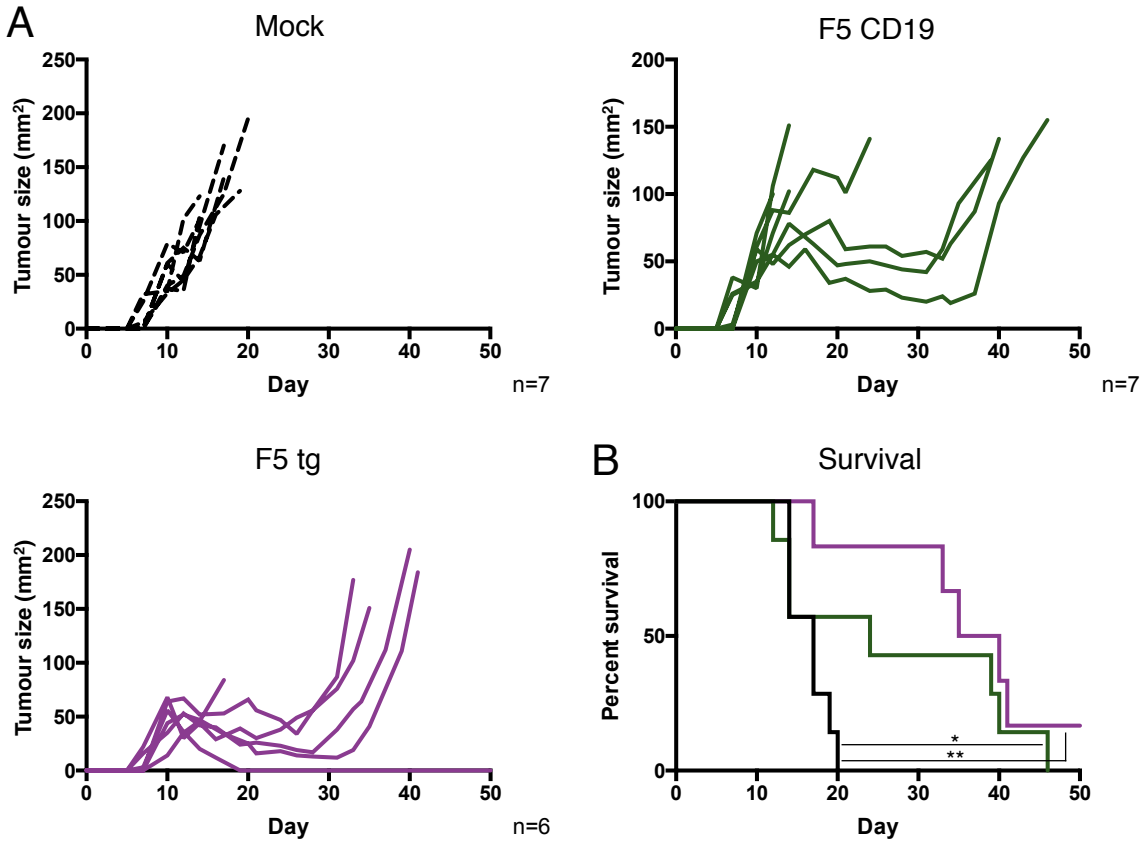


Figure 4.6: *F5 TCR transduced T cells control B16 NP GFP in vivo.*
(A) Tumour protection by transduced T cells was assessed by measuring tumour size in mice treated with $CD8^+$ mock or F5 CD19 transduced, or F5 transgenic (tg) T cells.
(B) Kaplan Meier survival graph. Significance measured by Log Rank test based on survival after T cell transfer. *: $p \leq 0.05$, **: $p \leq 0.01$. Results are from 6-7 mice per group from 2 independent experiments

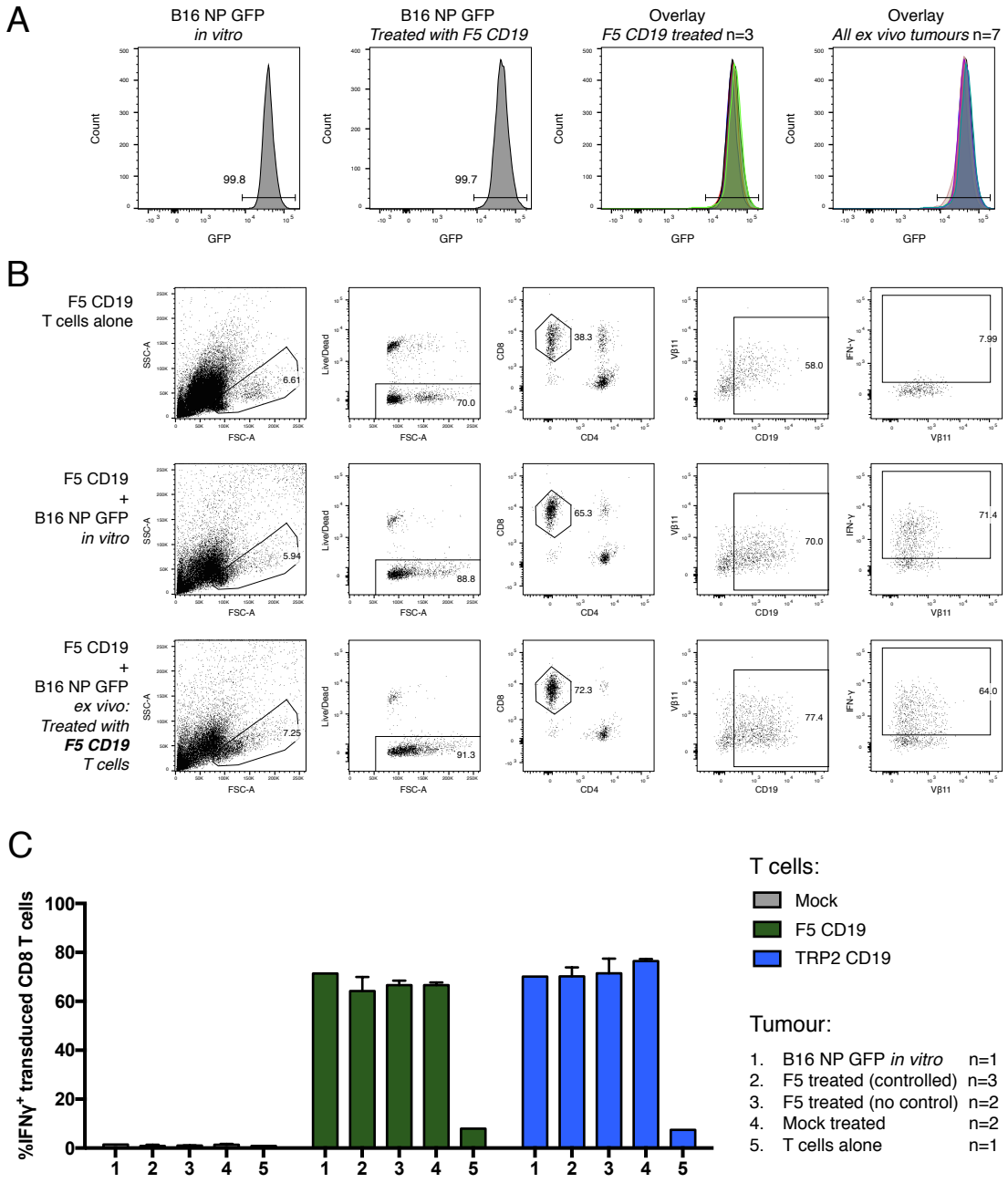


Figure 4.7: *ex vivo B16 NP GFP tumours still present NP to F5 transduced T cells.*
(A) GFP expression in *in vitro* B16 NP GFP or *ex vivo* B16 NP GFP tumours after treatment with F5 CD19 or mock transduced T cells. Overlaid histograms show F5 CD19 controlled tumours, or all T cell treated tumours.
(B) Representative FACS plots for *in vitro* IFN-γ production by F5 CD19 transduced T cells. Cells were gated on live, $CD8^+$, $CD19^+$ T cells.
(C) Summary data for co-cultures of *ex vivo* B16 NP GFP tumours with mock, F5 CD19 or TRP2 CD19 transduced T cells. 'F5 treated (controlled)' indicates mice that were sacrificed on day 33-35, 'F5 treated (no control)' and mock treated were tumours from mice sacrificed on day 15-20.

4.4 Summary and conclusion

Together, the data in chapters 3 and 4 show that TCR transduced T cells are stimulated by tumour cells *in vitro*, and control tumour growth *in vivo*. In line with previous reports using these TCRs (Abad et al. 2008; Chinnasamy et al. 2013; Kerkar et al. 2011), the TRP2 and Pmel-1 TCRs were only able to induce transient tumour control. The TRP1 TCR however was more effective and was able in some cases to completely clear the tumour burden. This, again, is in line with previous studies, which have reported the efficacy of CD4 T cells transduced with this TCR (Kerkar et al. 2011). Interestingly these transduced CD4 cells are able to induce tumour regression in the absence of CD8 T cells. The same result has been shown in transgenic TRP1 CD4 T cells, which are able to acquire effector functions and directly lyse B16 tumour cells *in vivo* (Quezada et al. 2010). In addition CD4 T cells expressing a different transgenic TCR have been shown to indirectly kill B16 tumours by expressing IFN-γ, TNF-α and IL-2 (Shklovskaya et al. 2016).

Whilst the enhanced control of B16 by TRP1 TCR transduced cells could be explained by the potential differences in CD4 vs CD8 mediated control, there is an alternative explanation. Unlike the TRP2 and Pmel-1 TCRs, the TRP1 TCR was originally generated in mice lacking *TRP1* gene expression (Muranski et al. 2008). Therefore the TRP1 TCR, although it recognises a self-antigen, has not been through central tolerance in the same way as the TRP2 and Pmel-1 TCRs have. TPR1 has more in common with a foreign-reactive TCR, which are able to generate high-avidities without being deleted in the thymus. This separation between high avidity 'foreign' TCRs and low avidity 'self' TCRs could neatly explain the differences seen in tumour control.

In line this, T cells transduced with the foreign-antigen specific F5 TCR also efficiently controlled growth of the B16 NP GFP tumour. This could be as a result of NP expression in these cells being driven by a constitutive viral promotor, resulting in a greater amount of NP being presented than TRP2. However these data together suggest that the efficacy of the F5 TCR is mediated by a

potentially enhanced affinity for antigen. These models are also beneficial for future experiments as it will allow us to compare the difference in responses when T cells have the potential to be tolerised to a self antigen or not. This may have important consequences for the role of DC as, unlike NP, the antigens for the TRP2, Pmel-1 and TRP1 TCRs will be chronically expressed in host tissue. The context in which DC present antigen is crucial in the subsequent T cell response, so restricting antigen expression to the tumour (as is the case with NP) may affect induction of T cell anergy, effector function or memory formation.

Lastly F5 transduced T cells can also be compared to transgenic T cells expressing the F5 TCR. In these tumour control experiments, F5 transgenic T cells were more effective at controlling tumour growth than F5 transduced T cells. One potential explanation is that whereas 100% of transgenic T cells express the F5 TCR, in the transduced population there is an untransduced, polyclonal fraction. Co-transfer of these untransduced cells with antigen-specific T cells has been shown to reduce expansion and tumour control by the anti-tumour TCR expressing T cells (Abad et al. 2008; De Witte et al. 2008a). In addition these transgenic T cells also differ from transduced cells in that they were not pre-activated *in vitro*. The differentiation status of transferred T cells affects their function *in vivo*. Adoptively transferred, cells that are less differentiated/more naive proliferate more and have improved anti-tumour immunity (Hinrichs et al. 2009; Klebanoff et al. 2005). In addition transgenic T cells may differ in that we would also predict that they would be dependent on antigen presentation *in vivo*. However whether this is required for activated, transduced T cells is unclear.

Overall these data show that in these B16.F10 and B16 NP GFP models, T cells transduced with anti-tumour TCRs are able to induce some level of tumour control. It is possible that T cell expansion could be enhanced by vaccination with tumour antigen however this is less effective than pre-conditioning irradiation and little synergy has been reported when vaccination is used in combination with irradiation (De Witte et al. 2008a). This has the added benefit

for this project in establishing a tractable model in which the role of endogenous DC can be addressed without the complication of an additional vaccine. With these models for partial tumour control by transferred T cells set up the next chapter will establish a murine model in which DC can be depleted, to allow the effect of DC depletion on transferred T cells to be assessed.

Chapter 5 Defining the impact of CD11c$^+$ cell depletion in tumour bearing mice

To examine the role of endogenous DC in the anti-tumour response mediated by TCR transduced T cells we chose to use a model in which we could transiently deplete DC. Conditional DC depletion was achieved using the well described CD11c.DTR model (Jung et al. 2002). In this model the high-affinity diphtheria toxin receptor (DTR) is placed under the control of the CD11c promotor. Injection of CD11c.DTR mice with diphtheria toxin (DT) results in the specific depletion of CD11c$^+$ cells, primarily the DC compartment. The advantages of such a model as opposed to a constitutive DC depletion/knockout model are threefold; it allows for temporal control of DC depletion and it avoids both developmental concerns or the appearance of compensatory mechanisms that are both associated with mice permanently depleted of cell subsets (Sapoznikov and Jung 2008). However one limitation of this model is that aberrant CD11c expression on non-haematopoetic tissue leads to lethality after multiple DT injections (Zaft et al. 2005). Whilst generation of CD11c.DTR bone marrow chimeras can restrict DTR to the haematopoetic system and therefore allow multiple DT injections, presence of radio-resistant cells can obfuscate results. An alternative therefore is to use the closely related CD11c.DOG model (Hochweller et al. 2008). These mice express the same high-affinity DTR but under the control of the whole CD11c locus control region, rather than just the CD11c promotor. This results in a tighter restriction of the DTR to CD11c$^+$ haematopoetic cells and mice can be injected with DT multiple times without toxicity.

Whilst DC depletion with the CD11c.DTR or CD11c.DOG models are powerful tools to study DC biology there are numerous caveats and limitations to using these depletion models. These include the expression of CD11c on other cell types and the appearance of monocytosis/neutrophilia following DT injection (Sivakumaran et al. 2016; Tittel et al. 2012; Van Blijswijk et al. 2013). Therefore in order to accurately assess the ability of transduced T cells to control tumour

growth in the absence of DC these limitations must be acknowledged. Moreover whilst these models have frequently been used to deplete DC in lymphoid organs, little has been done to show the efficacy or effects of DC depletion in the tumour. An array of different myeloid cells infiltrate the complex tumour microenvironment, including numerous DC subsets (Broz et al. 2014; Gajewski et al. 2013). In order to address the effect DC depletion has on the ability of transferred T cells to control tumour growth, it is paramount that the effect of DC depletion in the tumour is addressed. The aim of this chapter therefore is to:

1. Establish the CD11c.DTR model and define the extent of DC depletion in tumour bearing mice.
2. Assess the depletion of other $CD11c^+$ cell types including:
    - Neutrophils and macrophage/monocyte populations
    - Activated T cells
    - NK cells
3. Establish the CD11c.DOG model in tumour bearing mice

5.1 Depletion of CD11c$^+$ cells by DT injection in CD11c.DTR mice

To establish the DC depletion model, we first tested the efficiency of DC depletion from the spleen of CD11c.DTR mice. 1 day after injection of 100ng DT or PBS, mice were sacrificed and DC depletion in the spleen was assessed by FACS. The CD11c.DTR mice carry the high affinity DTR fused to GFP under the control of the CD11c promotor (Fig 5.1A). CD11c^{high} cells are therefore both GFP and MHCII positive, and either marker can be used alongside CD11c to identify DC (Fig 5.1B). Following DT injection, 91$\pm$6% of GFP$^+$ or 90$\pm$7% of MHCII$^+$ CD11c cells were depleted (Fig 5.1C). As DC return to homeostatic levels after 3 days post-DT injection, DC depletion was also measured in the spleen 48 hours after DT injection. Whilst depletion was lesser by this time-point, the majority of DC (74$\pm$14%) were still depleted (Fig 5.1C).

In addition to DC depletion, significantly higher frequencies of neutrophils and monocytes have also been noted in the spleens of DT injected mice (Sivakumaran et al. 2016; Tittel et al. 2012). To examine this in our experiments, spleens were stained for CD11b$^+$ Ly6C^{high} monocytes and CD11b$^+$ Ly6C^{int} neutrophils. As expected, 48 hours after DC depletion there were significantly higher frequencies of neutrophils and monocytes present in the spleen. Together these results were consistent with previously published DC depletion experiments from our laboratory using this model (Sivakumaran et al. 2016).

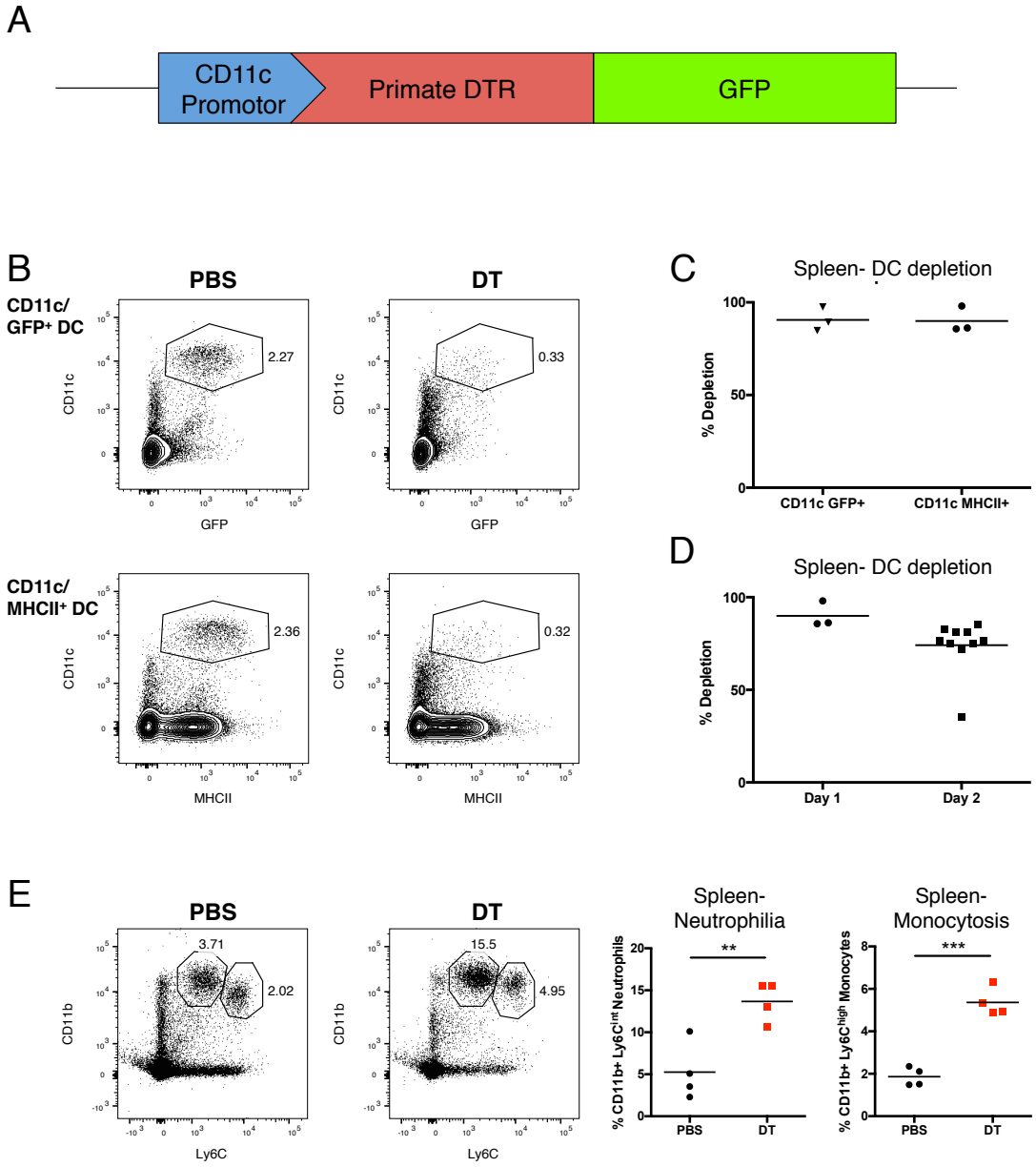


Figure 5.1: *Depletion of splenic CD11c⁺ cells by DT injection in CD11c.DTR mice.*
(A) Schematic representation of the DTR/GFP transgene.
(B) CD11c.DTR mice were injected i.p. with 100ng DT or PBS. The next day mice were sacrificed and spleens stained for expression of CD11c, GFP and MHCII. DC were gated on scatter and PI- cells, identifying DC as either $CD11c^+$ GFP^+ or $CD11c^+$ $MHCII^+$.
(C,D) Summary data from 2-3 experiments showing % depletion of GFP^+ or $MHCII^+$ CD11c cells (C) or the % depletion on day 1 and 2 post-injection (D).
(E) $CD11b^+$ $Ly6C^{high}$ monocytes and $CD11b^+$ $Ly6C^{int}$ neutrophils were pre-gated on scatter, PI- cells and $CD45^+$ cells, then distinguished by Ly6C expression as shown in the representative FACS plots. Summary data shows frequency of monocytes or neutrophils in the spleen after DT injection. Data is from 4 mice per group from 1 experiment.
Statistical test: unpaired T test. **: $p \leq 0.01$, ***: $p \leq 0.001$.

5.2 Frequency and phenotype of tumour-resident DC

To assess whether DT injection efficiently depletes DC from the tumour we firstly needed to establish a method of identifying tumour-resident DC. C57BL/6 mice bearing B16.F10 tumours were sacrificed on day 17 (as outlined in Fig 5.2A). Tumour infiltrating myeloid cells were identified according to a recent panel described by Broz et al. 2014, and as shown in Fig 5.2B. Tumour cells could largely be excluded based on size, then live singlets were selected and any remaining tumour cells were excluded by gating on $CD45^+$ cells. These then split into a $CD11b^+$ $Ly6C^+$ monocyte/neutrophil population, and a $Ly6C^-$ population. In contrast to the spleen, it is difficult to separate tumour infiltrating neutrophils and monocytes based on Ly6C expression, although the tumour appears to be dominated by $Ly6C^{high}$ monocytes. The $Ly6C^-$ cells contained a broad $CD11c^+$ $MHCII^+$ population, referred to here as 'APCs'. However as this APC population contains both DC and macrophage populations, DC were further selected by expression of the DC specific marker CD24. These $CD24^+$ DC themselves then split into either $CD103^+$ cDC1 or $CD11b^+$ cDC2, mirroring the DC populations present in tissues (Merad et al. 2013).

Summary data showing the infiltration of these cell types in B16.F10 tumours is shown in Fig 5.2C, either as the frequency of the total $CD45^+$ population or as the corresponding absolute number. These data show that whilst the vast majority of myeloid cells in the tumour are of monocyte origin, there is a consistently detectable $CD24^+$ DC population, which is primarily composed of $CD103^+$ DC. The low tumour infiltration of cDC1 and cDC2 compared to monocytic cells has been confirmed in other recent studies (Broz et al. 2014; Guilliams et al. 2016; Salmon et al. 2016). However despite their low frequency, these $CD103^+$ DC have been previously identified as the dominant tumour-resident population capable of cross-presenting tumour antigen to CD8 T cells, both in the tumour and in tumour draining LNs (Broz et al. 2014; Salmon et al. 2016).

To assess how these myeloid cells infiltrate the tumour over time, the same panel was run on samples taken at different points in the development of the tumour, as outlined in Fig 5.3A. These data suggest that from day 7 to 17 tumours become increasingly cellular as the frequency of both the monocyte/neutrophil population and the APC/CD24$^+$ DC population increases (Fig 5.3B). Within the DC population this increase appears to be driven by increased infiltration of the CD103$^+$ population, as the amount of CD11b$^+$ DC remains level. When tumours become large and well established by day 21 there appears to be less infiltration by all myeloid cells. By this time point the tumour-infiltrating DC had also started to express PD-L1. This appeared to be largely absent on DC at day 7 and began to increase as tumours became more established (Fig 5.3C). However these data have been generated from relatively few mice, so these experiments would need to be repeated to confirm this.

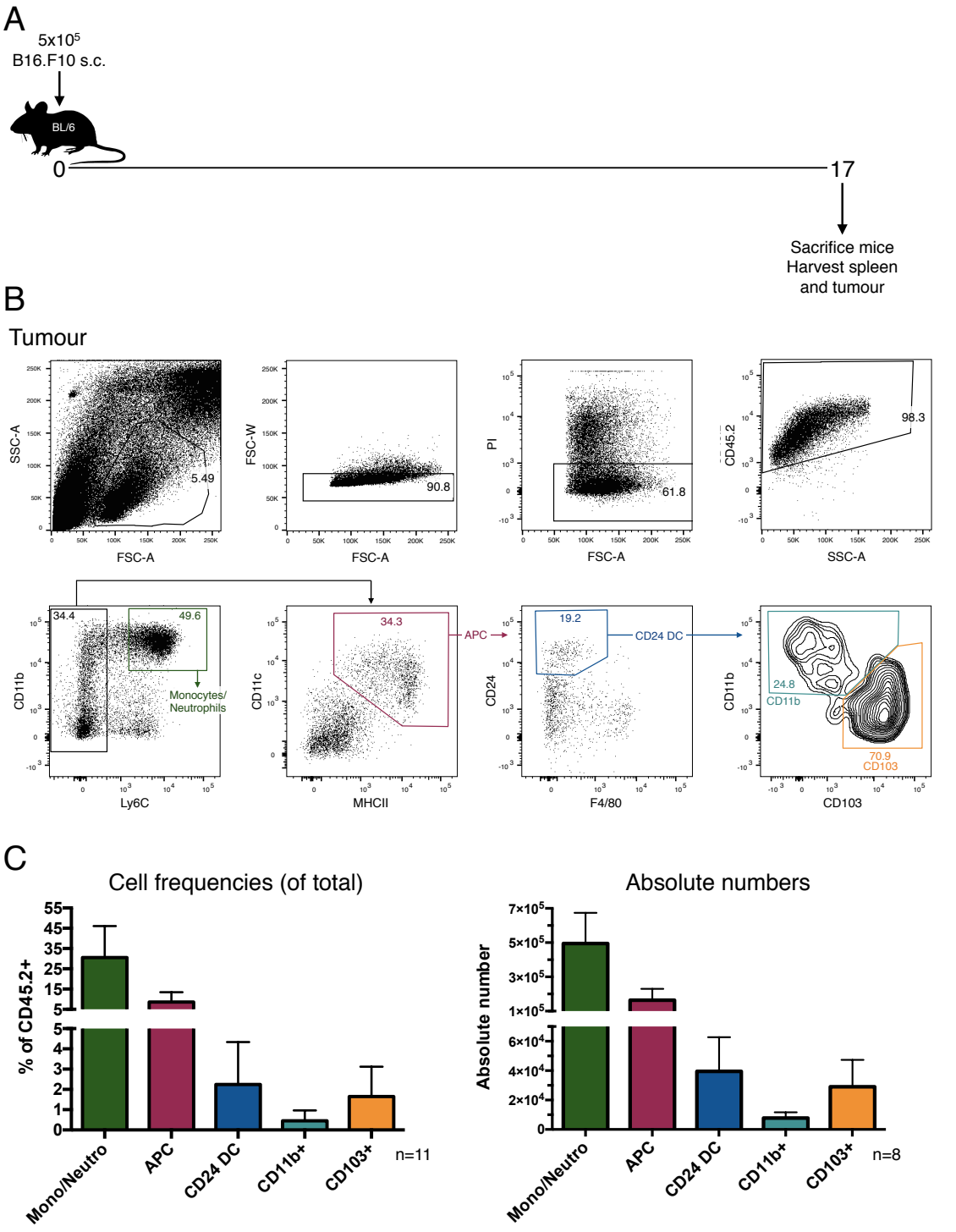


Figure 5.2: *Frequency and phenotype of DC in established B16.F10 tumours.*
(A) Experiment outline. C57/BL6 mice were injected with B16.F10 and sacrificed 17 days later once tumours were well established.
(B) Representative FACS plots showing gating strategy to identify tumour-resident DC. Live, CD45$^+$ cells were gated as shown to identify a monocyte/neutrophil population and a CD103 or CD11b$^+$, CD24$^+$ DC population.
(C) Summary data showing either the mean frequency of cell types from the total CD45$^+$ population or the corresponding absolute number. Frequencies are from a total of 11 mice, from 3 independent experiments ±SD. Absolute numbers are from a total of 8 mice, from 2 independent experiments ±SD.

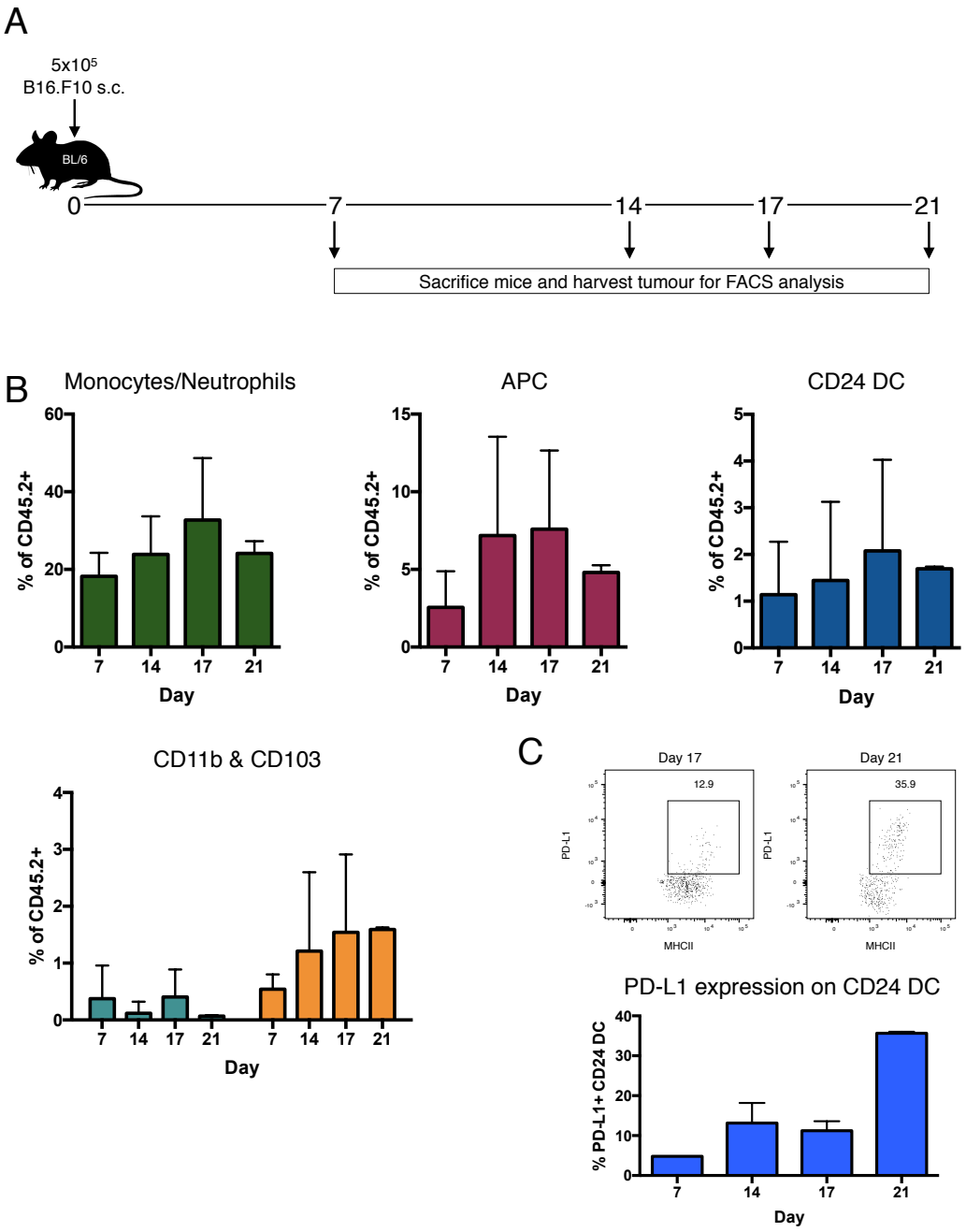


Figure 5.3: *Frequency and phenotype of DC in developing B16.F10 tumours over time.*
(A) Experiment outline. Tumour bearing mice were sacrificed on day 7, 14 and 21 and compared to previous tumours taken from day 17.
(B) Summary data showing the mean frequency of cell types from the total $CD45^+$ population. Data is from a total of 2-4 mice per group, from 2 independent experiments $\pm$SD. (day 7: n=2, day 14: n=4, day 21: n=2).
(C) Expression of PD-L1 on $CD24^+$ DC. Representative FACS plots of $CD24^+$ DC at day 17 and day 21. Summary data shows the mean frequency of PD-$L1^+$ DC with 2 mice per group from 1 experiment.

5.3 Depletion of tumour-resident DC by DT injection

With a strategy to identify tumour-resident DC now established, the degree to which these cells are depleted in CD11c.DTR mice following DT injection can be assessed. As shown in Fig 5.4A, CD11c.DTR mice were injected with B16.F10 tumours. On day 15, when the tumour was well-established, mice were injected i.p. with DT or PBS and 2 days later tumours were harvested and stained to measure the depletion of DC. Whilst there was no overall change in the frequency of the broad APC population, DT injection caused specific depletion of $CD24^+$ DC (Fig 5.4B). As summarised in Fig 5.4C, DT injection depleted on average $56\pm16\%$ of $CD24^+$ DC. This depletion appears to be mainly due to loss of the more abundant $CD103^+$ DC, rather than depletion of the $CD11b^+$ DC. This is reflected in the ratio of CD103:CD11b DC, which is 4:1 in PBS treated mice and falls to 1:1 after DT injection (Fig 5.4D). Interestingly, despite DC depletion mediated neutrophilia and monocytosis in the spleen, there was no difference in the infiltration of neutrophils/monocytes into the tumour. This suggests that although these cells may expand in the spleen, they do not traffic to the tumour.

To give a greater reflection of the loss of these $CD24^+$ DC following depletion, absolute numbers of the myeloid cell populations were also calculated (Fig 5.4E). As with the change in frequency, there were markedly fewer $CD24^+$ DC and $CD103^+$ DC in the tumours of DT treated mice. However whereas the change in frequency only affected this $CD24^+$ DC population, there was a significant loss in cell number in all the myeloid populations. This suggests that, while overall fewer cells infiltrate tumours after DT injection, this primarily affects $CD24^+$ DC.

The preferable depletion of $CD103^+$ rather than $CD11b^+$ DC was surprising as both populations would be expected to express CD11c and be depleted in this model. To address this directly, CD11c expression on the isolated CD103 or

CD11b DC was measured. Fig 5.4F shows the median CD11c expression, or the separate GFP expression from the DTR/GFP transgene. In PBS treated mice both CD103 and CD11b DC expressed similar amounts of endogenous CD11c. However whereas CD11c expression is closely linked to GFP expression in the CD103 DC, this is not true of CD11b DC. Surprisingly these cells express significantly lower GFP, relative to their CD11c expression. Likewise the CD103 and CD11b cells that persist after DT injection express similar levels of CD11c. However as in PBS treated mice, GFP expression in CD11b DC is significantly lower than CD11c expression. Together these data suggest that the introduced CD11c promotor faithfully expresses the DTR/GFP transgene in $CD11c^+$ CD103 DC which are then susceptible to depletion. However the $CD11c^+$ CD11b DC are able to express CD11c without expressing the DTR transgene. The decrease in GFP MFI in CD11b cells after depletion however suggests that those cells that do express the DTR will be depleted, but this is only a subset of the CD11b population. This differential DTR expression is in line with previously published data which has suggested that the CD11b DC, unlike CD103 DC, are a heterogenous population that may have a varied ontology (Guilliams et al. 2016; Merad et al. 2013). To further highlight this heterogeneity a similar result has been found in zDC.DTR mice, in which the DTR is expressed in all *Zbtb46*$^+$ DC. Although both $CD103^+$ cDC1 and $CD11b^+$ cDC2 derive from *Zbtb46*$^+$ pre-DC, DC depletion in this model is specific to cDC1 (Broz et al. 2014). In the CD11c.DTR context, it appears that some of the heterogenous CD11b DC express CD11c without activating the minimal CD11c promotor which drives DTR/GFP expression.

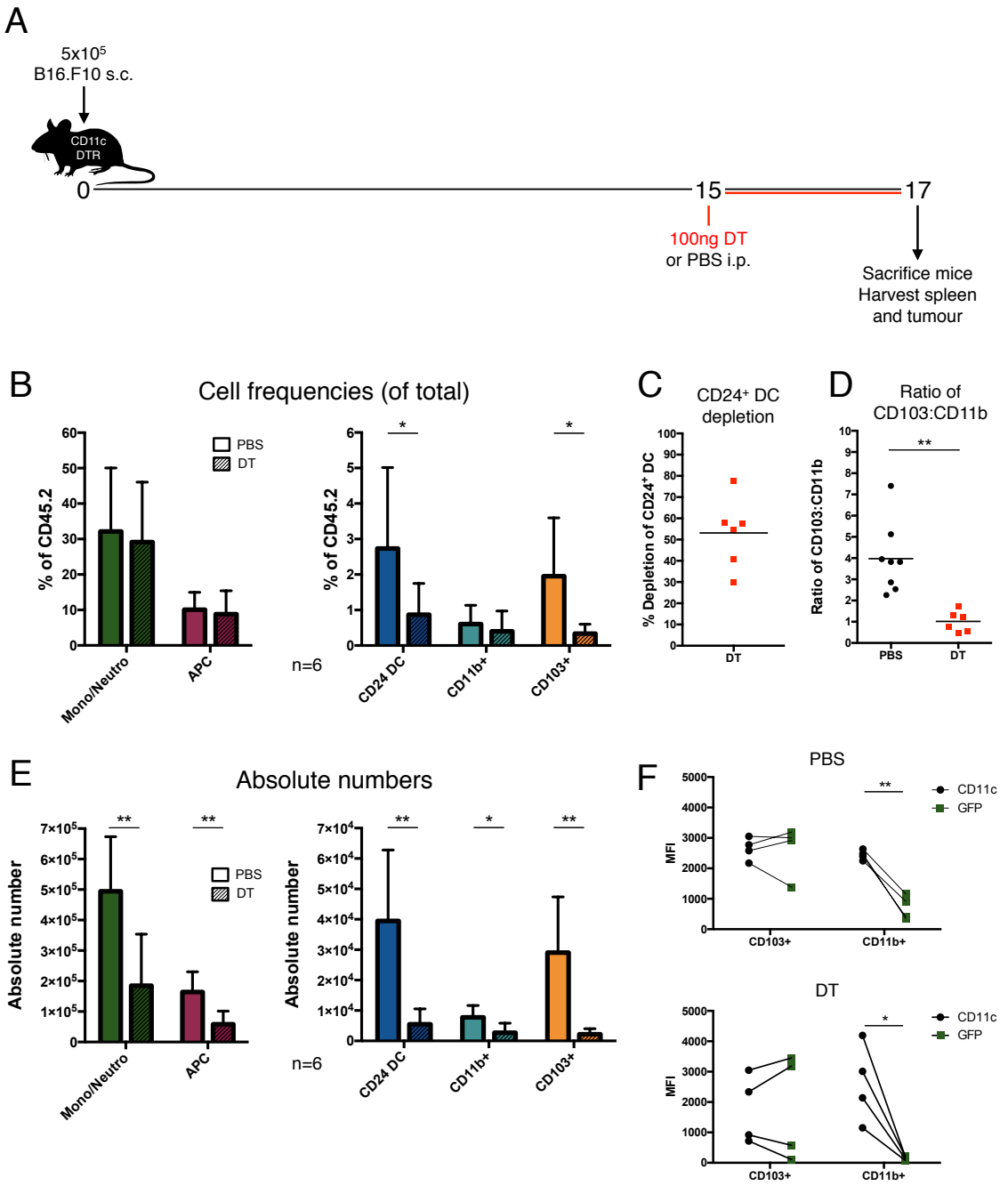


Figure 5.4: *DT injection depletes the tumour-resident CD24⁺ DC population*
(A) Experiment outline. Tumour bearing CD11c.DTR mice were injected with DT or PBS on day 15 and sacrificed 48 hours later.
(B) Summary data showing the mean frequency of myeloid cells from the total tumour-resident CD45⁺ population after injection of DT or PBS.
(C) Summary data showing the depletion of CD24⁺ DC from the tumour.
(D) Summary data showing the ratio of CD103:CD11b DC in the tumour-resident CD24⁺ DC population after injection of DT or PBS.
(E) Absolute numbers of tumour-resident myeloid cell populations after injection of DT or PBS. Data are from a total of 6-8 mice per group, from 2-3 independent experiments (mean ±SD). (8 PBS treated mice, from 3 experiments, 6 DT treated mice from 2 experiments). Statistical test: unpaired T test. *: $p \leq 0.05$, **: $p \leq 0.01$.
(F) Median fluorescence intensity of CD11c expression (black) and GFP expression (green) in CD103 or CD11b DC. Data is from 4 mice per group from 1 experiment. Statistical test: paired T test. *: $p \leq 0.05$, **: $p \leq 0.01$.

5.4 Depletion of other CD11c$^+$ cells beside DC

Whilst CD11c.DTR mice are valuable models for depleting DC, there are some concerns over whether other CD11c$^+$ cells are also depleted. Although DT injection has been shown not to deplete splenic macrophages and B cells (Jung et al. 2002), CD11c expression on some NK cell populations (Blasius et al. 2007) and activated T cells (Beyer et al. 2005; Lin et al. 2003) has been reported. Published data has shown that NK cells aren't depleted in this model (Lucas et al. 2007) however we confirmed this in our experiments. FACS staining for these cells was also performed from the experiments as described in Fig 5.4A. Concordant with previous studies there was no difference between the frequency of NK1.1$^+$ cells in the spleens of mice treated with DT or PBS (Fig 5.5A and B). However in the LN there appeared to be a trend towards a greater frequency of NK1.1$^+$ cells following DT injection, although this wasn't significant. In contrast, NK cells appeared to be at a lower frequency in the tumour following DT injection. These tumour-resident NK cells differ from those found in the LN and spleen in that they express higher levels of CD11c (Fig 5.5C). Interestingly, after DT injection the NK cells present in the tumour express significantly lower levels of CD11c than after PBS injection, suggesting that the CD11c^{high} NK cells are susceptible to depletion.

Although T cells have been shown to express CD11c, this is primarily after viral infections (Beyer et al. 2005; Lin et al. 2003), and little is known about CD11c expression in tumour-infiltrating lymphocytes. To address this we stained for CD11c expression on T cells in tumour bearing mice to see if these cells were susceptible to killing by DT injection. CD11c expression on CD8 and CD4 T cells was distinguished from CD11c$^+$ CD8 and CD4 DC by pre-gating live, CD45$^+$ cells on the T cell markers Thy1.2 and CD3 (Fig 5.6A). Whereas only relatively few T cells expressed CD11c in the spleen and LN, up to 40% of T cells in the tumour were CD11c$^+$ (Fig 5.6B). Despite this mixed expression, following DT injection the frequency of CD11c$^+$ CD4 T cells was significantly lower in the spleen, LN and tumour. CD8 T cells followed a similar pattern but only had

significantly lower $CD11c^+$ frequency in the tumour. This suggests that, similar to the NK cell depletion in Fig 5.5, depletion of T cells occurs primarily in the tumour where CD11c expression is highest. As shown in Fig 5.5C, after depletion the expression of CD11c on the remaining CD8 or CD4 T cells is significantly lower than compared to PBS treated mice. This is true of the T cells found in the tumour and LN, but not spleen.

These data suggest that depletion of $CD11c^{high}$ cells in the CD11c.DTR model can result in NK cell and T cell depletion. In the steady state, CD11c expression on these cells is low, however infiltration into the tumour is associated with increased CD11c expression on NK and T cells. Therefore depletion of these cells may need to be addressed when using tumour-bearing CD11c.DTR mice.

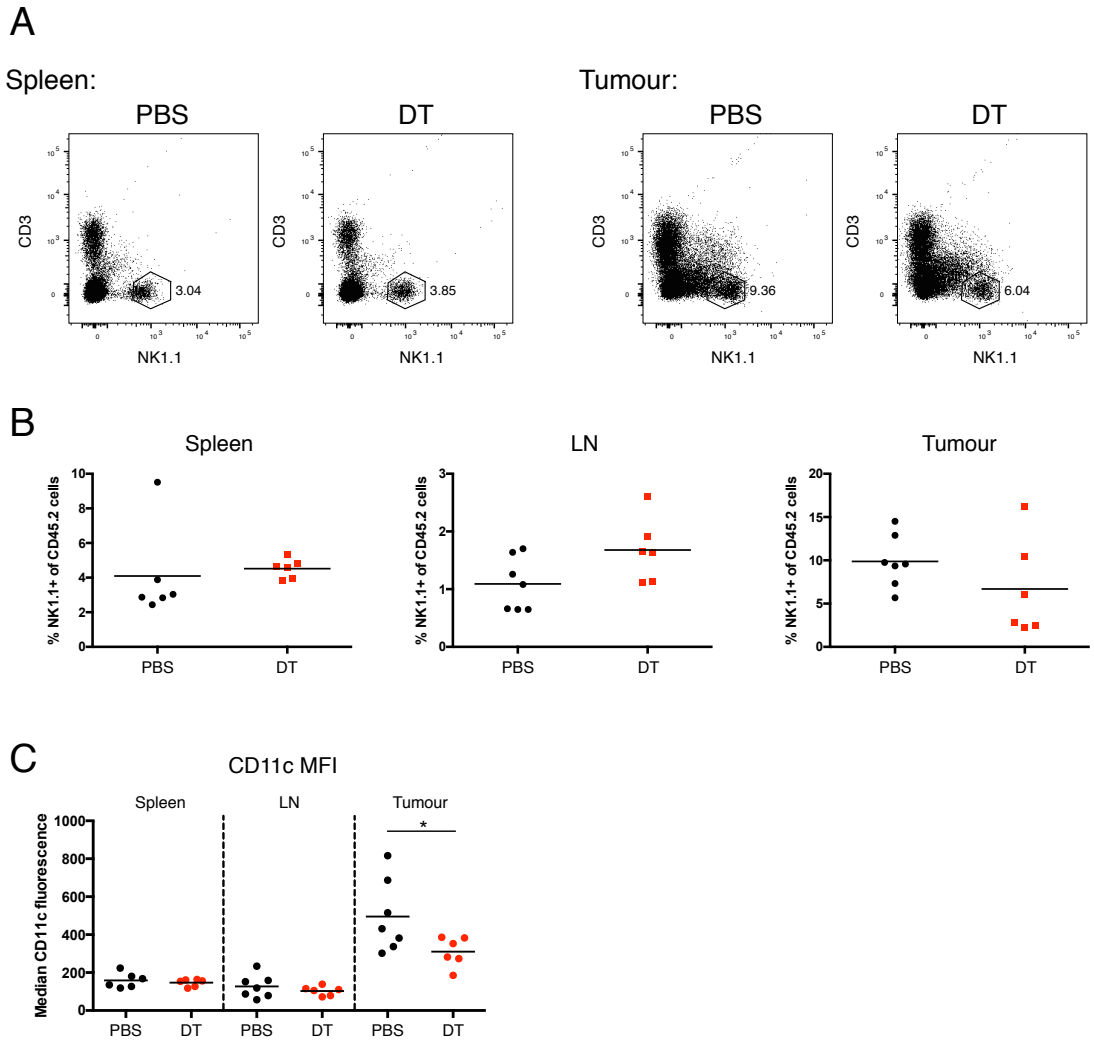


Figure 5.5: *Effect of DT injection on NK cells.*
(A) Representative FACS plots showing gating of NK cells. Cells were pre-gated on live (FSC/SSC and PI^-) and $CD45^+$ cells. NK cells were then identified as $NK1.1^+$ and $CD3^-$ cells.
(B) Summary data showing the frequency of NK cells in spleen, LN and tumour following DT or PBS injection.
(C) Summary data showing median fluorescent intensity of CD11c on NK cells.
Data is from a total of 6-7 mice per group, from 2 independent experiments. Statistical test: unpaired T test. *: $p \leq 0.05$.

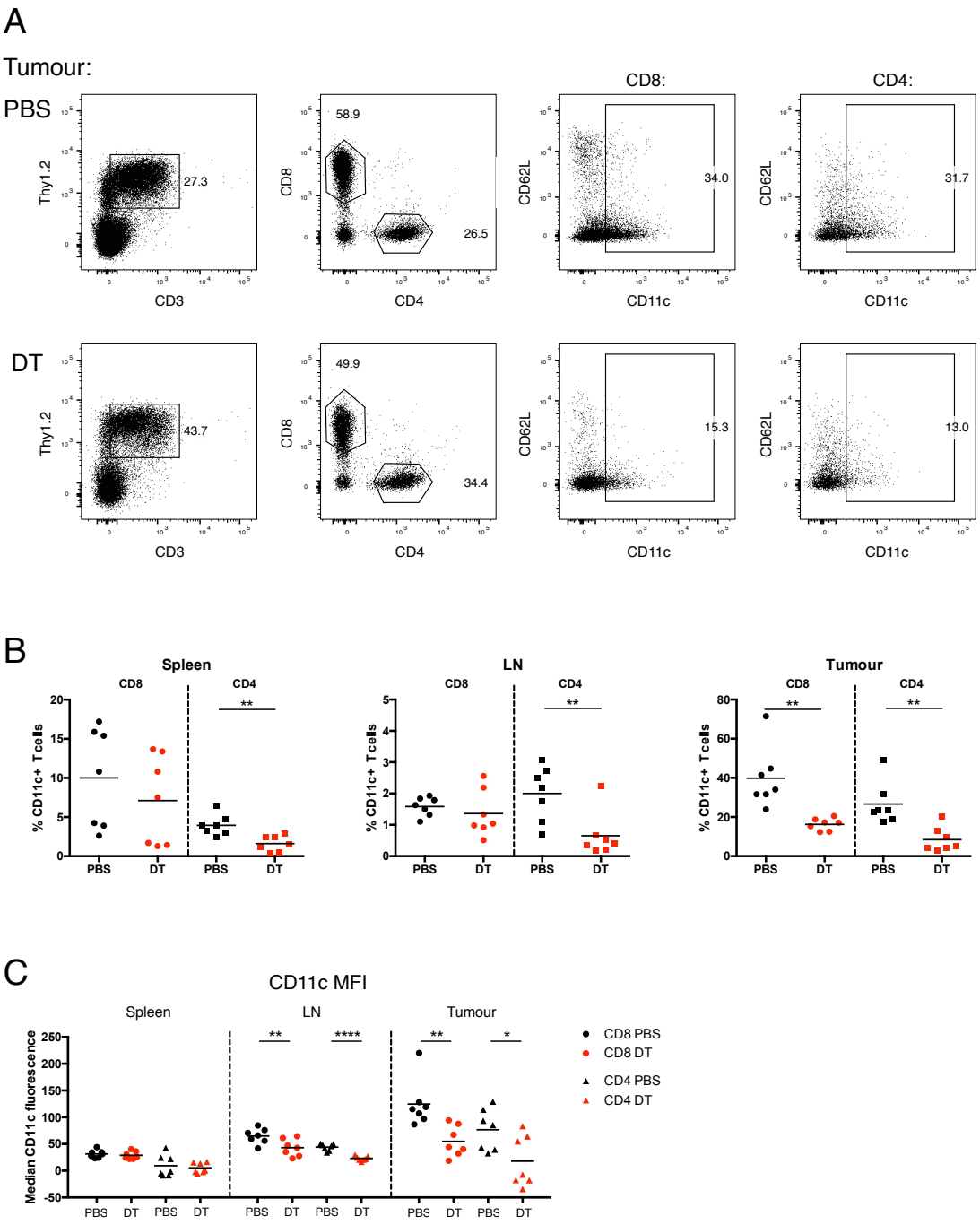


Figure 5.6: *Depletion of CD11c⁺ tumour-resident T cells.*
(A) Representative FACS plots showing gating of CD11c⁺ T cells. Cells were pre-gated on live (FSC/SSC and PI⁻) and CD45⁺ cells. T cells were then identified by expression of Thy1.2 and CD3, from which CD8 and CD4 T cells were identified.
(B) Summary data showing the frequency of CD11c⁺ CD8 and CD4 T cells in spleen, LN and tumour following DT or PBS injection.
(C) Summary data showing median fluorescent intensity of CD11c on CD8 or CD4 T cells. Data is from a total of 6-7 mice per group, from 2 independent experiments. Statistical test: unpaired T test. *: $p \leq 0.05$, **: $p \leq 0.01$, ****: $p \leq 0.0001$

5.5 Effect of DT injection on B16.F10 phenotype

As the B16.F10 tumour is from C57BL/6 origin and transplanted into CD11c.DTR mice, it will not express the high affinity DTR like the endogenous cells of these mice. However we wanted to check whether the changes to the tumour microenvironment described above would have an effect on the B16.F10 tumour itself. After DT injection into B16.F10 tumour bearing CD11c.DTR mice, we stained for the expression of MHCI and II on *ex vivo* isolated tumour cells. As before (Fig 3.4B) *in vitro* B16.F10 cells were also activated with CASM to induce MHCI upregulation, although in this experiment MHCII expression only marginally increased (Fig 5.7A). Expression of both MHCI and II on *ex vivo* tumour cells was much higher than *in vitro* B16.F10, comparable to after activation with CASM. However in mice injected with DT, MHCII expression is significantly lower and there is a trend towards lower MHCI expression ($p=0.06$). A possible explanation for this decreased MHC expression on tumours of mice treated with DT is that activated $CD11c^+$ T cells in the tumour will be depleted (as shown in Fig 5.6). Therefore there are fewer cells secreting IFN-γ *in vivo* and therefore less upregulation of MHC.

To assess whether this reduction in MHC upregulation on B16.F10 cells *in vivo* lessens their ability to present antigen to transduced T cells, isolated B16.F10 tumours were cultured overnight with TRP2 or TRP1 transduced T cells. Both PBS and DT treated tumours induced an IFN-γ response from TRP2 or TRP1 transduced T cells *in vitro*, comparable to the *in vitro* B16.F10 controls (Fig 5.7B). In fact although not significant there appears to be a slightly better IFN-γ response to DT treated tumours than to PBS.

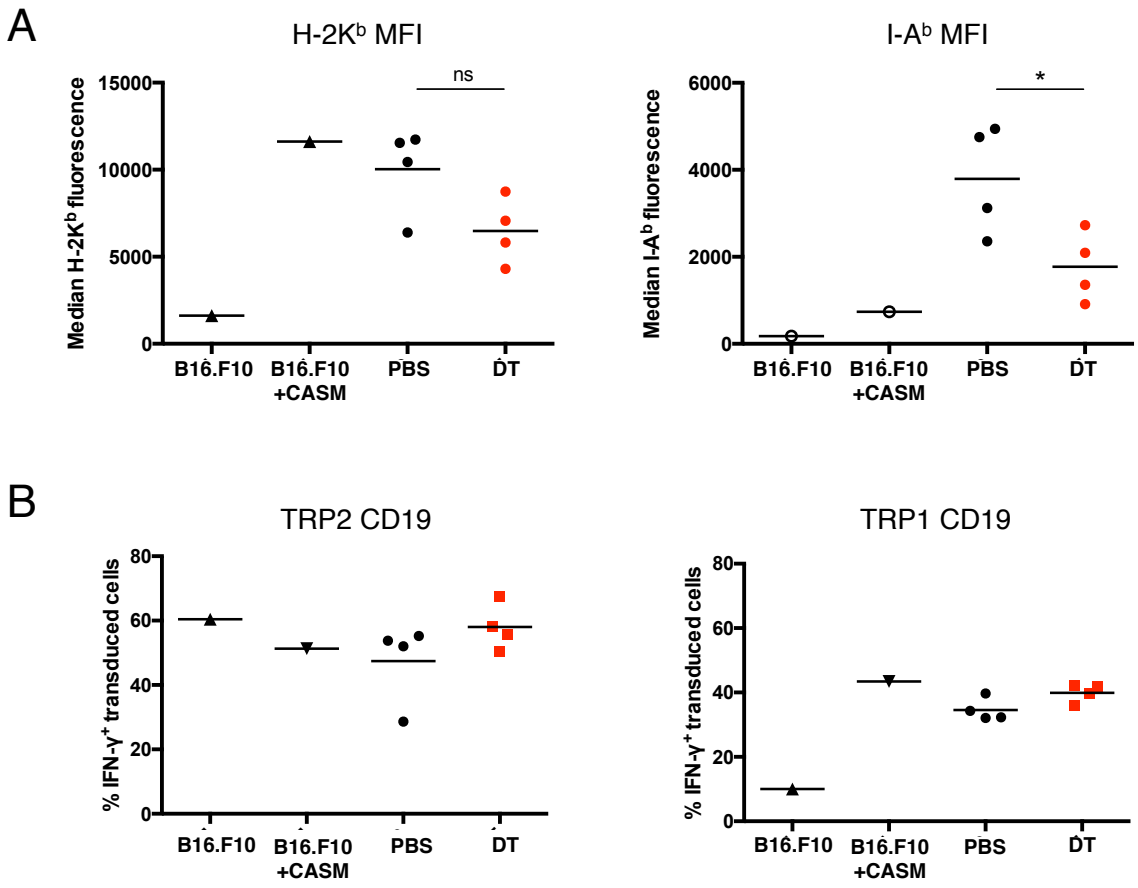


Figure 5.7: *Decreased MHC expression on tumours of DT treated mice.*
(A) Summary data of MHCI (H-2K^b) and MHCII (I-A^b) expression on *ex vivo* isolated B16.F10 tumours after injection of DT or PBS.
(B) Summary data of IFN-γ^+ TRP2 or TRP1 transduced T cells after overnight culture with *ex vivo* B16.F10. Cells were pre-gated on CD8$^+$ and CD19$^+$ lymphocytes.
Data is from 4 mice per group from 1 experiment. Statistical test: unpaired T test. n.s: non significant, *: $p \leq 0.05$.

5.6 Establishment of the CD11c.DOG DC depletion model

Although it is a very effective tool for studying DC, the CD11c.DTR model is limited by aberrant DTR expression on non-haematopoetic tissue (Zaft et al. 2005). Therefore the CD11c.DOG model, in which mice are able to be injected with DT multiple times without toxicity, was also established. Whereas in CD11c.DTR mice, the DTR transgene was placed under the control of the minimal CD11c promotor, CD11c.DOG mice were created using BAC transfer. This allowed transfer of the whole CD11c gene, including the associated enhancer and silencer elements, into which the DTR transgene was inserted (Fig 5.8A). This results in DTR expression being under the control of the CD11c locus control region and a much tighter restriction of DTR expression to $CD11c^+$ cells. As a result CD11c.DOG can be injected repeatedly and with much higher doses of DT without toxicity (Hochweller et al. 2008). The CD11c.DOG transgene also contains the model ovalbumin antigen (OVA) and GFP, although the GFP does not give a fluorescent signal in these mice.

To test whether DC were depleted in the spleen and tumours of CD11c.DOG mice after repeated DT injections, tumour bearing CD11c.DOG mice were injected with DT or PBS on days 6, 9 and 12 and sacrificed on day 13 (Fig 5.8B). Mice were injected with between 5-64 ng DT/g body weight (approximately 100-1200ng). As expected in all DT doses used, substantial depletion of splenic DC was noted (Fig 5.8C) comparable to that seen in CD11c.DTR mice. However although the highest dose of DT effectively depleted DC it was associated with increased weight loss in mice (Fig 5.8D). Whilst these mice did not lose enough weight to be sacrificed according to home office regulations, this may be a concern with longer experiments.

As well as depletion in the spleen, depletion of DC in the tumours was also measured. DC were identified as demonstrated previously (Fig 5.2B), although CD64 was used to identify macrophages rather than F4/80. Whereas DC

depletion in the CD11c.DTR mice primarily affected the $CD24^+$ DC population, in the CD11c.DOG model we see depletion of the broader $CD11c/MHCII^+$ APC population. Whilst this depletion occurs with a similar efficiency in mice injected with 16, 32 and 64 ng of DT/g, no depletion was seen with the lowest dose (<8ng/g). Of the remaining, undepleted $CD11c/MHCII^+$ cells, the frequency of $CD24^+$ DC remains broadly similar to PBS injected mice (Fig 5.9C). Therefore $CD24^+$ DC are not specifically targeted although they will still have a reduced frequency in DT treated mice because of the depletion of the parental $CD11c/MHCII^+$ population. Moreover whilst the ratio of $CD103:CD11b^+$ DC does slightly decrease after depletion, this is not significant like in the CD11c.DTR model (Fig 5.9D).

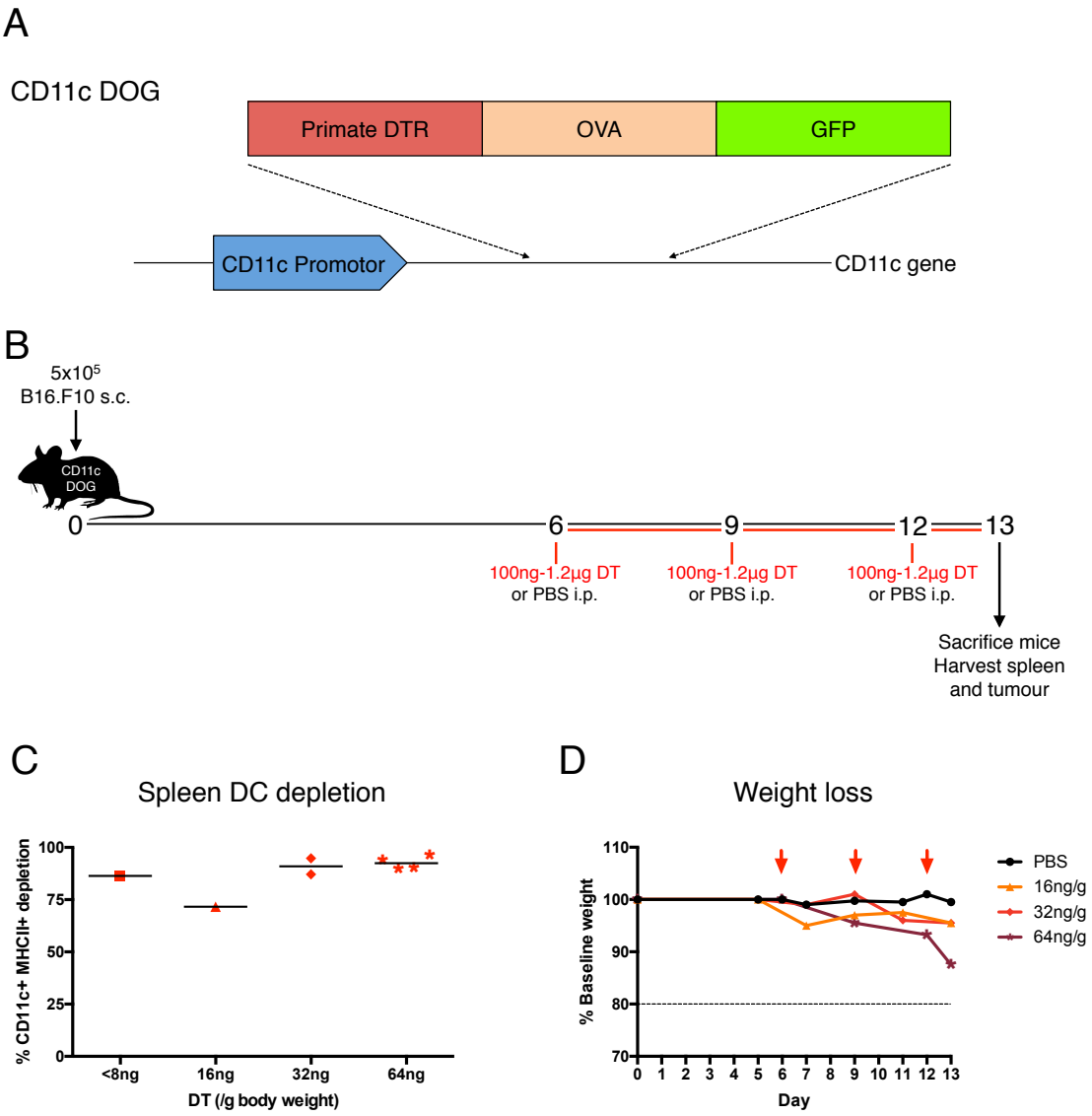


Figure 5.8: *DC depletion in the spleens of CD11c.DOG mice.*
(A) Schematic representation of the DTR/OVA/GFP transgene.
(B) Experiment outline for repeated DT injections in CD11c.DOG mice. Mice were injected with 100, 320, 640 or 1280ng of DT, relating to <8, 16, 32 and 64 ng/g body weight respectively.
(C) Depletion of DC in the spleens of CD11c.DOG mice. DC were gated on live, $CD45^+$ cells, then identified as DC by CD11c and MHCII expression.
(D) Weight loss in mice injected with PBS or DT.
Data is from 1-4 mice per group from 1 experiment. n= (PBS: 4, 8ng: 1, 16ng: 1, 32ng: 2, 64ng: 4).

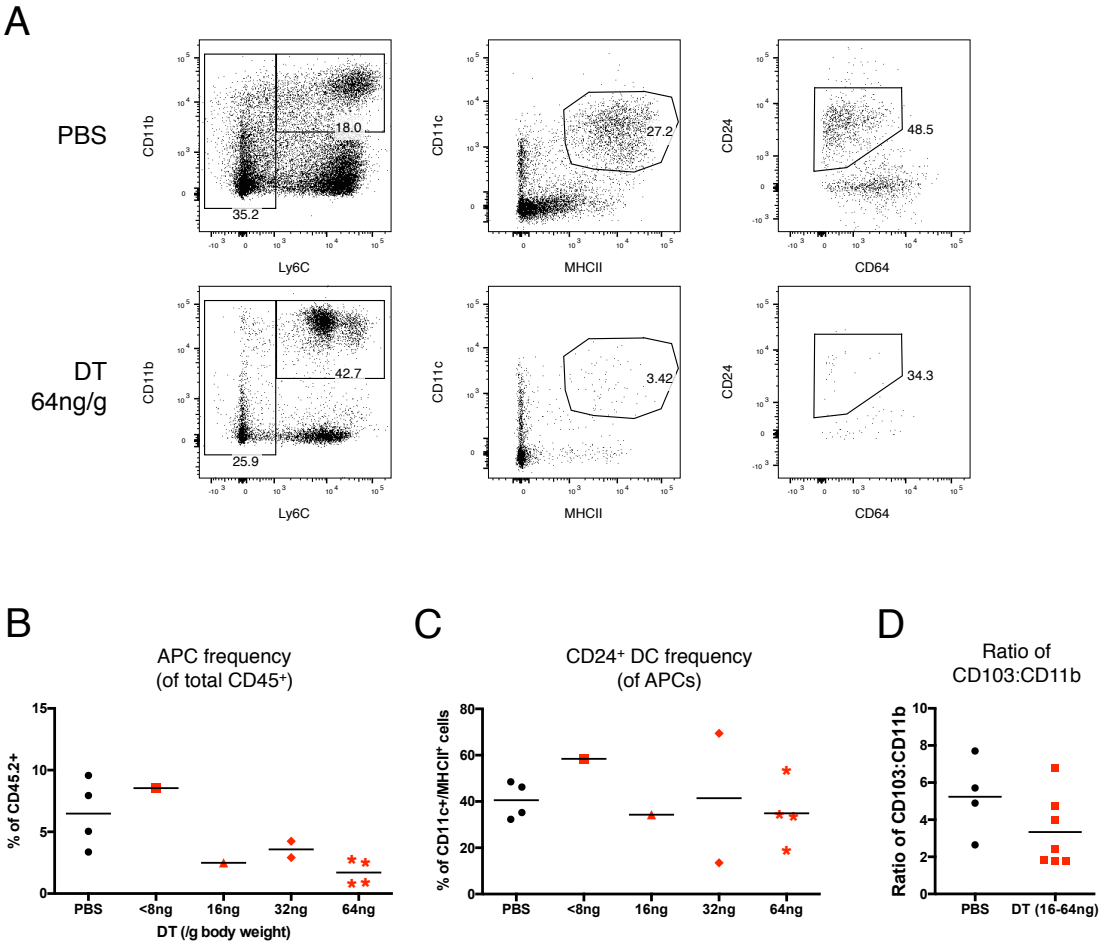


Figure 5.9: *DC depletion in the tumours of CD11c.DOG mice.*
(A) Representative FACS plots for depletion of DC in B16.F10 tumours. Cells were pre-gated on live, $CD45^+$ cells. $Ly6C^-$ cells were then gated on expression of CD11c and MHCII, then CD24 and CD64.
(B) Summary data for depletion of total $CD11c/MHCII^+$ APCs or $CD24^+$ DCs from the total $CD45^+$ population.

5.7 Summary and conclusion

We have established two models in which DC can be conditionally depleted; the CD11c.DTR and CD11c.DOG models. In these models tumours will develop normally in a DC replete setting, from which DC can then be transiently depleted. This will allow the interactions these cells have with transferred T cells to be investigated. As expected, DC were depleted in the spleens of CD11c.DTR and CD11c.DOG mice to similar extents, however the efficacy of DC depletion from the tumour varied. Depletion of tumour-resident DC in CD11c.DTR mice was specific to $CD24^+$ DC, particularly the $CD103^+$ population. In contrast CD11c.DOG mice show depletion of the broader $CD11c/MHCII^+$ cells. The reason for the difference in these depletion patterns is unclear, though it may reflect the different regulation of DTR expression on $CD11c^+$ cells between the two models. For example in CD11c.DOG mice, DTR expression is more tightly restricted to expression of the CD11c gene, due to the inclusions of the associated enhancers and silencers. This results in less toxicity after DT injection, which allows for a higher dose of DT to be injected. It appears however that this better association with CD11c expression also broadens the specificity of DTR expression. Whereas DC depletion in CD11c.DTR mice is focused on $CD11c^{high}$ cells, depletion in the CD11c.DOG model occurs in a wider range of $CD11c^+$ cells. Therefore this may allow depletion of tumour-resident $CD11c^{int}$ cells not depleted in the CD11c.DTR model. Depletion of DC in the spleen however is consistent across both models as splenic DC express consistently high CD11c and so are well depleted in both settings. Likewise as shown by the GFP expression, endogenous CD11c expression and expression of the DTR transgene are not necessarily linked in some cells in the CD11c.DTR mice. The tighter restriction of DTR expression to $CD11c^+$ cells in the CD11c.DOG model may result in a more equal expression CD11c and DTR. Unfortunately the GFP is not functional in the CD11c.DOG mice so more detailed experiments would be needed to accurately assess the differences in DTR expression between these mice and how that affects depletion of tumour-resident DC.

These data do suggest however that there is a ongoing endogenous immune response against the tumours of these mice. NK and T cell infiltration into the tumours was easily detectible and CD11c expression on these cells was higher in the tumour than lymphoid organs. CD11c expression is thought be expressed on T cells (Lin et al. 2003) and NK cells (Aranami et al. 2006) after activation in inflammatory settings. One obvious caveat with both these depletion models then is that DTR expression is not strictly limited to DC, and can be expressed on other $CD11c^+$ cells. We found that activated T cells and, to a lesser extent NK cells, were susceptible to depletion after DT injection in CD11c.DTR mice. This effect was primarily noticeable in the tumour, although LN and splenic CD4 T cells were also depleted. Although depletion of DTR expressing NK and T cells is the likely explanation for the reduced frequency of these cells in tumours, it remains plausible that this could instead be explained by DC depletion directly. DC have important roles in inducing activation, expansion and effector function of NK cells (Cooper et al. 2004) and T cells (Steinman 2007). The lack of $CD11c^+$ NK or T cells in the tumour might therefore be attributable to the depletion of DC, rather than the direct depletion of these cells. Either way the reduced frequency of T cells in the tumour following depletion could complicate experiments designed to investigate the role of DC in tumour-specific T cell responses. Of note however is that although DT injection can deplete endogenous T cells in CD11c.DTR mice, adoptively transferred T cells can be used from a BL/6 origin, and so won't express the high affinity DTR.

Like transferred T cells, the injected B16.F10 tumours did not originate from CD11c.DTR mice so they will not be depleted by DT injection. However the reduction in $CD11c^+$ cells in the tumour microenvironment does appear to reduce MHC expression on the tumour. This is likely caused by depletion of activated T cells, thereby limiting the expression of IFN-γ and reducing MHC up-regulation. The subsequent effect this MHC loss has on the ability of the immune response to recognise the tumour *in vivo* is unclear, although *ex vivo* B16.F10 tumours from DT treated mice, cultured with transduced T cells were able to present as well as, if not better than, tumours from PBS treated mice. This could be explained by the previously demonstrated IFN-γ mediated

upregulation of PD-L1 on tumours (Fig 3.4C). In this setting, diminished PD-L1 expression (mediated by reduced IFN-γ *in vivo*) may counteract the reduced MHC expression.

This endogenous immune response against the tumour is also suggested by the increase in tumour-resident DC as the tumour grows. However by day 21 when tumours are fully established DC frequency appears to decrease, whilst the remaining DC express more PD-L1. This may reflect the immuno-editing aspect of tumour growth as the tumour eventually becomes adept at subverting or avoiding the immune response. However the effect of DC depletion on this PD-L1-expressing DC population was not fully examined in these experiments.

Whilst the effects of DC depletion have been well examined in CD11c.DTR mice in this study, they have not been addressed in CD11c.DOG to the same extent. As DTR expression is more restricted in these mice, one might predict that T cells and NK cells are not as affected as in CD11c.DTR mice. Although conversely this allows for injection of higher-doses of DT which may enhance depletion, or allow for depletion of $CD11c^{int}$ cells. Moreover if the reduced frequency of NK and T cells is mediated more by depletion of DC themselves, then this should be similar in both models. These experiments would need to be repeated in the CD11c.DOG mice to determine these effects.

However these data have shown that we are capable of depleting DC from the spleens and tumours of B16.F10 tumour bearing mice. This detailed characterisation of the DC depletion models, together with the tumour models established in chapters 3 and 4 will allow us to examine the role of DC after the adoptive transfer of TCR transduced T cells.

Chapter 6 Investigating the effect of DC depletion on TCR transduced T cells *in vivo*

With the CD11c.DTR and CD11c.DOG models established, we could then investigate the ability of transduced T cells to control tumour growth with or without DC depletion. DC play a fundamental role in orchestrating T cell responses by priming naive T cells and by regulating effector T cell responses in the tumour and tissues. However in order to transduce adoptively transferred T cells with retrovirus, the T cells have to be activated *in vitro*. It is therefore unclear whether the effector function of pre-activated, transduced T cells depends on interaction with APC in the same way an endogenous T cell response would. We hypothesised that depletion of DC would lead to an inferior response by adoptively transferred T cells.

This chapter therefore aims to address these questions by using the CD11c.DTR model to elucidate the effect of DC depletion on tumour-specific TCR transduced T cells. We chose initially to use the B16.F10 model in combination with TRP2 transduced T cells as we and others have shown that TRP2 transduced T cells are capable of inducing transient tumour control. For the DC depletion we chose to deplete DC 1 day before T cells were injected, and this was then maintained for a further 8 days after T cell transfer. This was designed to assess the effect of DC depletion during the time the transferred T cells would normally mediate tumour control. However this would require repeated DT injections every 3 days, so CD11c.DTR BM chimeras were made to prevent the toxicity associated with repeated injections in CD11c.DTR mice. However in this model we chose to forgo the pre-conditioning irradiation before T cell transfer. Whilst this may limit the ability of the transduced T cells to control tumour growth, it will exclude the role of DC in the homeostatic expansion of transferred T cells during lymphopenia (Zaft et al. 2005). Moreover, an additional stage of pre-conditioning irradiation

in BM chimeras may be poorly tolerated, especially after DT injection. These experiments were therefore primarily focused on the effect of DC depletion on the expansion rather than tumour control, of TRP2 transduced T cells *in vivo*.

6.1 Transferred T cells accumulate in CD11c.DTR mice despite DC depletion

To address the effect of DC depletion on the ability of TRP2 transduced T cells to expand *in vivo*, the experiment as outlined in Fig 6.1A was performed. Bone marrow chimeras were made by transferring CD11c.DTR bone marrow into C57BL/6 recipients. After mice had reconstituted they were injected with B16.F10 tumour cells. 7 days later, mice were treated with a total of $4x10^6$ CD8 sorted splenocytes which congenically expressed Thy1.1 to allow easy tracking of transferred cells into Thy1.2 recipients. These cells were transduced with TRP2 CD19 and injected into mice 1 day after transduction. Remaining cells were cultured *in vitro* to assess transduction efficiency. Transduced cells were 93% CD8 and Thy1.1$^+$, and had an initial transduction efficiency of 17%, although this rose to 31% by day 3 post transduction (Fig 6.1B). As a total of $4x10^6$ Thy1.1$^+$ cells were injected, the total number of TRP2 transduced cells injected per mouse was ~$1.2x10^6$. Mice were injected with DT or PBS on days 6, 9 and 12 to deplete DC. Therefore in this setting DC would be depleted during the first week after T cell transfer, but then return to homeostatic levels by day 15. Control mice were routinely taken on day 13 to confirm DC depletion. Although injection of DT into mice induced relatively modest weight loss (<10%), this was not sufficient to necessitate their sacrifice (Fig 6.1C).

The ability of TRP2 transduced T cells to control B16.F10 growth did not appear to be different in either PBS or DT treated mice (Fig 6.1D). However in both settings this control was very limited and it appeared that neither group were able to control tumour growth. However in the absence of pre-conditioning irradiation this lack of control was not unexpected.

However as tumours grew to >15mm by days 18-25, mice were sacrificed. Tumours were harvested, alongside spleens and tumour-draining inguinal LN, and stained to identify the frequency of transferred T cells (Fig 6.2A). Transferred T cells could easily be identified from within the CD8 population due to

expression of the congenic marker Thy1.1. Despite DC depletion the frequency of total $CD8^+$ cells in the tumour and LN was similar between PBS and DT treated mice, however there did appear to be fewer CD8 T cells in the spleens of DT treated mice (Fig 6.2B), although this was not significant. However contrary to our initial hypothesis, the frequency of transferred ($Thy1.1^+$) T cells in this $CD8^+$ population was greater after DC depletion in all organs, albeit only significantly different in the spleen. As this population of transferred $Thy1.1^+$ T cells contains both transduced and untransduced T cells, the frequency of the transduced, $CD19^+$ Thy1.1 population was also measured. Like the total Thy1.1 population, the frequency of transduced T cells showed a similar trend towards greater accumulation after DC depletion. This suggests that this accumulation of transferred T cells occurred in both the transduced and untransduced population and was not dependent on whether the T cells expressed an anti-tumour TCR or not.

A potential explanation for this increased frequency of introduced T cells is that if endogenous T cells were being depleted from within the CD8 population, this could skew the percentages in favour of the transferred T cells. However the frequency of the endogenous ($Thy1.1^-$) CD8 T cells after depletion mirrored what was seen in the total CD8 population. There was a trend towards decreased frequency in the spleen, but no difference in the LN and tumour (Fig 6.3B). This may explain why the increase in transferred T cell frequency is the most prominent in the spleen, due to a reduction in the endogenous T cells from this population.

However to address this, the absolute numbers of transferred T cells were also calculated (Fig 6.3B). Surprisingly, the absolute number of endogenous CD8 T cells were lower in spleen, LN and tumour after DC depletion, although this was not significant. Whilst this may be expected in the spleen, the frequencies of endogenous CD8 T cells in the LN and tumour were the same after DC depletion. This suggests than in the LN and tumour particularly, there is an overall loss of cellularity after DC depletion. However in contrast to the endogenous CD8 T cells, the transferred $Thy1.1^+$ T cells appeared to accumulate in greater numbers in the

tumour and spleen, although not in the LN. However these differences were not significant in this experiment, so further repeats would be needed to confirm this. It is unclear why the response from T cells in the LN differs from those in the spleen or tumour, however in comparison very few T cells were collected from the LN which could affect the quality of this data.

As the transferred cells seemed to be accumulating to a greater extent after DC depletion, the differentiation status of these cells was also assessed. We characterised differentiation based on the expression of CD62L and CD127 (naive: $CD62L^+$ $CD127^+$, memory: $CD62L^-$ $CD127^+$ or effector: $CD62L^-$ $CD127^-$, as shown in Fig 6.2A). As expected there was a selective expansion of effector phenotype T cells in the tumour compared to the spleen or LN, however in all organs the differentiation status of the endogenous CD8 T cells was similar between PBS and DT treated mice (Fig 6.4). Likewise the phenotype of transduced T cells was similar between PBS and DT treated mice in the LN and tumour, but not in the spleen. After DT injection, transduced T cells in the spleen acquired a significantly less naive, more effector phenotype. It is unlikely that this is a systemic effect following DT injection, as it occurred only in the transferred T cell population, not in endogenous T cells. Moreover this enhanced effector phenotype presented in the spleen, where the greatest enhancement in the accumulation of transferred T cells was seen (Fig 6.2/6.3). This suggests that the acquisition of an effector phenotype is linked to the greater accumulation of transferred T cells.

Taken together, these data suggest that, whilst DC depletion limits endogenous T cell accumulation, it does not inhibit the transferred T cells. Indeed in the spleen and tumour, DC depletion may even enhance the accumulation of transferred T cells. However the majority of these differences are not statistically significant. Repeated experiments with more mice per group would generate more convincing data. However as shown by this and previous experiments, depletion of endogenous T cells, particularly in the tumour, may have a confounding effect on these experiments. Although the apparent increase in absolute numbers of transferred T cells suggests that the increase in Thy1.1

frequency isn't as a result of skewed CD8 frequencies following endogenous T cell depletion, there is an alternative explanation. Expansion of transferred T cells could be mediated by reduced competition for cytokines following endogenous T cell depletion. To address these concerns we chose to repeat these experiments in a DC depletion model that would remove the potential to deplete endogenous T cells. These experiments are discussed in more detail in the next section.

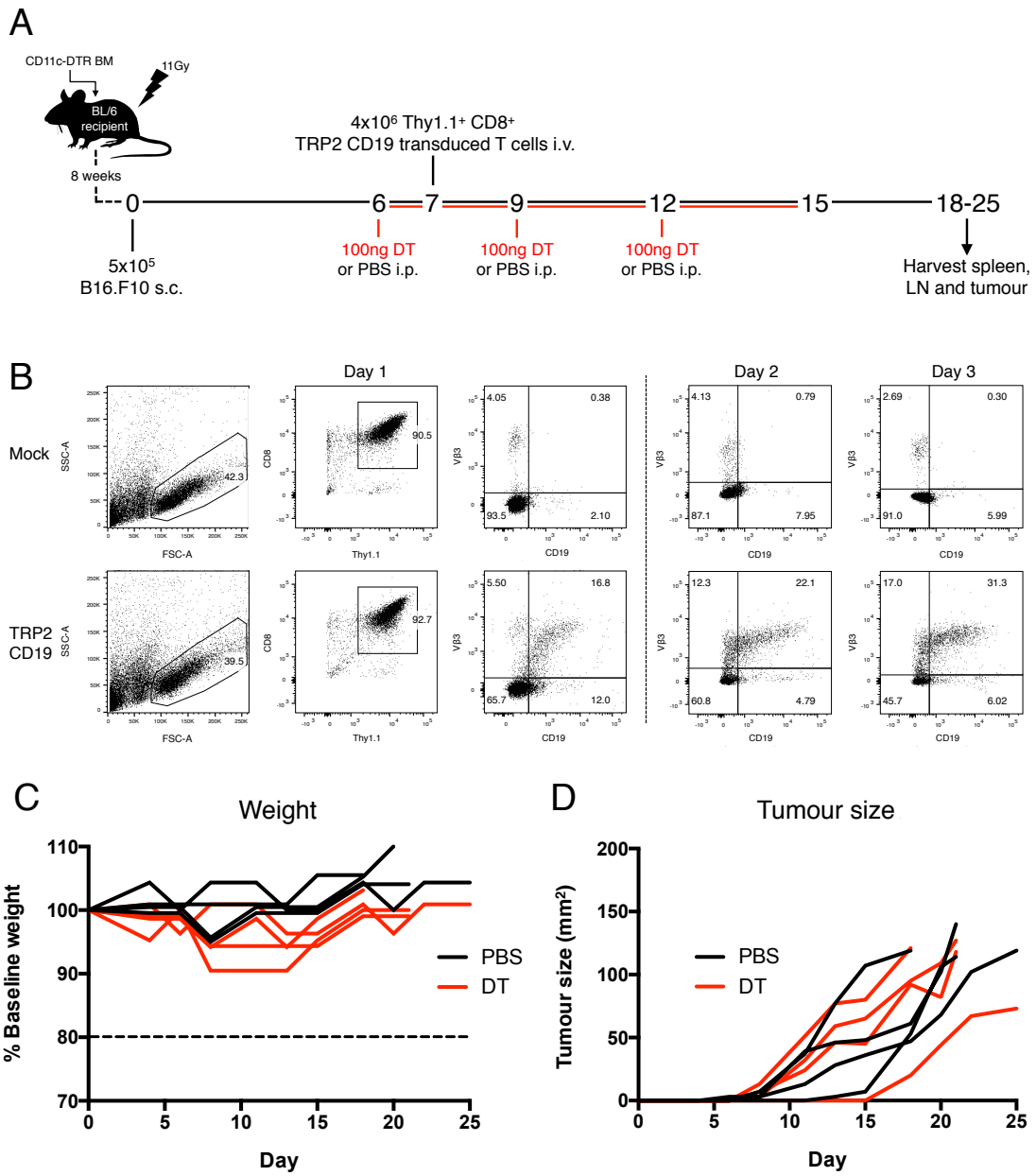


Figure 6.1: *Adoptive transfer of TCR transduced T cells in DC depleted mice.*
(A) Experiment outline. CD11c.DTR bone marrow chimeras were generated and once reconstituted, injected with B16.F10. Mice were treated with 4x10^6 Thy1.1$^+$ CD8$^+$ TRP2 transduced T cells. One day prior to T cell transfer (day 6) mice were injected with PBS or DT to depleted DC. DC depletion was maintained for 8 days after T cell injection by a further 2 DT injections, on days 9 and 12.
(B) FACS profile of CD8 sorted, TRP2 transduced T cells (as compared to mock transduced) on day 1, 2 and 3 after transduction.
(C) Change in weight after DT or PBS injection.
(D) Effect of DC depletion on B16.F10 tumour size in mice treated with TRP2 transduced T cells.

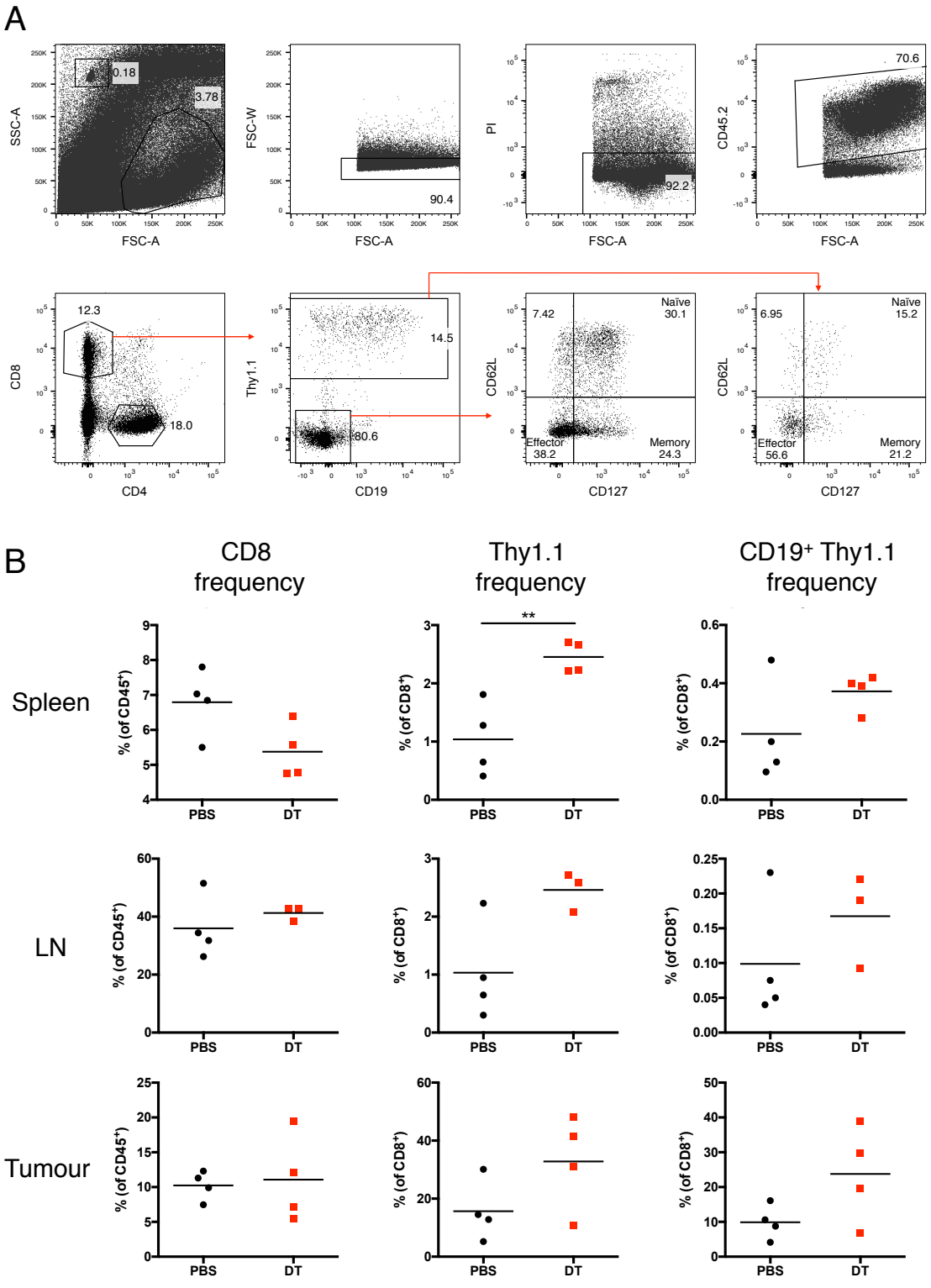


Figure 6.2: *DC depletion promotes enhanced frequency of transferred T cells.*
(A) Representative FACS plots for gating of tumour-resident, transferred T cells. Cells were gated on live, $CD45^+$ singlets. T cells were then identified by expression of CD8 and Thy1.1. Differentiation of T cells was measured by CD62L and CD127 expression. (B) Summary data shows frequency of $CD8^+$ cells from the total $CD45^+$ population, or the frequency of $Thy1.1^+$ or $CD19^+$ $Thy1.1^+$ cells from the $CD8^+$ population in spleen, LN and tumour. Data is from 4 mice per group, from 1 experiment. Statistical test: unpaired T test. **: $p \leq 0.01$.

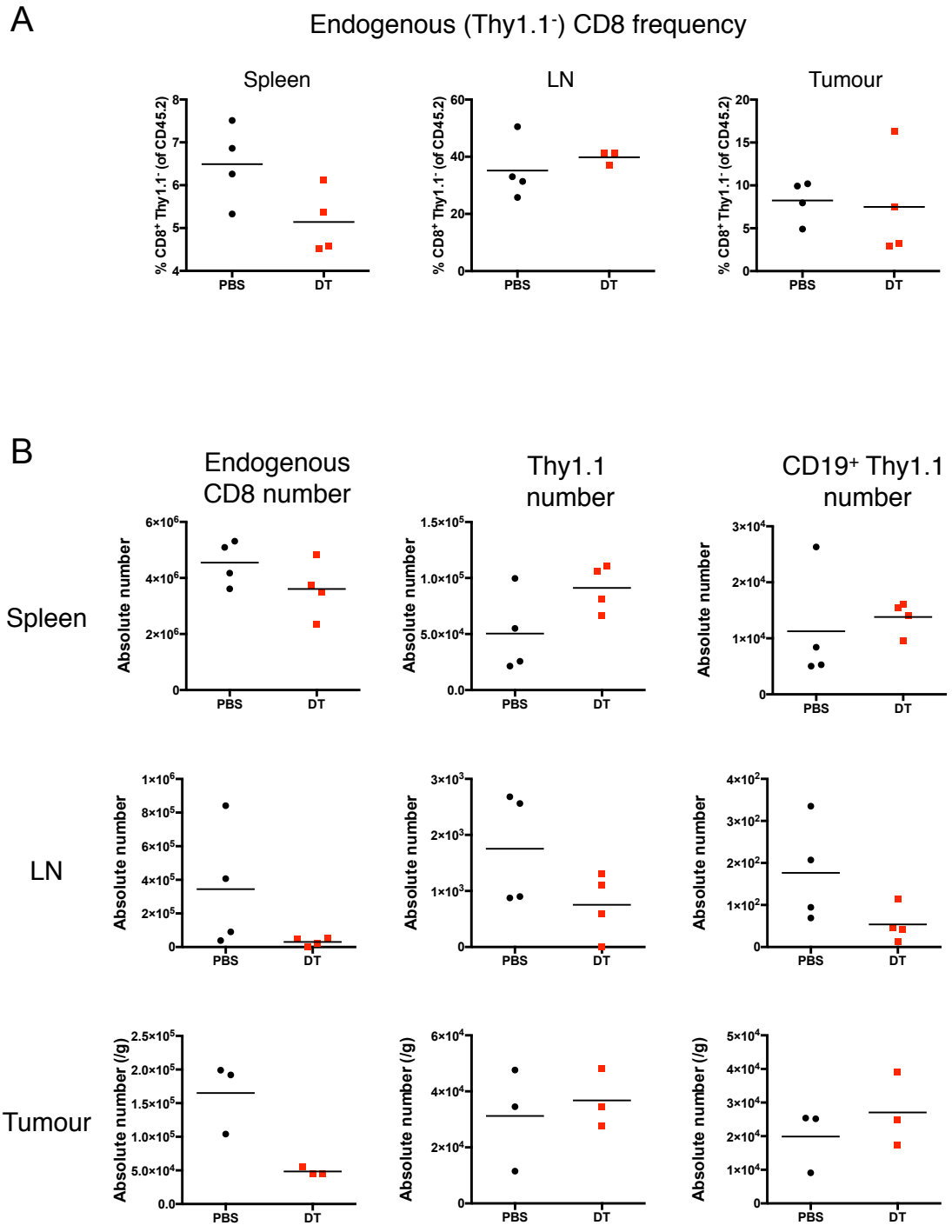


Figure 6.3: *DC depletion promotes accumulation of transferred T cells.*
(A) Summary data shows frequency of Thy1.1$^-$ CD8$^+$ cells from the total CD45$^+$ population in spleen, LN and tumour.
(B) Summary data of absolute numbers of endogenous CD8, transferred Thy1.1 or CD19$^+$ Thy1.1 cells in spleen, LN and tumour. Data is from 4 mice per group, from 1 experiment.

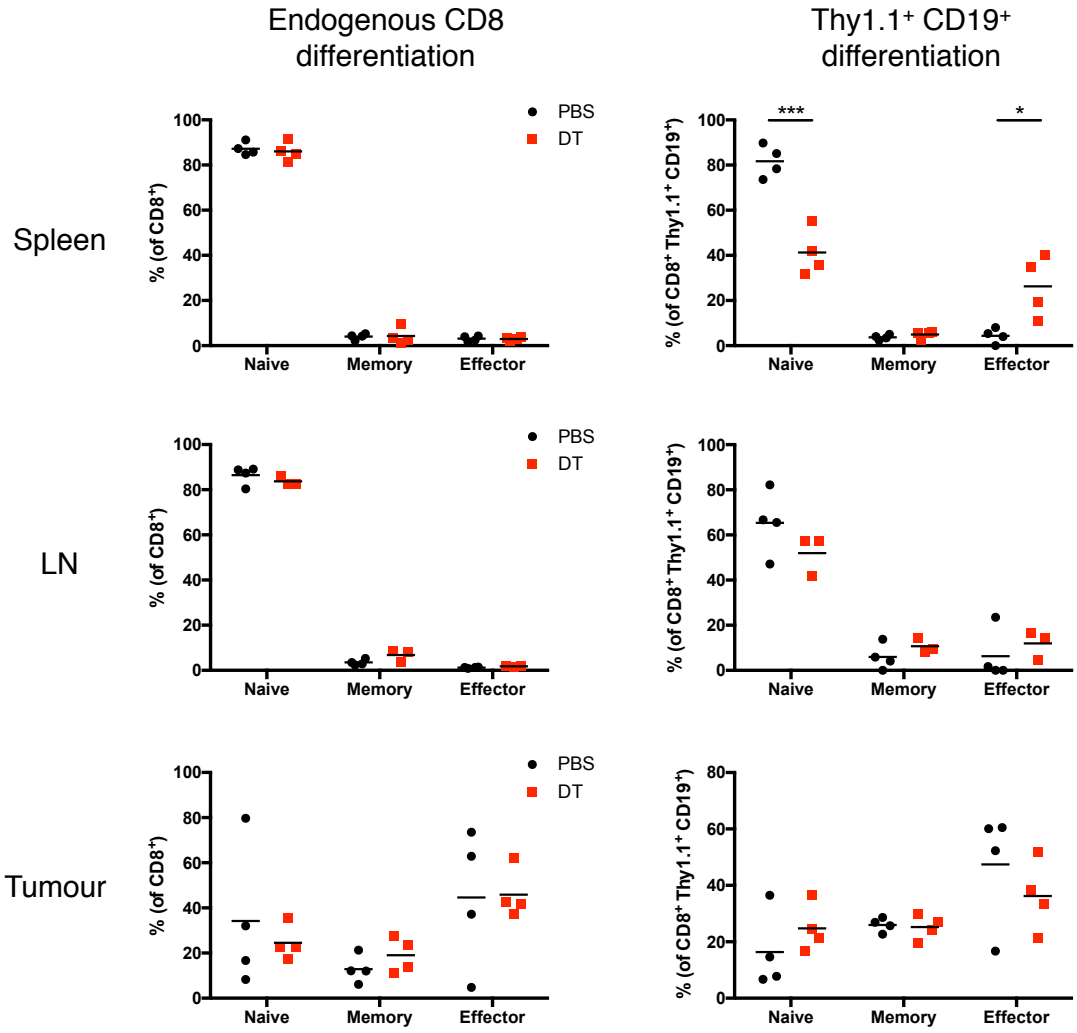


Figure 6.4: *T cell effector phenotype differentiation following DC depletion.*
Naive, memory or effector phenotype was determined by expression of CD62L and CD127 as demonstrated in Fig 6.2A. Data shows frequency of differentiated endogenous CD8 T cells, or transferred Thy1.1$^+$, CD19$^+$ T cells in the spleen, LN and tumour.
Data is from 4 mice per group, from 1 experiment. Statistical test: unpaired T test.
*: $p \leq 0.05$, ***: $p \leq 0.001$.

6.2 Depletion of DC leads to accumulation of transferred T cells in absence of endogenous T cell depletion

To determine the extent to which accumulation of transferred T cells was dependent on depletion of endogenous T cells, these experiments were repeated in a setting designed to minimise the effect of depletion of these cells (Fig 6.5A). To achieve this CD11c.DTR mice were crossed onto the RAG-$1^{-/-}$ background in which mice lack endogenous T cell expression. CD11c.DTR x RAG-$1^{-/-}$ bone marrow was used to generate bone marrow chimeras and repeat the experiment in Fig 6.1. The only T cells present in this setting are a small proportion of T cells that reconstitute from the BL/6 recipients and the transferred TRP2 transduced T cells, neither of which express the high affinity DTR. Whereas the DC compartment is reconstituted from the DTR expressing donor bone marrow and so is able to be depleted.

As before $4x10^6$ Thy1.1^+, $CD8^+$ T cells were injected into mice which had been injected with either DT or PBS. Transferred T cells were 91-94% CD8 and Thy1.1^+ (Fig 6.5B). Transduction efficiencies ranged from 35-56%, resulting in injection of 1.4-2.2$x10^6$ TRP2 transduced T cells (Fig 6.5C). To ensure that DT injection was depleting DC in these mice, control mice were sacrificed and 98-99% of splenic DC were depleted by day 13 (Fig 6.5D). Although transferred T cells may homeostatically expand in a T cell deplete setting, we again saw no evidence of tumour control by TRP2 CD19 transduced T cells, in both PBS or DT treated mice (Fig 6.5E.)

Despite an absence of T cells which were sensitive to DT in this model, the total frequency of CD8 T cells in the spleen was reduced after DT injection (Fig 6.6A). However the frequency of CD8 T cells in the LN and tumour remained similar. As before, specifically gating on the endogenous (Thy1.1^-) CD8 population showed a similar effect as the total CD8 (Fig 6.7A). This suggested that DC depletion, not depletion of endogenous T cells, was directly causing the decreased frequency

of CD8 T cells observed in the spleens of T cell replete (Fig 6.2B) and T cell depleted (Fig 6.6A) mice.

However although the frequency of T cells in the spleen was reduced after DC depletion, this was not true of the transferred fraction of these CD8 T cells. The frequency of Thy1.1$^+$ T cells in the spleen may even have increased following DC depletion, although this was not significant. LN resident T cells show a similarly mild and insignificant trend towards increased frequency of transferred T cells after depletion. Of note however was that this increase became significant in the tumour, where the mean frequency of transferred T cells increased from 20% to 33% after DC depletion. This appeared to be driven by, but not contained to, the transduced T cell fraction of the transferred cells. The mild (in LN and spleen) or significant (in tumour) increase of transferred T cells was enhanced in all organs by gating on the frequency of TRP2 expressing T cells (Vβ3$^+$ Thy1.1$^+$), rather than just the total transferred T cells (Thy1.1$^+$). In accordance with this, there were more transduced T cells in the total Thy1.1$^+$ population after DC depletion, although this was only significant in the spleen (Fig 6.6B).

To assess whether the changes in frequency of endogenous and transferred T cells were reflected in changes in the numbers of these cells, absolute numbers were recorded (Fig 6.7B). However although there was a reduced endogenous CD8 frequency in the spleen, the was no difference in absolute numbers. This was also true of the LN and tumour. This suggested that, as intended, endogenous T cells were not depleted by DT injection in this model. The reduced CD8 frequency (but not number) seen in the spleen after DC depletion may therefore be explained by a relative increase in other cell populations. Although this was not tested directly in these experiments, this could be as a result of neutrophilia or monocytosis that is associated with DC depletion in the spleen.

As before, depletion of DC in this model did not impair the accumulation of transferred T cells in spleen, LN or tumour, which remained relatively similar in PBS or DT treated mice. However as the frequency of Thy1.1$^+$ cells in the tumour were increased after DT depletion, it is surprising that this is not reflected

in the absolute numbers. This is particularly true as the stable numbers of endogenous T cells suggests this increase was not an effect of altered frequencies. However the numbers of transferred T cells show much greater variability than the frequencies, so it is possible this subtle difference is not seen as a noticeable change in the numbers.

Lastly, the differentiation status of endogenous or transferred T cells was also assessed after DC depletion (Fig 6.8). As in the T cell replete setting (Fig 6.4), DC depletion did not effect differentiation of the endogenous CD8 T cells in the spleen. However within the transferred population, the frequency of effector phenotype cells in the spleen was significantly higher following DC depletion. This appears to be at the expense of naive T cells which are less frequent after depletion, whereas memory cells remained constant. Moreover, this acquisition of an effector phenotype following DC depletion is more striking when gated on the transferred Vβ11 Thy1.1$^+$ T cells, rather than total Thy1.1$^+$ T cells.

Within the tumour a similar pattern emerges to the spleen. However here we do see a difference in the endogenous T cells, which see an enhanced memory formation after DC depletion. In the tumour the Thy1.1$^+$ population is nearly entirely composed of TCR transduced (Vβ3$^+$) T cells, so comparison between untransduced and transduced transferred cells is meaningless. However, like in the spleen, there were significantly more effector phenotype, tumour resident transferred T cells following DC depletion, again at the expense of the naive T cells (Fig 6.8).

These data show that although transferred T cells accumulated to a similar extent in the LN, spleen and tumours of mice in which DC were depleted, of the total CD8 T cells that infiltrated the tumour, a greater proportion of these were the transferred T cells. This may also be true of transferred T cells in the spleen and LN although this was unclear. Furthermore, in the spleen and possibly LN, after depletion there was a selective expansion of transferred T cells which expressed the anti-tumour TCR. This suggests that the accumulation of transduced T cells could have be driven by exposure to antigen *in vivo*. These data also suggest that the differentiation of transferred T cells was controlled by

DC *in vivo*, as transferred T cells acquired a more effector, less naive phenotype in the spleen and tumour after DC depletion. This effect was more prominent in TRP2 TCR expressing cells, but also affects activated but untransduced, transferred T cells. However it appears to be dependant on pre-activation *in vitro* as the endogenous T cell population was largely unaffected by DC depletion. Lastly, combined these data confirm that this accumulation of effector T cells occurred regardless of whether the endogenous T cells were present or not.

However the enhanced T cell accumulation shown in these experiments could have occurred as an indirect effect of DC depletion. Although DC were depleted for the first week after T cell transfer, this depletion was only transient. In our model DC will have returned to homeostatic levels by day 15, and would be able to engage with the transferred T cells for a further 5 days before mice were sacrificed. It is therefore feasible that this DC population that reconstitutes the depleted niche has a different phenotype from those DC that remained undepleted in PBS treated mice. This different population of reconstituted DC could therefore be driving the expansion of the transferred T cells.

To address whether reconstituted DC were enhancing the T cell accumulation seen previously, the frequency of the DC populations in the tumour was examined on day 20. Cells were gated as shown previously (Fig 5.2B), to identify monocytes/neutrophils, APCs and CD11b or $CD103^+$ $CD24^+$ DC. As expected, by this time point there was no evidence of depletion in either the frequency or absolute number of tumour-resident DC (Fig 6.9). Interestingly the $CD24^+$ $CD103^+$ DC population which was predominantly depleted in the tumour following DT injection, repopulated the tumour to a greater degree than non-depleted mice. Although these DC were found in a significantly higher frequency after reconstitution, this was only mildly reflected in the absolute numbers, which showed a similar trend but with more variability.

The $CD103^+$ DC have previously been shown to be crucial to cross-presenting tumour antigen and inducing T cell responses in the tumour (Broz et al. 2014; Salmon et al. 2016). These data therefore support the hypothesis that the

enhanced T cell accumulation in these experiments is driven by the reconstitution of DC after depletion. The ability of these different tumour-resident DC populations to present tumour antigen to transduced T cells was therefore assessed in the next section.

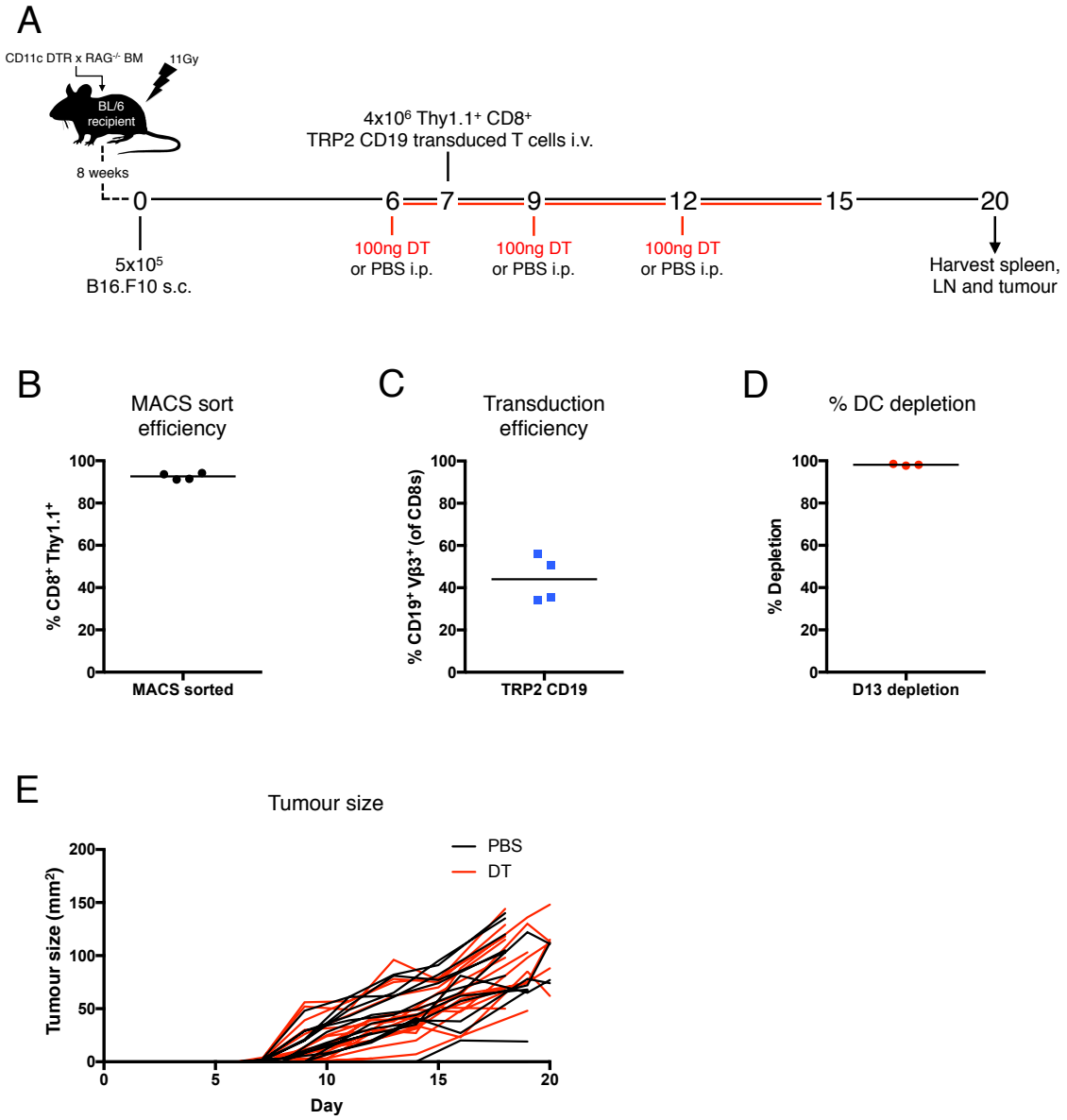


Figure 6.5: *Adoptive transfer of transduced T cells into CD11c.DTR x RAG-1$^{-/-}$ mice.*
(A) Experiment outline. Repeat of Fig 6.1 except irradiated BL/6 mice were reconstituted with CD11c.DTR x RAG-1$^{-/-}$ bone marrow.
(B) MACS sort efficiency. Data shows percentage of CD8^{+}, Thy1.1^{+} lymphocytes.
(C) Transduction efficiency of TRP2 CD19 transduced T cells on day 3-4 post-transduction. Data shows the percentage of Vβ13^{+}, CD19^{+} CD8 T cells. Data is from independent 4 experiments.
(D) Mice were sacrificed on day 13 to check depletion of DC in spleen. Data shows percentage depletion of splenic CD11c^{+}, GFP^{+} cells in DT treated mice, compared to PBS. Data is from 3 experiments.
(E) Tumour size in mice treated with TRP2 transduced T cells and DT or PBS. Data is from a total of 15-19 mice per group, from 4 independent experiments.

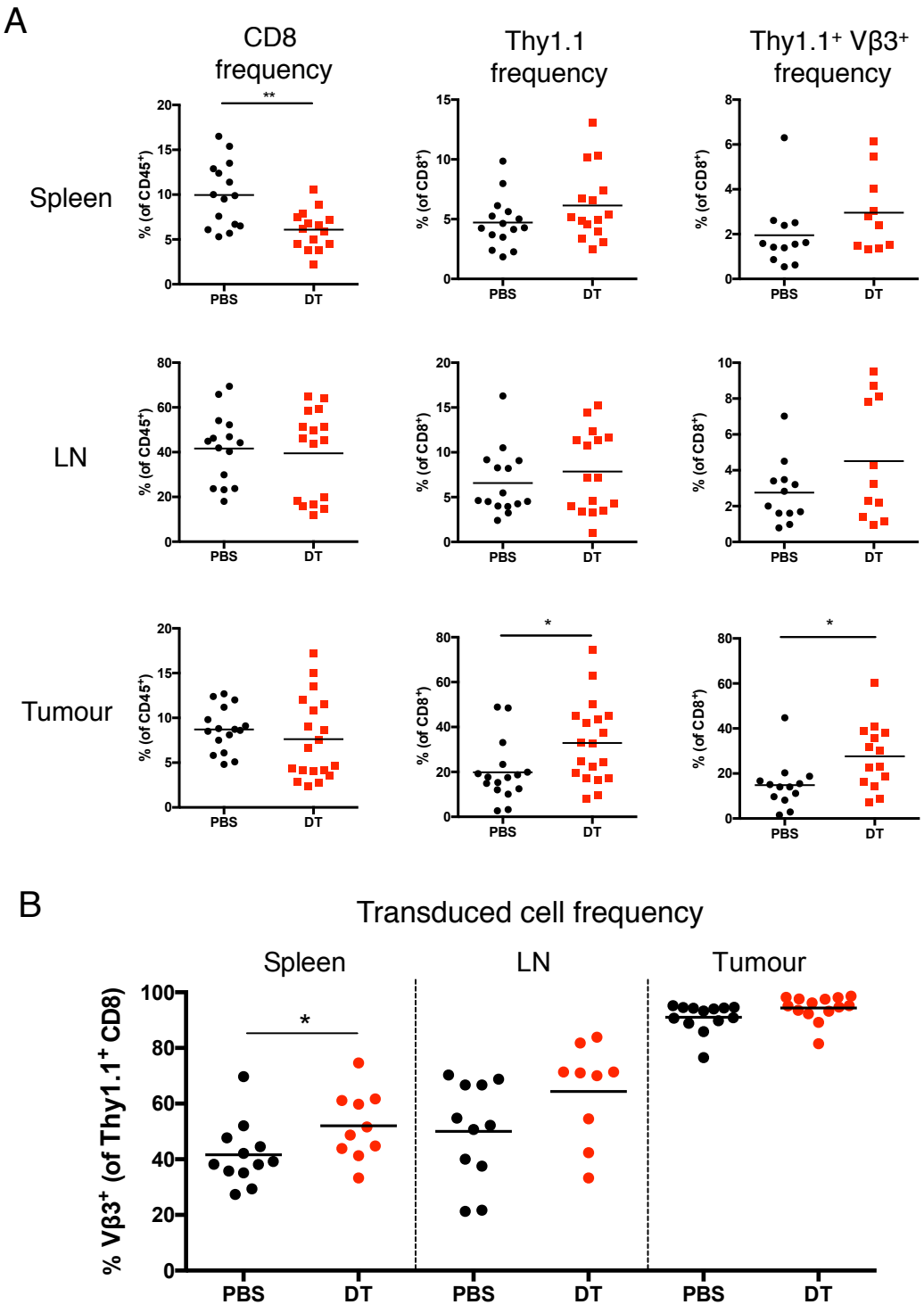


Figure 6.6: *DC depletion promotes enhanced frequency of transferred T cells in absence of endogenous T cell depletion.*
(A) Summary data shows frequency of $CD8^+$ cells from the total $CD45^+$ population, or frequency of Thy1.1 or $V\beta3^+$ Thy1.1 cells from the $CD8^+$ population in spleen, LN and tumour.
(B) Frequency of transduced ($V\beta3^+$) T cells from the $CD8^+$, $Thy1.1^+$ population.
Data is from a total of 15-19 mice per group, from 4 independent experiments. Statistical test: unpaired T test. *: $p \leq 0.05$, **: $p \leq 0.01$.

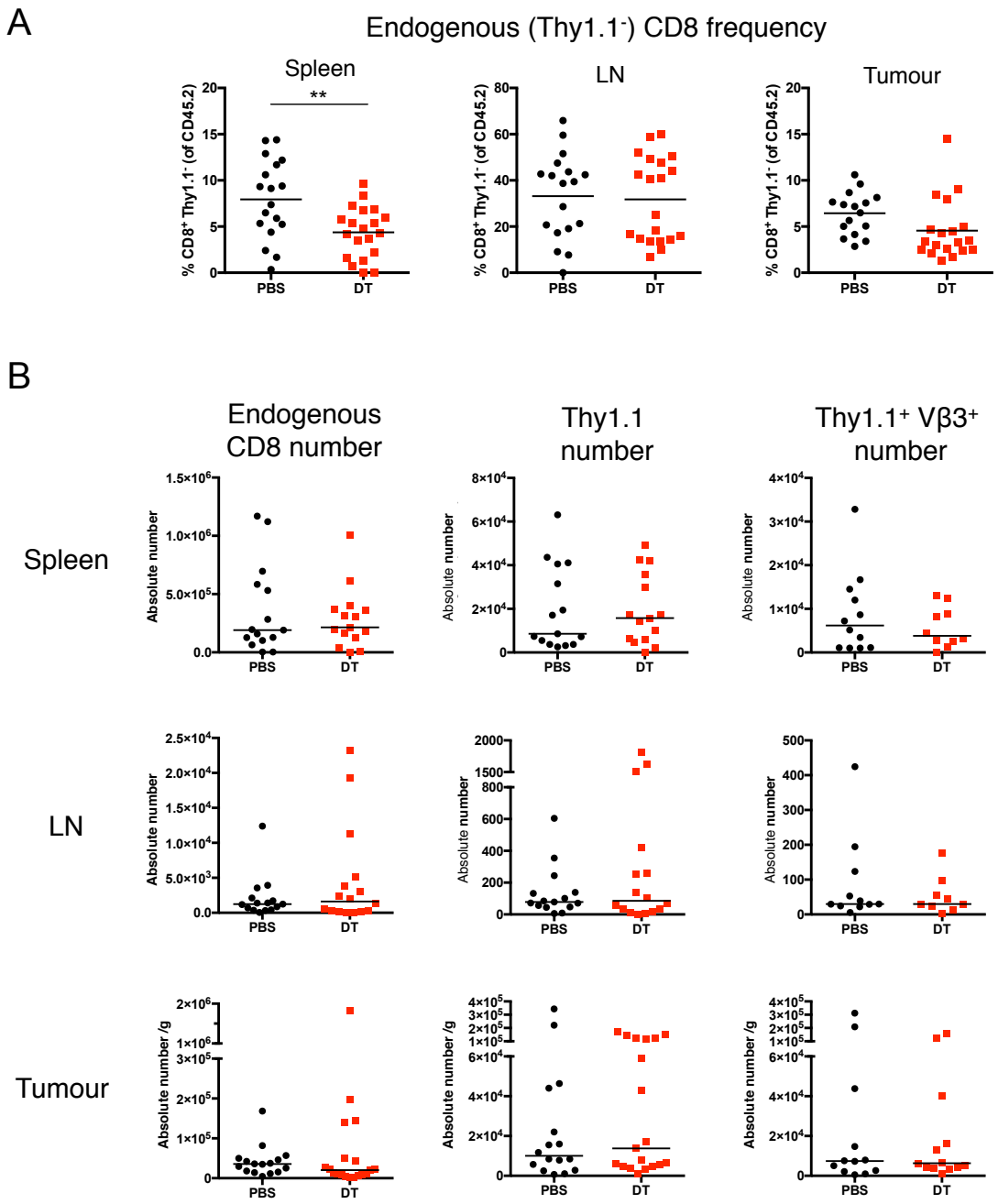


Figure 6.7: *DC depletion has limited effect on transferred T cell number in CD11c.DTR x RAG-1$^{-/-}$ mice.*
(A) Summary data shows frequency of Thy1.1^{-} CD8^{+} cells from the total CD45^{+} population in spleen, LN and tumour.
(B) Summary data of absolute numbers of endogenous CD8, transferred Thy1.1 or Vβ3^{+} Thy1.1 T cells in spleen, LN and tumour. Data is from a total of 15-19 mice per group, from 4 independent experiments.

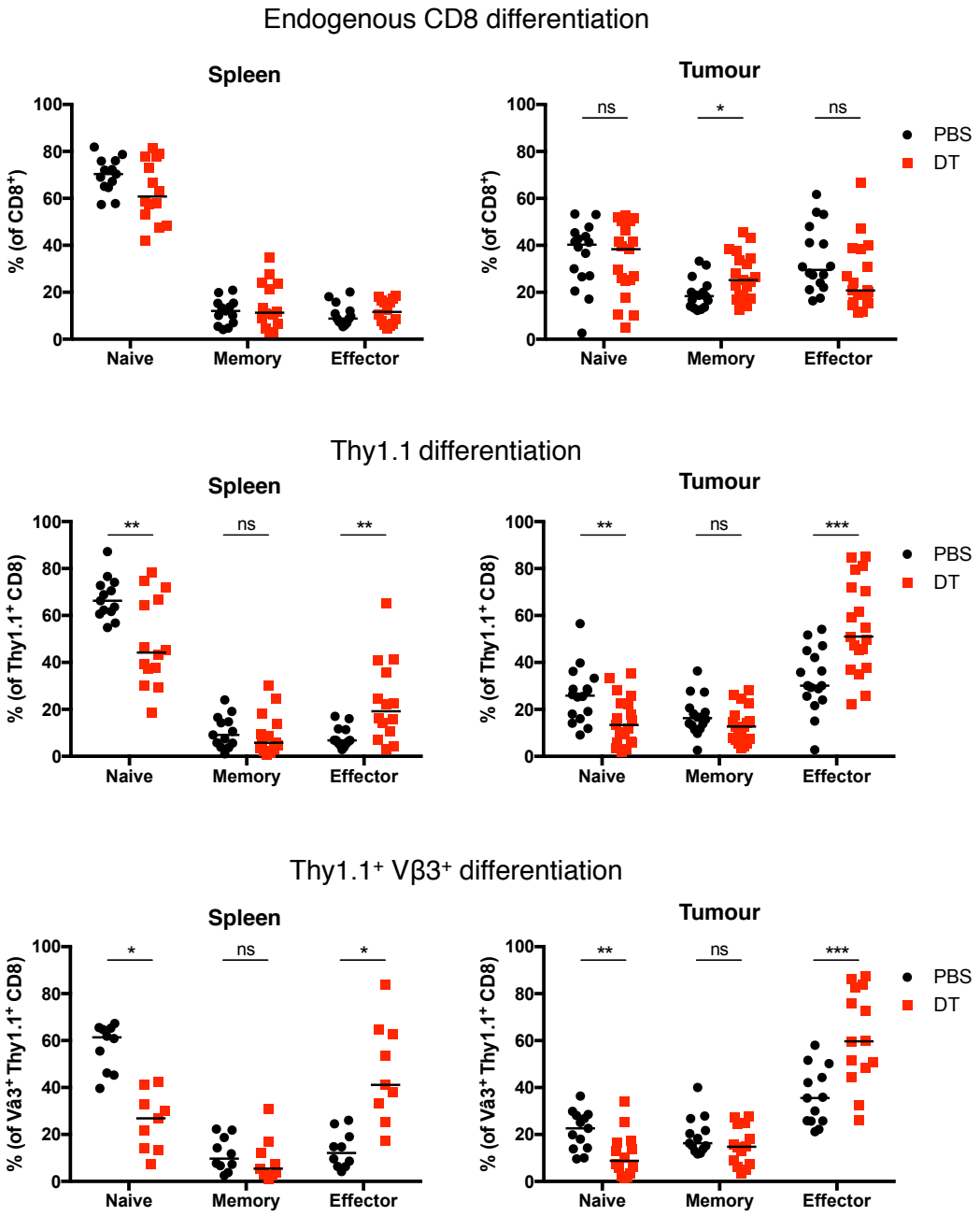


Figure 6.8: *T cell differentiation following DC depletion in CD11c.DTR x RAG-1$^{-/-}$ mice.* Naive, memory or effector phenotype was determined by expression of CD62L and CD127 as demonstrated in Fig 6.2A. Data shows frequency of differentiated endogenous CD8 T cells, transferred Thy1.1$^+$ T cells or transferred Thy1.1$^+$ transduced T cells (Vβ3$^+$) in the spleen and tumour. Data from endogenous CD8 and total Thy1.1 groups are from a total of 15-19 mice per group, from 4 independent experiments. Data from the transduced Vβ3$^+$ Thy1.1$^+$ groups are from 9-14 mice per group, from 3 independent experiments. Statistical test: unpaired T test. ns: non significant, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$.

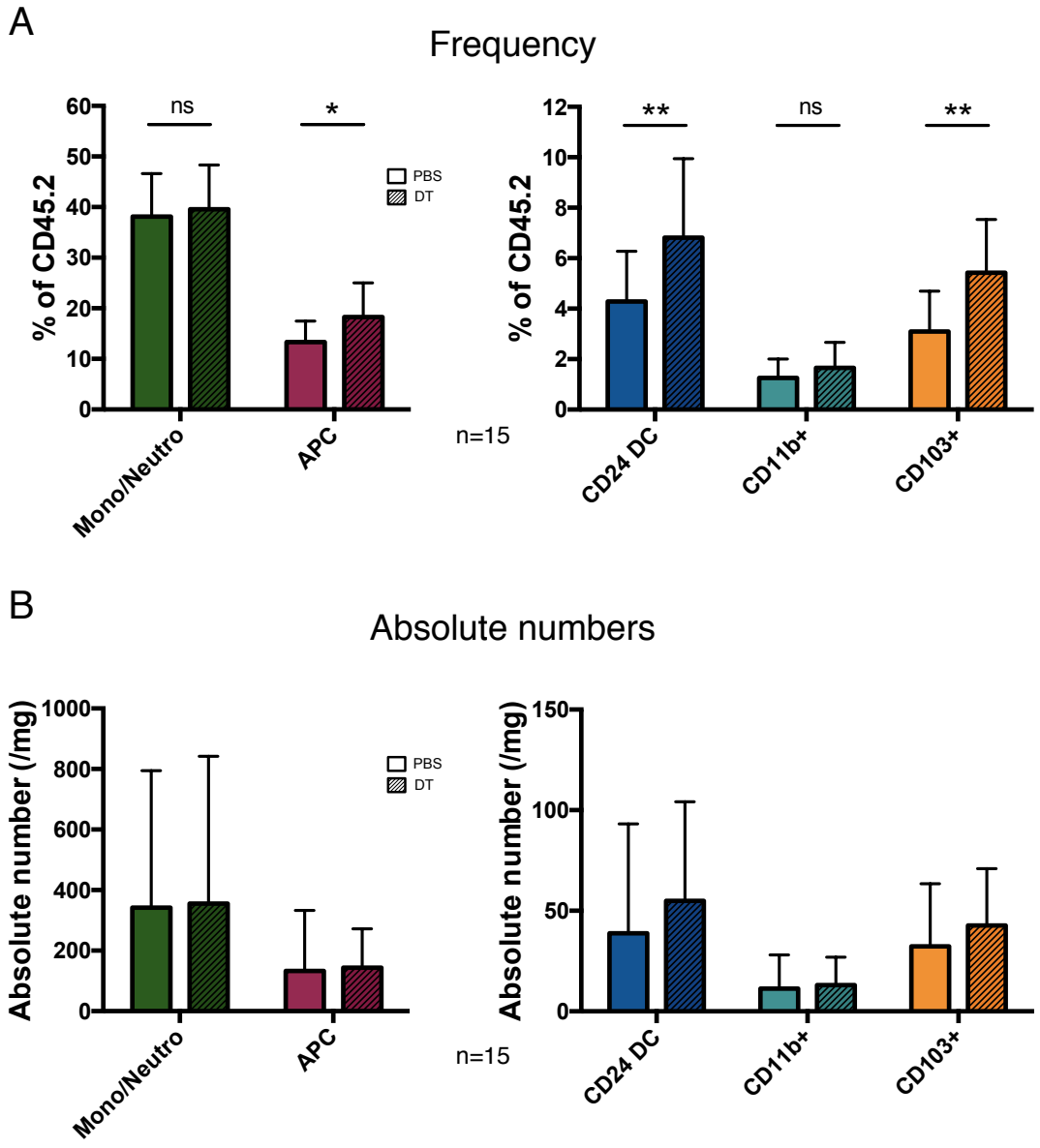


Figure 6.9: *Frequency and number of DC upon reconstitution following depletion.*
(A,B) Summary data from mice sacrificed on day 20 in Fig 6.5A. Tumour-resident myeloid cells were identified as demonstrated in Fig 5.2B. (A) shows the mean frequency of cells from the total $CD45^+$ population, (B) shows the related absolute number. Data is from a total of 15 mice, from 4 independent experiments $\pm$SD.
Statistical test: unpaired T test. ns: non significant, *: $p \leq 0.05$, **: $p \leq 0.01$

6.3 Reconstituted DC can cross-present tumour antigen *ex vivo*

The DC that reconstitute the tumour niche after depletion may not only be present in greater numbers but they may also have an enhanced ability to activate T cells. Whereas tumour-resident DC are normally subverted by the tumour and become dysfunctional, reconstituting DC could be less conditioned by this immuno-suppressive environment and so induce T cell expansion. The ability of these different DC populations to present antigen to transduced T cells *ex vivo* was therefore assessed. $CD11c^+$ cells from the tumours of non-depleted (PBS), transiently depleted (DT) or continuously depleted (DT DT) mice were MACS sorted on day 19 after tumour inoculation. The isolated $CD11c^+$ cells from PBS or DT treated mice were broadly similar. However the $CD11c^+$ cells sorted from the continuously depleted mice contained no $CD24^+$ DC as these had been depleted, however there was a population of $CD64^+$ macrophages (Fig 6.10A). These cells were peptide-loaded, or left to present *in vivo* acquired antigen to freshly transduced TRP2 CD19 T cells *in vitro*. Transduced T cells were then stained for IFN-γ to assess the ability of these isolated $CD11c^+$ cells to present tumour antigen (Fig 6.10B). When the $CD11c^+$ cells were peptide loaded, all groups were able to present to transduced T cells to similar extents. However when cells weren't peptide loaded differences between the groups began to emerge. Whereas the macrophage population from DC depleted mice (DT DT) should be able to present an *in vitro* loaded peptide, it is unlikely they will be able to cross-present tumour antigen directly. Indeed the IFN-γ response to these cells was very weak. However, surprisingly the non-depleted DC, which contain a $CD103^+$ population able to cross-present antigen, failed to do so in this setting. Yet the similar population of DC that reconstituted the tumour appeared to be able to induce a slightly better IFN-γ response than the other groups. These data suggest that repopulating DC either acquire and/or cross present tumour antigens more efficiently than the tumour-resident DC, although these experiments would need to be repeated to confirm this.

To investigate whether there was any phenotypic evidence for this improved capacity to stimulate T cells we stained the isolated CD11c$^+$ cells upon isolation from the tumour for the expression of the inhibitory receptor, PD-L1. However the frequency of PD-L1$^+$ reconstituted DC was similar to non-depleted DC. Furthermore, the frequency of PD-L1$^+$ CD11c$^+$ APC was also similar after PBS, DT or prolonged DT injection (Fig 6.10C).

Together these data suggests that DC do reconstitute the tumour to a greater extent after depletion and are better able to cross-present tumour antigen *ex vivo*, although this was not significant. Although PD-L1 expression is similar between these populations, it is possible that expression of pro-inflammatory molecules such as CD80/CD86 may vary, although this was not investigated. The next section attempts to address the effect of reconstituted DC directly *in vivo* by investigating the accumulation of transferred T cells in mice which were depleted of DC transiently or throughout the experiment.

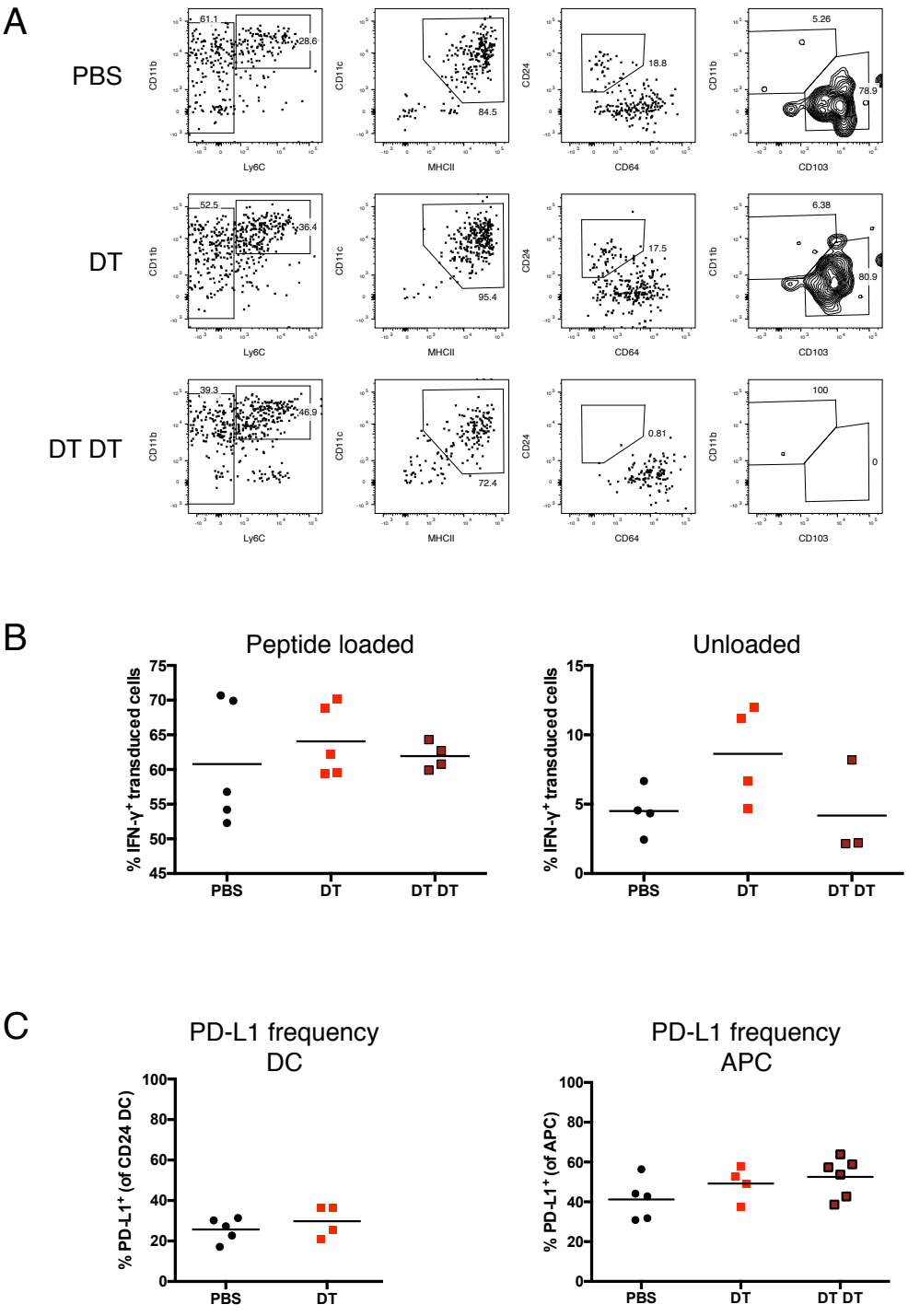


Figure 6.10: *ex vivo isolation of tumour-resident DC and in vitro antigen presentation.*
(A) Representative FACS plots of MACS-sorted, CD11c$^+$ cells from B16.F10 tumours.
(B) IFN-γ production by TRP2 CD19 transduced cells *in vitro* after culture with TRP2 peptide loaded or unloaded *ex vivo* isolated CD11c$^+$ cells. Data is from 2 independent experiments.
(C) PD-L1 expression on CD24$^+$ DC or APC after PBS, DT or prolonged DT injection. Data is from a total of 4-5 mice per group, from 2 independent experiments.

6.4 Enhanced T cell accumulation is not dependent on DC reconstitution after depletion

To test whether the skewed repopulation of DC in the tumour was driving T cell accumulation, DT was injected every 3 days throughout the experiment to prevent DC reconstitution. CD11c.DTR bone marrow chimeras were left undepleted (PBS), transiently depleted as before (DT) or depleted throughout (DT DT) (Fig 6.11A). Thy1.1$^+$ T cells were MACS sorted and were 98% CD8 and Thy1.1$^+$ (Fig 6.11B). A slightly higher dose of 5x10^6 T cells were injected 1 day after transduction with TRP2 CD19. By day 4 these were 67% Vβ3 CD19$^+$, meaning ~3.3x10^6 TRP2 transduced cells were injected (Fig 6.11C). As before the absence of pre-conditioning irradiation meant there was no tumour control evident in any group (Fig 6.11D).

To check the level of DC reconstitution or depletion, spleens and tumours were stained on day 19 for the various DC populations. As expected, continuous DC depletion resulted in significant depletion of DC in the spleen, both in frequency and absolute number (Fig 6.12). However there was a significantly higher frequency and number of DC in the spleen after they reconstituted the depleted niche. The same is true in the tumour where, as also shown in Fig 6.9, DC reconstituted the tumour to a greater extent than non-depleted mice (Fig 6.12). Although this was only significant in the increased frequency, the absolute numbers showed a similar trend. However compared to either non-depleted or reconstituted mice, the frequency and absolute number of DC in the tumour was significantly lower after continuous depletion. Prolonged DT injection depleted DC more efficiently than after a single dose. Whereas only 56% of CD24$^+$ DC were depleted in Fig 5.4, when depletion was extended, 93% of CD24$^+$ DC were depleted.

Based on our previous data, continued depletion of CD11c$^+$ cells should lead to loss of endogenous CD8 T cells in this setting. Indeed, this was evident in the spleen but not in the LN or tumour (Fig 6.13 and 6.14A). As expected, the

frequency of transferred T cells was greater in mice after transient depletion (DT) than in non-depleted mice (PBS). Whilst this wasn't significant in these experiments, the same trend was observed in spleen, LN and tumour.

However contrary to our hypothesis, prolonged DC depletion (DT DT) also resulted in the accumulation of transferred T cells. Indeed, this increase was more pronounced after prolonged depletion, rather than transient depletion/reconstitution (Fig 6.13). Due to this greater increase the trend towards enhanced frequencies of transferred T cells became significant after prolonged depletion in the spleen and LN. Transferred T cells also accumulated in the tumour after DC depletion (particularly after prolonged DC depletion), although this increase was not significant in these experiments. This trend was carried through when gating on the transduced fraction of the transferred T cells, which also tended to be present in greater frequencies after transient or prolonged depletion.

In the spleen this increase in the frequency of transferred T cells could be as a result of depleting endogenous T cells from this gate. Endogenous CD8 T cells were found in a significantly lower frequency (6.14A) and possibly lower number (6.14B) in the spleen after prolonged DC depletion. However the frequency and number of endogenous CD8 T cells in the LN remained unchanged after DC depletion and whilst there was some evidence for fewer endogenous CD8 T cells in the tumour, this wasn't significant. There were also significantly more endogenous T cells in the spleens of reconstituted mice when compared to mice with prolonged DC depletion. This pattern of increased or decreased endogenous T cells seems to follow a similar pattern to the frequency of DC in the spleen (Fig 6.12A). This could suggest that the T cell expansion/contraction in the spleen is controlled by the number of DC present.

The increased frequency of transferred T cells was also partially reflected in the absolute numbers, albeit less significantly. Whilst there were significantly more transferred T cells in the LN after prolonged depletion, a similar trend was not significant in the spleen. Moreover there appeared to be no difference in the number of transferred T cells in the tumour after depletion, in contrast to the

increased frequency. As before the number of transduced cells within the transferred population followed a similar pattern to the total transferred T cells.

Lastly, the differentiation status of the endogenous and transferred T cells was assessed after transient or prolonged DC depletion (Fig 6.15). In the spleen, depletion of DC appeared to promote a less naive, more effector phenotype in the endogenous CD8 T cells. While there was a mild difference after transient depletion, this was significant after prolonged depletion. A similar effect is seen in the transferred T cells in the spleen, which also acquired a significantly less naive phenotype, whilst possibly enhancing the memory and effector differentiation. However unlike the previous experiment, neither the endogenous nor transferred T cells in the tumour acquired a noticeably different phenotype after DC depletion.

Together these data suggest that although DC do reconstitute the tumour and spleen at higher numbers after transient depletion, DC depletion at the time of T cell transfer, rather than this reconstitution leads to the enhanced accumulation of transferred T cells. To gain a better understanding of how DC depletion drives accumulation of transferred T cells, we examined the process that governs how these T cells are able to accumulate more efficiently.

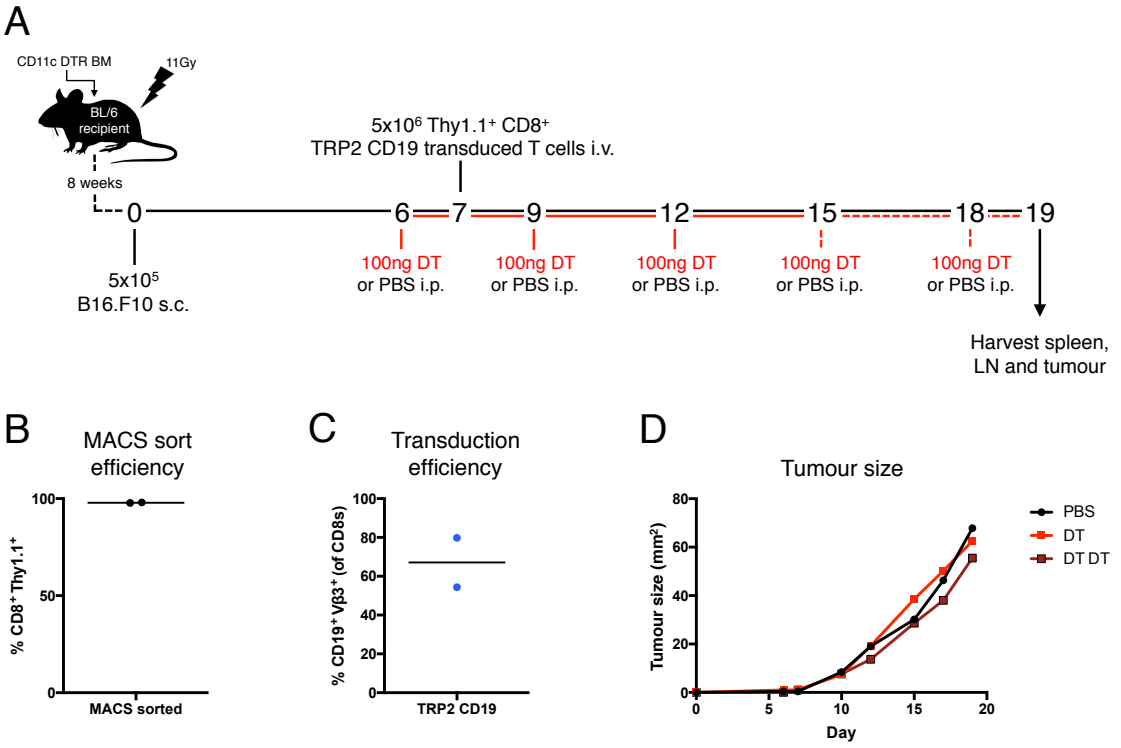


Figure 6.11: *Adoptive T cell transfer alongside prolonged DC depletion.*
(A) Experiment outline. Repeat of Fig 6.1 except an extra group had further DT injections on day 15 and 18, to ensure DC were depleted throughout the experiment.
(B) MACS sort efficiency. Data shows percentage of $CD8^+$, $Thy1.1^+$ lymphocytes.
(C) Transduction efficiency of TRP2 CD19 transduced T cells on day 4 post-transduction. Data shows the percentage of $V\beta13^+$, $CD19^+$ CD8 T cells.
(D) Mean tumour size in mice treated with TRP2 CD19 transduced T cells. DC were not depleted (PBS), transiently depleted (DT) or continuously depleted (DT DT) . Data is from a total of 5-6 mice per group, from 2 independent experiments.

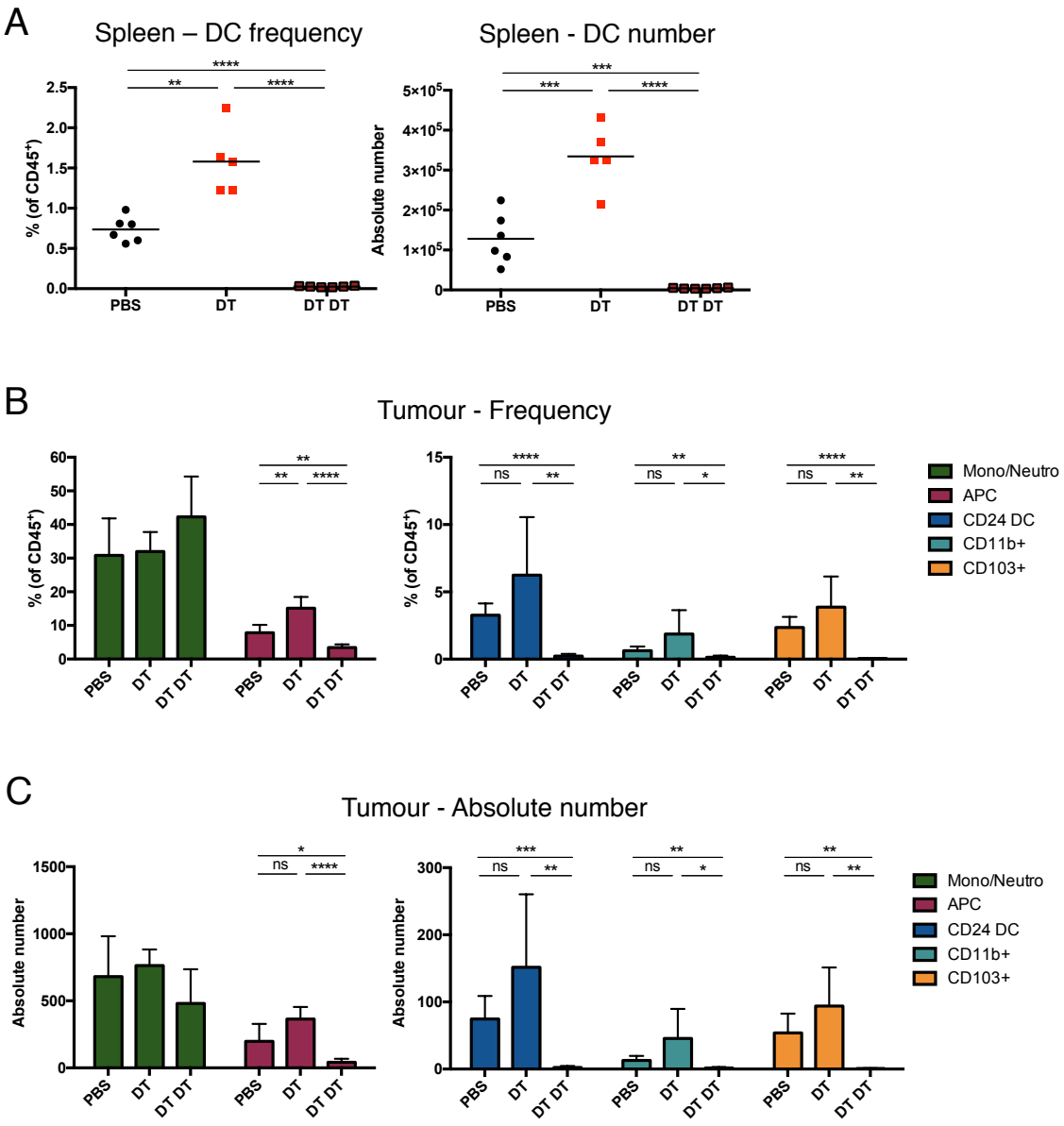


Figure 6.12: *DC reconstitution or prolonged DC depletion in spleen and tumour.*
(A) Frequency and absolute number of CD11c$^+$ MHCII$^+$ DC in the spleen.
(B,C) Frequency and absolute number of monocytes/neutrophils, APC and CD11b or CD103$^+$ DC in the tumour. DC were either not depleted (PBS), transiently depleted (DT) or continuously depleted (DT DT). Data is from a total of 5-6 mice per group, from 2 independent experiments. Statistical test: unpaired T test. ns: not significant, *: $p \leq 0.05$, **: $p \leq 0.01$,***: $p \leq 0.001$, ****: $p \leq 0.0001$.

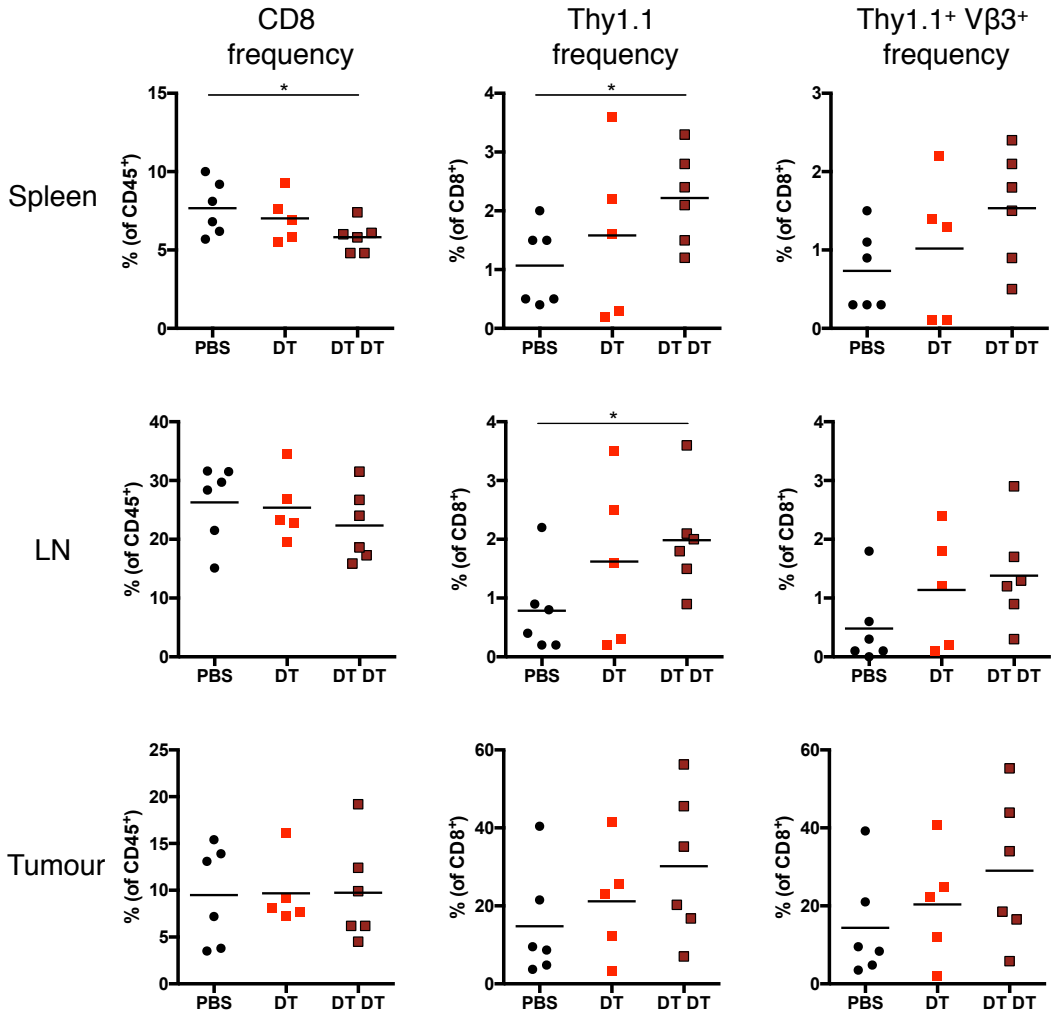


Figure 6.13: *Prolonged depletion further enhances the frequency of transferred T cells.* Summary data shows frequency of $CD8^+$ cells from the total $CD45^+$ population, or frequency of Thy1.1 or $V\beta3^+$ Thy1.1 cells from the $CD8^+$ population in spleen, LN and tumour. DC were either not depleted (PBS), transiently depleted (DT) or continuously depleted (DT DT). Data is from a total of 5-6 mice per group, from 2 independent experiments. Statistical test: unpaired T test *: $p \leq 0.05$.

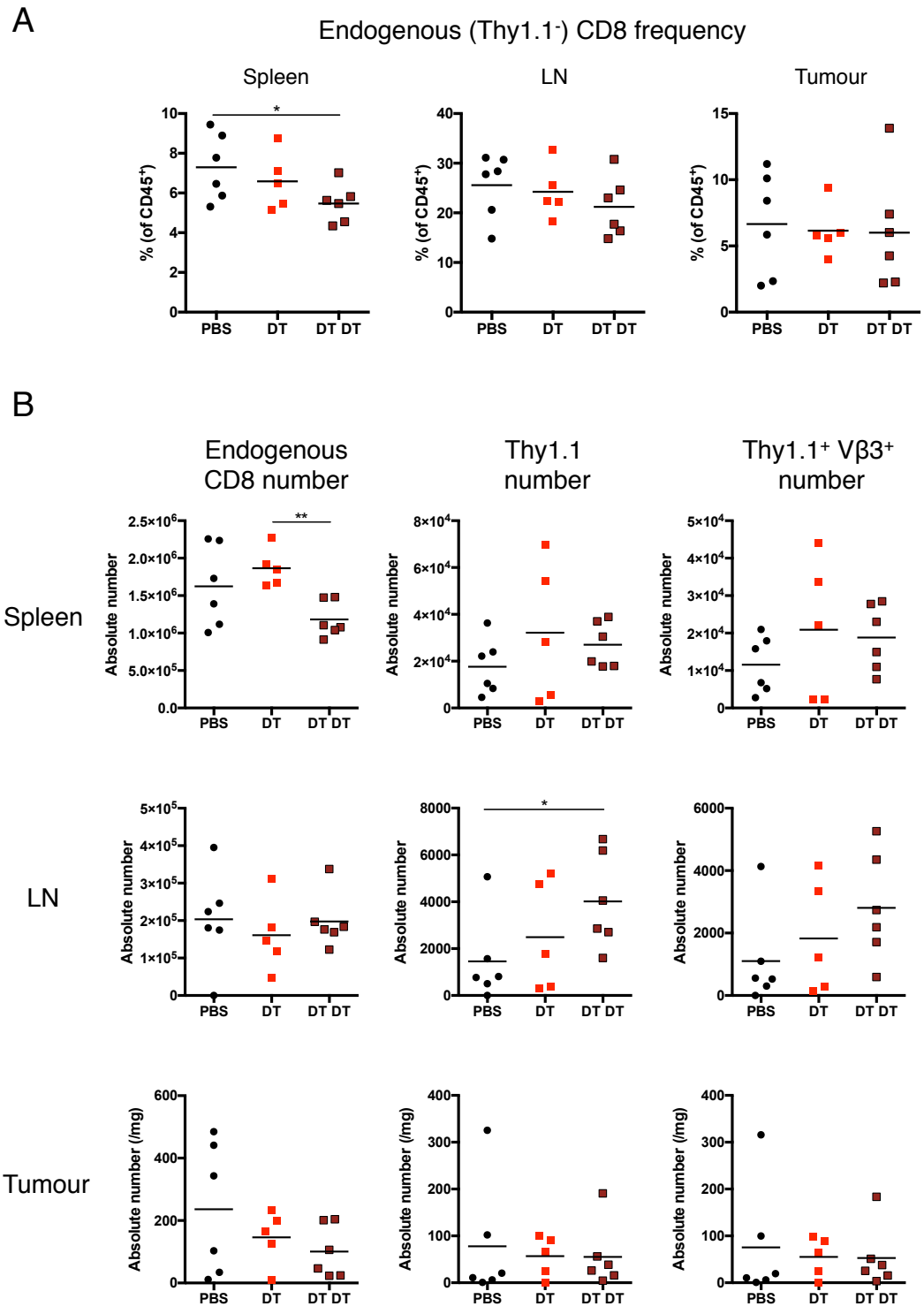


Figure 6.14: *Prolonged depletion partially increases the number of transferred T cells.* (A) Summary data shows frequency of Thy1.1$^-$ CD8$^+$ cells from the total CD45$^+$ population in the spleen, LN and tumour.
(B) Summary data of absolute numbers of endogenous CD8, transferred Thy1.1 or Vβ3$^+$ Thy1.1 cells in spleen, LN and tumour. DC were either not depleted (PBS), transiently depleted (DT) or continuously depleted (DT DT). Data is from a total of 5-6 mice per group, from 2 independent experiments. Statistical test: unpaired T test. *: $p \leq 0.05$, **: $p \leq 0.01$.

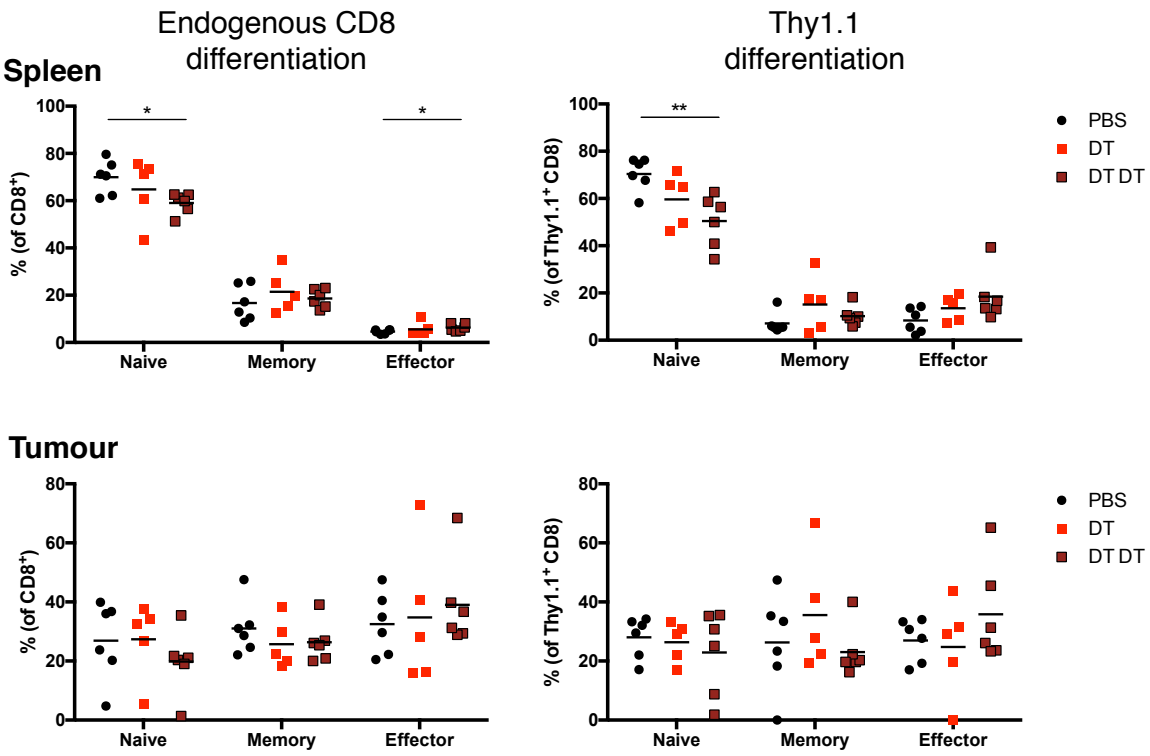


Figure 6.15: *T cell differentiation following prolonged DC depletion.*
Naive, memory or effector phenotype was determined by expression of CD62L and CD127 as demonstrated in Fig 6.2A. Data shows frequency of differentiated endogenous CD8 T cells or transferred Thy1.1$^+$ T cells in the spleen, LN and tumour. DC were either not depleted (PBS), transiently depleted (DT) or continuously depleted (DT DT). Data is from a total of 5-6 mice per group, from 2 independent experiments. Statistical test: unpaired T test. *: $p \leq 0.05$, **: $p \leq 0.01$.

6.5 Investigating the effect of DC depletion on transferred T cell proliferation

Depletion of DC at the time of T cell transfer appeared to be driving the enhanced accumulation of transferred T cells, which suggests that these DC normally exert a regulatory effect on transferred T cells. To investigate this hypothesis further, the mechanism that drives this T cell accumulation was assessed. Depletion of a regulatory DC population may be driving the accumulation of transferred T cells by removing the constraint to their proliferation and expansion. To address this, transferred T cells were stained for Ki-67 to mark proliferating cells (Fig 6.16A). Ki-67$^+$ endogenous or transferred CD8 T cells were identified in the spleens and tumours of mice receiving PBS, transient depletion or prolonged depletion (Fig 6.16B). In the spleen, the frequency of Ki-67$^+$ endogenous T cells was low (8%), as expected for unactivated T cells. This was unaffected by DC depletion. The *in vitro* activated, transferred T cells in the spleen were slightly more Ki-67$^+$ (14%), however the majority were still Ki-67$^-$. There was possibly a greater frequency of Ki-67$^+$, transferred T cells in the spleen after depletion (either transient or prolonged) although this was not significant due to high variability.

In the tumour the endogenous T cells had a higher Ki-67 frequency (35%) than in the spleen, and this appeared to increase after transient depletion and further increase after prolonged depletion. This suggests that depletion of regulatory DC was able to promote T cell proliferation. However it is not possible to tell whether transferred T cells in the tumour are more proliferative after DC depletion as nearly all transferred T cells were already Ki67$^+$ (91%). These data suggest that whilst increased proliferation may be responsible for the enhanced accumulation of transferred T cells, this was inconclusive.

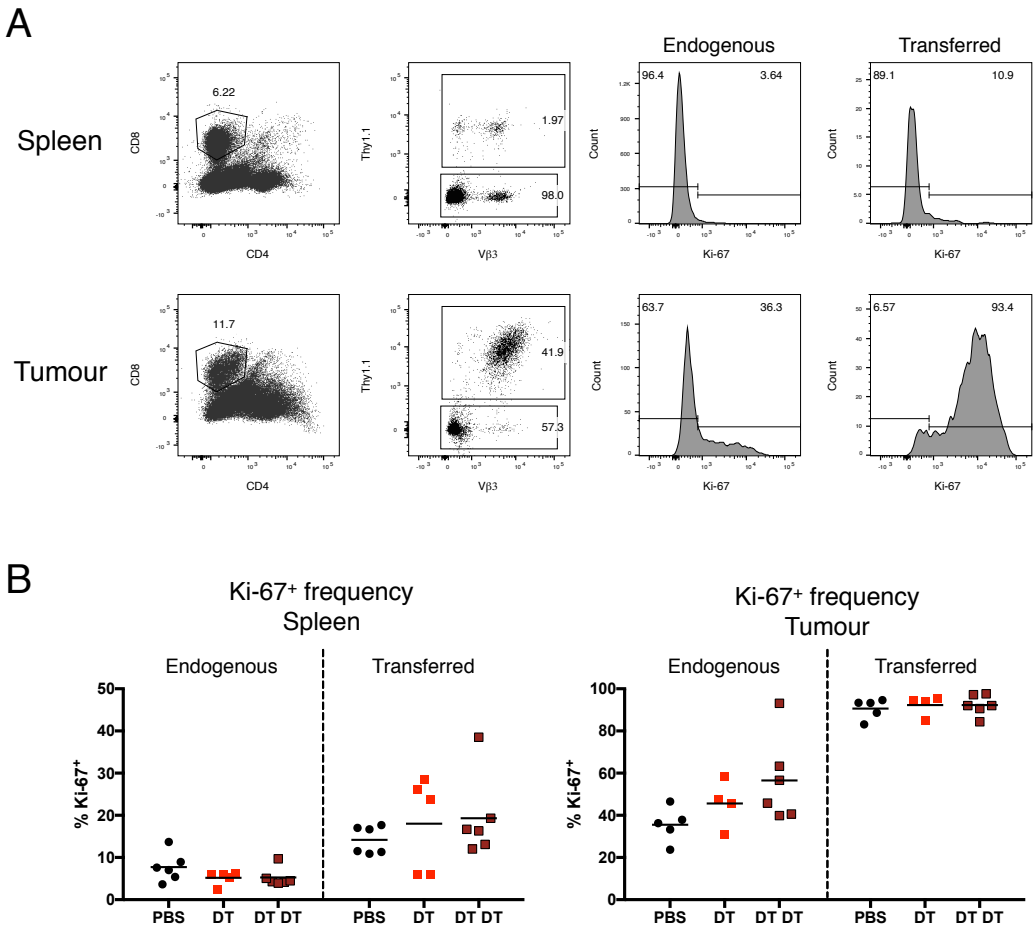


Figure 6.16: *Ki-67 expression on endogenous and transferred T cells.*
(A) Representative FACS plots showing gating of Ki-67$^+$ CD8 T cells. Cells are pre-gated on live, CD45$^+$ lymphocytes.
(B) Summary data showing frequency of Ki-67$^+$ endogenous or transferred CD8 T cells after PBS, DT or prolonged DT injection. Data is from a total of 5-6 mice per group, from 2 independent experiments.

6.6 Summary and conclusion

Contrary to our initial hypothesis, these data showed that DC depletion led to an enhanced accumulation of transferred T cells. This was not true of endogenous T cells and so appeared to be dependent on pre-activation *in vitro*, although non-activated transferred T cells were not assessed. However this accumulation appeared to be further enhanced in the population of transferred T cells that were transduced with anti-tumour TCR. This accumulation occurred independently of endogenous T cell depletion and favoured the acquisition of a more effector, less naive phenotype. We hypothesised that this T cell accumulation would either be driven by depletion of a regulatory DC population or by DC that reconstitute the depleted niches. In line with this latter hypothesis, we found that following DC depletion, the returning DC did reconstitute the tumour and spleen to a greater extent than in non-depleted mice. When these DC populations were sorted from the tumour, it appeared that reconstituted DC were better able to cross-present tumour antigen to transduced T cells *ex vivo*. However this data remains inconclusive and examination of PD-L1 expression on these cells did not show any differences.

To investigate the role of reconstituted DC in the accumulation of transferred T cells, the model was adapted to ensure DC were depleted throughout the experiment. In this setting we found that transferred T cells accumulated more readily after prolonged depletion than in non-depleted mice and possibly more than transiently depleted mice. This strongly suggested that instead of reconstitution of a pro-inflammatory DC population, the enhanced accumulation of transferred T cells was mediated by depletion of a regulatory DC population. Indeed tumour resident DC are largely thought of in a tolerogenic capacity (Pinzon-Charry et al. 2005), so depletion of these dysfunctional DC would likely lead to increased T cell accumulation. The ability of these regulatory DC to limit cell proliferation was therefore tested. This seemed to show that DC depletion promoted the proliferation of endogenous T cells in the tumour. However Ki67 staining may not be sensitive enough to discriminate differences in proliferation

in tumour resident transferred T cells which were nearly all Ki67$^+$. Use of a more sensitive method of tracking cell proliferation, such as CFSE dilution may be useful in further experiments as it can provide a variable readout rather than the binary description of cell proliferation provided by Ki67 staining.

Of note however is that despite DC depletion, anti-tumour TCR transduced T cells were still substantially proliferative in the tumour, much more so than transferred T cells in the spleen, or endogenous T cells in the tumour. This suggests that tumour-antigen presentation by a non-depleted APC population in the tumour is capable of driving the proliferation of these transferred T cells. This may then be further enhanced by the depletion of a regulatory DC population that would otherwise limit T cell proliferation.

However these results are surprising as although the tumour does contain tolerogenic DC, a large population of these cells in the tumour are pDC (Wei et al. 2005), which should not be depleted by DT injection in this model. Furthermore the CD103$^+$ DC population in the tumour that are specifically depleted in this model have been shown to effectively cross-present tumour antigen and induce pro-inflammatory T cell responses (Broz et al. 2014; Salmon et al. 2016). However DT injection is likely responsible for depletion of a broader range of DC subsets than has been accounted for here. Alternatively as a specific regulatory DC population has yet to be identified, it may be that tolerogenic DC in the tumour instead comprise a heterogenous population of DC that have similar regulatory functions *in vivo*. A better understanding of the activation status of these tumour resident DC subsets in this study would aid the identification of these regulatory DC. Of particular importance would be careful examination of the combinations of costimulatory molecules and inhibitory receptors that DC use to control the T cell response.

However a third possible explanation for this T cell accumulation also exists. In this model C57BL/6 recipients are reconstituted with CD11c.DTR bone marrow, to allow repeated DT injection. However radio-resistant DC populations have been identified in the skin that are capable of self-renewal (Bogunovic et al. 2006). Therefore it is possible that after depletion of donor DC, this non-DTR

expressing DC population of recipient origin expands and creates a pro-inflammatory environment not seen in undepleted mice. To test whether T cell accumulation is specific to the use of chimeric mice we exploited the CD11c.DOG model. This model allows for repeated DT injection into non-chimeric mice and so will allow us to test this hypothesis. The next chapter therefore examines the effect of DC depletion on transferred T cells in CD11c.DOG mice.

Chapter 7 Effect of DC depletion on transferred T cells in CD11c.DOG mice

Unlike in CD11c.DTR mice, the CD11c.DOG model allows multiple DT injections without toxicity. In this model therefore we could test whether transferred T cells accumulate after DC depletion in non-chimeric mice. Alongside this we could also assess whether this enhanced T cell accumulation is capable of inducing superior control of tumour growth. As mentioned previously pre-conditioning irradiation appears to be critical for effective responses from transferred T cells. Therefore we chose to use the B16 NP GFP model to assess the ability of F5 CD19 transduced T cells to control tumour growth after DC depletion. We have shown previously that despite T cell injection and pre-conditioning irradiation being separated temporally, F5 CD19 transduced T cells can mediate pronounced tumour control *in vivo* (Fig 4.5,4.6). This would lessen the possible accumulative toxicity of DT injection in direct combination with irradiation.

In addition, the F5 model also allows us to question whether transduced T cells interact with host DC differently than non-transduced T cells. To this end we can use transgenic T cells which express the F5 TCR and compare these unactivated but tumour specific T cells, with the activated, F5 CD19 transduced T cells. This chapter will therefore describe the use of the CD11c.DOG model to investigate the effects of DC depletion on:

1. Transferred T cell accumulation in non-chimeric mice
2. Accumulation of transferred T cells recognising foreign antigens
3. Tumour control by either TCR transduced or TCR transgenic T cells

7.1 Establishing the transferred T cell expansion model in CD11c.DOG mice

To investigate whether transferred T cell accumulation was dependent on DC depletion in chimeric mice, we repeated the T cell accumulation experiments in the CD11c.DOG mice (Fig 7.1A). As before mice were injected with $5x10^5$ B16.F10 cells s.c., however here the mice were heterozygous CD11c.DOG mice, rather than CD11c.DTR bone marrow chimeras. As before, these mice were injected with $4x10^6$ Thy1.1$^+$, CD8 T cells, which had been transduced with the TRP2 CD19 vector. To deplete DC, mice were injected with 100ng DT or PBS.

Surprisingly, unlike in the CD11.DTR model, transferred T cells in the CD11c.DOG mice did not accumulate in spleen, LN or tumour after DC depletion. The frequency of Thy1.1$^+$ cells remained similar after DC depletion (Fig 7.1B) and the numbers of transferred cells may even have fallen after depletion, particularly in the tumour (Fig 7.1C).

These data could suggest that the generation of chimeras was an important facet of the enhanced T cell accumulation seen after DC depletion. However alternatively, it could be explained by differences between the depletion of DC in the CD11c.DTR and CD11c.DOG models. As shown in Fig 5.8 and 5.9, whilst splenic DC in CD11c.DOG mice were well depleted after injection of 100ng DT, depletion of tumour-resident DC was poor. As the CD11c.DOG mice are less susceptible to DT injection, this experiment was repeated after injection of 1µg DT (~64ng/g), with which tumour-resident DC had been largely depleted. However again, high-dose DT injection in CD11c.DOG mice failed to replicate the enhanced T cell accumulation seen previously (Fig 7.2B). Again, in contrast to the CD11c.DTR model, CD11c.DOG mice injected with high-dose DT may even have a lower frequency of transferred T cells than non-depleted mice.

As the CD11c.DTR mice could not be used without first making bone marrow chimeras, to compare the two models accurately either CD11c.DOG bone

marrow or CD11c.DTR bone marrow was used to make chimeras (Fig 7.3A). Both sets of chimeras were injected with the same dose of DT (100ng) and treated with $4x10^6$ TRP2 CD19 transduced, Thy1.1$^+$ CD8$^+$ T cells. As shown previously, Thy1.1$^+$ T cells transferred into CD11c.DTR chimeras accumulated to a significantly greater extent in the LN and tumour after DC depletion (Fig 7.3B). However, despite chimerism in both models, transferred T cells did not accumulate after DC depletion in CD11c.DOG chimeras. Furthermore, as expected, transferred T cells in the tumours of CD11c.DTR chimeras appeared to acquire a more effector phenotype after DC depletion. In contrast DC depletion in the CD11c.DOG chimeras seemed to have the opposite effect. Both the endogenous and transferred tumour-resident T cells acquired a less effector phenotype after depletion in CD11c.DOG chimeras, although this was only significant for the endogenous cells (Fig 7.3C).

These data show that the generation of CD11c.DOG chimeras is insufficient to replicate the enhanced accumulation of transferred T cells seen in CD11c.DTR chimeras. Instead it further suggests that this enhancement is mediated by depletion of a regulatory DC population, rather than the perseverance of a radio resistant, non-depletable DC population of BL/6 origin. As transferred T cell accumulation is not repeated in CD11c.DOG mice however it appears that depletion of this regulatory population only occurs in the CD11c.DTR mice. In contrast DC depletion in the CD11c.DOG mice may even have a negative effect on the transferred T cells, in line with our original hypothesis. However the effect of high-dose DT injection in CD11c.DOG chimeras has not been tested here, which could be required to replicate the phenotype seen in CD11c.DTR mice, although this seems unlikely. The difference in response between the CD11c.DTR and CD11c.DOG mice is likely to be driven by the varied depletion pattern seen between these two models. For example depletion of CD24$^+$ DC in the CD11c.DTR mice, rather than depletion of the broader CD11c/MHCII$^+$ population may be driving this result, although the reason why is unclear. Alternatively the depletion of other CD11c$^+$ cells such as NK and T cells has not been as well defined in the CD11c.DOG model. It is possible this could have an unknown effect on the transferred T cell response.

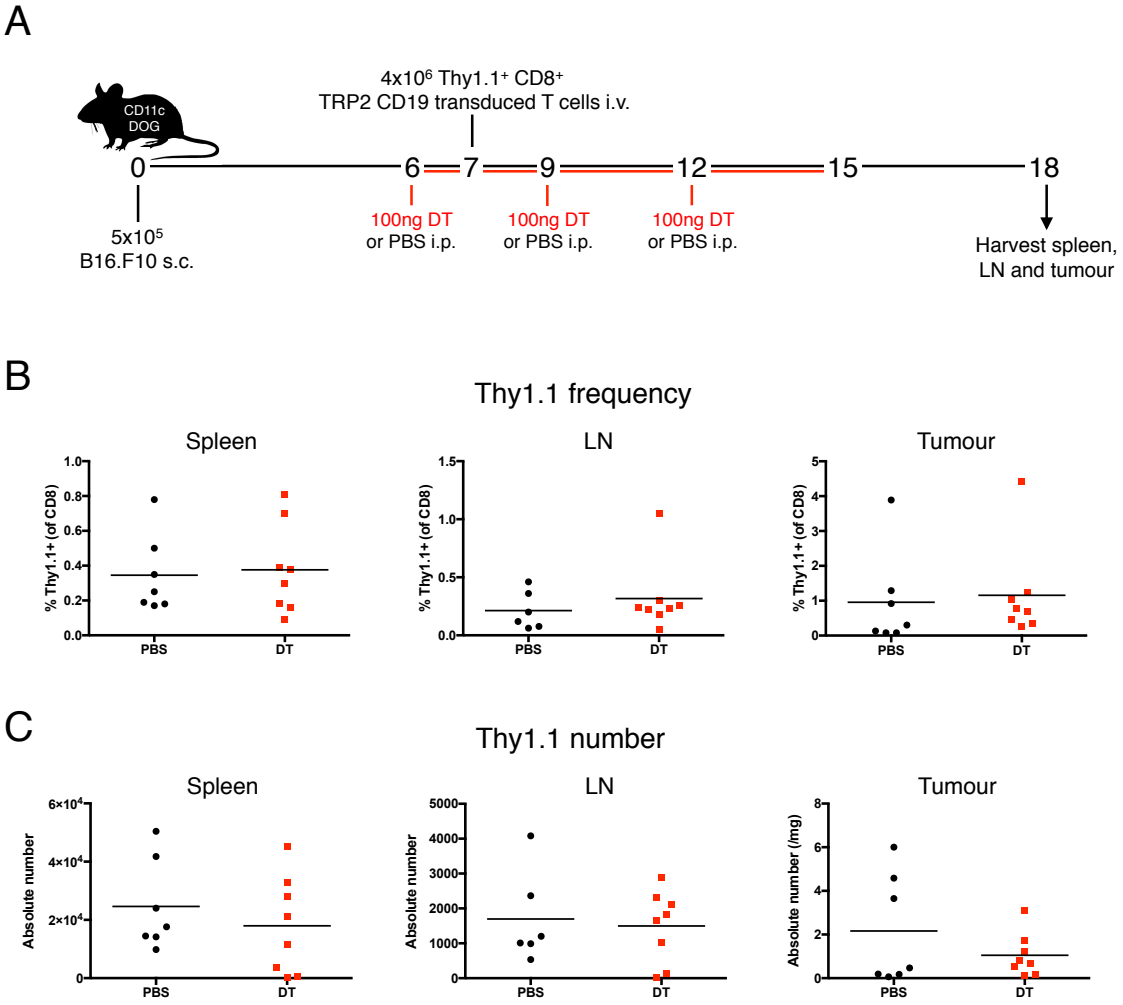


Figure 7.1: *Adoptive transfer of TCR transduced T cells in CD11c.DOG mice.*
(A) Experiment outline. CD11c.DOG mice were injected with B16.F10. Mice were treated with 4x10^6 Thy1.1$^+$ CD8$^+$ TRP2 transduced T cells. One day prior and for 8 days after T cell injection, mice were injected with PBS or 100ng DT to deplete DC.
(B,C) Transferred T cells were identified by expression of Thy1.1 on CD8$^+$, CD45.2$^+$ live, lymphocytes. Summary data shows the frequency of Thy1.1$^+$ cells from the CD8 population (B) or the total number of Thy1.1 T cells (C) in the spleen, LN or tumour. Data is from a total of 7-8 mice per group from 2 independent experiments.

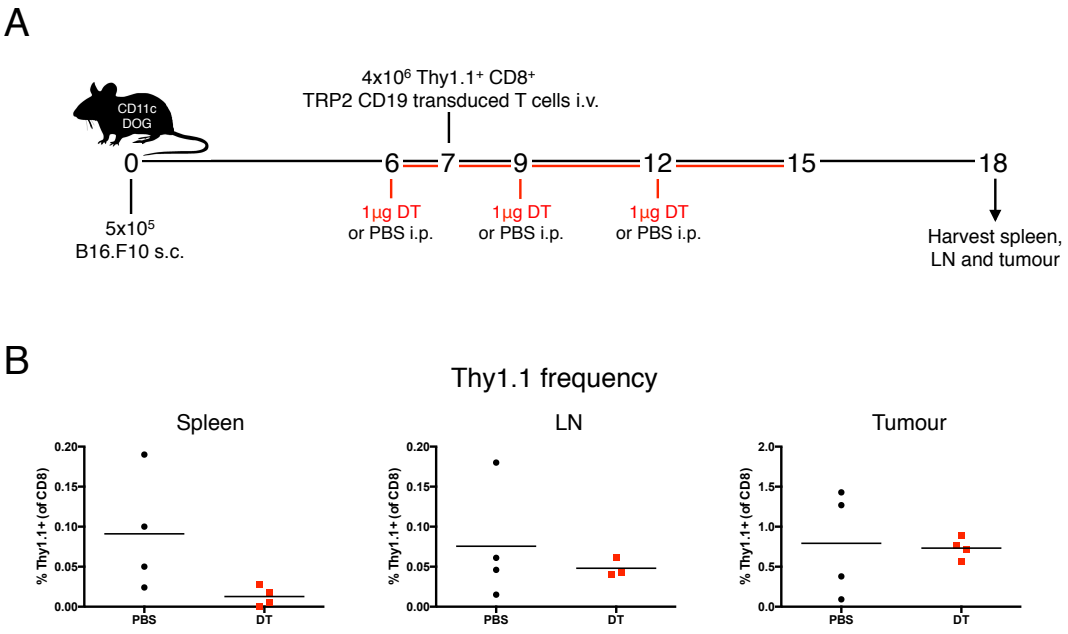


Figure 7.2: *Adoptive transfer of TCR transduced T cells after injection of high-dose DT.* (A) Experiment outline. CD11c.DOG mice were injected with B16.F10. Mice were treated with 4x10^6 Thy1.1$^+$ CD8$^+$ TRP2 transduced T cells. One day prior and for 8 days after T cell injection, mice were injected with PBS or 1µg DT to deplete DC.
(B) Transferred T cells were identified by expression of Thy1.1 on CD8$^+$, CD45.2$^+$ live, lymphocytes. Summary data shows the frequency of Thy1.1$^+$ cells from the CD8 population in the spleen, LN or tumour. Data is from a total of 4 mice per group from 1 experiment.

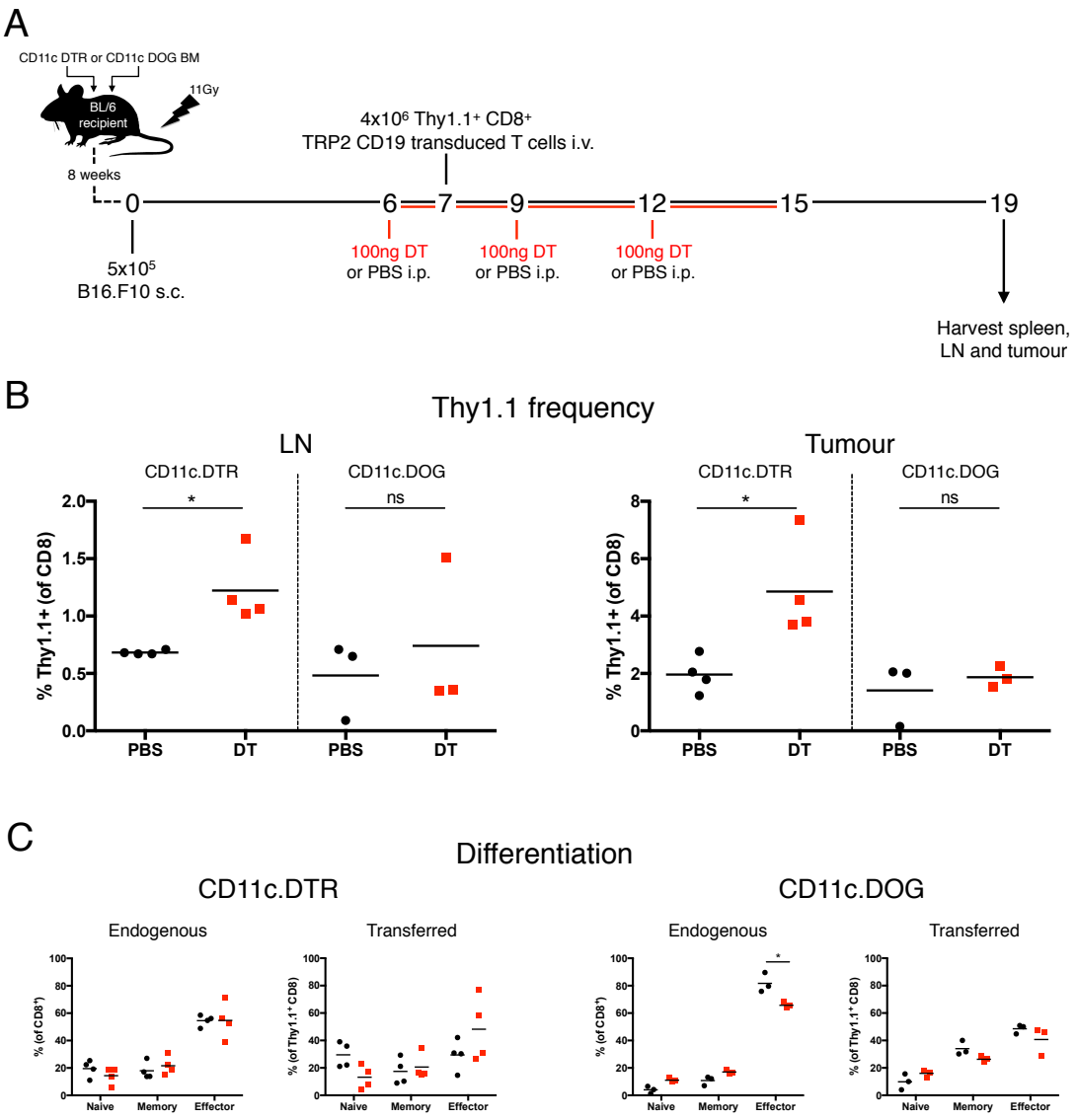


Figure 7.3: *Transferred T cell expansion after depletion in CD11c.DTR but not CD11c.DOG chimeras.*
(A) Experiment outline. CD11c.DTR or CD11c.DOG bone marrow chimeras were generated and once reconstituted, injected with B16.F10. Mice were treated with $4x10^6$ $Thy1.1^+$ $CD8^+$ TRP2 transduced T cells. One day prior and for 8 days after T cell injection, mice were injected with PBS or 100ng DT to deplete DC.
(B) Transferred T cells were identified by expression of Thy1.1 on $CD8^+$, $CD45.2^+$ live, lymphocytes. Summary data shows the frequency of $Thy1.1^+$ cells from the CD8 population in the LN or tumour.
(C) Differentiation status of endogenous CD8 or transferred CD8 T cells in the tumours of CD11c.DTR or CD11c.DOG chimeras. Differentiation is assessed by expression of CD62L and CD127.
Data is from 4 CD11c.DTR or 3 CD11c.DOG mice per group, from 1 experiment. Statistical test: unpaired T test. ns: not significant, *: $p \leq 0.05$.

7.2 Expansion of transferred T cells in irradiated CD11c.DOG mice

As DC depletion in the CD11c.DOG model appeared to be leading to reduced accumulation of transferred T cells, we investigated whether this would affect the ability of these T cells to control tumour growth. As tumour control with TRP2 CD19 transduced T cells was minimal, we instead turned to the F5 CD19 model. F5 TCR transduced T cells were capable of inducing prolonged tumour control after transfer into minimally irradiated hosts (Fig 4.3). However this model is also similar to the previously used TRP2 model in that it uses the same B16.F10 tumour (albeit one that expresses NP), and unlike the TRP1 TCR, it is also class I restricted. Moreover the availability of F5 TCR transgenic mice in this model also allowed us to question whether the effect of DC depletion on *in vitro* activated, transduced T cells is the same as for unactivated, transgenic T cells.

CD11c.DOG mice were irradiated with 4 Gy on day 0 and injected with B16 NP GFP (Fig 7.4A). To ensure robust tumour control $9x10^6$ $CD8^+$ F5 CD19 or mock transduced T cells were injected 1 day after transduction. $1.5x10^6$ unactivated $CD8^+$ F5 transgenic T cells were also injected into a separate group. T cells were kept *in vitro* until day 4 after transduction to assess transduction efficiency (Fig 7.4B) and 70% of F5 CD19 transduced T cells were Vβ11 and $CD19^+$, meaning these mice received $6.3x10^6$ tumour specific T cells. The F5 transgenic T cells were nearly all $V\beta11^+$, although as they are not transduced, they do not express CD19.

To ensure significant DC depletion, CD11c.DOG mice were injected with 1µg DT. Although this would deplete all splenic DC, depletion of tumour-resident DC is more variable so control mice were taken on day 13, 1 day after the last DT injection to check DC depletion. As with previous depletion experiments in the CD11c.DOG model, injection of high-dose DT substantially depleted the CD11c $MHCII^+$ APC population in the tumour (Fig 7.4C).

Tumours were measured following T cell transfer to assess whether DC depletion in this model affects tumour control by transferred T cells (Fig 7.5A). Regardless of DC depletion, both F5 CD19 transduced and F5 transgenic T cells appeared able to control tumour growth to a similar extent. However although mice treated with mock transduced T cells did not control the tumour without DC depletion, those mice injected with DT showed marked tumour control. This was unexpected as tumour growth had been unaffected by DT injection previously. As the B16 NP GFP tumour expresses the foreign NP peptide, it is possible that an naturally occurring anti-NP T cell clone from within the mock transduced T cell population has mediated tumour control. However this doesn't explain why this only occurred in DT injected, not PBS injected mice. Alternatively the DT itself could have been directly controlling tumour growth, however the B16 NP GFP tumours cells do not express the high affinity DTR, so should not be directly depleted by DT. Likewise, direct DT mediated tumour control had not been noted in other experiments.

As well as controlling tumour growth however, from day 12 onwards, all DT injected mice lost a significant amount of weight culminating at day 17 when these mice had to be sacrificed as they had lost >20% of their body weight (Fig 7.5B). It is possible that this systemic toxicity caused by DT injection had inhibited tumour growth. Either way, mice were culled at this point and infiltration of transferred T cells into the tumours was measured.

Tumour-resident $CD8^+$ T cells were identified as shown in Fig 7.6A. Although transferred T cells could not be identified by expression of a congenic marker in this experiment, NP-specific T cells (either F5 CD19 or F5 transgenic) could be identified by expression of CD8 and Vβ11. Compared to mice treated with mock transduced T cells, mice treated with F5 CD19 or F5 tg T cells showed a significant expansion of these cells in the tumour (Fig 7.5A,B). Despite receiving ~4-fold higher NP-specific T cells, F5 CD19 T cells were found at a lower frequency and absolute number than F5 transgenic T cells. This may reflect the improved proliferative potential that unactivated, naive T cells have over pre-activated T cells. However in both F5 CD19 and F5 transgenic groups, DC depletion led to a reduced frequency and number of transferred cells. Possible

because of the enhanced potential of the unactivated F5 transgenic cells to proliferate, this depletion-mediated reduction in their proliferation was more severe, compared to F5 CD19 T cells. As these mice were irradiated before T cell transfer there are not likely to be many endogenous CD8 T cells in the tumour. Indeed $CD8^+$ T cells were stained for expression of Vβ11 and >93% of $CD8^+$ T cells in F5 CD19 or F5 transgenic treated mice were $V\beta11^+$. This appeared to fall slightly after DC depletion, however this was not significant.

The differentiation status of tumour-resident $CD8^+$ T cells was also assessed after DC depletion (Fig 7.6C). Surprisingly in mice treated with F5 transgenic T cells, despite much less T cell expansion in DC depleted mice, there was no difference in T cell differentiation. In contrast, both mock and F5 CD19 transduced T cells had a less effector, more memory phenotype after DC depletion. This suggests that irrespective of antigen-specificity, cells that are pre-activated *in vitro* are more likely to form memory populations in the absence of DC. This occurred at the expense of effector differentiation. In contrast, although unactivated T cells undergo much less proliferation after DC depletion, this did not effect their ability to differentiate in effector cells.

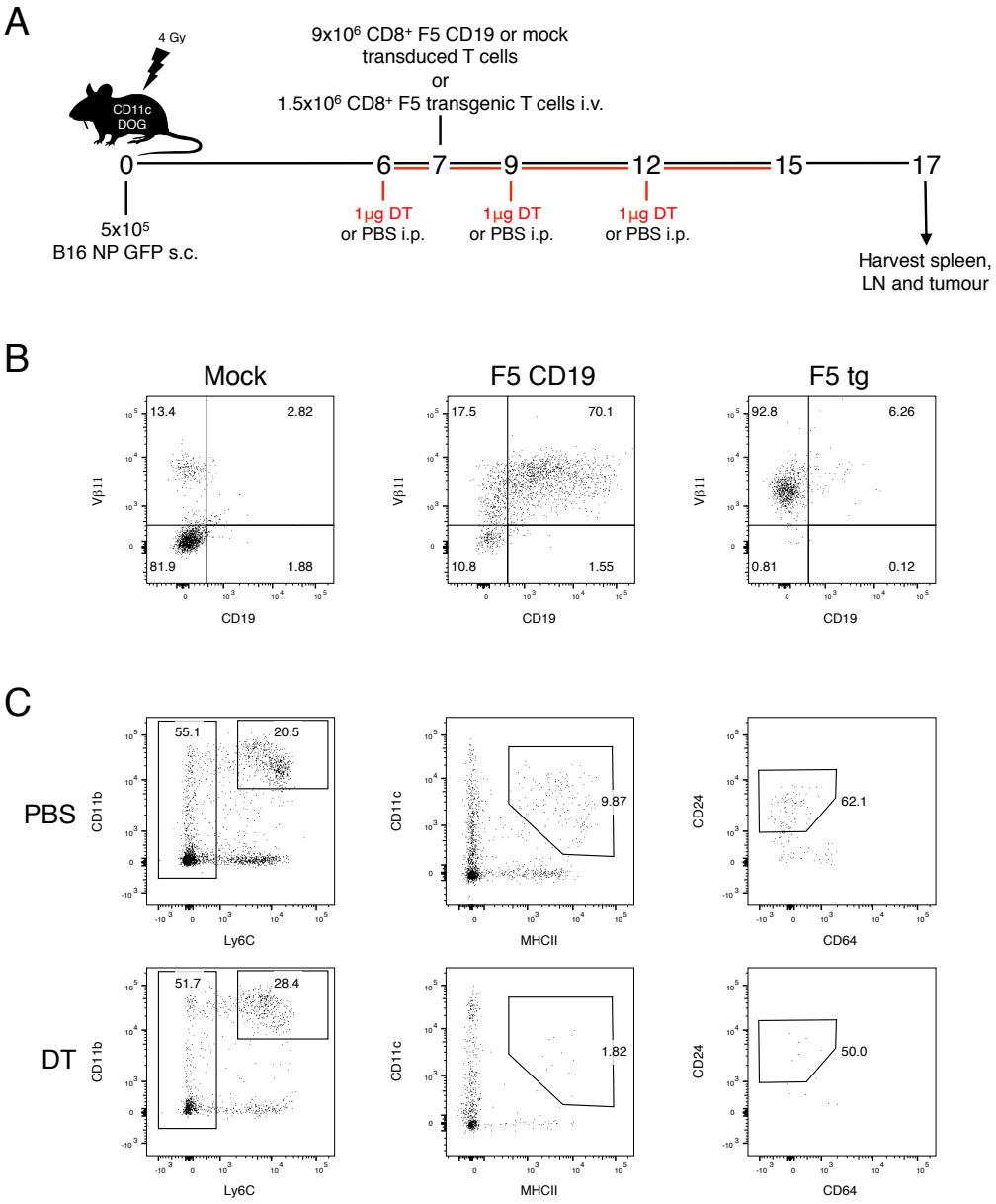


Figure 7.4: *Control of B16 NP GFP growth by transduced T cells after DC depletion.*
(A) Experiment outline. CD11c.DOG mice were injected with B16.F10 and treated with $9x10^6$ $CD8^+$ mock or F5 CD19 transduced T cells, or $1.5x10^6$ $CD8^+$ F5 transgenic T cells. One day prior and for 8 days after T cell injection, mice were injected with PBS or 1µg DT to deplete DC.
(B) TCR expression on mock, F5 CD19 transduced or F5 transgenic CD8 T cells on day 4 after transduction. Cells were pre-gated on $CD8^+$, live lymphocytes.
(C) Depletion of $Ly6C^-$, CD11c $MHCII^+$ APC in the tumours of DT injected mice. Cells were pre-gated on $CD45^+$, live cells.

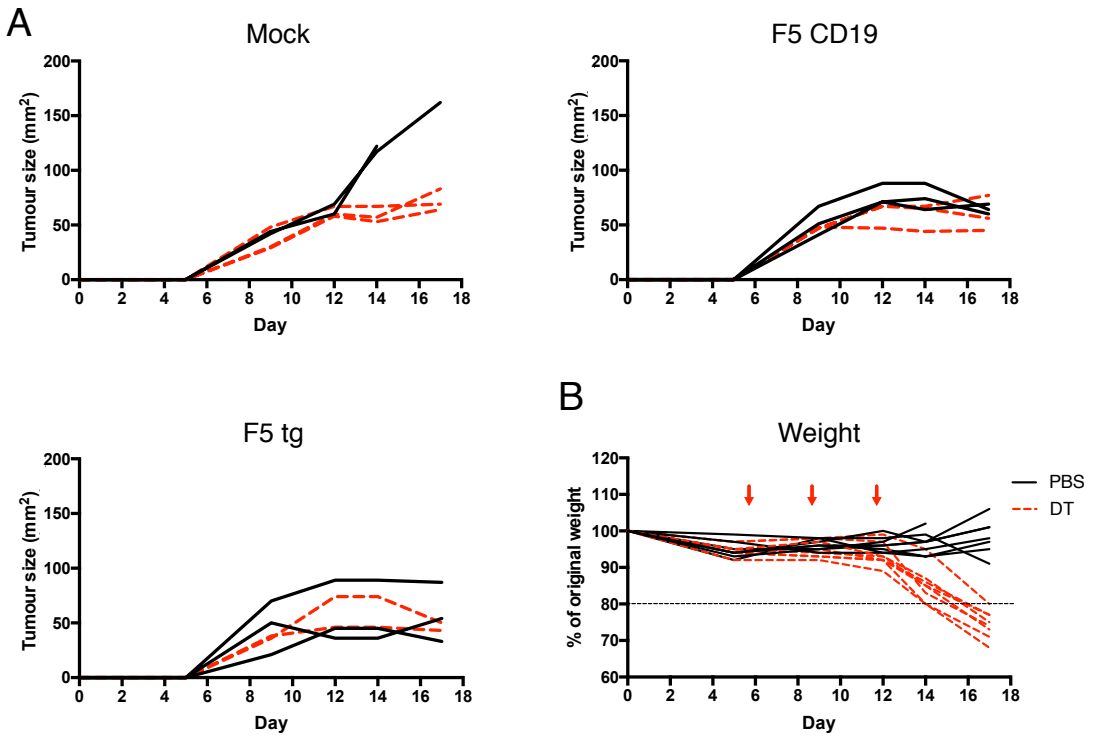


Figure 7.5: *Tumour size and weight change in mice treated with T cells after DC depletion.*
(A) Tumour size in mice treated with mock, F5 CD19 transduced or F5 transgenic T cells after PBS or DT injection.
(B) % weight change in all mice injected with either PBS or DT. Red arrows indicate PBS/DT injection.

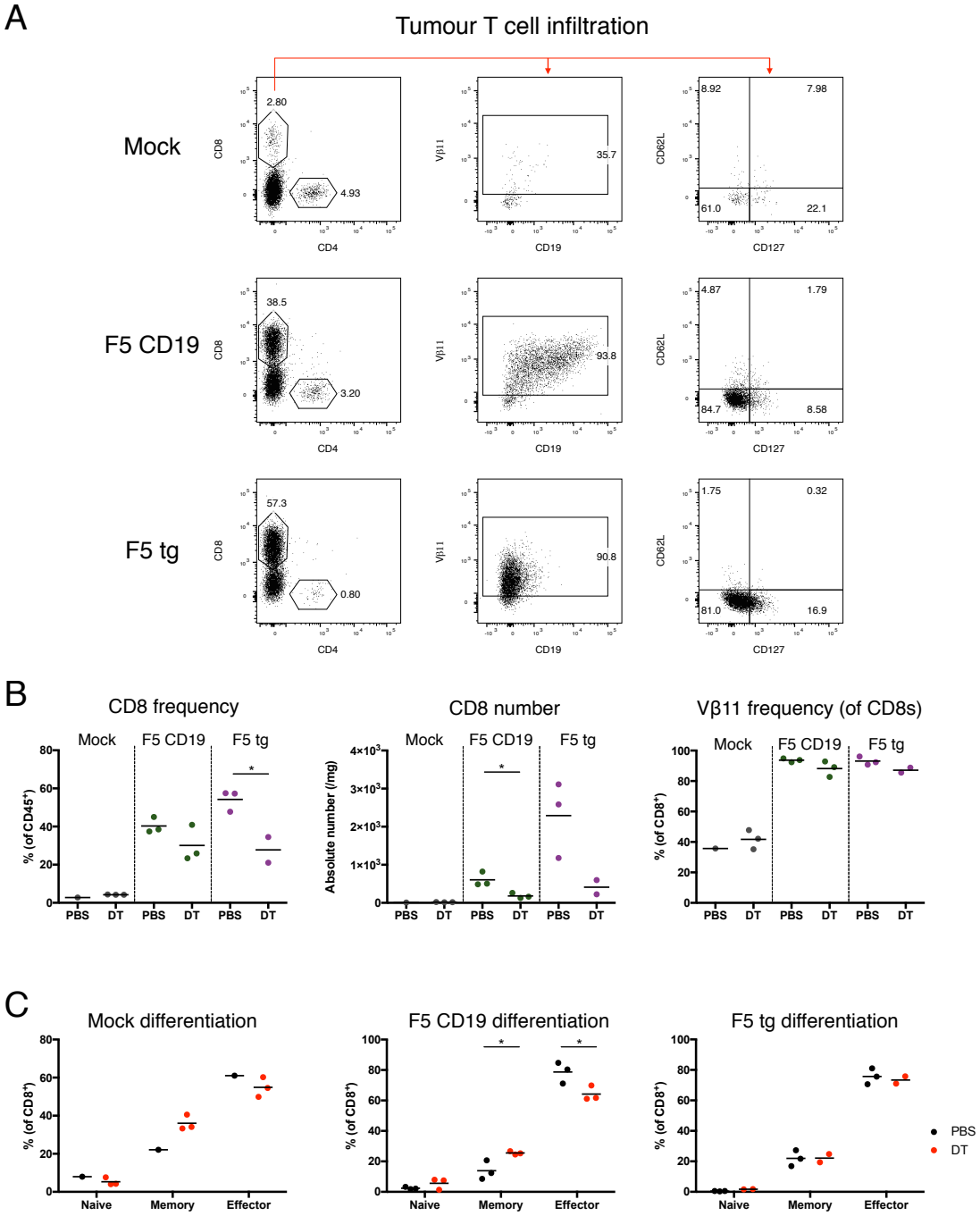


Figure 7.6: *T cell infiltration into tumours of DC depleted mice.*
(A) Representative FACS plots. $CD8^+$ T cells were gated on $CD45^+$, live lymphocytes.
(B) Summary data showing CD8 frequency, CD8 number and the Vβ11 frequency of $CD8^+$ cells, in mock, F5 CD19 transduced or F5 transgenic T cells after PBS or DT injection.
(C) Differentiation status based on expression of CD62L and CD127 in mock, F5 CD19 transduced or F5 transgenic CD8 T cells after PBS or DT injection. Statistical test: unpaired T test. *: $p \leq 0.05$.

7.3 Tumour control by transferred T cells after DC depletion

In order to get a more accurate assessment of tumour control in this model, the experiment was repeated but mice were injected with a lower dose of DT (650ng) to reduce DT-associated toxicity (Fig 7.7A). As before, CD11c.DOG mice bearing B16 NP GFP tumours, were treated with $9x10^6$ $CD8^+$ mock or F5 CD19 transduced cells, or $1.5x10^6$ $CD8^+$ F5 transgenic T cells. Vβ11 expression on day 4 after transduction is shown in Fig 7.7B. F5 CD19 transduced cells again showed a high level of transduction (79%), resulting in injection of ~$7.1x10^6$ antigen specific T cells. Despite the lower dose of DT used in this experiment, injection of 650ng DT resulted in similar depletion of tumour-resident $Ly6C^-$, CD11c $MHCII^+$ APC (Fig 7.7C).

The DT-mediated tumour control in mock transduced T cell treated mice noted previously was not evident when using this lower dose of DT (Fig 7.8A). In this setting, both mock treated groups failed to control tumour growth, regardless of DC depletion. As expected, F5 CD19 transduced T cells were able to offer significant tumour protection. Despite the previous experiment suggesting that DC depletion leads to fewer transduced T cells in the tumour, the ability to control tumour growth remained similar in DC depleted and non-depleted mice. However whilst some mice were able to control tumour growth until day 49, in others tumour control was lost after ~30 days as tumours rapidly regrew. In the PBS treated mice only 1/4 controlled the tumour, with the other 3 outgrowing. However in DT treated mice this was complicated by the return of DT associated toxicity in 2/4 mice treated, which had to be sacrificed by day 20 (Fig 7.7B). However of the 2 that survived, both were able to control tumour growth, and neither had subsequent tumour outgrowth. This suggests that although initial DC depletion led to fewer T cells in the tumour, there were still sufficient numbers to control tumour growth. As DC returned by day 15 however, the enhanced differentiation of memory T cells after DC depletion (as shown in Fig 7.6C) could aid long term tumour protection and prevent subsequent tumour outgrowth.

Surprisingly, mice treated with F5 transgenic T cells, showed relatively poor tumour control. Whilst they did receive much fewer cells than F5 CD19 treated mice, $1.5x10^6$ should be sufficient for tumour control. Of the 2 mice injected with PBS, one completely failed to control tumour growth, whilst the other showed mild tumour control. Of the 2 mice treated with DT, one had to be culled due to weight loss although it did appear to be controlling the tumour, whilst the other showed good tumour control until ~day 30 when the tumour outgrew. This was surprising as we expected unactivated cells to be dependent on DC for activation and tumour control. It may be that although DC were depleted at day 13 (Fig 7.7C), this was at the peak of DC depletion. DC depletion in this model is not complete so it may be that the few remaining DC were sufficient to prime F5 transgenic cells to induce tumour control. The F5 tg group in particular has only a few mice per group, so a larger repeat would be needed to confirm these data. However together these data suggest that although DC depletion results in less infiltration of T cells into the tumour, this is still sufficient to induce tumour control. Moreover in mice treated with F5 CD19 transduced cells whilst initially detrimental to the effector response, DC depletion may prime a T cell response typified by better memory formation and a more persistent, long-term anti-tumour response.

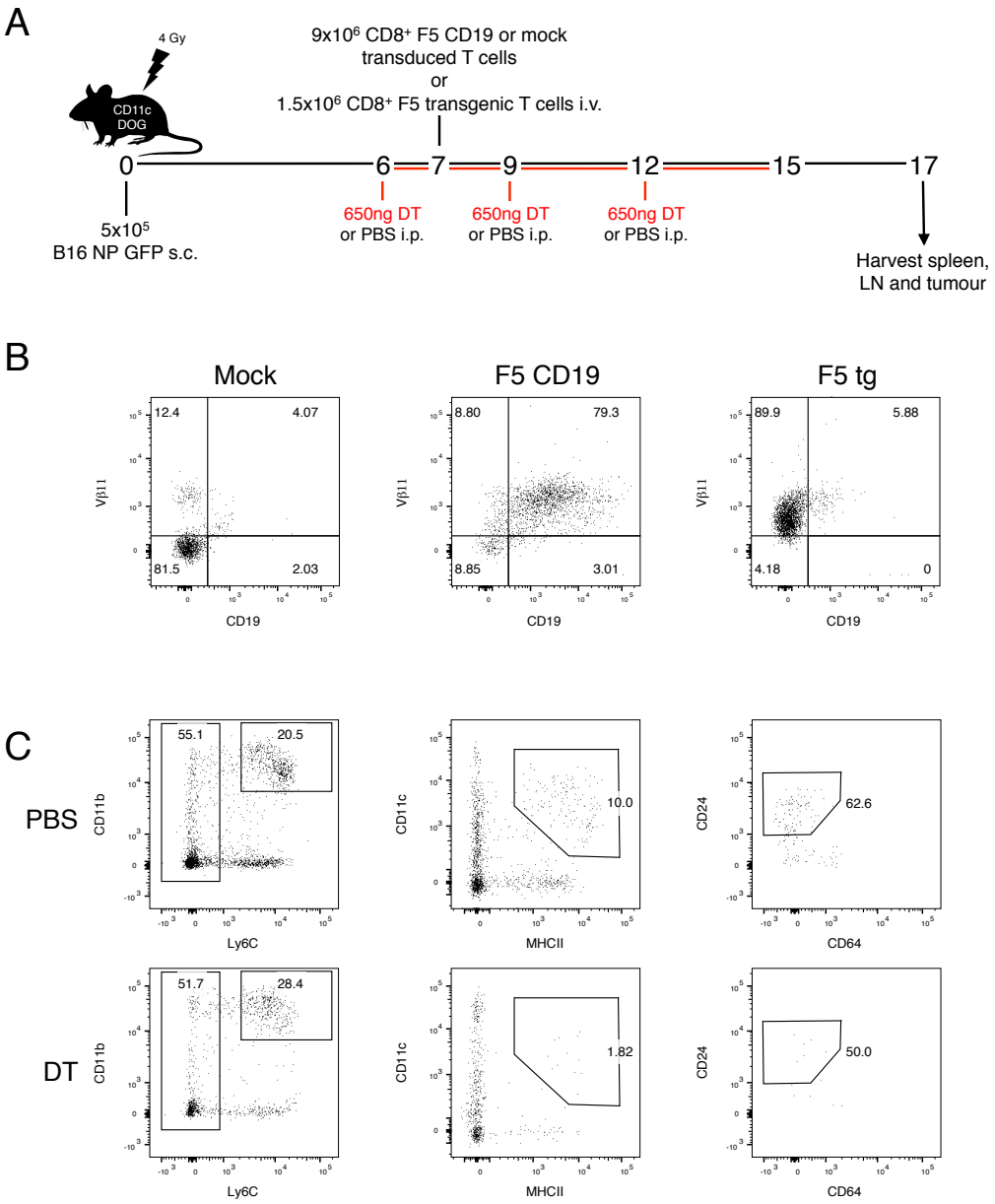


Figure 7.7: *Adoptive transfer of TCR transduced T cells after lower-dose DT injection.*
(A) Experiment outline. CD11c.DOG mice were injected with B16.F10 and treated with $9x10^6$ $CD8^+$ mock or F5 CD19 transduced T cells, or $1.5x10^6$ $CD8^+$ F5 transgenic T cells. One day prior and for 8 days after T cell injection, mice were injected with PBS or 650ng DT to deplete DC.
(B) TCR expression on mock, F5 CD19 transduced or F5 transgenic CD8 T cells on day 4 after transduction. Cells were pre-gated on $CD8^+$, live lymphocytes.
(C) Depletion of $Ly6C^-$, CD11c $MHCII^+$ APC in the tumours of DT injected mice. Cells were pre-gated on $CD45^+$, live cells.

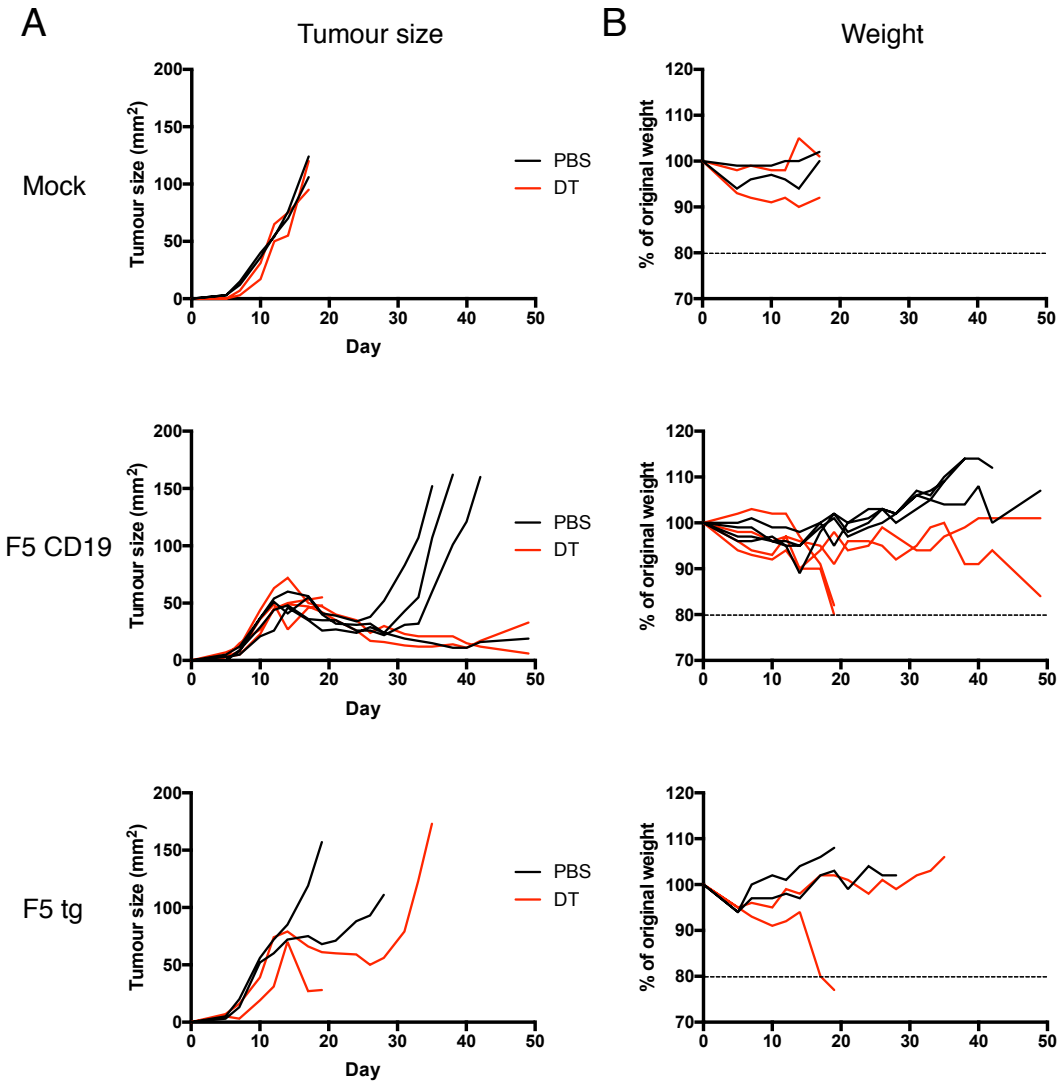


Figure 7.8: *Adoptive transfer of TCR transduced T cells in DC depleted mice.*
(A) Tumour size in mice treated with mock, F5 CD19 transduced or F5 transgenic T cells after PBS or DT injection.
(B) % weight change in mice treated with mock, F5 CD19 transduced or F5 transgenic T cells after PBS or DT injection.

7.4 Summary and conclusion

These data suggest that there are fundamental differences in the CD11c.DTR and CD11c.DOG models that are not explained by whether the mice are of a chimeric background or not. In contrast to the CD11c.DTR model, TRP2 CD19 transduced T cells do not accumulate or acquire a pronounced effector phenotype after DC depletion in the CD11c.DOG model. Indeed the opposite may occur, with transferred T cells in depleted CD11c.DOG mice being found in lower numbers and with a less effector phenotype than non-depleted mice.

This reduced response by TRP2 transduced T cells after DC depletion in CD11c.DOG mice was also true, and to a greater extent, in irradiated hosts treated with F5 T cells. However as the experimental models used in TRP2 and F5 treated mice differ, it is difficult to make direct comparisons between these responses or between the effect of DC depletion on T cells expressing a self- or foreign-antigen specific TCR. However taken together these data do suggest that as the proliferative capacity of transferred T cells increased, the effect of DC depletion on curbing T cell expansion became more pronounced. In line with this the unactivated, F5 transgenic T cells that greatly expanded *in vivo* appeared to be the most affected by DC depletion.

Of note however is that despite being pre-activated *in vitro*, the F5 CD19 transduced T cells still expanded less well after DC depletion. This suggests that transduced T cells do receive further activation signals from DC *in vivo*. However the details governing when and where DC interact with transduced T cells are still not clear. In addition, the nature of this interaction is also unclear as although DC were able to cause greater proliferation of transduced T cells, this did not seem to improve their effector function. This was shown by the similar tumour control mediated by F5 TCR T cells after DC depletion, despite these cells being fewer in number. Therefore in the absence of this interaction *in vivo*, activation of transduced T cells *in vitro* is sufficient to induce functional effector T cells that can control tumour growth. However due to the large amount of T cells used to control tumour growth in these experiments it may be that any

DC-mediated fine tuning of the T cell response is not noticed. For example as both F5 CD19 and F5 transgenic T cells were able to control tumour growth after DC depletion, this may reflect a T cell response that is already surplus to requirements and largely unaffected by reduced proliferation.

Lastly, F5 CD19 transduced cells may even be more effective at long-term tumour control in DC depleted rather than non-depleted mice. A possible explanation for this is that DC depletion appears to promote T cell acquisition of a memory phenotype. This may boost long term responses to the tumour as transferred central memory populations have been shown to persist long-term and induce improved T cell responses (Berger et al. 2008; Klebanoff et al. 2005). This hypothesis would fit with the linear model of T cell differentiation, where naive cells move from memory to effector phenotypes with increased antigen exposure. In this setting, antigen presentation by DC drives the proliferation and effector differentiation of naive/memory transferred T cells. However in mice in which DC are depleted, there is less antigen presentation *in vivo* and differentiation along this pathway is diminished. Transferred T cells still acquire effector function due to *in vitro* activation and antigen presentation from non-depleted DC, which is sufficient to mediate tumour control, however reduced exposure to antigen results in less terminally differentiated effector cells. These memory T cells are able to maintain tumour control for longer than short lived effectors and hence help prevent tumour outgrowth. However to confirm these hypotheses, the experiments presented above would need to be repeated.

Chapter 8 Discussion

Adoptive transfer of TCR transduced T cells is a promising field of tumour immunotherapy. However to translate pre-clinical work into an effective clinical treatment requires a greater understanding of the biology involved and how this can be exploited to improve T cell function. It has become clear that the tumour microenvironment is adept at subverting immune responses to avoid immune-mediated elimination. One of the mechanisms that tumours use to suppress anti-tumour T cell responses is to induce dysfunction of DC, which orchestrate this T cell response. DC have numerous potential roles in controlling T cell responses to tumours, including priming of naive T cells and regulating effector function *in situ*. However transduction of adoptively transferred T cells requires *in vitro* pre-activation, and little is known therefore about their requirements for interactions with DC. This project therefore aimed to understand the role that DC have in the context of tumour control by TCR transduced T cells. To achieve this we utilised two mouse models that allowed selective depletion of DC to interrogate their function after adoptive T cell transfer.

Due to their fundamental roles in promoting T cell responses, we hypothesised that depletion of DC would lead to an inferior T cell response in which cells proliferated less and exhibited weaker effector functions. However these experiments produced conflicting results. Contrary to this hypothesis, in the first depletion model (CD11c.DTR), DC depletion led to an enhanced accumulation of transferred T cells, which also acquired a less naive, more effector phenotype. Whereas T cells injected into CD11c.DOG mice conversely did not accumulate better after DC depletion and in fact were reduced in number. Interestingly though in this model neither DC depletion, nor the subsequent reduction in tumour infiltrating T cells affected the ability of these transferred T cells to control tumour growth. This section will discuss the implications of these findings in the CD11c.DTR model, then the CD11c.DOG model and finally examine possible differences between these models that could explain this contrasting effect.

8.1 CD11c.DTR model discussion

Contrary to our initial hypothesis we found that depletion of DC in the CD11c.DTR model was responsible for driving the enhanced accumulation of transferred T cells. Despite DC depletion in this model, the absolute number of T cells that infiltrated the tumour remained equal. However of the CD8 T cells in the tumour, a greater proportion of these were the TCR transduced, adoptively transferred T cells. Strikingly, significantly more of these transferred T cells that infiltrated the tumour, as well as those found in the spleen, had acquired an effector phenotype after DC depletion. However despite this enhanced accumulation, these cells were unable to provide tumour control. These data generated a number of different hypotheses that could explain this enhanced T cell response. These hypotheses included possible direct effects of DC depletion, but also the depletion of other $CD11c^+$ cells was addressed and both are discussed in more detail below.

8.1.1 Depletion of endogenous T cells

One caveat with this depletion model is that a wider range of cells are depleted than just DC. Of particular importance was the possible depletion of endogenous T cells which can express CD11c. We found that particularly in the tumour, endogenous T cells would upregulate CD11c expression and therefore be susceptible to depletion. This enhanced CD11c expression on the tumour-resident T cells corroborates with previous studies which have shown that activated T cells upregulate CD11c expression during viral infections (Beyer et al. 2005; Lin et al. 2003). This could therefore be important in experiments that aim to investigate the T cell responses to DC depletion. For example depletion of endogenous, activated T cells in a model of tumour control by transferred T cells may have an important bearing on the results. However the impact of the endogenous T cell response on tumour control in this setting is not fully understood. In the clinic the adoptive transfer of TILs does not seem to promote new T cell specificities from non-transferred T cells (Kvistborg et al.

2012). However 'epitope spreading' has been shown to induce novel B cell responses to tumours after CAR-T cell therapy (Beatty et al. 2014). In our model the absence of tumour control by either endogenous or transferred T cell limited the effect of endogenous T cell depletion. However this could still have enhanced transferred T cell proliferation by removing a 'cytokine sink'. To address this hypothesis we therefore utilised a DC depletion model in which endogenous T cells were not depleted. In the CD11c.DTR x RAG-1$^{-/-}$ BM chimeras, the only T cells present are those that reconstitute from the WT recipient, whereas the DC will still express the DTR. In this setting a similar expansion of transferred T cells was seen, certainly in terms of the frequency, if not necessarily in the absolute number. This suggested that this accumulation of transferred T cells was not dependant on endogenous T cell depletion.

However other aspects of the CD11c.DTR model were not fully accounted for, and these could have had an effect on T cell expansion. For example although DT injection did not deplete NK cells in the spleen or LN, they were depleted in the tumour. Interestingly, as with the endogenous T cells, this depletion seemed to correlate with upregulation of CD11c on the tumour-resident NK cells. However the effect of this NK cell depletion on the T cell response was not addressed in this study. NK cells are traditionally thought of as being part of the innate immune response that mediate tumour immunesurveillance (Waldhauer and Steinle 2008), however NK cells have also been shown to have a diverse range of functions, including the direct lysis of activated T cells (Pallmer and Oxenius 2016). It is therefore possible that the DT-mediated depletion of NK cells in the tumour prevented lysis of transferred T cells. As the NK cell depletion was minimal however, this was not directly assessed in this study, although further experiments could use NK cell depleting antibodies to equalise the depletion seen in DT-injected animals.

8.1.2 Reconstitution of a pro-inflammatory DC subset after transient depletion

As the enhanced T cell accumulation occurred in the absence of endogenous T cell depletion, we investigated whether the DC that return after depletion were capable of promoting this response. Isolated $CD11c^+$ cells from the tumours of transiently depleted mice did seem to show an enhanced ability to cross-present tumour antigen *ex vivo*, although this was not clear. This would be in line with data that shows the tumour produces immunosuppressive cytokines such as VEGF, M-CSF, IL-10 and IL-6 which prevent DC maturation (Gabrilovich 2004). In this setting the reconstituted DC would have been exposed to this immunosuppressive milieu for a shorter time than tumour-resident DC. Although PD-L1 expression was similar between tumour-resident and reconstituted DC, further analysis of other functional markers such as CD80/86 could prove informative. In addition, isolation of DC at different time points after reconstitution and therefore after varying exposure to the tumour may reveal phenotypic differences.

However the importance of these reconstituted DC in this tumour response is questionable as the enhanced accumulation of transferred T cells was prevalent when DC were depleted throughout the experiment. This suggests that DC depletion at the time of T cell transfer, rather than later reconstitution is the driving factor behind the enhanced accumulation of transferred T cells. However this continuous DC depletion also seemed to improve the transferred T cell response, more-so than transient depletion. This suggests that in this model, the DC have an inhibitory effect on the transferred T cells and by depleting these DC, the transferred T cells are de-regulated. However although DC depletion at the time of T cell transfer appeared to be important, DC were not depleted at different times during the transferred T cell response to test this. This could have an important impact on the subsequent effect as DC have been shown to have variable functions depending on the stage of tumour growth. For example in an inducible ovarian cancer model, during the earlier phases of tumour

development, DC promoted anti-tumour T cell responses and prevented rapid tumour growth. However later the phenotype of these tumour-infiltrating DC changed due to the immunosuppressive tumour environment, and this switch correlated with enhanced tumour growth (Scarlett et al. 2012). In the melanoma model described here it appears that DC are at this suppressive phase by the time of T cell transfer, although DC depletion at different time points after tumour inoculation could reveal different roles.

8.1.3 Depletion of a 'regulatory DC' subset

These data therefore suggested that the depletion of DC itself was responsible for the enhanced accumulation of transferred T cells. A regulatory function of DC is in line with the evidence that tumour-resident DC are often dysfunctional. This is as a result of tumours inhibiting the proper differentiation or maturation of DC in the tumour (Pinzon-Charry et al. 2005). This would explain why depletion of these regulatory DC would promote T cell responses in the tumour. However this is difficult to test directly because, as of yet, a specific regulatory DC population has not been identified. Nonetheless to gain a better understanding of the efficiency of DC depletion in the tumour we had identified various DC subsets before and after DC depletion. Although DT injection in these mice should deplete cDC equally we found that $CD103^+$ were preferentially depleted in the tumour following DC depletion. These cells are important for cross-presenting antigen to T cells in lymphoid organs as well as controlling T cell effector function in the tissue (Broz et al. 2014; Salmon et al. 2016). However this role is at odds with the regulatory role predicted of the DC in our model. This suggests either that their role in tumour control can vary or that a true 'regulatory DC' subset is depleted as well as these $CD103^+$ DC, which has been missed in this analysis. However this seems unlikely as 'regulatory DC' is instead thought to describe DC of various subsets that are tolerogenic because they are phenotypically immature, rather than a separate population of specialised regulatory DC (Reis E Sousa 2006). Therefore to investigate whether a regulatory DC population is depleted in the tumour, a more informative question may be to assess the maturation state of

those DC which are depleted, rather than their specific subtype. This could be assessed by staining for inhibitory receptors such as PD-L1, or co-stimulation molecules such as CD80/86, as well as measuring functional ability to promote T cell expansion *ex vivo*.

Depletion of regulatory DC could enhance the accumulation of transferred T cells by enhancing the proliferation. DC depletion did seem to enhance Ki67 expression on transferred T cells in the spleen, however Ki67 expression was saturated in the tumour. To gain a better understanding of cell proliferation these experiments could be repeated by transferring CFSE labelled T cells and measuring dilution. Interestingly despite DC depletion transferred T cells were still able to proliferate considerably. This proliferation was centred on the TCR-transduced population that infiltrated the tumour, strongly suggesting that it was dependant on tumour-antigen. It is unclear what population of APC is presenting this antigen however as the cDC should be depleted. Either a population of non-depleteable APC remain after DC depletion, or alternatively DC depletion is incomplete and remaining DC are able to present antigen. Certainly whilst depletion of $CD24^+$ DC reached >90% by day 19, depletion at the day of T cell transfer was only ~50%. Repeated DT injections before T cell transfer may enhance the DC depletion at this time and influence the proliferation of transferred T cells differently, although this has not been tested. However although transferred T cells may have an enhanced proliferation, they may alternatively be accumulating through improved survival.

8.1.4 Improved survival of transferred T cells

As the effect of DC depletion on T cell proliferation was inconclusive, an alternative explanation may be that transferred T cells undergo less cell death and so appear in higher frequencies. DC have been shown to enforce peripheral tolerance by presenting self-antigen and inducing either deletion or anergy of self-reactive T cell clones (Steinman and Nussenzweig 2002; Xing and Hogquist 2012). This mechanism can also be subverted by the tumour; DC dysfunction, rather than a specific loss of tumour immunogenicity, can be the primary cause

of tumour immune escape (Scarlett et al. 2012). Depletion of DC could result in reduced antigen presentation and therefore a reduction in activation induced cell death (AICD). This has been shown to limit T cell responses in tumours by inducing apoptosis either by Fas/FasL interactions (Zaks et al. 1999) or through a Bcl-2 regulated pathway (Strasser and Pellegrini 2004). In line with this hypothesis is that the population of transferred T cells that accumulate in the tumour and spleen after DC depletion have an enhanced effector phenotype. It is therefore possible that in this setting, reduced exposure to antigen after DC depletion leads to reduced AICD. These effector cells can therefore accumulate without being deleted.

Although this would seem to fit with the data presented here, whether T cells are deleted or anergised is determined by a combination of the level of antigen available (Redmond et al. 2005) and the avidity of the TCR to that antigen (Smith et al. 2014). CD8 T cells that receive a higher level of TCR signalling are more prone to anergy than deletion. Therefore in this model, after DC depletion there would be fewer DC to present self antigen to the transferred T cells, which would therefore receive less TCR signalling. However this should induce anergy rather than deletion in those transferred T cells. Nonetheless, the question remains over the mechanism of transferred T cell accumulation. In the absence of convincing data suggesting enhanced proliferation, the effect of DC depletion on T cell survival could be addressed. Analysis of factors such as Annexin V expression, PI incorporation, expression of FasL and activation of caspases can all be measured in the transferred T cells.

8.1.5 Radio-resistant DC promoting T cell expansion

As mentioned earlier, tumour-resident T cells were still able to proliferate in response to antigen, suggesting that some antigen-presenting function remained after DC depletion. Whilst this could be due to incomplete depletion of DTR expressing DC, alternatively a population of non-DTR expressing DC could be present. A population of radio-resistant DC in the dermis have been identified that are maintained after bone marrow transplantation (Bogunovic et al. 2006).

Unlike other DC populations, these have been shown to proliferate *in situ*. These cells could contribute to the enhanced T cell response seen in chimeric mice as they would be of WT origin and hence resistant to DC depletion. After DTR-sensitive cells are depleted these dermal DC could expand and compensate for the depleted DC. Dermal DC are an important cell for cutaneous immunity as they can acquire antigen and migrate to LN (Kissenpfennig et al. 2005). They are also able to initiate contact hypersensitivity in the absence of epidermal Langerhans cells (Bennett et al. 2005; Kaplan et al. 2005). However the impact of these DC on tumour immunity is unclear. Of note however is that a previous study using the CD11c.DTR model had found that whilst DC depletion abrogated lymphopenia driven expansion of T cells in CD11c.DTR mice, this was not true of CD11c.DTR BM chimeras (Zaft et al. 2005). This suggests that the effect of DC depletion in chimeras can be limited compared to non-chimeric mice.

To investigate whether these radio-resistant dermal DC of WT origin infiltrate the tumour, bone marrow chimeras could be made where host and transplant DC express separate congenic markers. Alternatively, unlike WT DC, DTR-expressing DC should also be GFP positive. However this expression may be more variable than a congenic marker. In this study we attempted to address whether the perseverance of WT DC after DT depletion was driving T cell expansion by utilising the CD11c.DOG model, in which similar experiments could be performed without making chimeras. However regardless of whether they were chimeric or not, experiments in these mice failed to repeat the T cell accumulation shown in the CD11c.DTR model. This suggests that the chimerism was not a driving factor, although this is not conclusive as other differences between these models could have affected the results. It would be interesting to assess whether a single injection of DT before T cell transfer is sufficient to increase T cell accumulation. If so then comparison between chimeric and non-chimeric mice could be performed in CD11c.DTR mice, using a single dose of DT to avoid toxicity. This would ensure the DTR model used is the same and remove the confounding factor of the different DTR models. The experiments that were performed in the CD11c.DOG models were informative

however and are discussed in more detail in the next section.

8.2 CD11c.DOG model discussion

Whereas DC depletion in the CD11c.DTR model was responsible for promoting transferred T cell accumulation, this was not the case in the CD11c.DOG model. DC depletion in these mice provided no such benefit. Indeed collectively the data suggested that fewer transduced T cells infiltrated the tumour after depletion in the CD11c.DOG mice.

To investigate whether this impaired expansion of transferred T cells would impact on their ability to control tumour growth in this depletion model we had to change the tumour model. This was because in the absence of pre-conditioning irradiation, TRP2 transduced T cells were incapable of controlling the tumour. The adoptive transfer of T cells into a lymphopenic environment has been shown to cause T cell proliferation and is important for tumour control (De Witte et al. 2008a). However T cell proliferation in these conditions requires $CD11c^{high}$ DC (Zaft et al. 2005). Therefore depletion of DC would need to be separated from irradiation to ensure the effect of DC depletion on homeostatic expansion is minimised. We therefore made use of F5 TCR transduced T cells which are able to mediate tumour regression with minimal pre-conditioning irradiation. The added benefit with this model was that the previous B16.F10 tumour model could be transduced with the NP antigen, and be recognised by F5 T cells. This therefore allowed these experiments to be performed using essentially the same basic tumour cells.

Unfortunately in these experiments, the high dose DT injection in the CD11c.DOG animals led to toxicity after the 3rd injection, presumably due to a build up in DT. When the dose of DT was reduced in the subsequent experiment, animals showed little toxicity. Data from the high-dose DT however showed that tumour antigen specific, introduced T cells expanded less well after DC depletion. However even after DC depletion, a large number of tumour-specific T cell still infiltrated the tumour. In line with this data, the subsequent experiment showed that these T cells that infiltrate the tumour after DC depletion are still able to mediate reliable tumour control. As in the CD11c.DTR model, this either

suggests that DC depletion is incomplete or that transduced T cells don't require interaction with DC to expand and infiltrate the tumour. Mice in these experiments received a high number of transduced T cells so the reduction in expansion seen previously may be irrelevant on this scale. It would therefore be interesting to titrate the number of T cells transferred down, to assess whether DC depletion has a noticeable effect on tumour control when the T cell response is more limited.

8.2.1 Comparison between transduced and transgenic T cells

Of note in these experiments however was that the B16 NP GFP tumour model allowed the comparison between F5 transduced and F5 transgenic T cells. This allowed us to investigate whether the activation of transduced cells *in vitro* diminished their need for DC interaction *in vivo*. As expected the requirement for priming *in vivo* was much greater for the unactivated, naive, transgenic T cells than for transduced T cells. However as for the F5 transduced T cells, a small amount of these cells did infiltrate the tumour, but this was much reduced when compared to DC replete mice. Interestingly, these cells seemed able to induce tumour control, even after DC depletion. However the results from this experiment would need to be repeated especially as so few mice were used. Taken together these data suggest that naive F5 transgenic cells and to a lesser extent, activated F5 transduced T cells, require DC interaction *in vivo* for optimal T cell expansion. However even with sub-optimal expansion, F5 transduced and possibly F5 transgenic T cells can mediate substantial tumour control after DC depletion.

Although F5 transgenic T cells were less expanded after DC depletion, their differentiation profiles were similar. This suggests that the DC that remain after depletion are able to induce differentiation of naive T cells into effector T cells *in vivo*. In contrast pre-activated F5 transduced T cells displayed a more memory, less effector phenotype after DC depletion. This might suggests that the

requirement for DC interaction in regards to controlling differentiation might vary between naive and activated T cells. Effector T cells are often thought of as terminally differentiated and therefore acquisition of this phenotype reduced proliferative capacity. Adoptive transfer of less differentiated cells is associated with improved anti-tumour efficacy (Hinrichs et al. 2009; Klebanoff et al. 2005). It is possible therefore that the reduced exposure to antigen after DC depletion limits the differentiation of adoptively transferred T cells. These therefore are more likely to form a memory population, as is seen in the data for the F5 transduced T cells.

The impact of this enhanced memory formation of transferred T cells may also be shown in the better long-term efficacy of tumour control after initial DC depletion. Although 3/4 tumours regrew after the tumours escaped immune control in the PBS treated mice, 0/2 of the DT treated mice that survived DT injection had tumours that escaped. The enhanced ability of these memory cells to proliferate and differentiate into effector cells may have prolonged immune control. Of note is that we had previously shown that the loss of immune control in this model was not through a specific loss of tumour immunogenicity such as a down-regulation of NP expression, as has been described in similar B16 tumour models (De Witte et al. 2006). It is therefore likely that tumour escape was either driven by a lack of persistence of the transferred T cells *in vivo* or by a shift in the tumour-resident DC phenotype as has been described during the development of a separate tumour model (Scarlett et al. 2012). Although this occurred after DC had reconstituted, the previous depletion of these cells may affect the ability of the tumour to induce later dysfunction. As in the CD11c.DTR model, depletion of DT at different time points may be revealing. It is possible that in this model DC depletion at the time of transfer limits T cell expansion, although there were sufficient T cells to mediate tumour control nonetheless. However as the tumour subverts the DC response and escapes immune control, DC depletion at this point may aid the continued control of tumour growth by transferred T cells. Alternatively the infiltration of transferred T cells in the tumour and their presence in the lymphoid organs would show whether tumour escape was instead driven by a failure of these cells to persist long-term *in vivo*.

8.3 Differences between the CD11c.DTR and CD11c.DOG models

Although ostensibly both the CD11c.DTR and CD11c.DOG models deplete $CD11c^{+/high}$ DC, there are clear differences in the response of the transferred T cells to DC depletion. The likely reason behind this is that DT injection in these mice have different patterns of depletion. In this study as with previous results using these mice, both models induce significant and similar depletion of $CD11c^{+}$ cDC in the spleen. However depletion in the tumour in either of these models had not been well documented. We have shown that in the CD11c.DTR model, DT injection induces the specific depletion of the $CD24^{+}$ DC in the tumour, particularly the $CD103^{+}$ population. It is unclear why $CD103^{+}$ DC1 are favourably depleted over $CD11b^{+}$ DC2, as both populations express similar levels of CD11c. However analysis of GFP expression suggested that whilst CD11c expression was closely related to GFP (and therefore DTR) expression in the homogenous $CD103^{+}$ DC, CD11c was often expressed on $CD11b^{+}$ DC without GFP expression. This suggests that these heterogenous DC2 are able to express their endogenous CD11c without expressing the DTR transgene. This should normally be controlled by the CD11c promotor, however in the CD11c.DTR mice, transgene expression is not additionally regulated by the CD11c associated enhancers and silencers (Jung et al. 2002). This suggests, as also suggested by the data from $CD11b^{+}$ DC, that DTR expression in CD11c.DTR mice is not as tightly regulated by CD11c expression as first thought. Of note is also that in these mice, aberrant DTR expression on non-haematopoetic tissue leads to lethality in mice after multiple DT injections (Zaft et al. 2005).

In contrast the CD11c.DOG mice made use of BAC transfer to transfer a larger amount of DNA containing the DTR transgene inserted into the whole CD11c gene. DTR expression in these mice is therefore controlled by expression of the CD11c promotor as well as the related control regions. Whereas these mice still have a separate CD11c gene that could be expressed differently from the DTR,

this is much less likely, due to this tighter restriction of DTR expression to $CD11c^+$ cells. Possibly due to this different control of DTR expression, DC depletion in the tumour of CD11c.DOG mice is different from in the CD11c.DTR model. Seemingly at odds with this more regulated DTR expression, depletion in the CD11c.DOG mice favours depletion of the broad $CD11c^+$ cells in the tumour, rather than specifically depleting $CD24^+$ DC. The reason why depletion in the CD11.DTR and CD11c.DOG mice differs is ultimately unclear, however it is reasonable to suggest that the differences in response to depletion from transferred T cells in these models stem from this difference in depletion.

However there are other factors that separate these models which have not been addressed. Whilst we have carefully evaluated the effect of DT injection of other $CD11c^+$ cells such as T cells and NK cells in the CD11c.DTR mice, this has not yet been characterised in the CD11c.DOG mice. Although depletion of these other cells seemed to have a minimal effect on the transferred T cell response, this effect may vary in the CD11c.DOG mice. Properly accounting for these various accessory effects after DT injection is of vital importance to generating reliable data using these depletion models. It will therefore be a useful exercise to properly document how other $CD11c^+$ cells are affected by DT injection in the CD11c.DOG model.

Lastly, regardless of DT injection, the LN of CD11c.DTR, but not CD11c.DOG mice show reduced accumulation of DC and an overall hypocellularity (Blijswijk et al. 2015). This was not directly measured in these experiments, however it could have had an impact on transferred T cells in these models. It is unclear how this would explain the difference in T cell response after depletion between these two models, however it does provide further evidence that the CD11c.DTR and CD11c.DOG models are not necessarily interchangeable.

8.4 Further work

Taken together these data show that DC do have a role in interacting with transduced T cells *in vivo* however this role is varied and is likely to encompass both stimulatory and regulatory effects. Identifying the mechanism behind the enhanced T cell accumulation after depletion in the CD11c.DTR model would be vital to determining more about the role of DC in this setting. The experiments mentioned previously, such as identifying whether T cells proliferate more or survive better would be important. Likewise identifying either the subtype of DC, or what state of maturation the DC that are depleted are would be essential to identifying which DC are responsible for this regulatory function in the tumour. In addition although this project has suggested that DC do interact with transferred T cells, the nature of this interaction is unclear. The enhancement of this accumulation in TCR-expressing T cells suggests that this interaction is dependant on antigen presentation. However the importance of this can be judged by transferring T cells into $MHCI^{-/-}$ mice. Alternatively, if co-stimulation (signal 2) or cytokine production (signal 3) is important then the use of inhibitors of these interactions could be informative. This could be achieved through specific gene deletions such as mice that lack CD80/86 expression, or by transferring transduced T cells that can't respond to these signals, such as those that lack expression of IL-12R. These experiments could shed some light of the mechanisms behind the DC-transferred T cell interaction.

Likewise although these experiments have been performed in models that deplete broad cDC populations, other models exist in which specific DC subsets are depleted. These could be used to assess whether the effect of DC depletion in the CD11c.DTR/DOG models is similar to when certain subtypes are depleted. For example the importance of depleting the $CD103^{+}$ DC1 in the CD11c.DTR model could be assessed through use of either the zDC.DTR model which has been shown to deplete tumour resident DC1 (Broz et al. 2014) or through use of *Batf3*$^{-/-}$ mice, which constitutively lack CD8/$CD103^{+}$ DC.

As the use of TCR transduced T cells for cancer immunotherapy becomes more

widespread the need to understand why this approach is effective in some patients but not in others becomes more important. A solid understanding therefore of the ways in which these *in vitro* transduced T cells interact with the host immune system is vital. DC are therefore of obvious interest due to their ability to activate, control and regulate T cell immunity. In line with these various functions, the interactions of transduced T cells with DC *in vivo* appears to display a similar level of diversity. However this study has suggested that these cells do interact *in vivo*, further work however will be needed to determine the exact nature of these interactions and how they can be best exploited to improve the anti-tumour response of transferred T cells.

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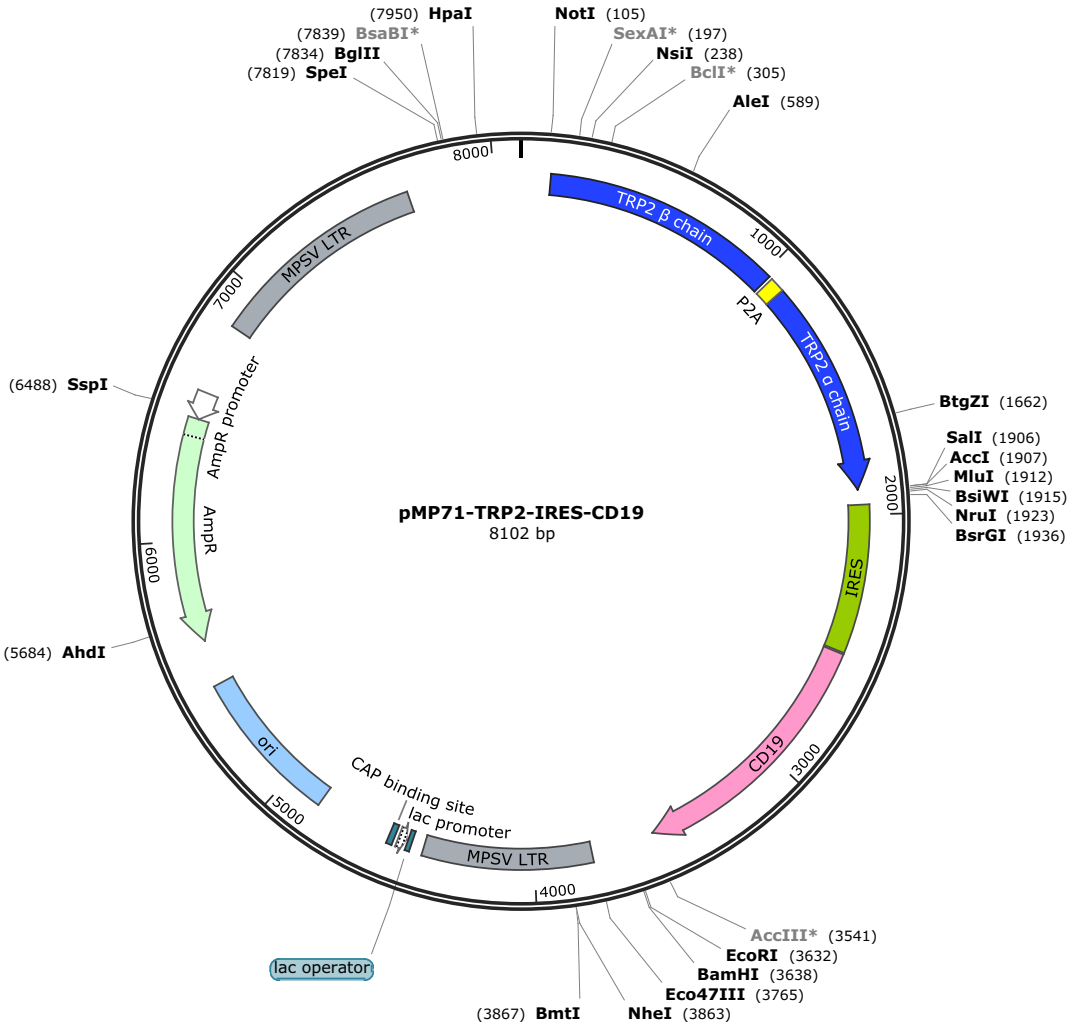
Appendix

pMP71 TRP2 IRES CD19 retroviral construct



TRP2 TCR

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 781 ACCAGCGCCA GCTACCACCA GGGCGTGCTG TCCGCCACCA TCCTGTACGA GATCCTGCTG
 841 GGCAAGGCCA CACTGTACGC CGTGCTGGTG TCCGGCCTGG TGCTGATGGC CATGGTGAAG
 901 AAGAAGAACA GCAGCGGCAG CGGCGCCACC AACTTCAGCC TGCTGAAGCA GGCCGGCGAC
 961 GTGGAGGAAA ACCCTGGGCC CATGAGGCCC GTGACCTGCA GCGTGCTGGT GCTGCTGCTG
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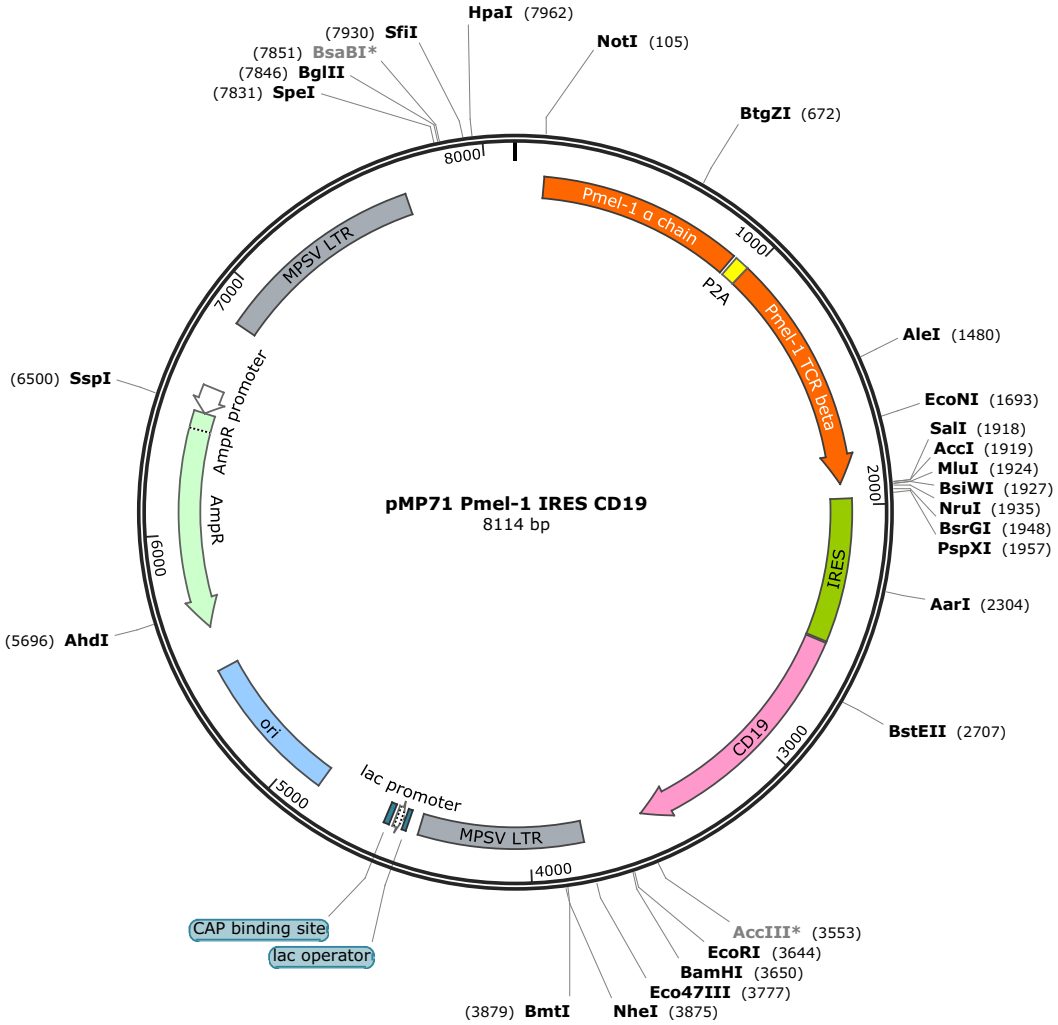
pMP71 Pmel-1 IRES CD19 retroviral construct



Pmel-1 TCR

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 121 CTGAACTGCA CCTTCAGCGA CAGAAGCAGC GACAACTTCA GGTGGTACAG GCAGCACAGC
 181 GGCAAGGGCC TGGAGGTGCT GGTGTCCATC TTCAGCGACG GCGAGAAGGA GGAGGGCAGC
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 961 CCCAGATACG CCGTGCTGCA GGAGGGCCAG GCCGTCAGCT TTTGGTGCGA CCCCATCAGC
1021 GGCCACGACA CCCTGTACTG GTATCAGCAG CCCAGGGACC AGGGCCCCCA GCTGCTGGTG
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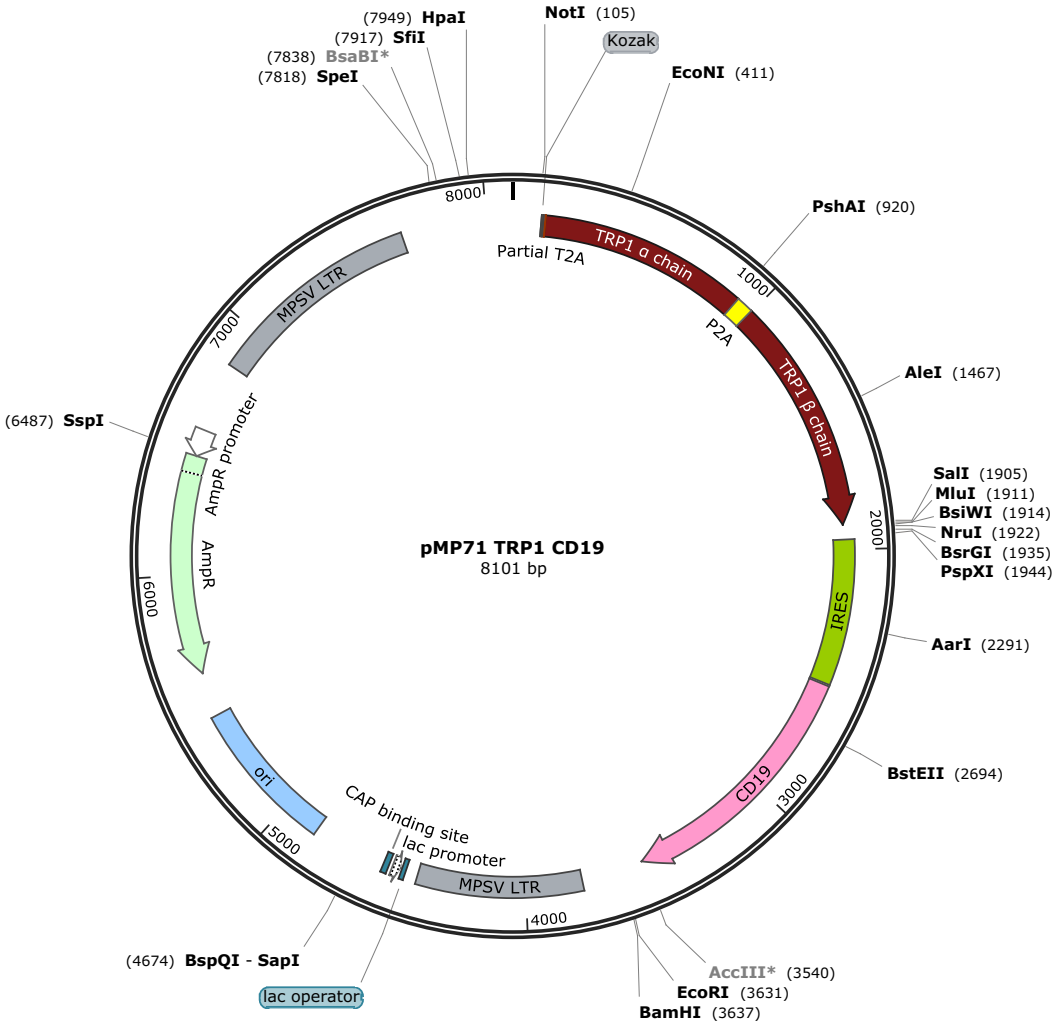
pMP71 TRP1 IRES CD19 retroviral construct



TRP1 TCR

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 841 GCAGGAGACG TGCAAGAAAA CCCCGGTCCC ATGCTGTACT CCCTGCTGGC TTTCCTGCTG
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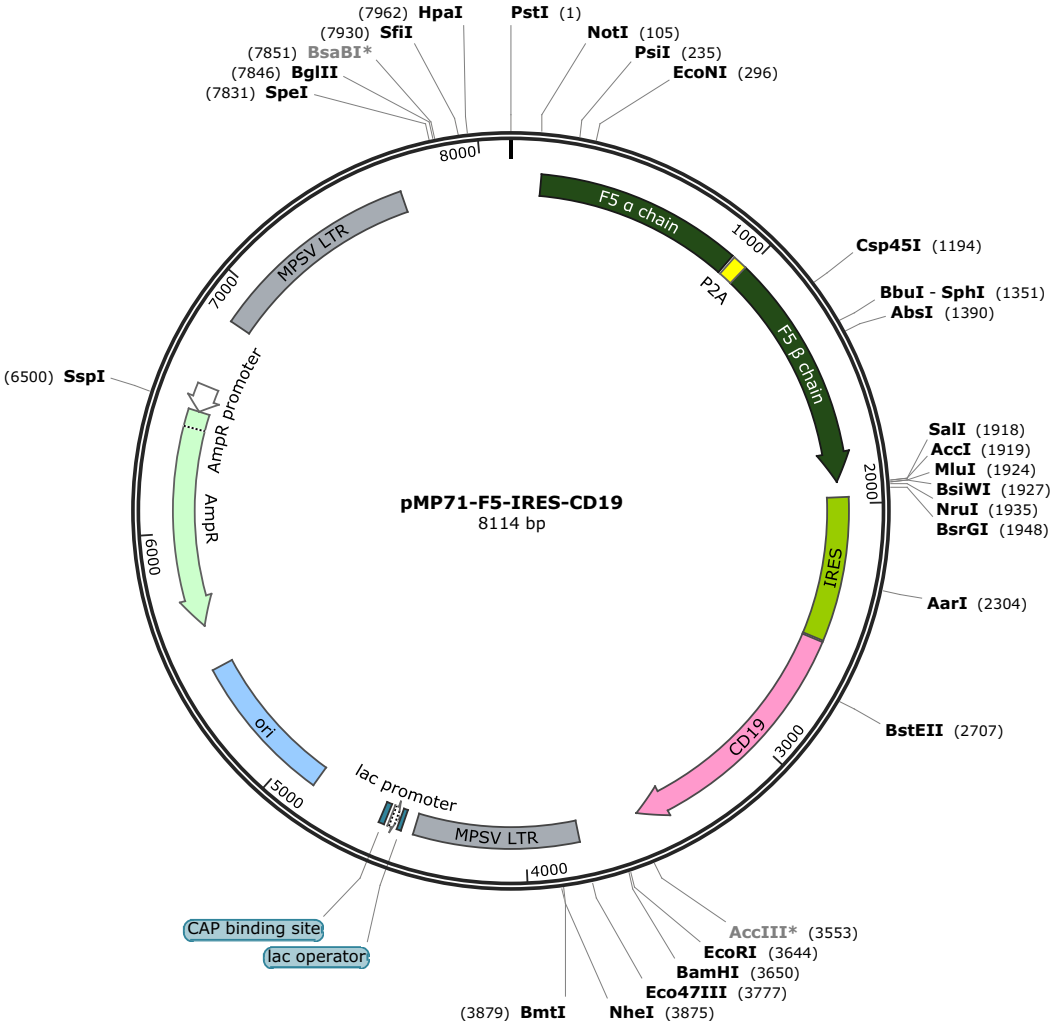
pMP71 F5 CD19 IRES retroviral construct



F5 TCR

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