T cell cancer immunotherapy: Characterisation and manipulation of tumour antigen-specific T cell subsets for adoptive immunotherapy in mouse models

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Matthew White

I dedicate this thesis to my friends and family particularly my mother, Marie Butterworth, my father, John Rooney and my devoted partner Dee Townsend. I thank you for the years of inspiration and motivation that have culminated in the submission of this thesis for the degree of Doctor of Philosophy.

Abstract

Understanding the behaviour of T cells in context of tumour responses is crucial for designing adoptive immunotherapy strategies. The aim of the study is to characterise tumour-specific T cells, including conventional and regulatory T cells (Tregs), in tumour-bearing mice after adoptive transfer. Male-specific minor histocompatibility antigen HY was used as the tumour-associated antigen, expressed by MB49 murine bladder carcinoma and recognised by TCR transgenic CD4 (Marilyn) and CD8 (Matahari) T cells. Unlike male skin grafts, MB49 tumours express a panel of immune-suppressive molecules including PD-L1, FAS-L, TGF-β, IL-10 and IDO which contribute to the formation of a tolerogenic microenvironment. As a result, HY-specific CD8 T cells reject syngeneic male skin grafts but not MB49 tumours. In response to MB49 tumours, HY-specific Tregs underwent expansion**.** A fraction of proliferating Tregs also lost FoxP3 and became ex-Tregs, which upregulated IFNγ, and downregulated a panel of Treg-specific genes. In addition**,** it was observed that preferential ex-Treg differentiation took place in an IL-6-enriched microenvironment, such as in the mesenteric lymph nodes. The antigen-specific CD8 response to MB49 is insufficient for rejection. Retroviral modification of MB49 to express hIL-2 allows for induction of effective antigen-specific CD8 responses, providing a potent whole cell *in vivo* vaccine strategy for exploring factors mediating immune evasion. Finally, the role of anti-HY TCR transgenic T cells in GvHD is assessed. Lymphopenic male recipients lose weight after adoptive transfer of CD4 but not CD8 T cells. With radiation preconditioning, full-blown GvHD ensues, raising questions about this combination for clinical therapy.

This study advances knowledge of antigen-specific T effector and Treg responses to HY in a range of environments, including MB49 tumours, male skin grafts and GvHD. It highlights the importance of understanding not only induction of effector responses, but potentially harmful side-effects of adoptive cell transfer.

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- **Distinct in vivo CD8 and CD4 T cell responses against normal and malignant tissue.**, *The Journal of Immunology (under revision).*

- **Depletion of regulatory T cells by anti-GITR mAb as a novel mechanism for cancer immunotherapy**., Cancer Immunology, Immunotherapy Volume 59, Number 9, 1367-1377, 2010

- **The roles of antigen-specificity, responsiveness to transforming growth factor-β and antigen-presenting cell subsets in tumour-induced expansion of regulatory T cells**., Immunology Volume 131, Issue 4, pages 556–569, December 2010

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Abbreviations

Ab	Antibodies
Ag	Antigen
ACT	Adoptive cell transfer
APC	Allophycocyanin
APCs	Antigen-presenting cells
B6	C57BL/6 background mice
BMDCs	Bone Marrow Dendritic Cells
bp	Base Pair
CD	Cluster of differentiation
CFSE	Carboxyfluorescein Succinimidyl Ester
CTL	Cytotoxic T Lymphocyte
CTLA-4	Cytotoxic T Lymphocyte Antigen
ConA	Concanavalin A
CO_2	Carbon Dioxide
^oC	Degrees Celsius
DC	Dendritic cell
DNA	Deoxyribose Nucleic Acid
DT(R)	Diphtheria toxin (receptor)
Ex-Treg	Treg lost FoxP3 expression
FACS	Fluorescent activated cell sorting
FCS	Fetal Calf Serum
FITC	Fluorescein isothiocyanate
FoxP3	Forkhead box P3
G (RCF)	Relative centrifugal force
GFP	Green Fluorescent Protein
GITR(L)	Glucocorticoid Induced TNFR related protein (Ligand)
GvHD	Graft vs host disease
H	Histocompatibility
HBSS	Hank's Balanced Salt Solution
HSC	Haematopoietic stem cells
ICOS	Inducible T cell co-stimulator
IDO	Indoleamine 2,3-DiOxygenase
IG	Immunoglobulin
IFNγ	Interferon Gamma
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
i.p.	intraperitoneal
iTreg	induced regulatory T cell
i.v.	Intravenous
Kb	Kilo Bases
KO	Knock Out
pLN	Peripheral Lymph node
mLN	mesenteric Lymph node
dLN	Draining Lymph node
ndLN	Non-draining Lymph node
mAb	Monoclonal antibody
MACS	Magnetic activated cell sorting
MB49	Murine Bladder Carinoma-49

MB/IL-2	MB49 containing rhIL-2 and GFP transgene
MB/GFP	MB49 containing GFP transgene
MHC	Major Histocompatibility Complex
MHC-I	Major Histocompatibility Complex Class I
MHC-II	Major Histocompatibility Complex Class II
peptide-MHC	peptide-MHC
nTreg	natural Treg
O/N	Overnight
PBL	Peripheral Blood Lymphocytes
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PD-1	Programmed Death 1
PE	Phycoerythrin
PerCP	Peridinin chlorphyll protein
PE-cy5	Phycoerythrin cyanine 5
PDL-1/2	Programmes Death Ligand 1/2
PMA	Phorbol 12-myristate 13-acetate
qPCR	quantitative PCR
Rag	Recombination activating gene
RBC	Red Blood Cell
Rcf	Relative centrifugal force (g)
rpm	Revolutions per Minute
RPMI	Rosewell Park Memorial Institute Medium
RT	Room Temperature
RT-PCR	Real Time PCR
s.c.	subcutaneous
SP	Spleen
TAA	Tumour-associated Antigen
Tc	T cell
Tcm	Central memory T cell
TCR	T cell receptor
Tem	Effector memory T cell
Tg	Transgenic
TGFβ	Transforming growth factor beta
TIL	Tumour Infiltrating Lymphocyte
TNFα	Tumour necrosis factor alpha
Treg	regulatory T cell
TOPO	Topoisomerase
WT	Wild Type
μg	Micro Grams
μl	Micro litres
ml	Milli litres
mM	Milli Molar
μM	Micro Molar
ng	Nano Grams
nM	Nano Molar

Chapter 1: Introduction

1.1: The Immune System

The higher vertebrate immune system is possibly the most highly evolved system known to biology. Immune systems and cells undergo constant selective pressures from the environment and pathogen interactions, driving genetic diversity. Over vast time periods this has lead to a plethora of cellular and molecular networks designed to protect against all forms of threat to the survival of an organism (Du Pasquier 1992). The oldest branch of the immune system, known as the innate system, is universal to the animal domain and has evolved to protect the host against early phases of infection. The innate system is comprised of both soluble mediators such as cytokines and chemokines as well as cellular components that include phagocytitic cells, natural killer cells and some antigen-presenting cells (APC) (Azuma 2006). This system relies primarily on a restricted number of germ line-encoded receptors such as the Toll-like receptors designed to recognise infectious agents based on pathogen associated molecular patterns (PAMPS) (Fraser, Koziel et al. 1998). This system allows the organism to respond effectively to a wide range of infections and natural damage and is often sufficient in protecting short-lived organisms (Medzhitov and Janeway 1997).

Although the innate system is able to mount potent early stage responses, it is not able to recognise the plethora of disease causing agents, nor is it able to retain 'memory' of these events. A low reproduction rate coupled with a long life span and high energy usage in vertebrates has lead to the evolution of an adaptive immune system. The adaptive immune system has evolved to enable directed responses against 'non-self' or infectious/malignant 'self' by modulating the components of the innate system and by activating antigen-specific T and B lymphocytes (Janeway 2005). The ability to specifically tailor an immune response, such as by the production of target-specific antibodies, underlies the adaptive system and its ability to provide lifelong protection for the host.

1.2: Major Histocompatibility Complex (MHC)

A vital component of the adaptive immune system is antigen presentation through MHC molecules. The MHC was discovered by Gorer and Snell in the early 1950s as a locus responsible for the rejection of transplantable tumours and tissues (Gorer 1948). Subsequent studies revealed two classes of MHC genes encoding glycoproteins involved in antigen presentation; MHC class I glycoproteins are expressed on almost all nucleated cells and can present antigen to CD8 T cells, MHC class II glycoproteins are expressed on APCs and present antigen to CD4 T cells (Potts and Wakeland 1990). MHC class I and class II genes encode structurally distinct but homologous proteins with the common feature of a cleft running across the membrane distal surface where a vast variety of small peptides are bound. MHC genes are co-dominantly expressed in each individual and are some of the most polymorphic genes known. The human MHC complex (HLA) spans 3.6 megabases and encodes 140 genes (MHC sequencing consortium1999). The DNA encoding HLA Class I and II genes has a diversity of around 5-10% between individuals.

The MHC class I pathway of antigen presentation is present in almost all cells and processes mainly intracellular proteins for presentation on the surface; MHC class I is often referred to as the cell's 'window'. MHC I proteins are assembled in the endoplasmic reticulum (ER) where the polymorphic MHC I heavy chain α, encoded by the MHC complex, associates with the non-polymorphic $\beta 2$ microglobulin, forming a heterodimer. Peptides that are generated in the cell, mainly through proteasome mediated degradation, are translocated into the ER by the transporter associated with antigen processing (TAP1/2) proteins. TAP forms a complex with MHC I and several other proteins to optimise loading of stably bound peptides. Once a peptide has bound MHC and assumes the correct conformation, it is released from the loading complex and moves from the ER to be transported to cell surface where the loaded peptide is presented (Cresswell 2005). CD8 T cells can recognise non-self peptide-MHC I complexes through their specific T cell receptor (TCR) and, if previously primed, can respond; for example by killing the cell through release of cytotoxic granules (Garcia and Adams 2005).

Professional APCs can also present peptides using the MHC II molecules. This pathway primarily processes proteins derived from extracellular sources through phagocytosis, endocytosis and pinocytosis and subsequent digestion in endosomes

and lysosomes. Like MHC I molecules, MHC II is also a heterodimer which initially assembles in the ER and presents peptides on the cell surface. However, MHC II is formed by the α and β chains, each composed of two domains. Polymorphism is mainly present in the class II beta chain (Nakagawa and Rudensky 1999). CD4 T cells recognise non-self peptide-MHC II complexes through their specific TCRs. If sufficient secondary signals are present T cells are activated to mediate a variety of effector functions appropriate to the response, including cytokine production and priming of other cell types such as CD8 T cells and B cells (Garcia and Adams 2005).

1.3: T cell subsets

1.3.1: T cell Development

T cells are an essential component of the adaptive immune system. T cell lineage progenitors (TLP) migrate from the bone marrow and enter the thymus where they progress into the inner cortex and interact with resident cortical epithelial cells (Bhandoola, Sambandam et al. 2003). Notch signalling commits TLPs to the T cell lineage where they become double negative thymocytes that do not yet express CD4 or CD8 (Harman, Jenkinson et al. 2003) but do express a pre-TCR (Shortman, Vremec et al. 1991). Ligand independent signalling through the pre-TCR ensures survival and directs proliferation and differentiation to the double positive (DP) $CD4^+$ $CD8^+$ stage when the mature αβTCR heterodimer is expressed (von Boehmer, Aifantis et al. 2003). The vast diversity of the αβTCR repertoire is generated by assembly of each chain via rearrangement of multiple variable (V), diversity (D, β only) and joining (J) gene segments. Recombination is mediated by several enzymes including the Recombination Activating Genes 1 and 2 (Rag1/2) nucleases (Fugmann, Lee et al. 2000). Rag creates double stranded breaks at sites containing recombination signal sequences that flank the gene segments. Once rejoined and translated this produces a TCR with unique CDR3 loop structures which generally engage with the peptide component of the peptide-MHC complex (Bodmer, Marsh et al. 1991; Bassing, Swat et al. 2002).

At the DP stage, the mature TCR interacts with complexes on cortical thymic epithelial cells as well as thymic B cells and dendritic cells (DCs). If interaction occurs with sufficient affinity to MHC-I, cells are directed into CD8 single positive (SP) lineage. If the TCR favourably reacts with MHC-II, cells are directed into the CD4 SP lineage. At the DP stage, thymocytes recognising self peptide with a high affinity are deleted by negative selection, preventing autoreactive T cells from entering the periphery, known as central tolerance (Davey, Schober et al. 1998). DP cells then migrate to the medulla where terminally differentiated medullary thymic epithelial cells (MTEC) present a variety of tissue specific gene products under the control of the autoimmune regulator gene (AIRE). This facilitates presentation of peptides from a large proportion of extra-thymic self on MHC-I and II broadening the extent of thymic central tolerance (Kyewski and Klein 2006). However, it is this tolerance mechanism which acts to prevent autoimmunity that allows for the evasion and persistence of many tumours. Many tumour-associated antigens (TAAs) are derived from self proteins expressed in the thymic medulla; as a consequence, T cells that are highly reactive to TAAs are also deleted (see chapter 3) (Overwijk 2005).

DP thymocytes that successfully audition for positive selection mature into SP CD4 (TCR-specific to MHC-II) or SP CD8 (MHC-I) thymocytes and migrate to the periphery as naive T cells (CD62 high; CD44 low). αβ TCRs are highly variable and the T cell compartment has a potential repertoire of up to 10^{12} structurally distinct TCRs (Davis and Bjorkman 1988), however the human TCR repertoire in adults is estimated to comprise 10^{7} structually distinct forms (Arstila, Casrouge et al. 1999). It is this process of selection that allows αβ T cells, as a population, to monitor and regulate other cells within the host. Having specific binding features for a given peptide bound to MHC allows for recognition of a huge number of peptides never before encountered by the immune system. These features allow an individual organism to respond to a wide range of structurally distinct pathogens. Polymorphism at the MHC locus also enhances immune defences across the population as a whole by ensuring wide variation in the T cell response to a broad array of epitopes (Lawlor, Zemmour et al. 1990).

1.3.2: $CD4^+$ T cells

CD4 T cells can recognise peptide MHC-II complexes expressed by APCs, known as signal 1. If other congruent signals (signal 2) are provided, such as positive co-stimulation, commonly through CD28 (Harding, McArthur et al. 1992) (see co-stimulation below), CD4 T cells can activate and clonally expand. If signal 1 is provided in the absence of signal 2, T cells fail to become fully activated and can become anergic or adapted. Adaptation is a state of functional unresposniveness to antigen induced by chronic exposure, this is not a sate of anergy (Choi and Schwartz 2007). This is a common outcome associated with anti-tumour responses (Walunas, Bakker et al. 1996).

Activated CD4 T cells can also develop cytotoxic effector function (Williams and Engelhard 1996), however their primary role is to maintain and regulate B cells, NK cells and CD8 T cells (Janssen, Lemmens et al. 2003). CD4 cells can activate, modulate and maintain other immune cells by the secretion of cytokines such as IL-2 (Antony, Paulos et al. 2006). Once antigen is cleared from the host, a small proportion of CD4 cells become persistent memory cells capable of activation and clonal expansion at a dramatically accelerated rate upon re-encounter of antigen. The precise function of CD4 T cells depends on the manner in which they are activated by APCs, with multiple lineage options being available at the naïve stage including Th1, Th2, Th17 and Treg (see later sections).

1.3.3: $CD8^+$ T cells

CD8 T cells, also known as cytotoxic T lymphocytes (CTLs), are the other major subset of T cells. CD8 T cells can recognise peptide MHC I complexes which are expressed by most nucleated cells. CD8 T cell priming, however, requires engagement of specific peptide MHC I complexes presented by activated dendritic cells (signal 1). Again, if secondary congruent signals are present, such as positive co-stimulation (signal 2), then the CD8 T cell will clonally expand, while incomplete activation signals induce anergy or adaptation (Choi and Schwartz 2007).

Following binding of the specific peptide-MHC-I complex to which they have been primed, activated CD8 T cells upregulate perforin, granzyme B and FAS-L all of which can be used to induce the death of target cells (Cao, Cai et al. 2007). In addition, activated CD8 T cells secrete cytokines such as IFN-γ or IL-4 (Sad,

Marcotte et al. 1995) capable of modulating the local environment and recruiting and activating other cell types in an immune response, and like their CD4 counterparts, CD8 T cell responses can be defined as Tc1, Tc2 or Tc17 (see later sections). Cytotoxicity is a common mechanism by which CD8 T cells kill tumour cells (Hinrichs, Gattinoni et al. 2006). CD8 cell function is modulated by the microenvironment in which they are activated: IL-2 (Yron, Wood et al. 1980), IL-7 (Schluns, Kieper et al. 2000) and IL-15 (Schluns, Williams et al. 2002) are all known to enhance CD8 T cell activation and proliferation. Following the primary immune response, the antigen-specific CD8 T cell population contracts, and like CD4 T cells, a small proportion of high affinity CD8 T cells remain as memory cells capable of rapid proliferation in response to secondary antigen exposure (Harty and Badovinac 2008).

1.3.4: $CD4^+$ $FoxP3^+$ Regulatory T cells

A subset of $CD4^+$ T cells (~5-10%) are known as regulatory T cells (Tregs); they express the transcription factor Forkhead box P3 (FoxP3) (Fontenot, Rasmussen et al. 2005) and often the surface IL-2 receptor α chain (CD25) (Sakaguchi, Sakaguchi et al. 1995). Tregs are primarily suppressive to T cell responses through the release of suppressive cytokines such as IL-10 and TGF-β (Sakaguchi, Ono et al. 2006), as well as directly causing T cell death and anergy through perforin-dependent cytotoxicity and through negative co-inhibitory molecules (see later sections). Tregs can be classified as natural or induced/adapted (Bluestone and Abbas 2003).

Natural Tregs (nTreg) develop and mature in the thymus where TCR and self peptide-MHC interaction falls into an avidity range between positive and negative selection. This is thought to produce peripheral regulatory T cells specific for particular self-antigens (Fontenot, Gavin et al. 2003), capable of controlling self-reactive T cells that have escaped central tolerance, as shown through studies using CD25 depletion (Kohm, McMahon et al. 2006) and verified using $FoxP3^{DTR}$ mice (see later) (Kim, Rasmussen et al. 2007). Induced or adaptive Tregs (i/aTregs) form in the periphery by conversion from conventional CD4 T cells in conditions of suboptimal antigen presentation, however, they can be specific for mutated self-antigens as well as foreign antigens. iTregs often develop under conditions of poor activation caused by low levels of antigen presented by immature APCs, generally

coupled with negative co-stimulation through CTLA-4 (Horwitz, Zheng et al. 2008) (see co-stimulation), such conversion as is seen with many low dose antigens that induce suboptimal APC activation (Kretschmer, Apostolou et al. 2005). The process of expanding nTregs and reprogramming of conventional CD4 T cells into iTregs is a common immunoevasive mechanism associated with tumour microenvironments (Curiel 2007).

1.3.5: Th1, Th2 and Th17 T cell responses

The environment in which T cells are activated is critical in determining the type of T cell response to be induced. The type of T helper (Th) response induced is dependent on the manner in which antigen is presented in combination with co-stimulation and cytokine signals from APCs. Th1 responses are often generated in response to viruses, bacteria and intracellular pathogens. A Th1 response by CD4 T cells is characterised by the production of IL-2, INFγ and TNFα leading to activation and induction of IFNγ, TNFα and by induction of cytotoxic granules in CD8 T cells, along with the activation of macrophages, NK cells and B cells. IFNγ induces the production of IL-12 by DCs and macrophages which in turn induces further IFNγ production by Th1 cells in a positive feedback loop (Mosmann and Sad 1996). It has been suggested that the most effective anti-tumour responses are driven by the Th1 lineage (Surman, Dudley et al. 2000).

Th2 responses are generated against multi-cellular pathogens with the primary function of activating B cells. Activated Th2 cells produce IL-4, 5, 6, 10 and 13, inducing maturation of B cells and the production of antibodies in a humoral response (Mosmann and Sad 1996). In general, Th2 responses are associated with a poor tumour prognosis (Hung, Hayashi et al. 1998).

Recently, an additional Th subset has been characterised in which activated CD4 T cells were identified as secreting IL-17 (Yao, Fanslow et al. 1995). This has therefore become known as a Th17 response. Th17 cells are often found in proinflammatory environments similar to those that produce Th1 responses. Th17 cells are induced in environments where TCR stimulation occurs in the presence of TGF-β and IL-6 (Kryczek, Wei et al. 2007; Bettelli, Korn et al. 2008). Th17 cells secrete IL-17 and IL-22 capable of producing a wide range of responses from surrounding tissues due to the broad expression of IL-17 and IL-22 receptors (Korn,

Bettelli et al. 2009). The inflammatory environment within tumours often contains high levels of TGF-β and IL-6, giving the potential for favourable induction of a Th17 response. The prognosis associated with Th17 anti-tumour responses is mixed, with several conflicting reports in the literature (Hinrichs, Kaiser et al. 2009; Wang, Yi et al. 2009). The precise role of Th17 and its relation to Th1 and Th2 responses remains to be fully elucidated, particularly in response to tumours. CD8 T cells primed under conditions of Th1, 2 or 17 also have distinct cytokine profiles. These are known as Tc1 (IFNγ, TNFα producing), Tc2 (IL-4, IL-10 producing) and Tc17 (IL-17, IL-22 producing) cells.

1.3.6: T cells and Co-stimulation

Naive T cells require two signals to become fully activated, the first, which defines specificity, is TCR engagement with peptide-MHC and the second is through the ligation co-stimulatory molecules and their ligands expressed on mature APCs (Janeway 2005). Proinflammatory environments, for example following engagement of PAMPS by pathogen recognition receptors, can upregulate positive co-stimulatory molecules. The nature of the activation signals received by the APC often defines the type of co-stimulation molecules that are upregulated and the consequent immune response (Wang and Chen 2004), see table 1. Toll like receptors (TLR) on APCs detect PAMPS, for example TLR4 detects lipopolysaccharide (LPS). Binding of TLR4 for example induces high levels of CD80/86, IL-12 and IL-18 expression by APCs resulting in stong Th1 priming of T cells (Iwasaki and Medzhitov 2004).

The most characterised co-stimulatory and co-inhibitory molecules expressed by T cells are members of the CD28 Imuunoglobulin superfamily. CD28 binds B7.1 (CD80) and B7.2 (CD86), part of the B7 Immunoglobulin superfamily, expressed on APCs, thus inducing a positive signal to T cells (Lanier, O'Fallon et al. 1995). When combined with antigen stimulation this signal allows T cells to be activated with a Th1 phenotype, to proliferate and survive. CTLA-4 is a co-inhibitory molecule on T cells and Tregs that can also bind CD80/86, an antagonist of CD28 (Greenwald, Boussiotis et al. 2001). There are other co-stimulatory molecule pairs, such as Glucocorticoid Induced Tumour necrosis factor Receptor family-related gene (GITR (L)), Inducible Co-stimulatory Ligand (ICOS (L)), CD40 (L), 4-1BB (L) and Ox40 (L), which are generally believed to act to further expand

or maintain survival of already activated T cells. The effects of these molecules are summarised in Table 1. Another family of death receptor molecules, in the CD28 and B7 Immunoglobulin superfamily, can act as co-inhibitory molecules, namely Programmed Death Receptor-1 (PD-1) expressed on activated T cells and its ligand PD-L1 and PD-L2 expressed on APCs (Butte, Keir et al. 2007). The PD-1 pathway is commonly used by tumours to induce programmed cell death or exhaustion in tumour-specific T cells and is a common mechanism of immune evasion (Blank, Gajewski et al. 2005).

Table 1: Co-stimulatory molecules and Ligands

Receptor/Ligand (T cell)	**Cognate Receptor/Ligand (Expressed by)**	**Effect on T cell**	**Reference.**
CD28 (CD4; CD8)	CD80/86 (Bc;DC;Tc)	Activation; Proliferation; Cytokine secretion	(Lanier, O'Fallon et al. 1995)
CTLA-4 (CD4; CD8; Treg)	CD80/86 (Bc;DC;Tc)	Inhibition; Tolerance Th1	(Rudd, Taylor et al. 2009)
ICOS (act CD4; act CD8; Treg)	ICOS-L (Bc;DC)	Increased IL-10. Th2	(Hutloff, Dittrich et al. 1999)
GITR (Treg; act CD4; act CD8)	GITR-L (Bc; DC; Mθ)	Th1 or Th2	(Kwon, Yu et al. 1999)
CD40L (CD4)	CD40 (Bc; Mθ)	Th2	(Banchereau, Bazan et al. 1994)
4-1-BB (CD8 more than CD4)	4-1-BBL (Bc; DC; Mθ)	Increased CD4/8 proliferation. Th1 or Th2.	(Watts 2005)
OX-40 (mainly CD4; some CD8)	OX-40L (Bc; DC; act Tc)	Th2, but sustains Th1 proliferation and survival	(Watts and DeBenedette 1999)
PD-1 (act CD4; act CD8)	PD-L1/PD-L2 (Bc; DC; Mθ; Tc)	Negative regulation of PD-1 expressing cell	(Probst, McCoy et al. 2005; Butte, Keir et al. 2007)
Bc - B cell, Tc - T cell, DC - dendritic cell, Mθ - macrophage, act - activated			

1.4: Antigen-presenting Cells (APCs)

MHC Class II is constitutively expressed by a set of cell types collectively known as professional antigen-presenting cells, which includes dendritic cells, macrophages and B cells. The principle function of APCs is to capture antigens from the environment by endocytosis, phagocytosis and pinocytosis. Complex antigens are processed and some of the resulting peptides are presented on the APC surface in the context of MHC-II where specific CD4 T cells are capable of recognising and becoming activated in response (Boniface, Reich et al. 1999).

1.4.1: Dendritic Cells

DCs derive from progenitor cells in the bone marrow and were originally discovered by Paul Langerhans, the term dendritic cells coined by Steinman and Cohn in 1973 (Steinman and Cohn 1973). DCs have a dishevelled membrane morphology with a high degree of motility that provides a large surface area, allowing efficient uptake and presentation of antigen. DCs are broadly classified as either lymphoid, $CD11c^+$ $CD8\alpha^+$ $DEC205^+$ $CD11b^-$, myeloid (mDC) $CD11c^+$ $CD8\alpha^-$ $DEC205^+$ $CD11b^+$ or plasmacytoid (pDC) as mDC with $CD123^+$ (IL-3Rα) as a marker (Shortman and Liu 2002). Although similar to other DCs, pDCs look more like plasma cells and generally produce IFN-α and IL-6 upon maturation while lymphoid and mDCs broadly produce IL-1, IL-10, IL-12 and TNFα (Cella, Sallusto et al. 1997).

DCs circulate through lymph nodes, spleen and peripheral tissues. Upon antigen encounter and activation in the periphery, they migrate to secondary lymphoid organs where they present peptide antigen to T cells by via MHC-I and II molecules along with co-stimulatory signals as discussed earlier inducing activation of naïve T cells. DCs will induce different T cell responses depending on the secondary signals recieved during maturation. For example DCs express a range of toll-like receptors (TLRs) capable of interacting with a wide range of microbial PAMPs such as LPS and CpG that can induce the DC to produce high levels of IL-12 and CD80/86 inducing a strong Th1 response by T cells (Macatonia, Hosken et al. 1995).

1.4.2: Macrophages and Monocytes

Macrophages are produced by the differentiation of monocytes deriving from granulocyte/monocyte progenitor cells in the bone marrow. These progenitors develop into pro-monocytes and migrate to the blood stream where they become monocytes and eventually into peripheral tissues where they mature into macrophages. Macrophages are phagocytic cells that express a range of receptors that allows them to phagocytose dying or dead cells, microorganisms and cell debris. Macrophages are able to present antigenic peptides to T cells by MHC I and II molecules, along with co-stimulatory signals and cytokines to enable activation (Aderem and Underhill 1999). As with DCs, the environment in which the macrophage scavenges antigen is critical to the type of responses to be induced in T cells.

1.4.3: B cells

B cells differentiate from lymphoid stem cells to progenitor B cells within the bone marrow where they proliferate and further differentiate into precursor B cells. Formation of immature B cells is then dependent on the rearrangement of the DNA encoding immunoglobulin, driven by the RAG genes and IL-7. In a highly similar mechanism to T cell development, immunoglobulin chains are rearranged and expressed on the cell surface as a B cell receptor (BCR), each with a unique structure and range of specificity. Self-reactive B cells are deleted in the bone marrow while non-self reactive cells are released into the periphery as naive B cells (Janeway 2005).

B cells are activated upon engagement of the BCR by cognate antigen, inducing the upregulation of peptide-MHC II for presentation to CD4 T cells. B cells can cross-talk with T cells through co-stimulation of CD40 (B cell) engaging CD40L (T cell). This leads to the activation and proliferation of B cells and the generation of plasma cells that ultimately secrete high levels of antibody, the isotype of which depends on the cytokine environment (Banchereau, Bazan et al. 1994). B cells therefore can act as APCs for CD4 T cells, although prior activation by DCs is often required (Parker 1993). B cells are considered as poor APCs, mainly due to poor co-stimulation, although B cells can be highly effective at endocytosing antigen through their surface BCR for representation at the surface in the context of MHC-II

molecules. In addition, the large B cell number in the periphery of lymphoreplete hosts can often equilibrate this effect (Janeway, Ron et al. 1987).

1.5: Cytokines and Chemokines

Cytokines are small cell-signalling molecules extensively produced and used by lymphocytes and glial cells. They are immunomodulating agents with similar properties to many hormones. Cytokines are often sub-classified as lymphokines, interleukins and chemokines based on their source, mode of action and target. Most cytokines are soluble or membrane-bound and all have a corresponding receptor that when bound by the cytokine can convey a signal to the target cell. These signals induce a variety of responses, such as specific upregulation or downregulation of gene expression, structural and morphological changes, differentiation, activation and even inhibition. The effects of a cytokine on a given target cell depend on the context in which it is delivered and cytokines often act synergistically. Cytokine genes and families are often highly homologous, with many cytokines evolving through gene duplications, for example IL-2 and IL-21 are believed to have diverged recently in terms of evolution (Hinrichs, Spolski et al. 2008). The highly homologous nature of cytokines and their receptors also means they tend to work in pathways and feedback loops (Casciari, Sato et al. 1996), for example CD4 T cell activation can result in the production of IL-2, IL-2 is required for activated CD4, CD8 T cell and Treg survival (Janeway 2005). Common cytokines associated with immune responses to tumours are described below and in table 2.

Table 2: Common cytokine effector functions

Cytokine	Source	Effect on T cells	Effect on APCs	Other effects
IL-2	Th1 CD4 and some CD8	Growth T cells, Growth and survival of Tregs	-	activation of NK cells
IL-4	Th2, mast cells	Growth, survival, inhibits IFNg	Inhibits macrophage activation	IgE isotype switching
IL-6	Macrophage, DC, Th2, endothelium	-	Inhibition of IDO synthesis by pDC	B cell proliferation, synthesis of acute phase proteins

IL-10	Th2, macrophage	Growth	Inhibition of IL-12	inhibition of proinflammatory cytokines
IL-12	Macrophage, DC, B cells	CD4 Th1 differentiation, IFNγ synthesis, CTL activation	-	NK cell activation, inhibition of IL-4
IL-17A	CD4 Th17, some CD8	Th17 development	Macrophage activation	Neutrophil activation, secretion of IL-6 and IL-8 in fibroblasts, epithelial cells and keratinocytes
GM-CSF	T, B cells, NK cells, endothelial, macrophage	-	Monocyte differentiation, DC maturation and survival	granulocyte and mononuclear cell growth and survival
IFNγ	Th1 CTL, NK, some CD4	Th1 development, activation	Maturation of macrophage, DC	IgG isotype switching
TNFα	Macrophage, DC, Th1	Th1	Activation, Nitric Oxide (NO) production by macrophages	synthesis of acute phase proteins, endothelial activation, increased vascular permeability, fever, ↑adhesion molecules
TGF-β	Treg, T cells, monocytes	Treg growth, survival, CD4 T cell conversion, inhibits CTL	Inhibits maturation	suppression of inflammation, increased fibrosis

1.5.1: Interleukin-2 (IL-2)

IL-2 was the first mitogenic factor described for lymphocytes (Kasakura and Lowenstein 1965) and is discussed in detail in chapter 6. IL-2 is a proinflammatory cytokine produced by CD4 and CD8 T cells upon TCR engagement and activation when it acts in an autocrine fashion to induce proliferation, differentiation and cytotoxic functions (Villarino, Tato et al. 2007). The IL-2 receptor alpha (CD25), one of several IL-2 receptors, is transiently expressed on both CD4 and CD8 T cells following activation and is ubiquitously expressed on nTregs. IL-2 is required for the effective priming of typical Th1 T cell response, however, nTregs can sequester excess IL-2 from the local microenvironment in a co-evolved system to limit autoimmunity. In fact IL-2 knockout mice have lymphoproliferative disorder and autoimmunity rather than immunodeficiency (Sadlack, Lohler et al. 1995).

1.5.2: IL-10

IL-10 is often classed as an anti-inflammatory cytokine commonly secreted by monocytes, mast cells, Tregs and Th2-cells. IL-10 can modulate T cell responses either directly or indirectly through APCs (Sharma, Stolina et al. 1999) and is often termed an immune regulator. IL-10 acts mainly by inhibiting cellular Th1 responses through downregulation of MHC II (de Waal Malefyt, Haanen et al. 1991) and positive co-stimulatory molecules CD80/86 on APCs (Ding and Shevach 1992). Additionally, acting on the JAK-STAT signalling pathway and blocking NF-κB, IL-10 inhibits the production of proinflammatory cytokines such as IL-12 by APCs and IL-2 by CD4 T cells limiting T cell activation (de Waal Malefyt, Yssel et al. 1993). Like IL-2, IL-10 has a dual role in the controlling development and function of T regulatory type 1 (Tr1) cells, an independent Treg population that does not express FoxP3 (Vieira, Christensen et al. 2004) but can inhibit T cell responses.

1.5.3: IL-12

IL-12 is a heterodimer produced by DCs, macrophages and B cells in response to antigen stimulation. IL-12 is another proinflammatory cytokine that can direct T cells towards a Th1 phenotype, inducing proliferation and expression of other Th1 cytokines, such as INFγ and TNFα, while inhibiting IL-4-mediated suppression. IL-12 is required for the effective induction of cytotoxic responses in CD8 T cells and NK cells, which is enhanced by co-exposure to IL-2 (Wang, Frank et al. 2000).

1.5.4: IL-17A

IL-17A is a proinflammatory member of the IL-17 cytokine family first described in 1993 (Rouvier, Luciani et al. 1993). IL-17A has been shown to promote cytotoxicity in T cells, angiogenesis and has been implicated as a mediator of rheumatoid arthritis (Korn, Bettelli et al. 2009). It is primarily produced by CD4 T cells and some CD8 T cells, while the five receptors of IL-17 are broadly expressed by most cell lineages. IL-17 receptor signalling can induce the secretion of IL-6 and IL-8 in fibroblasts, epithelial cells and keratinocytes (Yao, Fanslow et al. 1995). IL-17, IL-22 co-producing T cells are often described as differentiated Th17 cells (Bettelli, Korn et al. 2008).

1.5.5: Interferon Gamma (IFNγ)

IFNγ is a soluble homodimer and the only known member of the type II interferon family (Gray and Goeddel 1982). It is a signature cytokine produced by Th1 activated CD8 T cells and NK cells and induces the upregulation of MHC I and II in many cell types (Morris and Tomkins 1989). It has also been shown to increase lysosome activity and active inducible nitric oxide synthase in macrophages (Schroder, Hertzog et al. 2004). IFNγ promotes anti-microbial and anti-tumour responses in macrophages through the JAK-STAT pathway by activating NF-κB. Inhibition of this cytokine using blocking antibodies *in vivo* can abrogate anti-viral and anti-tumour responses (Spitalny and Havell 1984). IFNγ is also implicated in immunoglobulin production and isotope class switching in B cells (Schroder, Hertzog et al. 2004).

1.5.6: Tumour Necrosis Factor alpha (TNFα)

TNFα was discovered in the late 1960s as a macrophage product able to mediate apoptosis in mouse fibrosarcoma L-929 cells (Carswell, Old et al. 1975). It is a proinflammatory cytokine of the TNF family and is associated with the acute phase reaction along with IL-1, INFα and β. The acute phase reaction is a response to local inflammation and injury and is discussed in detail in Chapter 7 (Hattori, Hirano et al. 1998). While TNF receptor 1 (TNFR1) is broadly expressed on most cells, TNFR2 is mainly restricted to immune cells. TNFα can mediate a Th1 phenotype by the induction of NF-κB MAP kinase pathways, leading to production of other proinflammatory factors (Wajant, Pfizenmaier et al. 2003). TNFR1 contains a death domain that triggers apoptosis in a caspase-dependent manner, using the same intracellular pathways as FAS (Gaur and Aggarwal 2003).

1.5.7: Transforming Growth Factor Beta (TGF-β)

TGF-β is soluble cytokine that exists in at least three isoforms (β1-3) and is expressed to some degree by most cells, specifically by T cells and monocytes, and is an important mediator of immune suppression. TGF-β can induce apoptosis, inhibit proliferation, control inflammatory responses (Li, Sanjabi et al. 2006) and prevent the action of CD8-mediated cytotoxic responses (Fukuyama, Ichiki et al. 2007).

TGF-β1 signals through TGF-βRII and propagates apoptotic signals through the Smad pathway (Wahl and Chen 2005). TGF-β is powerfully suppressive to T cells and can induce the conversion of conventional CD4 cells to $FoxP3^+$ iTregs (Chen, Jin et al. 2003; Coe, Addey et al. 2010). TGF-β is often expressed to a high level by tumours and induced by certain pathogens such as mycobacterium tuberculosis (Reed 1999).

1.5.8: Chemokines

Chemokines are a family of small cytokines (8-10 kDa) named for their ability to induce chemotaxis in motile target cells such as T cells and APCs. Chemokines have a definitive 3D shape conferred by four conserved cysteine residues but can function in a variety of ways with some members being proinflammatory, others largely suppressive and many involved in homeostasis (Fernandez and Lolis 2002). Chemokines can induce migration of resting and activated lymphocytes and leukocytes to sites of inflammation or injury. Due to the large numbers of chemokines, receptors and modes of action, specific chemokines are addressed when necessary in this study. A few notable chemokine receptors include CCLR5 and CXCR3 expressed on Th1 activated T cells. These bind the CXCL9, 10 and 11 chemokines which are primarily involved in the migration of T cells from secondary lymphoid organs to sites of inflamation (Smit, Verdijk et al. 2003). Other chemokine receptors, such as CCR3 and CCR4, are highly expressed on Th2-cells and bind CCL7 and CCL5 (RANTES) which are commonly expressed by many tumours (Luboshits, Shina et al. 1999).

1.6: Tumour biology and tumour: T cell interactions

Tumours arise principally through genetic abnormalities deriving from innate genetic instabilities, carcinogens and environmental factors including radiation. Progressive accumulation of mutations that enhance survival can ultimately lead to tumour development. Such enhancing mutations can allow cancers to provide their own growth signals, become resistant to inhibitory signals, avoid cell death, induce angiogenesis and in some cases metastasise (Hanahan and Weinberg 2011). These mutations can also allow cancers to evade immune elimination, a process termed

immunoevasion. Cancers that are able to evade the immune system often thrive and eventually come to represent the majority of cells within a tumour by immuno-selection (Zitvogel, Tesniere et al. 2006). Immunoevasion is not a necessary component for tumour development but it is certainly common to most cancers to some degree and enhances their development (Dunn, Old et al. 2004).

Tumours have several mechanisms associated with their ability to evade immune responses; these have been touched on earlier in this section. Briefly, tumour cells can reduce expression of MHC and co-stimulatory molecules, preventing immune recognition. Tumours are known to actively suppress APC function, preventing their maturation and upregulation of co-stimulatory molecules. Tumours themselves can make suppressive factors to modulate the microenvironment and even induce immune cells to produce inhibitory factors. In addition, tumours can express surface molecules such as FasL and PD-L1 that can actively induce apoptosis of infiltrating lymphocytes. Finally, tumours can downregulate the TCR of infiltrating T cells limiting their ability to recognise the tumour (Dunn, Old et al. 2004; Zitvogel, Tesniere et al. 2006).

Immune responses to solid tumours are mostly mediated by T cells and NK cells (Perez-Diez and Marincola 2002). Tumour development is also favoured by hijacking natural mechanisms of immune regulation including Tregs. As the primary function of Tregs is to prevent autoimmune responses to self-antigens they also mediate suppression of responses to common tumour-associated antigens (TAAs). TAAs are generally over expressed or altered self antigens. These factors are discussed in more detail here.

1.6.1: Immunoevasion and Immunomodulation

Defects in antigen processing and presentation confer an advantage for tumour development due to reduced T cell recognition. The classical MHC I pathway presents intracellular peptides on the cell surface, loss of MHC I or its related accessory proteins can confer a protective advantage to tumours. Human colorectal and ovarian carcinomas have been shown to downregulate β2-microglobulin and the TAP heterodimer, reducing surface MHC I (Atkins, Breuckmann et al. 2004; Vitale, Pelusi et al. 2005). Coupled with these defects in antigen presentation, tumours are generally poor stimulators of T cells, due to the

limited level of co-stimulatory molecules on their surfaces. This is evident in the effective immunity stimulated by CD80 transfected melanoma cells, which can lead to subsequent rejection of untransfected melanoma cells (Townsend, Su et al. 1994).

Tumours can downregulate co-stimulatory molecules such as CD80 on their surfaces; however this is a double-edged sword as they can express abundant levels of T cell co-inhibitory molecules. The interaction between PD-L1 expressed on APCs and PD-1 on activated T cells is essential for peripheral CD8 T cell tolerance (Ansari, Salama et al. 2003), through inhibition of T cell proliferation, cytotoxic function and induction of apoptosis (Butte, Keir et al. 2007). Many tumours upregulate PD-L1 as a mechanism to induce apoptosis in tumour-specific activated T cells expressing PD-1 (Blank, Gajewski et al. 2005), as described on human colorectal, lung, ovarian carcinomas and melanomas (Dong, Strome et al. 2002). CTLA-4 (CD152) can also be induced on human T cells, B cells and APCs; ligation of this molecule can inhibit T cell responses (Sica, Choi et al. 2003). Many tumours also express CTLA-4 which can be increased by IL-6 and IL-10 and decreased in environments of GMCSF and IL-4. Kryczek *et al.* has shown CTLA-4 expression on human ovarian carcinoma infiltrating macrophages is associated with an increase in Treg infiltration and a poor prognosis. This is also correlated with CTLA-4 expression on the tumour itself (Kryczek, Wei et al. 2007). Further dynamics of tumours and co-stimulation are discussed in chapter 3.

Tumour environments also often contain immunosuppressive factors secreted by the tumour or induced from APCs and T cells. Indoleamine-2,3-dioxygenase (IDO) is an enzyme that catabolises tryptophan in the kynurenine pathway and IDO-induced depletion of tryptophan can cause reduced T cell proliferation and render DCs tolerogneic to tumour-specific T cells (Grohmann, Fallarino et al. 2003). Tumours can induce IDO expression by DCs in the tumour-draining lymph node (dLN) (Belladonna, Puccetti et al. 2007). Tumours can also directly secrete suppressive factors that include prostoglandins, gangliosides, GMCSF, IL-6, IL-10, TGF-β and VEGF into the local microenvironment (Ghiringhelli, Puig et al. 2005). IL-10 and TGF-β are two of the best characterised suppressive cytokines found in the tumour microenvironment (see sections 1.5.2 and .6). Further details of tumour-related cytokines are discussed in chapter 3. Briefly, IL-10 modulates T cell responses directly or through APCs and controls the development of Tr1 (non-FoxP3

expressing Tregs) (Sharma, Stolina et al. 1999). TGFβ is directly secreted by many tumour lines *in vitro* and *in vivo* (Fukuyama, Ichiki et al. 2007) and can convert conventional CD4 T cells to FoxP3^{+} Tregs (Chen, Jin et al. 2003) and inhibit tumour-specific CD8 T cells (see chapter 3). Tumours are therefore capable of modulating naturally occurring immunosuppressive mechanisms at both the cellular and molecular level in order to enhance their survival. Better understanding of these mechanisms allows for rational design of new cancer immunotherapies.

1.6.2: T cells and cancer

Tumour resident T cells, specific for TAAs, are found in most tumours, such as human melanomas (Mortarini, Piris et al. 2003) and colorectal cancers (Wagner, Koch et al. 2008). As discussed in section 1.3, T cells can mediate cytotoxic responses against tumour cells when sufficiently primed (Perez-Diez, Joncker et al. 2007). Despite this, many tumours are not controlled by these T cells, partly due to the immunomodulatory mechanisms discussed above. Tumour-specific T cells are also often subject to TCR downregulation (Abad, Wrzensinski et al. 2008). This effect can be caused by factors contained in the microenvironment and is a characteristic signature of T cell exhaustion/anergy induced by the tumour rather than tolerance to the TAA (Melchionda, McKirdy et al. 2004). T cells are therefore capable of activating and proliferating in response to TAA but cannot mediate effective anti-tumoural responses as a result of incomplete activating signals and inhibitory factors.

Other factors that inhibit anti-tumour cytotoxicity include an inability for large numbers of T cells to access solid tumour environments. Many progressive tumours also have poor tumour cell to T cell ratios of over 10,000:1. Lower ratios of T cell to tumour cells are associated with a worse prognosis (Yu, Lee et al. 2003). Established tumour environments are also highly hypoxic and induce T cell apoptosis through inhibition of CCR7 (Sun, Zhang et al. 2010). T cells must overcome a vast range of inhibitory mechanisms in order to induce successful tumour regression. Therefore, pro-effector modulation of tumour-specific T cells remains a challenging target for cancer immunotherapy.

1.6.3: Tregs and cancer

Tregs can have a major role in preventing sufficient immune responses to tumours by direct suppression of the immune response (Curiel 2007).
As discussed above, established tumours are rarely rejected by their hosts despite the presence of detectable anti-tumour T cell responses. One major reason for this failure is the presence of tumour-specific regulatory T cells. Tregs play a key role in suppressing anti-tumour responses as many TAAs are aberrantly expressed normal self-peptides (Wang, Huang et al. 2004). nTregs develop in the thymus where they are thought to recognise self-peptides with moderate affinity (see section 1.3.3) and have the potential to recognise these ligands expressed in the tumour environment. *In vivo* use of anti-CD25 mAb to deplete nTregs can lead to improved anti-tumour immunity and inhibition of tumour growth (Shimizu, Yamazaki et al. 1999; Turk, Guevara-Patino et al. 2004). Tumour-specific Tregs are found at elevated levels in the peripheral blood and tumour samples of patients with invasive breast or pancreatic cancer, with higher numbers of Treg and enhanced Treg function associated with poor prognosis (Liyanage, Moore et al. 2002). Tumour infiltrating Tregs are also able to inhibit the effector functions of CD4 Th cells (Chaput, Darrasse-Jeze et al. 2007) and CD8 CTLs (Chen, Pittet et al. 2005). Tregs can also be induced (see section 1.3.3) in tumour microenvironments, leading to direct suppression of anti-tumour T cell responses (Zhou and Levitsky 2007).

Tregs require activation via their TCR in order to suppress anti-tumour responses; tumours themselves can often induce this activation, via direct and indirect antigen presentation. Activated Tregs can also be maintained by several factors produced directly by the tumour *in vivo,* such as TGF-β (Yang, Nurieva et al. 2008). The molecular mechanism of Treg-induced immune modulation remains to be fully defined. However, the expression of TGF-β (Daley, Ma et al. 2007), the production of adenosine and the catabolism of tryptophan, have all been shown to play a role (Mellor and Munn 2004). IDO is an immuno-regulatory enzyme which catalyses the first rate-limiting step of tryptophan catabolism. IDO is expressed by plasmacytoid DCs (pDCs) that produce IDO in a GCN-2 kinase-dependent fashion that can activate Tregs in the draining lymph node of tumour-bearing mice (Munn, Sharma et al. 2004; Munn and Mellor 2007). This regulation check-point can be suppressed by IL-6 which can then induce Tregs to become proinflammatory Th17/Th1-like cells (Sharma, Hou et al. 2009), a process called Treg reprogramming.

1.7: Cancer Immunotherapy

The strongest risk factor for cancer is age (Coleman and Tsongalis 2009) and with greater life expectancy it is an increasing burden on society. In 2008, 12.7 million cases of cancer were recorded resulting in 7.6 million deaths, the most common forms being lung (1.3M), stomach (0.8M) and colorectal cancer (0.64M) (Jemal, Bray et al. 2011).

Cancers are caused largely by chemical/radiation exposure, diet, physical trauma and hormones although ~5% arise from genetic susceptibility. Many common cancers are carcinomas of epithelial origin, such as prostate, breast, lung, pancreas and colon. Other cancer groups include sarcomas, lymphomas, germ-cell tumours and blastomas (Jemal, Bray et al. 2011). Whatever the cause and type, cancers are characterised by their failure to regulate tissue growth. Genetic predisposition and spontaneous genetic contributions often involve genes (oncogenes) that regulate growth and differentiation (Croce 2008). Overexpressed gene products (often oncognes) provide markers for distinguishing tumours as different from normal tissue and are so termed tumour-associated antigens (TAAs).

In recent decades, many TAAs have been identified in murine and human tumours. TAAs are antigens derived from aberrantly expressed non-mutated proteins, aberrantly expressed intronic sequences, alternative reading frames of normal genes and cancer-specific mutated proteins (Rosenberg 1999). While TAAs are not tumour-specific, they provide a target for immunotherapy as their limited distribution makes targeted therapies more specific than chemo/radiotherapy. The identification of TAAs and TAA-specific T cells for a range of tumours has allowed development of immunotherapies which have shown promise in several melanoma trials (Abad, Wrzensinski et al. 2008). Tumour-specific antigens are soley expressed on the tumour and are therefore rare in naturally arising tumours, although these can be common in virally induced tumours (Coulie, Hanagiri et al. 2001).

1.7.1: Cancer immunotherapeutic strategies

There are four general strategies for cancer immunotherapy. 1) Monoclonal antibodies (mAb) to directly target tumour cells or modulate the immune system. 2) Vaccination including tumour-specific peptides, peptide loaded DCs and/or novel adjuvants designed to activate endogenous DCs and T cells. 3) Direct genetic

modification of tumours using gene therapy. 4) Adoptive transfer of TAA-specific lymphocytes. This last approach, from now on called adoptive immunotherapy, has yielded encouraging clinical responses in melanoma patients (Dudley and Rosenberg 2003). This strategy will be discussed in the next section.

1.7.1.1: Monoclonal antibody therapy

mAb therapy is utilised in two ways. Firstly, immuno-targeting therapy that directs mAbs to TAAs and other factors associated with tumour growth can lead to antibody-mediated immunity. An example of this class of mAbs includes Alemtuzumab, a humanised antibody against CD52 that induces the destruction of leukaemia by antibody mediated immunity (Hale, Hoang et al. 1983). mAbs, such as Mylotag™ have also been engineered to carry toxins sufficient to directly kill target cells (Reichert and Valge-Archer 2007).

Secondly, immuno-modulating mAbs binding to receptors, cytokines and chemokines that naturally regulate anti-tumour immune responses. For example, these mAbs can block inhibitory pathways such as PD-1/L1/L2 or, conversely, activate stimulatory pathways for APCs and T cells. Clinically relevant antibodies include anti-CTLA4 (Ipilimumab™), constitutively expressed on Tregs. *In vivo* blockade using this antibody has led to successful therapy in prostate cancer (Small, Tchekmedyian et al. 2007). GITR is highly expressed on activated T cells. Ligation of GITR using the DTA-1 mAb (Shimizu, Yamazaki et al. 2002) is sufficient to cause upregulation of proinflammatory factors. DTA-1 has been shown to eliminate large established tumours (Turk, Guevara-Patino et al. 2004).

While several clinical mAb approaches are now in use, they are of limited value. Most mAb therapies target single pathways or cells types, which can limit their efficacy and effectiveness for many cancers. In addition, it can often be difficult to ensure mAbs can enter into the tumour mass due to tumour-induced exclusion (e.g metastatic pressure). The use of mAbs in conjunction with other therapeutic approaches may be a more promising approach in the future.

1.7.1.2: Vaccination therapy

Cancer vaccines fall into two general categories: those that target immunity to TAAs, such as use of peptide loaded DCs, and those that modulate enhanced immune

responses to TAAs, such as novel adjuvants. Due to the nature of cancer it is difficult to vaccinate pre-emptively unless the cancer is associated with specific infectious diseases, e.g. cervical cancer rates and liver cancer rates are reduced by vaccination against HPV and Hepatitis B respectively (Doren 2010). Cancer vaccine designs must therefore aim to generate or enhance immune responses to already established tumours.

Cancers of a particular lineage develop from the same cell type and can involve mutations to a limited set of genes and gene families, for example human melanomas often express the antigen MART1MelanA, against which T cell clones have been expanded for use in clinical immunotherapy (Yee, Thompson et al. 2002). Such TAA prediction allows for identification of potentially useful vaccines. Clinically relevant vaccines such as Provenge™ are used in the treatment of advanced prostate cancer. This vaccine uses APCs activated using the prostatic acid phosphatise (PAP) - a prostate cancer antigen linked to granulocyte-macrophage colony-stimulating factor (GM-CSF), an immune cell activator which is currently undergoing clinical trials (Cheever and Higano 2011). Other approaches such as the use of synthetic peptides are currently also being developed with varying levels of success (Perez-Diez, Martinez-Crespo et al. 1996).

The other major vaccine approach is designed to enhance the immune responses to tumours using novel adjuvants. As discussed in previous sections tumours can be highly immunoevasive and can escape control by either preventing antigen recognition or by creating a suppressive microenvironment (Melchionda, McKirdy et al. 2004; Kim, Emi et al. 2006). Adjuvants against cancers have been known for many decades. For example, it was noted in 1975 that serum from BCG inoculated mice can induce necrosis in transplanted tumours (Carswell, Old et al. 1975). Currently many new therapeutic approaches are being investigated, particularity using immuno-modulatory cytokines such as IL-2, GMCSF and others (Elias, Zapas et al. 2005). However, in a clinical setting only BCG, aluminium based salts and squalene-oil-water emulsifications are approved for use. Cancer vaccines, such as anti-cancer mAbs, are currently of limited use and often insufficient when used on their own. Only two therapeutic vaccines of this sort are currently at phase II or above at this present time.

1.7.1.3: Genetic modification and gene therapy

Genetic modification of tumours is a novel approach to directly alter tumour cells and the tumour-related factors released into the microenvironment. For established tumours, *in vivo* delivery of genes can be achieved through local injection of viral vectors containing the gene into the tumour itself or to specific immune cells (Dummer, Hassel et al. 2004; Dummer, Rochlitz et al. 2008). Many vectors are now available for *in vivo* or *ex vivo* modification of the immune system, including lentivirus (Poeschla, Wong-Staal et al. 1998), adenovirus (Bramson, Hitt et al. 1996), retrovirus (Miller, Miller et al. 1993), herpes simplex virus (Rees, McArdle et al. 2002), vaccinia (Carroll, Overwijk et al. 1998), and fowlpox (Kim, Cormier et al. 1998). Transduction of the IL-2 gene into rat gliosarcoma 9L cells *in vivo*, in combination with treatment with an irradiated 9L cell vaccine, was sufficient to induce tumour rejection (Iwadate, Yamaura et al. 2001). Other studies using TNFα transduced B16 melanoma tumours *in vivo* have also resulted in reduced tumour growth and increased infiltration of CD4 and CD8 T cells (Kianmanesh, Hackett et al. 2001). Clinical trials using canarypox viral vectors to express cytokines in murine prostate cancer has shown positive results, inhibiting tumour growth (Griffith, Kawakita et al. 2001). OncoVEX GM-CSF is currently in a phase III trial using modified herepes simplex engineered to selectively replicate in melanoma tissue and express GM-CSF to enhance the anti-tumour response. This has shown a very high degree of efficacy in Phase II trials.

Ex vivo genetic modification of tumour cells for use as a whole cell vaccine is a recent approach to provide exposue to TAAs under pro-immune conditions. This is a promising approach that avoids the logistical problems faced in trying to direct vectors specifically to tumour cells as well as ethical issues associated with gene therapy (Ettinghausen and Rosenberg 1995). The use of a genetically modified tumour as a whole cell vaccine is discussed in more detail chapter 6.

1.8: T cell Adoptive Immunotherapy

Adoptive immunotherapy for the treatment of cancer was first utilised in mice in the late 1950s and 60s, where it was demonstrated that transfer of leukocytes, before tumour inoculation, could lead to rejection (Mitchison 1955). It was later

found that leukocytes from immunised mice transferred to tumour-bearing mice could also lead to tumour regression (Borberg, Oettgen et al. 1972). Shortly after these observations, IL-2 was discovered to be an important cytokine in the maintenance and proliferation of T cells, allowing generation of large numbers of T cells *in vitro* (Robb, Munck et al. 1981). Subsequently, IL-2 was used to expand tumour infiltrating lymphocytes for use in adoptive transfer to reduce tumour burden (Rosenberg and Lotze 1986).

Special note must be made of Rosenberg and colleagues in applying the system of *ex vivo* expansion of melanoma-specific T cells for adoptive immunotherapy. Initially lymphocytes were harvested from surgically removed melanomas and expanded *in vitro* for adoptive transfer back to the patient, combined with high doses of IL-2. This produced promising results with some patients entering into complete remission, however many experienced significant 'cytokine storm' side effects associated with high doses of IL-2, causing this approach to be dropped or modulated by others (Rosenberg, Mule et al. 1985; Rosenberg 1988). This has been furthered by Dudley and colleagues who have demonstrated that lymphocyte depletion using cyclophosphamide and other drugs prior to adoptive cell transfer significantly improved the anti-tumour response (Dudley, Wunderlich et al. 2005). Following the early success of adoptive immunotherapy utilising polyclonal tumour responses, more recent trials have focused on transfering tumour-specific lymphocytes. The most recent clinical adoptive therapy strategies have been utilised against melanomas using well defined melanoma TAAs such as Melan-A/Mart-1 (Dudley and Rosenberg 2003). Recently however, identification of many other TAAs in association with other cancers has increased the clinical relevance of adoptive immunotherapy. Table 3 highlights some of the most recent adoptive immunotherapy trails.

Table 3: Clinical trials using adoptive T cell transfer

Date	Cell Type	Treatment regimen	Tumour	Responses	Reference
1988	*In vitro* expanded Tumour infiltrating Lymphocytes (TIL)	High IL-2 (7.2 x10^5 IU/Kg)	Melanoma	11/15 Objective response (OR)	(Rosenberg, Packard et al. 1988)
1994	*In vitro* expanded TIL	Cyclo. High IL-2 TIL and IL-2 in two week	Melanoma	24/86 Partial response (PR)	(Rosenberg, Yang et al. 1994)

		cycles		5/86 Complete response (CR)	
1990	*In vitro* expanded TIL	High IL-2. TIL and IL-2 in two week cycles	Melanoma		(Rosenberg, Anderson et al. 1993)
2002	*In vitro* expanded Mart-1 or gp-100 T cells from Peripheral blood mononuclear cells (PBMC)	Increasing doses of IL-2 s.c.	Melanoma	5/10 Stable disease (SD) 1/10 Major response (MR) 2/10 Minor response (MiR)	(Yee, Thompson et al. 2002)
2002	*In vitro* expanded TIL (7.8×10^{10} cells i.v.)	Cyclo and Flur for 7 days, cell transfer. High IL-2.	Melanoma	6/13 OR 4/13 MR	(Dudley, Wunderlich et al. 2002)
2005	*In vitro* expanded TIL	Cyclo (2d) then Flur (5d) transfer then high IL-2	Melanoma	15/35 PR 3/35 CR	(Dudley, Wunderlich et al. 2005)
2006	PBMC transduced with Mart-1 specific TCR.	Cyclo (2d) then Flur (5d) transfer then high IL-2 and Mart-1 vaccine	Melanoma	2/17 PR 1/17 MR	(Morgan, Dudley et al. 2010)
2008	*In vitro* expanded NY-ESO-1 specific CD4 from PBL	N/A	Melanoma	1/1 CR	(Hunder, Wallen et al. 2008)
2008	Genetically modified T cells expressing CD20 specific chimeric receptor	Varied cytoreductive therapy before cell transfer then 14 days low IL-2 (5×10^5 IU/m^2)	Non Hodgekins Lymphoma / Mantel cell lymphoma	2/7 CR 4/7 SD 1/7 PR	(Till, Jensen et al. 2008)
2010	PBMC transduced with CD19 chimeric antigen receptor, 66% CD8, 34% CD4.	Cyclo, 2 days and Flur for 5 days. Then cell transfer followed by high IL-2 i.v. every 8hrs	Folliculoar lymphoma	1/1 PR	(Kochenderfer, Wilson et al. 2010)

1.8.1: Adoptive immunotherapy using TCR transgenic T cells

Initial approaches to obtain tumour antigen-specific T cells were to selectively expand TILs using cytokines such as IL-2 in the hope of expanding tumour-specific lymphocytes (Rosenberg and Lotze 1986). Later identification of

specific TAAs has allowed for selective expansion, isolation and purification of tumour-specific T cells prior to adoptive transfer, as is the case with many melanoma trails (Dudley and Rosenberg 2003). Despite this, the resultant T cell responses *in vivo* are often limited by tumour-induced immunosuppressive networks, combined with the relatively low affinity of TCR/TAA interactions due to most TAAs being self-peptides. These factors can induce tolerance, adaptation and anergy in tumour-specific T cells, representing a major challenge for adoptive immunotherapy (Lohr, Knoechel et al. 2005). A more recent strategy is to genetically modify T cells, usually CD8 T cells, to counteract tumour-mediated suppression (Kershaw, Hsu et al. 2001).

The use of TCR transgenic mouse models has provided much insight in this area of research (Johnson, Morgan et al. 2009). Abad *et al.* used Pmel1 specific TCR transgenic CD8 T cells that recognise the gp100 B16 melanoma antigen in the context of $H2D^b$ (Overwijk, Theoret et al. 2003). Adoptive transfer of Pmel1 specific CD8 T cells caused tumour regression in a dose-dependent manner. This response was inhibited when Pmel1 T cells were co-transferred with unmodified lymphocytes (Abad, Wrzensinski et al. 2008). Similar results are also seen by DeWitte *et al.*, who showed OT-1 TCR transgenic CD8 T cells, which recognise the OVA antigen expressed by the B16-OVA cell line (Johnson, Morgan et al. 2009), can mediate tumour rejection. When transferred with unmodified lymphocytes, this response can also be inhibited. Conversely, irradiation before OT-1 transfer was found to increase the anti-tumour response (de Witte, Jorritsma et al. 2008), supporting initial observations by Dudley using immunosuppressive drugs (Dudley, Wunderlich et al. 2005).

Similar observations have also been reported in spontaneous tumour models. De Witte used transgenic adenocarcinoma of mouse prostate (TRAMP) mice in which transforming SV40 large T antigen is expressed under control of the probasin promoter resulting in the development of prostatic intraepithelial neoplasia between 8-12 weeks of age. T cells that are isolated and transduced with a TCR specific for an epitope of SV40 are able to induce tumour regression on adoptive transfer to tumour-bearing TRAMP mice; when combined with an $SV40_{IV}$ recombinant virus vaccine at week 10, long-term remission was achieved (de Witte, Bendle et al. 2008).

Similar observations have been reported using T cell adoptive transfer coupled with radiation pre-conditioning (Cao, Daniel et al. 2002). Irradiation is

beneficial to adoptively transferred T cells by increasing antigen shedding by tumours, and by creating 'space' for expansion, increasing cytokine availability by depletion of other T cells and by the removal of Tregs (Gattinoni, Powell et al. 2006). Together, these results indicate that genetic modification of T cells with TCR gene therapy can be sufficient to induce regression and this response is enhanced by radiotherapy, lymphopenia and vaccination. However, as an unwanted side effect, T cell responses specific for TAAs can have the potential to cause GvHD in irradiated hosts (see chapter 7) (Kaplan, Anderson et al. 2004).

1.8.2: Adoptive immunotherapy using CD8 T cells

Adoptive transfer of CD8 CTLs has been used extensively in mouse tumour models. As discussed above, TCR transgenic CD8 T cells can induce anti-tumour responses *in vivo* leading to tumour rejection (Abad, Wrzensinski et al. 2008). A plethora of new CTL targets and improved cell culture protocols have lead to a succession of clinical trails in humans, particularly in melanoma treatment (Yee 2010). Efficacy of these treatments however rarely exceeds 50% (Yee, Thompson et al. 2002) and the high costs associated with generating large numbers of CTL clones make widespread treatments impossible at this time. Approaches that produce lower numbers of CTLs with a more robust anti-tumour response are promising for future therapies. This can be achieved by *in vitro* conditioning and genetic modification.

Driving T cell differentiation *in vitro* prior to adoptive cell transfer is an approach to increase the efficacy of *in vivo* anti-tumour responses. Adoptive transfer studies using CD8 T cells have revealed that optimal anti-tumour function is dependent on their differentiation state. Effector memory (T_{EM}) CD8 T cells have up to 100 fold less effective anti-tumour activity than central memory (T_{CM}) CD8 T cells (Wang, Huang et al. 2004). This is because T_{EM} have reduced expression of lymphoid homing molecules ($CCR7^-$) and co-stimulatory molecules (CD28) (Klebanoff, Gattinoni et al. 2006), and lose their ability to produce IL-2 and TNFα and to respond to homeostatic cytokines, resulting in decreased survival and exhaustion *in vivo* (Zhou, Ou et al. 2004).

IL-15 drives T cells to a T_{CM} phenotype while IL-21 promotes a $CD45RO^+$ $CD28^{hi}$ stable phenotype that retains ability to produce IL-2 after antigen exposure (Hinrichs, Borman et al. 2009). Driving towards a T_{CM} phenotype is a promising

strategy to maintain effective anti-tumour immunity; however this is likely to require supplementary modification to overcome tumour immune evasion (Klebanoff, Gattinoni et al. 2006). IL-21 is the most recently identified member of the common gamma chain receptor cytokine family and has been used in clinical trials for treating cancer patients. *In vivo*, IL-21 is more potent than IL-15 at maintaining tumour-specific CD8 T cells and eliminating tumours (Moroz, Eppolito et al. 2004). *In vitro* activation of tumour-specific CD8 T cells with antigen plus IL-21 induces a less differentiated population that can make IL-2 following antigenic re-stimulation, representing potential helper independent CD8 T cells (Li, Bleakley et al. 2005). Interestingly, IL-21 primed Pmel1 specific T cells display a distinct gene expression profile in comparison with IL-15 conditioned cells (Zeng, Spolski et al. 2005). It is therefore very important to consider the activation conditions and phenotype of CD8 T cells before adoptive transfer.

1.8.3: Adoptive immunotherapy using CD4 T cells

CD4 T cells are critical for the anti-tumour response in a variety of murine models, largely based on their ability to produce IL-2 (Antony, Paulos et al. 2006) and IFNγ (Hung, Hayashi et al. 1998). CD4 cells have been shown to reject tumours alone or in combination with additional treatments, and can be more efficient at rejecting tumours that CD8 T cells (Perez-Diez, Joncker et al. 2007). Mumberg *et al.* also demonstrated that CD4 T cells were able to clear MHC-II$^{-/-}$ tumours *in vivo* by a mechanism that was dependent on the indirect effects of IFNγ (Mumberg, Monach et al. 1999). Despite this, most basic and clinical studies have focused on adoptive transfer of CD8 T cells alone, or those co-transferred with CD4 T cells (Antony, Piccirillo et al. 2005) (Cohen, Peng et al. 2000).

As for CD8 T cells, the differentiation status of CD4 T cells is important for their *in vivo* behaviour following adoptive transfer. CD4 T cells can be differentiated into Th1, Th2 or Th17 cells *in vitro*. Th17 cells produce IL-17A, IL-6 and GM-CSF, capable of activating other innate immune cells such as neutrophils, leading to local inflammation (Korn, Bettelli et al. 2009). Several groups have shown Th17 cells can be more effective at tumour eradication than Th1 cells (Muranski, Boni et al. 2008). This observation is relevant to recent findings describing antigen-driven loss of FoxP3 by Tregs and their subsequent differentiation towards Th1 or Th17 like

phenotypes in tumour-draining lymoh nodes (Sharma, Hou et al. 2009) (see chapter 5).

It is clear that a large variety of factors influence the anti-tumour activity of adoptively transferred T cells. These factors must be considered in the design of future adoptive T cell therapies. Despite many clinical trials, novel protocols, along with reports of donor T cell persistence (Rosenberg 1988; Kochenderfer, Wilson et al. 2010), and infiltration into the tumour mass (Yee, Thompson et al. 2002), the potential of adoptive immunotherapy has yet to be realised.

1.9: Project Aims

The key aim of this project is increase our understanding of the *in vivo* fate of adoptively transferred HY-specific CD4 and CD8 T cells in response to HY antigens in various models including tolerance induction and immunotherapy:

Specific questions to be addressed are:

1. Why do endogenous T cell responses fail to control tumours?
2. What are the processes that underlie the distinct responses of CD4 and CD8 T cells to male skin grafts, and male-derived tumour cells?
3. What is the role of antigen-driven FoxP3 downregulation in antigen-specific anti-tumour responses?
4. Why do polyclonal and antigen-specific CD8 T cells eliminate tumour cells expressing IL-2 but not wild type tumour cells?
5. Why do male antigen-specific CD4 but not CD8 T cells induce GvHD in radiation conditioned lymphopenic male mice?

Chapter 2: Materials and Methods

The materials and methods used in this project are described in this chapter. Buffers and chemical reagents are described first, followed by protocols. Experiment specific detail is described in Results.

2.1: Media and Reagents

Table 4: List of Reagents

Medium	Use	Composition
Phosphate Buffered Saline (PBS)	Resuspending of cells/reagents for *in vivo* use	Made from tablets (Oxoid, Hampshire, UK) in distilled water. NaCl, 137mmol/L; KCl, 2.7mmol/L; Na_2HPO_4, 8mmol/L; KH_2HPO_4, 2mmol/L. pH 7.3.
MACS buffer	Washing of single cell suspensions before MACS/FACS sorting	PBS, 2mM EDTA, 1% FCS
RBC lysis buffer (Qiagen, Hilden, Germany).	Depletion of red blood cells before counting/FACS	Unspecified concentrations of NaCl, EDTA and Sodium Bicarbonate
Complete medium	Maintenance of hybridomas / tumour cell lines / *in vitro* cultures	RPMI 1640 Medium (Invitrogen), supplemented with 10% foetal calf serum (FCS; Autogen Bioclear, Calne, UK), 10mM HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid) (Invitrogen), 100 IU/ml penicillin (Invitrogen), 100μg/ml Streptomycin (Invitrogen), 2mM L-glutamine (Invitrogen) and $2x10^5$ M β2-Mercaptoethanol.

Complete medium 2	Maintainance of phoenix cell line	IMDM (Iscove's Modified Dulbecco's Medium) supplemented as with RPMI 1640
Blood Buffer	Collection of blood	PBS, 100U/ml Heparin, 2mM EDTA
Ammonium Sulphate Solution	Purification of Antibodies from hybridomas culture supernatant	4.1M $(NH_4)_2SO_4$ in dH_2O
Freezing Medium	Storing cells in liquid nitrogen	8ml FCS, 2ml DMSO (20%) (1ml freezing medium + 1ml cell suspension)

Ingredients of complete culture medium (Invitrogen, Paisley, UK) except for FCS (Autogen Bioclear, Calne, UK).

2.2: Animals

Mice used in this project were housed under specific pathogen-free conditions at the Central Biological Services Unit in Hammersmith Campus, Imperial College London. Animals were used in accordance with the Home Office guidelines as defined in the project licence and Animal Procedures Act 1986. All mice were on a C57BL/6 background.

Table 5: Mouse strains used in this project

Strain	**Phenotype**	**Reference**
C57BL/6	Wild type, $Thy1.2^+$	(Russell, Neufeld et al. 1951; Hoag 1963)
C57BL/6 $Thy1.1^+$	Wild type, $Thy1.1^+$	(Russell, Neufeld et al. 1951)
$Rag2^{-/-}$	Deficient in B cells and T cells No TCR rearrangement due to a lack of Rag Enzyme.	(Shinkai, Rathbun et al. 1992)
Marilyn	TCR transgenic for HY-A^bDby	(Valujskikh, Lantz et al. 2002)

Marilyn dnTGFβRII	Double transgenic Marilyn TCR and dominant negative TGFβ receptor type II	(Gorelik and Flavell 2001)
Matahari	TCR transgenic for HY-D^bUty	(Lantz, Grandjean et al. 2000)
FoxP3-GFP	$FoxP3^+$ T cells co-express GFP which can be detected by FACS	(Wang, Kissenpfennig et al. 2008)
FoxP3-GFP Marilyn	TCR transgenic for HY-A^bDby with $FoxP3^+$ T cells co-express GFP	(Chai, Coe et al. 2008)
FoxP3-GFP-DTR	$FoxP3^+$ T cells co-express GFP and Diphtheria Toxin receptor	(Kim, Rasmussen et al. 2007)
FoxP3-GFP-DTR Marilyn	TCR transgenic for HY-A^bDby with $FoxP3^+$ T cells co-express GFP and Diphtheria Toxin receptor	(Developed by Chai, JG. Unpublished)
IL-10 KO	Defect of IL-10 production	(Kuhn, Lohler et al. 1993)
IL-17A KO	Defect of IL-17A production	(Nakae, Komiyama et al. 2002)

C57BL/6 wild type mice ($H2^{b+}$,$Thy1.2^+$) were originally produced in 1921 by Clarence Little and have since been maintained as an inbred strain. B6 have a high leukocyte count (Russell, Neufeld et al. 1951); a low frequency of tumour development (Hoag 1963), and a skew towards a Th1-like phenotype (Hsieh, Macatonia et al. 1995). The phenotype of B6 mice makes them useful for studying the cellular and molecular mechanisms of tumour development. B6 were purchased from Harlan Laboratories (Bicester, UK).

Rag deficient mice ($Rag2^{-/-}$) have an inactivated recombination activating gene 2 required for TCR and BCR rearrangement and without which B cells and T cells do not develop. B6 $Rag2^{-/-}$ mice were originally produced by Frederick Alt *et al.* (Columbia University, NY) (Shinkai, Rathbun et al. 1992). Marilyn and Matahari mice on a B6 background were kindly provided by Dr Oliver Lantz (Curie Institute, Paris). Marilyn mice express a HY-specific $V\beta6^+$ TCR which directs development of $CD4^+$ T cells recognising the Dby (NAGFNSNRANSSRSS) peptide presented by the MHC class II molecule H2-A^b (Valujskikh, Lantz et al. 2002). Marilyn mice

were provided on a Thy1.2^{+} Rag2$^{-/-}$ background, these were then crossed with B6 Thy1.1^{+} male mice to produce Thy1.1^{+} and/or Thy1.2^{+} Marilyn mice on a Rag2$^{-/-}$ or Rag2$^{+/-}$ background. Matahari mice express a HY-specific Vβ8.3^{+} TCR which directs development of CD8^{+} T cells recognising the Uty (WMHHNMDLI) peptide presented by the MHC class I molecule H2-D^{b} (Lantz, Grandjean et al. 2000). Matahari mice were provided on a Rag1$^{-/-}$ Thy1.2^{+} background and crossed with B6 Thy1.1^{+} male mice to produce Thy1.1^{+} and or Thy1.2^{+} Matahari mice on a Rag2$^{-/-}$ or Rag2$^{+/-}$ background.

FoxP3-GFP knock in mice on a Thy1.2^{+} B6 background were kindly provided by Dr B. Malissen (Centre d'Immunologie de Marseille-Luminy, Marseille, France) (Wang, Kissenpfennig et al. 2008). These mice were also bred with Rag2$^{-/-}$ Marilyn mice to give Rag2$^{+/-}$ F1 females that are TCR transgenic for HY-A^{b}-Dby with FoxP3^{+} T cells co-expressing GFP. FoxP3-GFP Marilyn mice produce double transgenic HY-specific Tregs that can be purified by FACS cell sorting. Those purified from the thymus represent nTregs whilst those purified from spleen (SP) and lymph node (LN) includes both nTreg and iTreg.

FoxP3-GFP-DTR knock in mice on a Thy1.2^{+} B6 background were kindly provided by Prof Daniel Altmann (Imperial College London) (with the permission of Prof Rudensky) (Kim, Rasmussen et al. 2007). These mice have a double fusion protein linked to FoxP3 that induces FoxP3^{+} cells to express DTR on their surface in addition to GFP. These cells were purified as above and selectively deleted by administration of Diphtheria Toxin (DT) *in vivo* (i.p) or *in vitro*. By mating FoxP3-GFP-DTR male mice with Rag2$^{-/-}$ Marilyn females we were able to produce Rag2$^{+/-}$ F1 females that are TCR transgenic for HY-A^{b}-Dby with FoxP3^{+} T cells co-expressing GFP and DTR. FoxP3-GFP Marilyn mice produce triple transgenic Tregs that can be purified by FACS cell sorting and selectively deleted using DT.

IL-10 knockout (IL-10 KO) mice do not produce IL-10 and were generated on a C57BL/6 background using gene targeting, by Ralf Kuhn *et al.* (University of Cologne) (Kuhn, Lohler et al. 1993). IL-10 KO mice were purchased from the Jackson Laboratory (Bar Harbor, ME). IL-17A knockout mice (IL-17A KO) mice do not produce IL-17A and were generated, on a C57BL/6 background by Susumu Nakae *et al.* (Institute of Medical Science, Tokyo) (Nakae, Komiyama et al. 2002).

2.3: Peptides

The HY-A^b-Dby peptide, NAGFNSNRANSSRSS (Scott, Addey et al. 2000), and the HY-D^b-Uty peptide, WMHHNMDLI (Simpson, Scott et al. 1997), were synthesised by the central research resource unit at the Clinical Sciences Centre (Hammersmith Hospital, London) using 9-fluorenylmethyloxycarbonyl-protected amino acids and (2-(1-H-benzotriazol-2-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)) activation chemistry.

2.4: Cell purification

2.4.1: Purification of Lymphocytes using MACS

Lymphocytes were purified from single cell suspensions of pooled LN and SP or from *in vitro* cultures. Suspensions were passed through a 70μm nylon cell strainer (Becton Dickinson, Oxford UK), using the base of a 1ml syringe plunger to force through cell suspensions. Cells were then purified using MACS® cell purification kits (Miltenyi Biotec, Surrey, UK). Firstly, cells were labelled with small chemically inert magnetic beads with a specific mAb antibody on their surface (MACS beads) (4^{o}C 15minutes 100-200μl volume). Cells were washed and re-suspended in ~2ml MACS buffer (PBS, 2mM EDTA, 1% FCS) and then passed 3 times through magnetic columns mounted on a powerful magnet. MS (medium) or LS (large) MACS separation columns were used initially in this study followed by AutoMACS® cell separation machine at later stages. Columns were washed by passing through a further 2ml of MACS buffer. Columns are then removed from the magnetic source and cells are forced out of the column using MACS buffer (~5-10ml) and the provided plunger. Cells were then washed using MACS buffer and pelleted by centrifugation (1800rpm 5mins).

2.4.2: Purification of Marilyn and Matahari conventional T cells

Matahari or Marilyn cells were purified from LN and SP cell suspensions or from *in vitro* culture. 30μl of anti-CD4 (Marilyn T cells) or anti-CD8 (Matahari T cells) MACS beads were added to cell suspensions and stained as described

previously. Samples were then purified using an MS or LS MACS separation columns or AutoMACS®. Following separation, cells were re-suspended in an appropriate volume of either PBS or complete culture medium for *in vivo* injection (i.v.) or cell culture.

Matahari and Marilyn T cells particularly from $Rag^{+/-}$ mice have CD8 or CD4 compartments that can contain T cells not expressing a surface transgenic TCR. In some experiments it was therefore important that cells were further purified using FACS sorting. For Matahari, cells are stained using 1-5 µl of anti-$V\beta8.3^{PE}$ or ^{FITC} and anti-$CD8^{PE/cy5}$ or ^{APC} (4^oC 15minutes 100-200µl volume) and sorted by the Medical Research Council (MRC) cell sorting facility (Hammermith Hospital, London) using cell sorters such as Becton Dickinson FACS Aria®. This gave cell preparations of >99% purity. These cells were then re-suspended in an appropriate volume of PBS or complete culture medium for *in vivo* injection (i.v.) or *in vitro* cell culture.

For Marilyn, cells were stained using 1-5 µl of anti-$V\beta6^{PE}$ or ^{FITC} and anti-$CD4^{PE/cy5}$ or ^{APC} (4^oC 15minutes 100-200µl volume) and sorted as described previously.

2.4.3: Purification of Tregs from B6 and Marilyn mice

Marilyn $CD4^+$ $CD25^+$ Tregs were purified from spleen and pooled LN of $Rag^{+/-}$ mice which produce ~5% Tregs (containing both nTreg and iTreg) in the CD4 T cell compartment. To purify nTregs alone, cell suspensions were made from thymus, and $CD8^+$ and $CD4^+CD8^+$ thymocytes were removed using anti-CD8 microbeads. Cells were isolated and suspended as described previously before staining using 5µl of anti-$CD25^{PE}$ antibody (e-Bioscience) (4^oC 15minutes 100-200µl volume). Cells were then washed (MACS buffer) and stained using 20µl anti-PE MACS beads (4^oC 15minutes 100-200µl volume). Cells were washed again and separated using columns or AutoMACS® as described previously. The negative fraction was used to generate a CD25 depleted conventional CD4 T cell fraction. This was then further purified by staining using CD4 beads as described previously. $CD25^+$ fractions were used as a pre-enriched suspension of Tregs. For further purification, cells were stained using 1-5 µl of anti-$V\beta6^{FITC}$, $CD25^{PE}$ anti-$CD4^{PE/cy5}$ or ^{APC} (4^oC 15minutes 100-200µl volume) and sorted by FACS as described

previously. To obtain a purified fraction of WT B6 Tregs, the same procedure was used as described above except cells were stained using anti-TCRβ^{FITC} instead of Vβ6. It is of note that not all CD4^{+} CD25^{+} cells are regulatory T cells.

2.4.4: Purification of green Treg cells from single or double transgenic mice.

To obtain reliable Tregs populations, FoxP3-GFP knock in mice were used as the FoxP3 transcription factor expression is required for the development of Tregs which can be sorted based on GFP expression (Williams and Rudensky 2007). For FoxP3-GFP knock in Marilyn double transgenic mice, cells were isolated and suspended as described previously. CD25-expressing cells were first enriched by MACS prior to staining with anti-Vβ6^{FITC}, anti-CD25^{PE} and anti-CD4^{APC} and FACS sorted for triple positive population CD4^{+} Vβ6^{+} and GFP^{+} (or GFP^{-} for conventional population) with >99% purity. For FoxP3-GFP knock in B6 mice the same procedure was followed except cells are stained using TCRβ^{FITC} instead of Vβ6^{FITC}. For the isolation of GFP^{+} nTregs, the initial cell suspension was thymocytes, which were subjected to CD8 depletion to removal of both CD8^{+} and CD4^{+}CD8^{+} thymocytes.

2.4.5: Purification of antigen-presenting cells (APCs)

Due to peripheral lymphoid organ architecture, SP and LN must be additionally digested prior to APC purification. SP and LN were initially diced using a size 22 scalpel. The resulting tissue was added to a 50ml Falcon tube and digested by the addition of 2 mg/ml Collagenase XI and 10 μg/ml DNase in 1ml PBS with 2% FCS. This was incubated by shaking at 37^{o}C for 30 minutes. 100μl 0.1M EDTA was added for the final 5 minutes to terminate the reactions. The tissue was then strained into a cell suspension as described previously. APCs were then further isolated by the addition of MACS beads; anti-CD11c for dendritic cells, anti-CD11b for macrophages and anti-B220 for B cells (4^{o}C 15minutes 100-200μl volume) and MACS purification as described previously. These cells were then used for further cell culture.

2.5: Proliferation Assays

2.5.1: T cell proliferation assays with peptide and cytokine

Cell suspensions were made as described previously. Either whole SP and LN cells were plated in 200µl volume complete media +/- HY peptide +/- cytokine (37^oC 5%CO_2). When purified T cells were used as responders, APC populations were also included. 48-72 hours later 0.5µlCi/well of tritiated thymidine (New England Nuclear) was added and cells were cultured for a further 8-24 hours. Plates were then harvested onto a filter mat and analysed using a Wallac Trilux 1450 Microbeta liquid scintillation and luminescence counter (PerkinElmer, Beaconsfield, UK).

2.5.2: Activation of HY-specific T cells with Male SP cells *in vitro,*

Cell suspensions were made as described previously. Either whole SP and LN or purified T cells (either CFSE labelled or not) were plated out in 1ml complete media +/- cytokine. Male SP cells were also re-suspended at 5 x 10^6cells/ml and added to 1ml of responders in a 24 well plate and cultured for 5-7 days (37^oC 5%CO_2). Cells were then harvested and analysed for *in vitro* phenotype, cytokine production and expansion or harvested for adoptive cell transfer.

2.5.3: Carboxyfluorescein Succinimidyl Ester (CFSE) dilution assay

T cell proliferation can be measured by dilution of CFSE. T cells were isolated *ex-vivo* or directly from culture as described previously. Following purification using MACS and or FACS cell sorting, cells were washed three times using PBS and re-suspended in 1ml PBS. CFSE (Molecular Probes, Cambridge, UK) was-added to the cell suspension with a final concentration of 1µM and incubated for 10 minutes RT in the dark. Following incubation, cells were washed in MACS buffer before being re-suspended in either PBS for injection (i.v.) or complete medium for *in vitro* culture. Following *in vitro* culture or *in vivo* experiment T cells were isolated from SP and LN as described previously and stained for surface markers using conjugated antibodies (see next section). CFSE dilution was

measured as a readout of T cell proliferation analysed using FACS™ Calibur (Becton Dickinson).

2.6: Flow Cytometry Analysis

2.6.1: Surface staining for Flow Cytometry

A wide variety of cell types were prepared for Flow cytometric analysis. *Ex-vivo* cells were harvested and washed and, if necessary, depleted of red blood cells using 2ml of red blood cells lysis buffer (Qiagen) and incubated (15 mins at RT). When staining APCs, LN and or SP may also be digested using collagenase and DNAase as described previously. All types of cells (*ex vivo* and *in vitro*) were then centrifuged (1800rpm 5mins) and washed (PBS) before centrifuging once more. Cells were incubated with conjugated antibodies (eBioscience, Hatfield, UK or R&D Systems, Abingdon, UK) and incubated (4^{o}C 15 minutes 100-200µl volume of PBS) in the dark. Antibody concentrations were variable and defined on the basis of previous data although generally 1µl of antibody is added per million target cells. Cells were washed once more analysed using a FACS™ Calibur (Becton Dickinson). When staining for tetramer, cells were treated as above but were stained with tetramer conjugated with PE and incubated for 15 minutes at 37^{o}C in 100-200µl complete medium followed by further surface staining of CD8 as described previously.

2.6.2: Intracellular staining for Flow Cytometry

Intracellular staining was used to detect protein targets that are not expressed on the cell surface, such as cytokines. If intracellular staining was required, cells harvested either from *in vitro* cultures or isolated *ex vivo* must be stimulated *in vitro* with 0.05µg/ml PMA and 0.25µg/ml Ionomycin. After 2 hours, 1.0µg/ml Brefeldin (BD Biosciences, Oxford, UK) was added for a further 2 hours. Following *in vitro* stimulation, live cells were harvested, re-suspended (~2ml complete media) and isolated by layering the cell suspension onto Ficoll (GE heathcare) and centrifuging for 20 minutes 400g at room temperature (RT). Live cells form a layer above the

Ficoll which can be pipetted off. Cells were then washed once in PBS. Cells were then washed once in PBS, then prepared and stained for surface molecules as described previously. Cells were then washed and fixed by removing all PBS and re-suspending cells in 300μl of Fix-Perm (e-Bioscience) and incubated for 30 minutes in the dark at RT. Then washed once in 1ml Perm buffer (e-Bioscience) and incubated with an appropriate volume of conjugated antibody (4^oC 15 minutes 100-200μl volume of Perm buffer). Cells were washed once more in Perm buffer and finally in PBS before analysis using a FACS™ Calibur (Becton Dickinson).

2.7: Antibodies

Some monoclonal antibodies (mAbs) were purified from hybridoma culture supernatant. Hybridomas were grown in rotating (0.02rpm) 1 litre roller bottles in 500ml complete medium at 37^oC for 10 days. Following incubation cells were removed by centrifugation (1800rpm 10 mins), supernatant was removed and carefully mixed with saturated ammonium sulphate solution (VWR, Lutterworth, UK) at a 1:1 ratio and left overnight 4^oC with a magnetic stirrer. A precipitate was formed which was pelleted by centrifugation (2000g for 30 minutes) and re-suspended in 10ml PBS. The resultant suspension was aliquoted into dialysis tubing and sealed at both ends before floating in 4 litres PBS with magnetic stirirng overnight at 4^oC, PBS was replaced once and left for a further overnight dialysis. The purified antibody was then isolated from dialysis tubing by first centrifuging (1800rpm 10 mins) to remove any remaining cells or particulates. The supernatant was then sterilised (0.2 μm) before being aliquoted for use *in vivo*.

mAbs were typically used as 200μl i.p. or further purified using a protein AG column (ThermoFisherScientific, UK). To purify further 10ml of supernatant was concentrated using a Centricon YM-10 centrifugal filter (Millipore, Southampton, UK). Antibodies were then purified from a 2ml concentrated supernatant using a protein A/G spin kit (ThermoFisherScientific), concentration was measured with a BCA™ Protein Assay Kit (ThermoFisherScientific). A table of mAbs used in this project is listed below.

Table 6: List of Hybridomas

Antibody/Hybridoma	Brief Description	Reference
DTA-1	Rat anti-mouse GITR, IgG2a	(Shimizu, Yamazaki et al. 2002)
PC61	Anti-CD25 (depleting)	(Lowenthal, Corthesy et al. 1985)
GL113	Anti-Galactosidase, control mAb	(Savelkoul, Seymour et al. 1991)
YTS169.4	Anti-CD4 (depleting)	(Benjamin, Cobbold et al. 1986)
YTS191	Anti-CD8 (depleting)	(Cobbold, Jayasuriya et al. 1984)
R46A/2	Anti-IFNγ (neutralising)	(Li, Sanjabi et al. 2006)

2.8: Microscopy and Imaging

2.8.1: Deconvolution microscopy

Tumour cells transduced with the GFP fluorescent transgene were imaged using a Deconvolution microcope Zeiss LSM-510 (Faculty for imaging by light microscopy, Hammersmith Hospital, Imperial College London). Cells were suspended at 10^3 cells/ml and 100µl was mounted on a slide (unfixed). Cells were then imaged using standard white light (brightfield), followed by imaging using a TRITC laser which excites cells at a frequency of 475nm. Cells containing the GFP transgene will emit light at a wavelength of 510nm which was imaged. The fluorescent image was overlaid onto the brightfield image to give a final image where genetically modified tumour cells were visible as green.

2.8.2: *Ex vivo* images

Photographic images were used in several experiments to reveal tumour structure and lymphoid organ architecture and size. A FugiFilm 5 mega pixel

camera was used in close-up mode fixed to a tripod. All photographs were taken at the same distance with similar lighting conditions. Photographs of tumours were taken in situ; photographs of lymphoid organs were taken on petri-dishes.

2.9: Skin Grafting

I am indebted to Dr David Coe and Dr Jian-Guo Chai for surgical grafting of tail skin onto the lateral thorax as described by Billingham and Medawar (Billingham, Krohn et al. 1951).

Tail skin from the donor was cut into sections ($1cm^2$). Recipients were anaesthetised using Isoflurane (Halocarbon Products Corp) until surgical anaesthesia was reached when 0.26μg of Metacam (Boehringer Ingelheim GmbH) was injected (s.c.). Mice were then immobilised giving access to the surgical site which was shaved and washed with 70% ethanol and Opsite spray dressing administered. A $1cm^2$ section of skin was removed from the right flank creating a graft bed. Any blood was removed and the donor skin was placed in the graft bed. Grafts were then covered in Vaseline (VWR)-soaked muslin and a plaster was wrapped around the thorax and graft. Mice were placed in a warming chamber at 32^oC to allow the plaster to dry and the mice to recover. Animals were inspected daily, plasters were removed after 10 days and grafts were then scored every other day

2.10: Tumour and T cell lines

2.10.1: MB49

The MB49 tumour cell line was derived from a 7,12 Dimethylbenz(a)anthracene (DMBA) induced bladder carcinoma arising in a male B6 mouse (Summerhayes and Franks 1979). Aliquots of MB49 were stored in liquid nitrogen. MB49 cells were rapidly thawed, washed with complete medium and cultured for 5-7 days. Once MB49 cells had grown to an optimal density, non-adherent cells were washed away with PBS and the remaining adherent cells were removed from the flask after 10 minutes incubation at RT with 2ml trypsin solution (Invitrogen), washed with PBS/2% FCS and centrifuged (1800rpm 5 minutes). Cells

were then plated out for continued culture (2 x 10^5 cells/25cm^3) or re-suspended at an appropriate concentration in 0.2-0.3ml PBS for *in vivo* injection. Cells were injected sub-cutaneously (s.c.) using a 0.5ml insulin syringe into the right-hand side dorsal lateral flank of the animal (unless stated otherwise). Mice were shaved in the area of injection and anesthetised using Isoflurane (Halocarbon Products Corp). The area of the tumour (mm^2) ((length (mm) X width (mm)) was then measured at regular intervals (3-4 times per week) using a digital calliper (Fisher Scientific). The humane end-point of this procedure was reached when any dimension reaches 14mm or if a tumour became ulcerated.

2.10.2: B16F10 and B16F10/HY

B16F10 (Fidler 1973) cell lines were maintained and used as for MB49 described previously. B16F10/HY cell line was developed within our lab by Dr Caroline Addey by transduction of HY-Dby transgene (pcDNA3.1/Zeo-*Dby*). Cells transduced with the HY transgene were selected *in vitro* using Zeocin (1mg/ml) (Invirtogen). Resistant colonies were selected after 2-3 weeks and tested for *Dby* expression by RT-PCR (Coe, Addey et al. 2010). These cells were also maintained as an *in vitro* culture as for B16F10 and MB49.

2.10.3: CTLL-2

CTLL-2 is an IL-2 dependent cytotoxic T cell line originally derived from C57BL/6 mice immunized with allogeneic (DBA/2, $H2^d$) Friend leukemia cells (Weston, Geczy et al. 1998). Cells were plated out in complete medium and recombinant human (rh) IL-2 at 10IU/ml at 1 x 10^5 in a 25cm^3 culture flask and split every 3 days or used at appropriate concentrations for thymidine proliferation assays.

2.11: Polymerase chain reaction (PCR)

2.11.1: Standard PCR

Standard PCR was used in this project to amplify genes of interest used for retroviral transduction of T cells and tumour lines. Much of this work was

performed by Dr Caroline Addey and Dr Istvan Bartok using the protocol outlined below:

2.5ul	10x buffer
1ul	DNA template
1ul	Forward primer
1ul	Reverse Primer
0.5ul	dNTPs (10mM)
0.7ul	$MgSO_4$ (50mM)
0.1ul	Taq polymerase
18.2ul	dH_2O

Denaturation

95 ^oC	5 minutes

PCR (30 cycles)

95 ^oC	30 seconds
(55 to 62 ^oC) ^oC	30 seconds
72 ^oC	30 seconds

Final extension

72 ^oC	7 minutes

Standard PCR was used to add *EcoR1* and *Bgl II* restriction enzyme sites to all products to facilitate cloning into the MIGR1 retroviral vector.

2.11.2: Reverse transcription PCR (RT-PCR)

I am again grateful to Dr Caroline Addey for her assistance in performing many of the RT-PCR techniques in this thesis. To assess expression of HY genes by tumour cells (MB49 and B16F10/HY), RNA was extracted from *in vitro* cultures or *ex vivo* excised tumour samples and male C57BL/6 SP cells by phenol-chloroform extraction using Trizol (Invitogen) according to the manufacturer's instructions. RNA was then quantified using a spectrophotometer (NaNoDrop® Technologies, USA) (Thermo Scientfic) prior to conversion into complementary DNA (cDNA) using BRL Superscript Reverse Transcriptase (Invitrogen) according to manufacturer's instructions. cDNA was then used as template in the PCR protocol outlined below, PCR products were then analysed on a 1% agarose gel electrophoresis containing Safeview (Invitrogen).

2.5ul	10x buffer
2ul	cDNA template

1ul	0.4 μM Forward primer
1ul	0.4 μM Reverse Primer
0.5ul	dNTPs 0.4 mM
0.7ul	$MgSO_4$ 50mM)
1.0ul	Taq polymerase
18.2ul	dH_2O

Denaturation

95 ^oC	5 minutes

PCR (30 cycles)

95 ^oC	60 seconds
52.5^oC	60 seconds
72 ^oC	60 seconds

Final extension

72 ^oC	7 minutes

Dby: Forward: CAGCAAGAGGTCTAGACA. Reverse: TCCTCTGTTGTGGCTACT

Smcy: Forward: TGAGACATAAGAAGCCCC. Reverse: GGCTTAATCCTTGAACTC

U*ty:* Forward: ACTTCGCCATATCCTCCT. Reverse: GTGCTTTCTGTCTCCTTC

2.11.3: StellARray® quantitative PCR (qPCR)

A commercial StellARray® was used for the analysis of a gene expression for a wider range of immune related genes. RNA was extracted from sorted cells using an RNAqueous 4PCR kit (Applied Biosystems, Warrington, U.K.) including a DNase I digestion step. cDNA was made from 300ng RNA using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems), which facilitates quantitative conversion of mRNA to cDNA.

For T Regulatory and MB49 gene expression analyses, 383 StellARray qPCR array plates (Lonza Sales AG, Basel, Switzerland) were used, with each plate containing 96 immune gene primer pairs. Power Sybr green master mix (Applied Biosystems) was mixed with cDNA and water following the manufacturer's instructions and 10 μl was added per well representing 1.5 ng cDNA. Plates were run on a 7900HT Real-Time PCR system (Applied Biosystems), under standard PCR

cycling conditions (50°C 2 min, 95°C 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min). The resulting cycle threshold (Ct) data were analyzed using a modification of Global Pattern Recognition algorithm, GPR 2.0 (Lonza), comparing three replicates of control cDNA with test cDNA. When there was no difference between the Ct values of both groups, the genes were considered as "normalizers". Each gene was compared with every normalizer in succession and the DCt was calculated (DCT Gene = CT Gene - CT Normalizer). The DCt for each group were then compared by a two tailed unpaired Student t test. Results were expressed as fold change, and those changes with a p value <0.05 were considered significant.

2.11.4: Taqman® RT-PCR

For the analysis of specific gene expression in a small set of samples, Taqman® RT-PCR assays were performed. 10 μl reactions were set up in 384 well plates, using Taqman MGB (minor groove binder) gene expression assays from Applied Biosystems. Target gene Taqman MGB probes were FAM dye labelled, whereas the 18S endogenous control probe was VIC dye labelled.

1-10ng 1st strand cDNA (in 4.5 μl H_2O)

0.5 μl 20x Taqman gene expression assay mix (primers and probe)

5 μl 2x Taqman master mix (Applied Biosystems)

Plates were run on a 7900 HT Real Time PCR System (Applied Biosystems), under standard cycling conditions (95^oC 10 min, followed by 40 cycles of 95^oC for 15 sec and 60^oC for 1 min). The resulting Ct data was analysed by the comparative Ct method $2^{-\Delta Ct}$, using RQ Manager 1.2 software (Applied Biosystems).

2.12: DNA amplification

To obtain sufficient DNA for retroviral transductions, *E.Coli* containing the plasmid of interest was plated out from glycerol stocks onto ampicillin LB agar plates. These colonies were then grown up in 100ml LB containing ampicillin at 37^oC overnight with shaking. A QIAGEN Maxiprep kit was then used to purify plasmid DNA from the bacterial culture according to the manufacturer's instructions. DNA concentrations were determined using a NANODROP spectrophotometer

(NaNoDrop Technologies, USA). Typically, around 250-500 ug of DNA was isolated using this procedure.

2.13: Transfection of Phoenix cell line using lipofectamine™ 2000

The Phoenix retroviral packaging cell line (Kinsella and Nolan 1996), was maintained by passaging every 2-3 days. This was done by removing adherent cells from a 75cm^3 flask by digesting with 2ml trypsin (Invitrogen) for 3-5minutes. Cells were then suspended in HBSS (Hank's Balanced Salt Solution) medium containing 10% FCS and penicillin/streptomycin and centrifuged (1800rpm 5 minutes) and re-suspended in 20ml IMDM (Iscove's Modified Dulbecco's Medium) containing L-Glutamine/25mM HEPES (Invitrogen), 10% FCS and penicillin/streptomycin. Cells were plated at 2 x 10^6 cells per 75cm^2 flask in 50ml IMDM and maintained at 37^{o}C in 5% CO_2. For plasmid transfections, 2 x 10^5 Phoenix cells were plated at in 2 ml/per well of IMDM in a 6 well plate and grown overnight (37^{o}C, 5%CO_2).

4 hours prior to transfection, media was discarded and 7.5ml of fresh IMDM added. Phoenix cells were transfected with appropriate DNA using a lipofectamine™ 2000 kit following the manufacturer's instructions (Invitogen). In summary two solutions were made for each transfection. Tube A contained 250µl Optimem with 3µg vector of interest and 1µg PCLE helper vector; tube B contains 250µl Optimem and 2 µl liopfectimine™ 2000 (incubate 5 minutes RT). Tubes A and B were then mixed and incubated for 20 minutes at RT. The mix was slowly added to the Phoenix cells on the 6 well plate and left overnight (37^{o}C, 5%CO_2). Media was then discarded and the cells washed twice with HBSS, 5ml of fresh IMDM media (without penstrep) was then added and the cells left overnight (37^{o}C, 5%CO_2). Viral supernatant was harvested (~5ml), centrifuged (1800rpm 5 minutes) and either used immediately or frozen in liquid nitrogen. As an indication of transfection efficiency and virus production, Phoenix cells were harvested and GFP expression determined by FACS. For larger scale transductions up to 30ml of virus was produced using cell culture Petri dishes (Becton Dickinson). For the transduction of T cells virus was usually concentrated by high speed centrifugation (13500rpm 90 minutes), 90% supernatant was removed and virus re-suspended at 10x concentration.

2.14: Retroviral transduction of tumour cells

MB49 cells were plated out at $2x10^5$ cells/well in a 24 well plate 48 hours prior to transduction. Conditioned media was removed and stored and cells were washed once with PBS. 1ml viral supernatant was added to each well and the plate was centrifuged at (2000 rpm for 120 minutes at RT). The 1 ml of conditioned media was removed before the spin infection was re-added and cells were left to grow for 48 hours (37^oC, 5%CO_2). Cells were harvested and washed with PBS. Cells were then analysed by FACs to measure transduction efficiency against an untransduced control. MIGR1 retroviral vectors containing GFP or a rat Thy-1 reporter genes (either empty or containing gene of interest) (kindly supplied by Douglas Fearon) were purified using an endofree MaxiPrep kit (Invitrogen). The resulting virus containing MIGR1 with the respective reporter and gene of interest was used to transduce MB49 cells. MB49 cells were left to grow for 48 hours before FACS sorting to select for GFP^+ or $RatThy\text{-}1^+$ (using anti $Rat\text{-}Thy\text{-}1^{FITC}$) populations.

2.15: Retroviral transduction of T cells

Whole lymph nodes and spleen suspensions of the relevant animal were plated out in a 24 well plate at 2ml/well (5×10^6 cells/well) in complete medium. Peptides were used as antigen to induce an appropriate T cell activation. HY-D^b-UTY peptide at a concentration of 0.1-1nM was used to specifically activate Matahari CD8 T cells and HY-A^b-Dby peptide was used at a concentration of 10-100 nM to activate Marilyn CD4 T cells. Cells were left to grow for 48 hours at (37^oC, 5%CO_2).

Cells were harvested, washed in complete media and layered onto Ficoll (GE heathcare), centrifuged (400g, 20 minutes). Live cells harvested and washed in PBS. Cells were then depleted of lymphocytes not required for retroviral transduction. For transduction of Matahari CD8 T cells, B cells and CD4 T cells were removed using B220 and CD4 dynabeads. For Marilyn CD4 T cell transductions, B cells and CD8 cells were removed (CD8 and B220 dynabeads). In brief, cells were re-suspended in 2ml PBS, 30ul anti-B220 and 10ul anti-CD4- or anti-CD8-dynabeads were added. Cells were incubated at 4^oC under a low speed centifugation (10 rpm) for 20 minutes.

Cells attached to dynabeads were attracted to the tube wall in a mageneic field and the negatively selected target population collected. Cells were then centrifuged (1800rpm 5 mins) and plated out in 24 well plates (1-5 x 10^5/well). 1ml of relevant concentrated virus supernatant was added, viral spin infection and was then conducted as described for MB49 above. Following spin infection, 1ml of conditioned media (S/N stored following primary culture) was added back to each well with 50IU/ml rhIL-2 to aid expansion. Cells were then FACS sorted or used directly for i.v. injection.

2.16: Adoptive cell transfer

Relevant cell populations were injected into mice i.v. in 0.2ml PBS using a 0.5ml insulin syringe. Briefly, mice were placed in a 37^{o}C warming chamber for 10 minutes to induce vasodilation. The mouse was restricted in a tail holder and injections were made into the either the ventral or dorsal tail vein. Pressure was applied to the injection site to prevent bleeding and loss of adoptively transferred cells.

2.17: Analysis of adoptively transferred donor cell populations

The representation of donor populations in various tissues including tumour dLN, ndLN, SP and tumour, control peripheral inguinal LN (pLN), mLN, kidney, liver, heart and lungs were analysed. Organs were harvested and excess fat tissue removed. Where necessary, organs were sliced and digested as described in section 2.6. Cells were then forced through a 70μm cell strainer to create a cell suspension. Cells to be analysed using FACS were treated with RBC lysis buffer (Qiagen) (RT 10 minutes) and washed with PBS before staining as described in section 2.6. Cells to be used for further cell culture were washed in culture medium and purified using MACS or FACS cell sorting where required. Cells to be used for secondary adoptive cell transfer were purified as described in section 2.5 and injected i.v.

2.18: Animal phenotyping

In order to ensure correct donor and recipient mice were used for each experiment, mice bred internally were phenotyped between 4-8 weeks of age. Mice were placed in a 37^{o}C warming chamber for 10 minutes and restricted in a tail holder, 1-2 drops of blood were taken using a 25g needle to puncture the tail vein and collected in an eppendroff tube containing 0.3 ml blood buffer. Red blood cells were then lysed as described previously. Cells were washed in PBS and stained as described in section 2.6. Mice were phenotyped for surface expressed proteins including Thy1.1, Thy1.2, CD4, CD8, Vβ6 (Marilyn), Vβ8.3 (Matahari), GFP, B220, CD44 and CD62L.

2.19: Irradiation of cells and mice

In certain assays, cells were exposed to gamma radiation from a caesium source to prevent proliferation that may interfere with the responder population (e.g. assays using male SP see section 2.5.2). 30Gy (3000Rads) was used to induce DNA damage preventing replication but not killing the cells. For certain reagents and materials used *in vivo* or for sterile *in vitro* cultures, irradiation was also used for sterilisation at 30Gy. Animals were irradiated in order to induce lymphopenia at a rate between 5-9Gy. For the purposes of our experiments a sub-lethal dose of 5Gy was used. Mice were then adoptively transferred with donor T cells for therapy or induction of GvHD.

2.20: Generation of bone marrow dendritic cells (BMDCs)

For vaccination purposes, male B6 BMDCs were generated by culturing bone marrow cells with GM-CSF (Inaba, Inaba et al. 1992). Bone marrow was harvested by the epiphyses of the femur, tibia and fibula and flushing the marrow using a 22g needle, 1ml syringe and PBS. The resultant suspension was forced through a 70μm cell strainer and washed once in PBS. Cells were re-suspended in complete media with 10% GMCSF* and 4μg/ml Gentamycin (to prevent microbial infection) (Invitrogen) and plated out at 1 x 10^{5}/ml in 10 ml in a 25cm^{3} cell culture flask. 72

hours later the media was replaced. On day 6, cells were harvested, and replated as above. On day 7, lipopolysaccaride (LPS) was added at 10ng/ml in order to induce DC maturation. On day 8, cells were harvested and centrifuged with Ficoll (400g 20 minutes) to separate live cells. Cells were counted and used for injection at 1 x 10^6 i.v. or i.p.in 0.2ml PBS.

* GM-CSF was produced from the murine encoded gene cloned into the mammalian expression vector BCMGSNeo and transfected into the X63-Ag8 plasmacytoma cell line. Cells were selected in media further supplemented with G148 (1 mg/ml). When ready for supernatant harvesting, cells were washed twice and cultured for a further 2-3 days in media lacking antibiotics. Supernatant was harvested and stored at -20°C.

2.21: Enzyme-linked immunosorbent assay (ELISA)

A mouse anti-TNFα sandwich ELISA (Biolegend LEGEND MAX™) was used in the analysis of murine blood serum samples. Blood samples were incubated at 4^{o}C overnight, pelleted (3000rpm 5 mins) and serum pipetted off and stored at -80^{o}C. ELISA was conducted as per the protocol provided, briefly: serum was serially diluted from 1/10 to 1/10000 and standards serially diluted from 1000pg/ml to 15.6pg/ml. Plates were washed four times in >300µl wash buffer and diluted samples and standards added to the plate (in 50µl) and incubated (2hrs, RT, shaking). Plates were washed (x4) and 100µl mouse TNFα detection antibody added and incubated (1hr, RT, shaking). Plates were washed (x4) and 100µl Avidin-HRP solution added and incubated (30 min, RT, shaking). Plates were washed (x5) and 100µl substrate solution added and incubated (15mins RT, in the dark). 100µl stop solution was added/well and absorbance at 450-570nm was read within 30 minutes (SpectramaxM2, Molecular Devices).

2.22: Quantification and statistics

All statistics were carried out using non-parametric tests. Error is presented as ± standard error mean where possible, or as ± standard deviation. All P values were generated using paired and unpaired two sample students T test or two-tailed non-parametric Mann-Whitney U-test.

Chapter 3: Characterisation of the phenotype for the MB49 tumour

3.1: Introduction

3.1.1: Recognition of tumour-associated antigens (TAA)

Most tumours express tumour-associated antigens (TAAs). In addition to tumour cells, TAAs can be expressed on some non-malignant tissues. Tumour antigens can be derived from cancer-specific mutated genes, abnormally expressed intronic sequences, viral genes or from non-mutated genes that are expressed in a tumour-specific pattern. Antigens restricted solely to tumour cells are known as tumour-specific antigens (TSAs) (Wortzel, Philipps et al. 1983). Antigen-presenting cells (APCs) particularly dendritic cells (DCs) can cross present TAAs to T cells (Shurin 1996). As the majority of TAAs derive from host DNA, most of these antigenic components will be expressed in the thymic epithelium or by thymic DCs during primary T cell selection, leading to the deletion of high avidity self-reactive T cells. T cells in the periphery that are responsive to TAAs therefore have a low to moderate avidity, resulting in a poor proliferation and effector function in response to TAAs (Overwijk 2005).

In addition, tumours have several mechanisms by which they evade host responses to TAAs. Factors released by tumours can act on the host immune system to downregulate T cell receptor (TCR) expression on T cells thereby preventing activation of these T cells in response to TAAs (Takahashi, Kono et al. 2001). Conversely, tumour cells often have defects in antigen processing and presentation. This mechanism is selective as those tumour cells that are less immunogenic will survive and proliferate (Zitvogel, Tesniere et al. 2006). Tumour cells can down regulate MHC I and II directly by deletion, or indirectly by deletion of chaperone and accessory molecules. For example, many human primary cervical carcinomas show strong downregulation of MHC Class I and TAP, with a potent upregulation of MHC Class II (Cromme, van Bommel et al. 1994).

3.1.2: Tumours, APCs and co-stimulation

Tumours can modulate host APCs by downregulating expression of co-stimulatory molecules while upregulating inhibitory molecules (Zitvogel, Tesniere et al. 2006). Many cancers can downregulate positive T cell co-stimulatory molecules that initiate immune responses and can upregulate T cell inhibitory molecules to remain immunoevasive. Tumours also commonly release or express factors which allow escape from immune system, for example tumours often express high levels of apoptotic promoting molecules such as Fas ligand (FasL) that can induce apoptosis in T cells by binding to the Fas molecule (Bennett, O'Connell et al. 2001). Programmed Death Ligand 1 (PD-L1) is expressed on APCs and is able to bind the PD-1 receptor on T cells, transducing an inhibitory signal preventing T cell proliferation and cytokine production that can ultimately lead to anergy or cell death (Fife and Bluestone 2008). This mechanism is used physiologically to remove auto reactive T cells in the periphery that have escaped central tolerance (Probst, McCoy et al. 2005). PD-L1 is therefore upregulated by many tumour cell lines with the effect of abrogating effector T cell function through binding to the PD-1 receptor on T cells and inducing cell death (Blank, Gajewski et al. 2005; Okazaki and Honjo 2007). Research has shown that tumours that express high levels of PD-L1 are associated with decreased survival, decreased CD8 T cells in the tissue and a high level of T cell anergy (Willimsky and Blankenstein 2005). Studies using antibody blockade of PD-1 and PD-L1 have shown increased tumour responsiveness with improved survival (Iwai, Ishida et al. 2002; Iwai, Terawaki et al. 2005).

Commonly DCs upregulate B7-1 (CD80) and B7-2 (CD86) molecules in response to danger signals (Lanier, O'Fallon et al. 1995) these factors are required for primary T cell activation. Once activated, CD8 T cells become effector cells and produce perforin and Granzyme B, on encounter with antigen in the periphery they are activated by ligation of the T cell receptor and co-stimulatory molecules (e.g. on the tumour). In murine trials, transduction of melanoma with B7-1 is sufficient to stimulate rejection; consequently it is common for tumours to downregulate positive co-stimulatory molecules (Townsend, Su et al. 1994). Once activated, effector T cells can remain reactive or differentiate into memory T cells for an effective secondary response in the correct environment of IL-2 and other proinflammatory cytokines (Antony, Piccirillo et al. 2005).

3.1.3: Tumour-related cytokines and immunomodulatory factors

Tumour microenvironments can augment the cytokine milieu, making it difficult to achieve an effective and sustained T cell response. Tumour cells can direct the host immune system to release factors that abrogate responses to the cancer. For example the indoleamine-2,3-dioxygenase (IDO) enzyme can be induced in DCs. IDO is the first enzyme of tryptophan catabolism in the kynurenine pathway and IDO induced depletion of tryptophan can cause reduced T cell proliferation and render DCs tolerogneic to the TAA specific T cells (Grohmann, Fallarino et al. 2003). Tumours can also induce or secrete immunosuppressive factors such as prostoglandins, gangliosides, GMCSF, IL-6, IL-10, TGF-β and VEGF. IL-10 and TGF-β are particularly well characterised immunosuppressive cytokines in the tumour microenvironment (Ghiringhelli, Puig et al. 2005). TGF-β is associated with the induction or activation of Tregs from naïve CD4 T cells, with upregulation of the transcription factor FoxP3 (Chen, Pittet et al. 2005; Coe, Addey et al. 2010). In the tumour microenvironment a high level of TGF-β can prevent CD8 T cell activation, leading to anergy (Fukuyama, Ichiki et al. 2007). IL-10 has a similar effect by modulating host APCs, crucially, IL-10 is important in the production of non-FoxP3 producing Tr1 Tregs (Vieira, Christensen et al. 2004).

Collectively, cancers can produce or induce a wide range of growth and immune-inhibitory factors to modulate their environment to an immunosuppressive milieu. By investigating and understanding these modulation mechanisms it is possible to design new cancer immunotherapy strategies that focus not just on the killing of the tumour but also on modulating the local tumour environment to maintain T cell activity.

3.1.4: MB49/HY as a model TAA for antigen-specific T cell responses

Minor histocompatibility (H) antigens are self peptides derived from polymorphic genes that are presented to T cells in the context MHC class I and II molecules (Scott, Ehrmann et al. 1995). Like minor H antigens, tumour antigens are exclusively self proteins (except for virally induced tumours) that share commonalities with minor H antigens in their molecular composition and immunogenic properties. Both tumour antigens and minor H antigens consist of a limited set of MHC-binding peptide epitopes. The proteins from which they are

derived are often expressed at low levels (Spierings, Wieles et al. 2004; Spierings 2008) giving a low cell surface density of peptide-MHC complexes that is generally insufficient to trigger optimal T cell activation. As T cell responses to minor H and tumour antigens are guided by the same rules, the response to minor H antigens has been regarded as an excellent surrogate to study tumour immunity. One of the best characterised minor H antigens is the male-specific HY transplantation antigen which has been identified at molecular level (Scott, Ehrmann et al. 1995; Simpson, Scott et al. 1997).

In C57BL/6 $H2^{b+}$ male mice, HY T cell epitopes are derived from 3 genes located on the Y chromosome: two $H2D^b$ restricted peptides are encoded by the *Smcy* and *Uty* genes, while one $H2A^b$ restricted epitope derives from the *Dby* gene (Greenfield, Scott et al. 1996; Scott, Addey et al. 2000). One of the key reagents in this project are the TCR transgenic Marilyn and Matahari mouse strains that have been backcrossed to $Rag1^{-/-}$ and $Rag2^{-/-}$ mice respectively, thus preventing rearrangements of their endogenous TCR genes (Lantz, Grandjean et al. 2000). These TCR transgenic mice produce monoclonal T cells recognising the $H2D^b$ restricted Uty peptide (WMHHNMDLI) for Matahari $CD8^+$ T cells and the $H2A^b$ restricted Dby peptide (NAGFNSNRANSSRSS) for Marilyn $CD4^+$ T cells (Valujskikh, Lantz et al. 2002; Valujskikh, Baldwin et al. 2010). This makes them attractive T cell populations for adoptive transfer in the treatment of male B6 derived tumours such as murine bladder carcinoma 49 (MB49). Matahari mice harbour functional HY-specific CD8 T cells that can induce rejection of male skin grafts although they fail to control HY-expressing tumours *in vivo* (Perez-Diez, Joncker et al. 2007), suggesting that Matahari T cell function is inhibited directly in this system by the tumour.

The other component of this system is MB49, a chemically induced murine bladder carcinoma cell line generated by administration of the DMBA (7,12-Dimethylbenz(a)anthracene) carcinogen to male mice. A mouse that became tumour positive was used to establish a stable *in vitro* tumour cell line that expresses all three HY genes naturally (Summerhayes and Franks 1979) (Loskog, Ninalga et al. 2005). MB49 cells also express $H2D^b$ molecules that present Uty peptide to CD8 T cells and $H2A^b$ that presents Dby peptide to CD4 T cells, although the MB49 cell line is MHC class II negative *in vitro*, $H2A^b$ is upregulated *in vivo* or upon IFNγ treatment.

The use of HY system for the study of anti-tumour T cell responses has several unique features which provide advantages to existing model systems (Chen, Pittet et al. 2005; Yang and Baltimore 2005). MB49 cells express HY peptides at a physiological level relevant to the expression levels of naturally occurring TAAs (Perez-Diez, Joncker et al. 2007), therefore it is likely that the HY system mirrors the *in vivo* T cell response to true tumour antigens. Other models rely on transduced antigen such as cOVA (Yang and Baltimore 2005) or viral antigens such as gp100 (Abad, Wrzensinski et al. 2008) that are often over expressed in a non-physiological manner. While MB49 has also been shown to express other male TAA such as prostate cancer stem cell antigen (PSCA) and transmembrane epithelial antigen of the prostate (STEAP) (Loskog, Ninalga et al. 2005), the primary response to the tumour by female C57BL/6 mice is against the HY minor antigens. This has been demonstrated previously as male mice fail to control MB49 at doses females do, suggesting that HY responses make a significant contribute to anti-MB49 immunity in female mice (Melchionda, McKirdy et al. 2004).

3.1.5: MB49 tumour growth and wildtype T cell responses

The growth of MB49 tumour *in vivo* has been well characterised in C57BL/6 female mice. Models have included use of intravenous (i.v.), and subcutaneous (s.c.) injection. Inoculation of MB49 cells i.v. induces a metastatic tumour that is difficult to quantify and often leads to rapid morbidity and mortality (Weiss, Wong et al. 1987; Loskog, Ninalga et al. 2005). Inoculation of MB49 cells s.c. to female mice at doses between 0.7-1 million cells produces an progressive solid tumour that can be measured externally with little morbidity to the animal (Loskog, Ninalga et al. 2005). This result also demonstrates the immunogenicity of the tumour is insufficient to induce rejection at high doses of MB49. Other studies have shown that lower does (0.1 million MB49 cells) female mice can reject the tumour but not at higher doses (0.6 million cells) (Melchionda, McKirdy et al. 2004). In addition, T cells isolated from female mice with large MB49 tumours, adoptively transferred to athymic mice, are capable of rejecting male skin grafts, indicating that tumour-bearing mice generate a functional immune response to the HY antigen (Melchionda, McKirdy et al. 2004).

3.1.6: MB49 Characterisation and phenotype

Tumour cell lines often change their expression profiles following tumour formation by the mechanisms discussed earlier (e.g immuno-editing of MHC or TAAs). It is important therefore to understand the natural molecular and cellular phenotype of the MB49 tumour *in vivo* in order to evaluate the effects of therapeutics, such as the HY-specific T cell adoptive cell transfer discussed later. Little published data exists regarding the phenotype of this tumour cell line, for example MHC expression levels, cytokine production and surface marker expression. It is therefore my aim in this section to give an overview of the MB49 phenotype both *in vivo* and *in vitro* to provide a base line to evaluate tumour responses in later sections.

3.2: Results

3.2.1: MB49 cell line expresses all 3 HY genes (encoding HY-specific T cell epitopes in the context of MHC I and II)

The MB49 cell line has been maintained *in vitro* for nearly two decades. Prolonged culture has the potential to cause down regulation or even loss of HY gene expression. To test this possibility RNA was extracted from MB49 cells or from freshly isolated female B6 splenocytes, as a negative control. Following this DNA was amplified by RT-PCR using primers specific for *Uty*, *Smcy*, *Dby* and *HPRT* genes. All 3 HY genes are expressed by the MB49 cell line but not by female splenocytes (Fig. 3.1a) demonstrating MB49 cells constitutively express HY genes, which is consistent with other reports (Halak, Maguire et al. 1999; Perez-Diez, Joncker et al. 2007).

Many tumours downregulate Class I and Class II MHC molecules and co-stimulatory molecules as a mechanism of immune escape, particularly *in vivo* where the tumours are under greater selection pressures, termed immune-editing (Zitvogel, Tesniere et al. 2006). Long term cultured MB49 cells and *ex vivo* fresh MB49 tumour cells (from a WT B6 female mouse) were analysed for surface expression of MHC class I (D^b and K^b) and MHC class II (A^b) (Fig. 3.1b) as well as co-stimulatory

molecules (CD40, CD80 and CD86) (Fig. 3.1c). Both *in vitro* and *ex vivo* MB49 cells express MHC I although there is a clear upregulation of class II and downregulation of class I *in vivo*. Freshly isolated *ex vivo* MB49 tumour cells expressed significant levels of CD40, CD80 and CD86, in comparison with the *in vitro* cell line that had a much lower level of expression (Fig. 3.1c). These data show that MB49 cells expresses all 3 HY genes and both MHC I and II, also upregulating positive co-stimulatory molecules *in vivo,* indicating that MB49 tumour is potentially immunogenic.

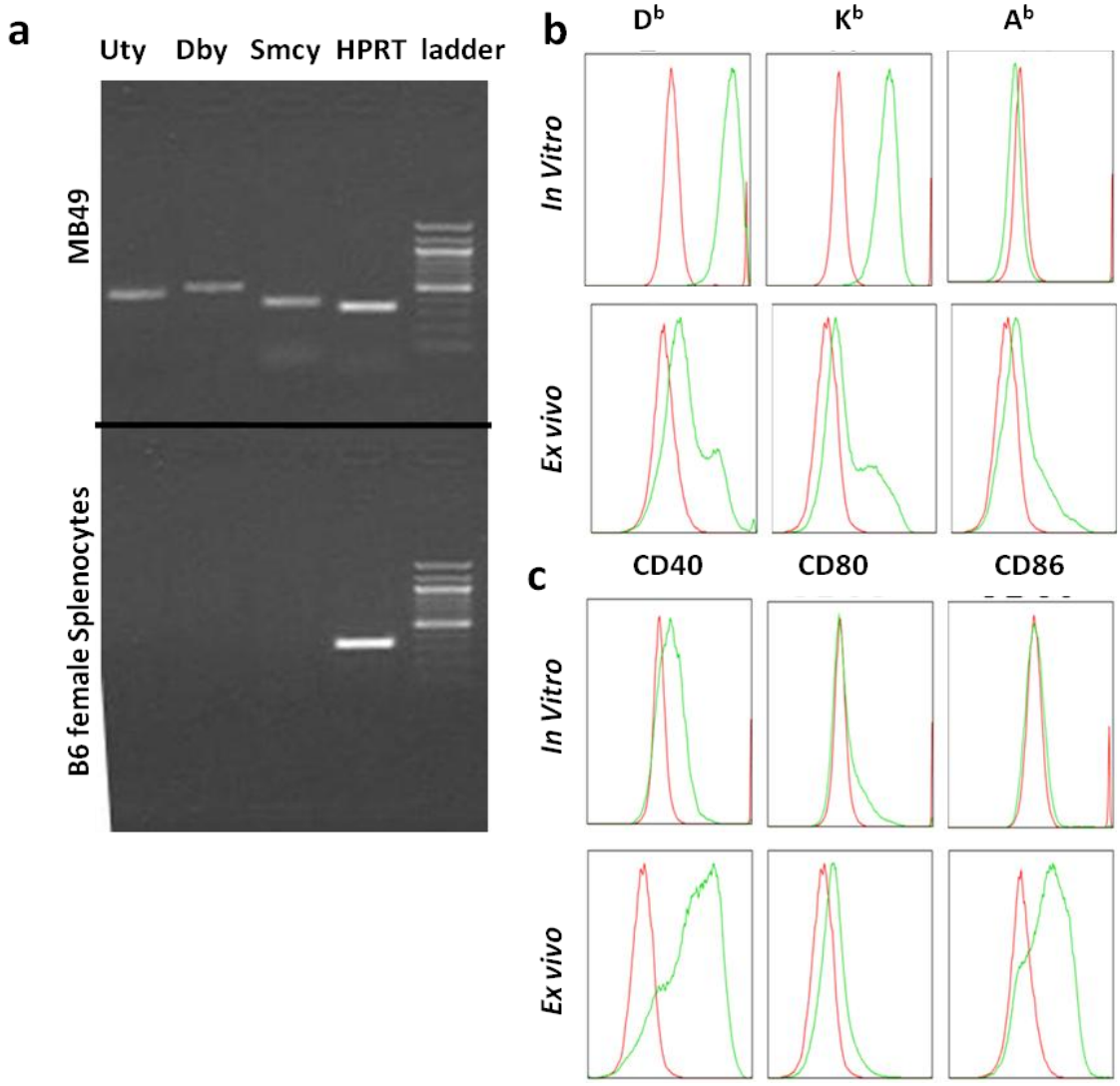


Figure 3.1. MB49 cells express HY genes, MHC and co-stimulatory molecules
a. Expression of HY genes by cultured MB49 cells. RNA was isolated from cultured MB49 cells and female B6 splenocytes and was amplified by RT- PCR using primers specific for *Uty*, *Smcy*, *Dby* and HPRT genes. PCR products were analysed on a 1% Agarose gel.
b and c. Analysis of expression of MHC (**b**) and co-stimulatory molecules (**c**) by fresh *ex vivo* and cultured *in vitro* MB49 cells. *In vitro* or *ex-vivo* MB49 cells were stained with PE or FITC conjugated antibodies (green line) or isotype control mAb

(red line) and analysed by FACS for the expression of MHC Class I (D^b and K^b), Class II (A^b) (**b**), co-stimulatory molecules (CD40, CD80 and CD86) (**c**).

3.2.2: MB49 tumour expresses an array of immunological markers

Tumour identity in the context of the immune system can be defined by two broad categories, immunological and non-immunological. Non-immunological tumours tend to evade the immune system by downregulation of immunological molecules and often grow slowly in non-inflammatory environments. In contrast, immunological tumours are visible to the immune system and so often exist in more inflammatory environments. These tumours therefore escape control by other methods such as having a very fast growth rate and through the expression of co-inhibitory molecules and other suppressive factors such as cytokines (Zitvogel, Tesniere et al. 2006).

MB49 cells were tested for a range of immunological markers to define their immunogenicity. An *in vitro* culture was set up with MB49 cells either in media alone or in media containing IFNγ for 4 days. Cultures were then harvested and stained for a range of surface markers (Fig. 3.2a). IFNγ treated cultures were designed to resemble a similar affect to an *in vivo* Th1 immune response, IFNγ secretion is associated with a Th1 viral immune response and consistent with a Th1 biased C57BL/6 T cell mouse line. MHC class II A^b, MHC class I D^b and K^b, co-stimulatory molecules CD80, CD86, CD40 and co-inhibitory molecules PD-L1 and PD-L2 were upregulated by IFNγ, B7H4 was reduced and GITR-L was unchanged. These data indicate the MB49 phenotype can change dramatically dependent on the cytokine microenvironment.

In a separate experiment MB49 cells were cultured for 4 days in medium alone and tested for various phenotypic markers ranging from cell surface adhesion molecules, chemokine receptors, cancer stem cell markers and TNF family ligands and receptors (Fig. 3.2b). The expression of MHC, positive and negative co-stimulatory molecules by MB49 cells was described previously. Importantly MB49 cells also showed expression of the TNF family molecule FAS-L (~11%), which can serve as an altered mechanism to mediate apoptosis other than the PD-1 pathway. MB49 also expresses lymphocyte adhesion molecules CD44 and CD62L as well as CD49b which binds collagen and associates with CD29 on leukocytes. MB49

universally expresses stem cell antigen-1 (Sca-1 99.8%), a molecule that is downregulated when haematopoietic stem cells (HSC) differentiate into myeloid progenitors (Bradfute, Graubert et al. 2005; Zitvogel, Tesniere et al. 2006). Interestingly MB49 has some expression of CD48 (~8%), this molecule is a putative ligand of CD244 involved in the regulation of NK cells, believed to be the major cytotoxic killer of tumour cells (Perez-Diez, Joncker et al. 2007). Finally MB49 cells express the chemokine receptor CCR9, but no other chemokine receptors tested for were expressed *in vitro*. This result confirms MB49 is highly immunogenic and visible to the immune system but able to employ various inhibitory molecules to mediate escape. MB49 can also be defined by the expression of other molecules such as CD49b and sca-1.

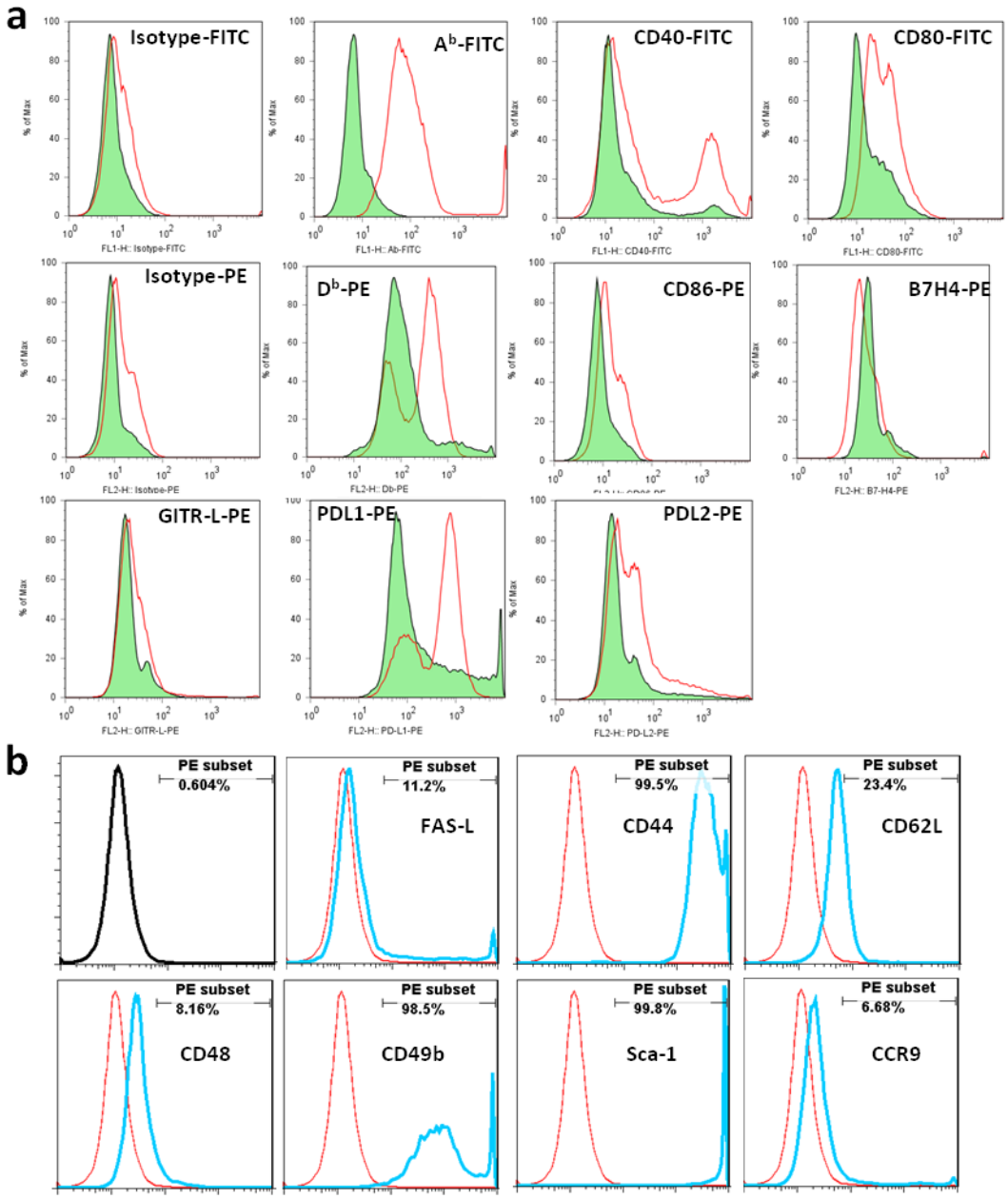


Figure 3.2. MB49 is immunogenic
a. MB49 cells were plated out at 5 x 10^5 cells/well for 5 days in the presence of IFNγ (10ng/ml) or in media alone. Cells were harvested and aliquoted in equal amounts for surface staining using a range of PE- and FITC- conjugated mAbs and analysed using FACS. MB49 cells were gated as a single population and results displayed as histograms. Media alone (filled green histograms) and INFγ cultured (open red histograms) were compared for expression of A^b, D^b, CD40, CD80, CD86, B7H4, GITR-L, PD-L1 and PDL2 along with FITC and PE isotope controls.
b. MB49 cells were plated out as above for 4 days in media alone. PE isotype control (red histograms) cultured MB49 (blue histograms) were compared for expression of a range of surface expressed molecules.

3.2.3: MB49 tumour maintains immunogenicity *in vivo*

The analysis shown in Fig. 3.1b and c was conducted using cells from MB49 tumour tissue, which contain MB49 tumour cells and tumour-infiltrated lymphocytes. Next, CD45 was used as a marker to distinguish host lymphocytes from MB49 cells. CD45 is a lymphocyte common antigen (Arendt and Ostergaard 1995) and is not expressed by MB49 cells which are of epithelial origin (Summerhayes and Franks 1979). MB49 tumours were isolated *ex vivo* and CD45-negative fractions were analysed for various molecules described previously (MHC I, CD86, GITR, PD-L1/2) with the addition of chemokine receptors (CXCR3 and 4, CCR5,7 and 9), which are of interest in determining recruitment of lymphocytes.

Ex vivo MB49 tumour cells clearly expressed MHC class I molecules D^b and K^b, and co-inhibitory molecules PD-L1 and PD-L2, they also expressed a low level of GITR but not 4-1BB. CD80 expression by gated MB49 was low, thus previous a high CD80 profile was likely to due to the contribution by tumour-infiltrating lymohocytes. Among five chemokine receptors tested, MB49 expressed CCR9 but not other markers CXCR3 and 4 and CCR5 and 7.

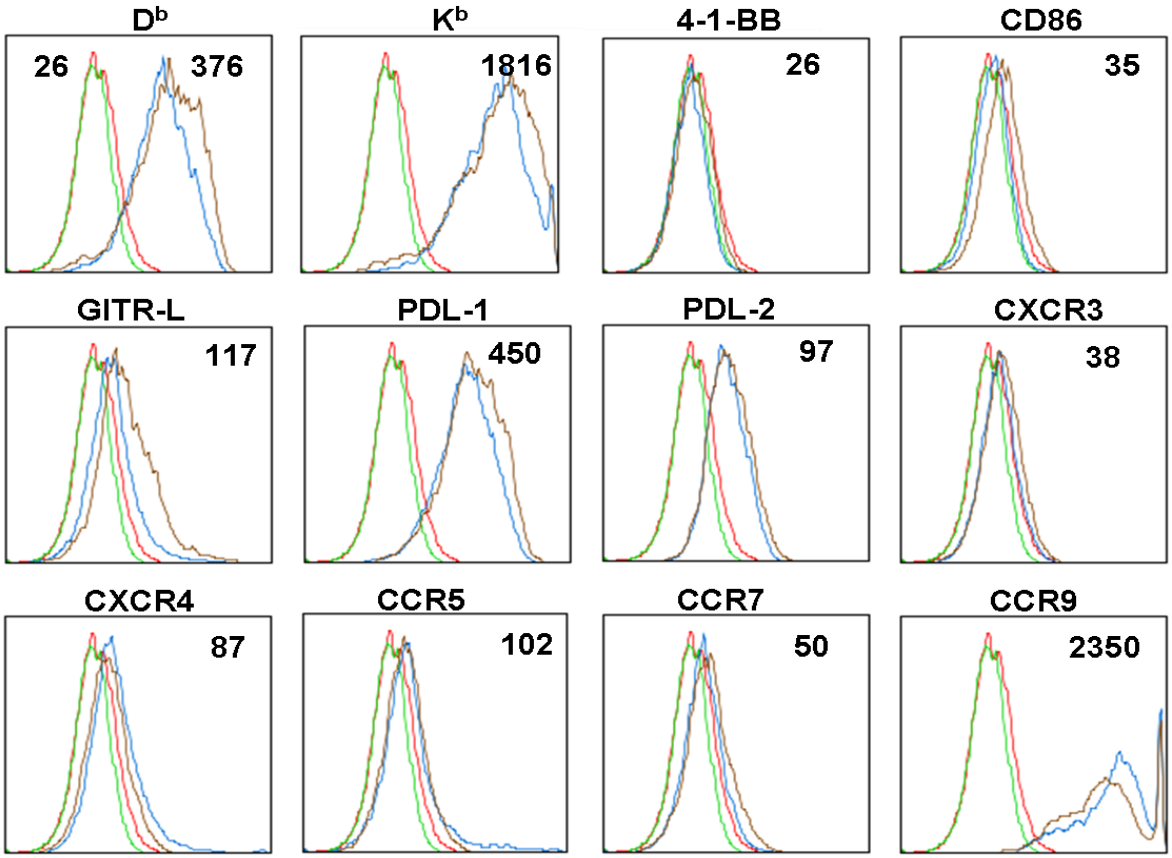
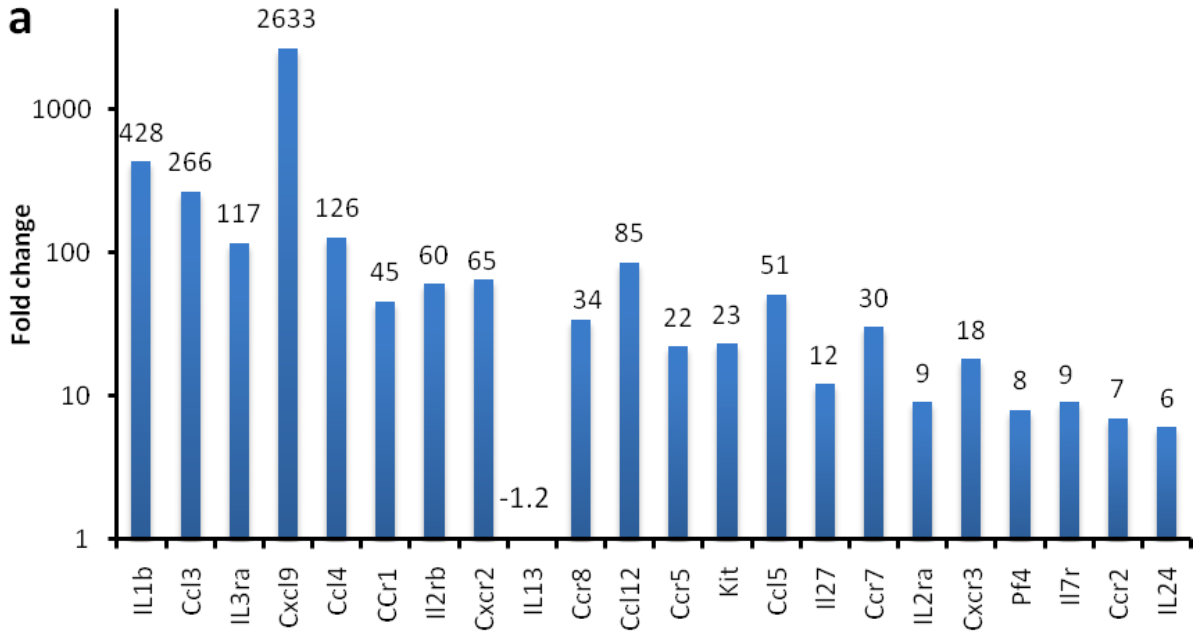


Figure 3.3. Phenotype of MB49 tumour cells *ex vivo*
MB49 cells ($5x10^5$) were injected (s.c) to 2 WT B6 female mice. 20 days later, two MB49 tumours were isolated and digested with collagenase before staining with anti-$CD45^{FITC}$ and PE-conjugated antibodies against respective cell surface markers or PE-conjugated isotype control mAb. CD45-negative and intermediated cells were gated and analysed for cell surface marker expression. Isotype control from tumour 1 (red line) and from tumour 2 (green line), PE-conjugated stained from tumour 1 (Blue line) and from tumour 2 (brown line). Inserted numbers in each histogram represent mean fluorescence intensity (MFI).

3.2.4: Cytokine and chemokine gene expression changes in the *in vitro* cell line and *in vivo* tumour

In order to quantify cytokine and chemokine gene expression changes between the *in vitro* cell line and the *in vivo* tumour, the two populations were compared by qPCR using a 383 StellARray™. StellARay™ is a collection of PCR assays designed in this case to measure immunological cytokines and chemokines. MB49 tumour cells *ex vivo* (as described in Fig. 3.1) as well as *in vitro* cultured MB49 tumour cells were subjected to RNA extraction using an RNAqueous 4PCR kit and cDNA conversion. Both populations were analysed for the expression of a

range of 96 selected cytokine, chemokine and their receptor genes and significant changes are shown as fold change from a 'normaliser' 18sRNA control gene (Fig. 3.4). Fig. 3.4a shows 21 genes were significantly upregulated by MB49 tumour *ex vivo*, in comparison to MB49 cell line. The one gene downregulated by MB49 tumour *ex vivo* was *IL13* (Fig. 3.4a). 28 genes were highly expressed by both the cell line and the tumour (Fig. 3.4b). It is of note that several molecules tested in surface phenotyping (Fig. 3.2 and 3.3) were not included on StellARray™ such as Sca-1, MHC and many co-stimulatory and adhesion markers.



b

HIGH EXPRESSION IN CELL LINE AND TUMOUR (ct < 25)
Tgfb1, Kitl, Csf1, Il2rg, Il2rb1, Il1r1, Il1rn, Il18, Ccl2, Cx3cl1, Cxcl2, Cxcl1, Cxcr4, Cxcl10, Il6, Il6ra, Il6st, Lif, Lifr, Il7, Il4ra, IL10, Il10rb, Il11ra1, IL15ra, IL17ra, IL27ra *IL4 just over ct25*

Figure 3.4. Comparison of cytokine and chemokine expression profiles between MB49 tumour and cell line
Ex vivo MB49 cells were harvested at day 17 from B6 tumour-bearing female mice (inoculated (s.c) with 5 x 10^5/mouse). MB49 cell line was also harvested from culture and both populations were subjected to RNA extraction and cDNA conversion before running qPCR array assay using 383 StellARray™ plates, which assays 96 murine chemokine and cytokine genes.
a. 21 genes were significantly upregulated following tumour formation.
b. Genes highly expressed by both cell line and tumour.

3.2.5: Male and female B6 mice show a disparity in the anti-MB49 response

MB49 is immunogenic and expresses all 3 HY genes and can present these T cell epitopes in the context of MHC I and II with sufficient co-stimulation. To evaluate the immune response to these HY antigens, MB49 was injected (s.c.) to male and female WT B6 mice at a range of doses and percentage survival was measured (Fig. 3.5 a-d). Tumour size is measured daily and mice were culled and recorded as dead when they reach humane end point (any dimension of tumour reaches 14mm) as stipulated by our project licence and defined by the animal procedures act 1986. At all concentrations of MB49 tested, there was a statistically significant protective effect in female B6 mice. This effect was able to ensure survival at lower concentrations (1×10^4 and 3×10^4) (Fig. 3.5a and b) where 60 and 80% of females survived compared with 0 and 20% males respectively. At higher concentrations (1×10^5 and 6×10^5) (Fig. 3.5c and d) all mice were culled but again a clear protective effect is seen in female mice which survived ~ 10 days longer.

To further evaluate the HY response to MB49, male and female B6 mice were immunised with HY and other TAAs antigens by s.c. injection with a high dose of irradiated (30Gy) MB49 cells (9×10^5). 21 days later mice were re-challenged with a high dose of MB49 (5×10^5). All female mice rejected the MB49 tumour with 100% survival, only 60% of male mice were able to mount an effective anti-tumour response (Fig. 3.5e) this was statistically significant. Male mice are able to mount effective tumour responses to TAAs other than HY although this is insufficient to provide the same protection as seen in the female response to HY antigen.

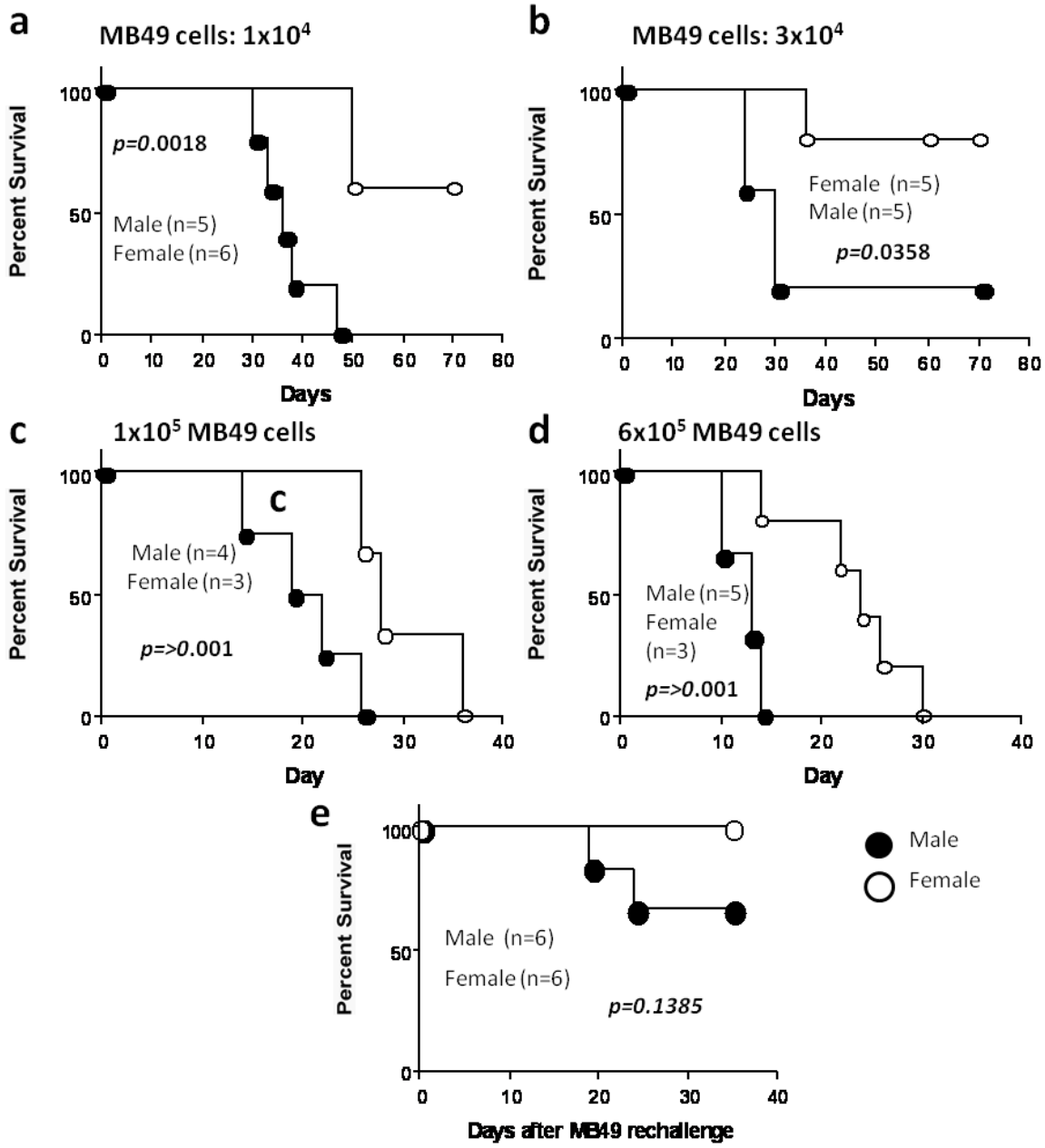


Figure 3.5. Male vs female disparity in the *in vivo* anti-tumour response
a-d. Male and female B6 mice were injected (s.c.) with $1x10^4$ (**a**), $3x10^4$ (**b**), $1x10^5$ (**c**) and $6x10^5$ (**d**) MB49 cells. Tumour growth was measured over 70 days (**a** and **b**) or 40 days (**c** and **d**) and data were presented as % survival in a Kaplan-Meyer plot, with p value calculated by using students T-Test. Tumour area was measured daily until a humane end point was reached (any dimension >14mm) at which time mice were culled and recorded as dead.
e. Male and female B6 mice were injected (s.c.) with $9x10^5$ irradiated MB49 cells. 21 days later the mice were challenged (s.c.) with $5x10^5$ MB49 cells at the same injection site. Tumour growth and survival of mice were measured as above.

3.2.6: The kinetics of MB49 tumour growth in female B6 mice

The growth profile of MB49 has previously been characterised at high doses of $7x10^5$ and $1x10^6$ that produced a progressive solid tumour in female B6 mice (Loskog, Ninalga et al. 2005). In order to establish an MB49 dose that would be appropriate for therapeutic interventions, groups of female B6 mice were challenged with titrated doses of MB49 cells ($1x10^5$, $3x10^5$or $6x10^5$) injected (s.c.) to the right lateral flank. Tumours become visible at around 2mm in 3-5 days after inoculation, tumours are then measured daily as described previously, until humane end point is reached.

At lower doses of 1 x 10^5 cells (Fig. 3.6a), MB49 grew in all mice, with >60% of mice reaching humane endpoint by day 36. At moderate doses of 3 x 10^5 cells (Fig. 3.6b) 80% of mice developed tumours which grew bigger and faster. With high doses of MB49 6 x 10^5 cells (Fig. 3.6c) 100% of mice developed tumours to humane endpoint with some mice being culled due to ulceration, an effect only seen at higher dose tumour inoculations. These results are typical of tumour growth of MB49 in our lab; to obtain reliable tumour growth in all recipients, the dose of MB49 cells has been carefully selected as 5 x 10^5 cells (s.c.) to WT B6 mice and 1 x 10^5 cells for $Rag^{-/-}$ B6 mice, based on data from several representative experiments from this lab and others (Perez-Diez, Joncker et al. 2007). Fig. 3.6d shows a typical MB49 tumour image (isolated at day 21 from a female mouse inoculated (s.c.) with 5 x 10^5 MB49 cells). MB49 forms a solid progressive tumour (top left) with high angiogenesis (top right), metastatic pressure and blood volume shown by cutting the tumour with a scalpel (bottom).

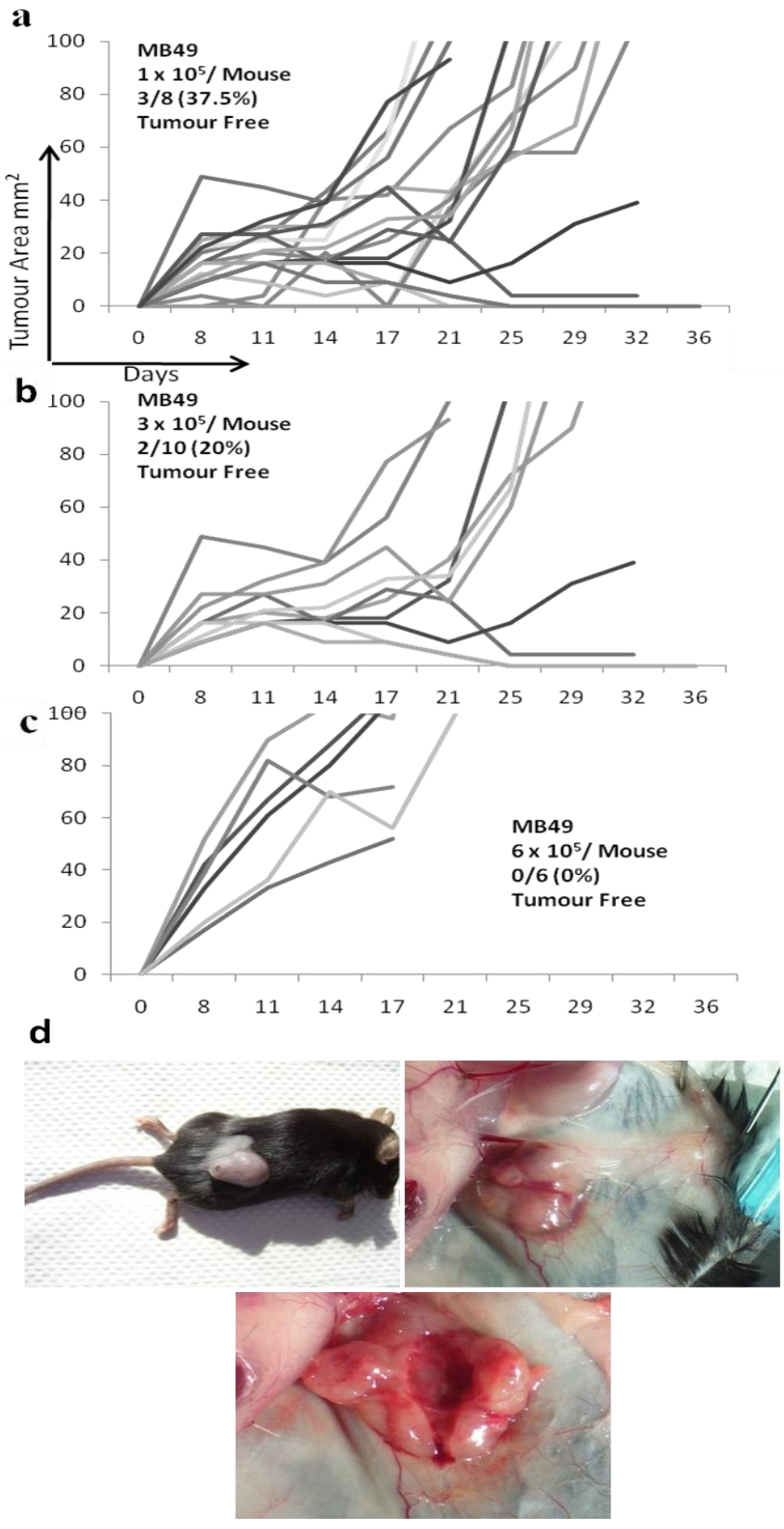


Figure 3.6. MB49 tumour growth profile in C57BL/6 WT female mice
a. MB49 cells were injected at 1 x 10^5 cells s.c. to the right flank of B6 female mice (n=8) tumour growth was measured on consecutive days using digital callipers and tumour area calculated (length x width (mm^2)).

b. MB49 cells were injected at 3 x 10^5 cells s.c. to the right flank of B6 female mice (n=10) tumour growth was measured as above.
c. MB49 cells were injected at 6 x 10^5 cells s.c. to the right flank of B6 female mice (n=8) tumour growth was measured as above.
d. MB49 cells were injected at 5 x 10^5 cells s.c. to the right flank of B6 female mice. Tumours were imaged on day 21. Tumours were also cut using a 22 grade scalpel (bottom image).

3.2.7: $CD11c^+$ cells from tumour-draining lymph node are able to stimulate HY-specific CD4 T cells *in vitro*

In principle, to induce an anti-tumour response sufficient to cause regression, tumour derived antigens must be persistent in the tumour-draining lymph node (dLN). These antigens are presented by APCs to prime adaptive responses, however little is known regarding the relative contribution of B cells, macrophages and DCs in this response to these tumour cells. To test the priming abilities of these APC subsets, 3 groups of B6 female mice (n=2/group) were inoculated with 5 x 10^5 MB49 cells on day -24, -17 or -10 along with a group of naive female B6 mice not given tumour cells that were culled on day 0 as HY-negative controls. $CD11c^+$ DCs were purified from the tumour dLN using MACS and co-cultured with MACS-enriched Marilyn CD4 T cells as responders (see chapter 4). Resting Marilyn CD4 T cells were purified from a $Rag^{-/-}$ Marilyn transgenic female and plated out with APC in a tritiated thymidine proliferation assay (Fig. 3.7a). Marilyn CD4 T cells did not proliferate in response to $CD11c^+$ DCs from naive female pLN (peripheral LN) due to lack of HY antigens, but showed a significant level of proliferation in response to DCs from the dLN of MB49-bearing females, with a peak response induced by $CD11c^+$ cells from mice challenged on day -17. These results indicate that $CD11c^+$ cells from tumour dLN display HY peptide/MHC complexes.

In order to compare the magnitude of this response to that seen with B cells and macrophages, individual APC populations were sorted from female mice challenged with MB49 on day -17. B cells ($B220^+$), macrophages ($CD11b^+$) or DCs ($CD11c^+$) were plated out with Marilyn responders in a tritiated thymidine proliferation assay (Fig. 3.7b). Marilyn T cells cultured with DCs showed the most potent proliferative response compared to macrophages and B cells, indicating that they are the most effective APC in priming CD4 T cells *in vitro*.

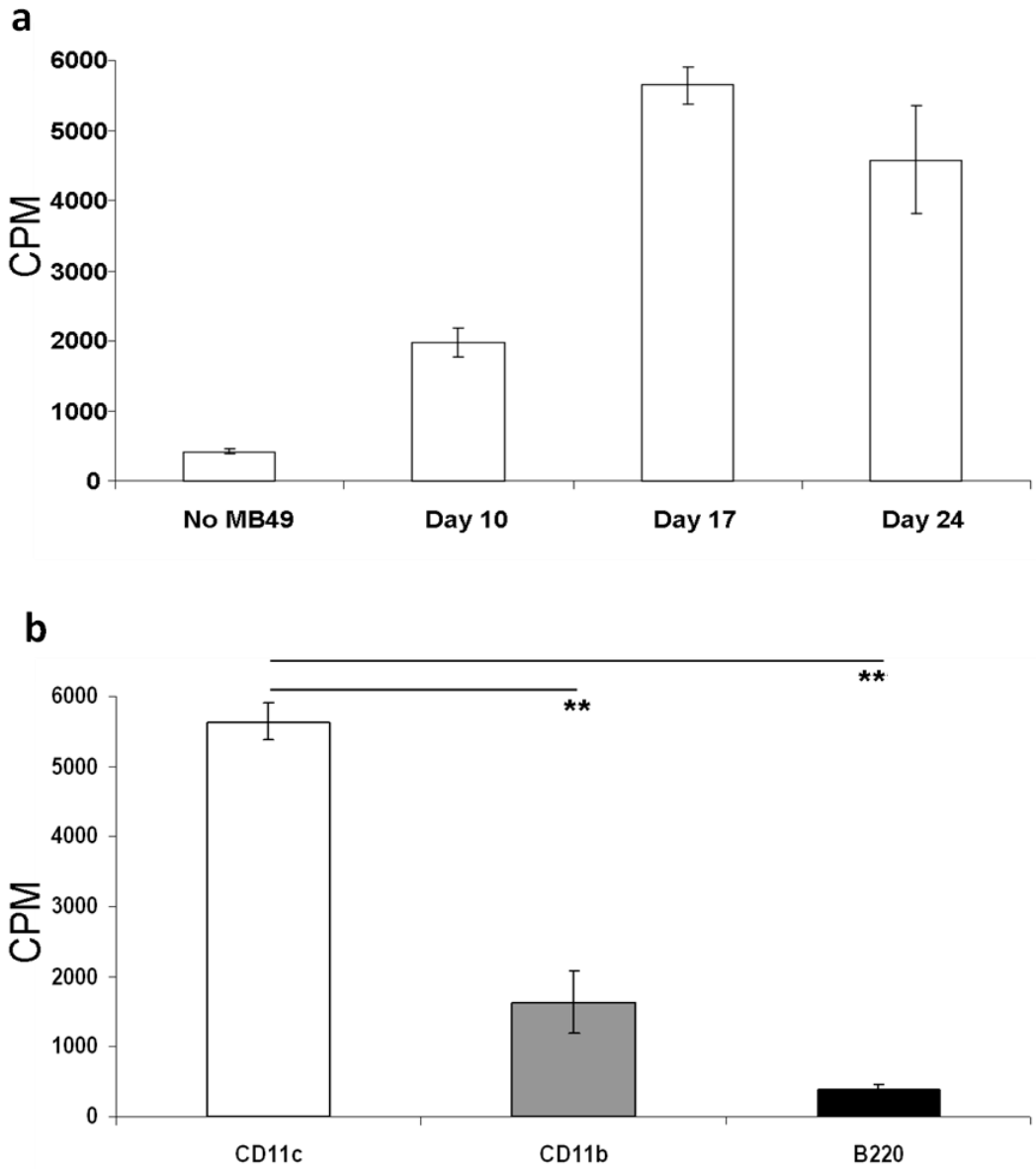


Figure 3.7. CD11c$^+$ cells from tumour dLN of MB49-bearing B6 female mice are capable of stimulating resting HY-specific CD4 T cells *in vitro*

a. 3 groups of female B6 mice (n=2/group) were inoculated with MB49 (s.c) at 5 x 10^5 on day -24, -17 and -10. On day 0 tumour dLN as well as pLN from a tumour free naive B6 female were harvested and CD11c$^+$ cells were purified using MACS. CD4$^+$ T cells were purified from SP + LN from a Rag$^{-/-}$ Marilyn female mouse by MACS. DCs were plated at 1 x 10^5 cells and Marilyn at 4 x 10^5 in a 96U bottom plates. Tritiated thymidine (0.5 μCi/well) was added on day 3 and proliferation was evaluated 24 hours later as counts per minute (CPM).

a. CD11c$^+$ DCs (white bar), CD11b$^+$ macrophages (grey bar) and B220$^+$ B cells (black bar) were isolated and MACS enriched from tumour dLN of B6 female mice inoculated with MB49 cells 17 days earlier. Cells were plated with MACS purified Marilyn responders for a tritiated thymidine proliferation assay as described in 'a'. (** = p value <0.01) Error is represented as ± standard error mean.

3.2.8: Endogenous T cell responses to MB49 tumours *in vivo*

In general terms CD8 T cells and NK cells are the major cytotoxic effectors in tumour killing, although optimal CD8 T cell activation requires CD4 help (Antony, Piccirillo et al. 2005). In order to characterise the antigen-specific anti-tumour response it is important to derive the kinetics of the endogenous T cell response, antigen-specific tumour responses will be discussed in a separate chapter in this thesis. To evaluate the endogenous T cell response an *in vivo* T cell depletion strategy was designed whereby female B6 mice were depleted of CD4 T cells, CD8 or both using a single injection (i.p.) of 1mg of depleting monoclonal antibody (YTS 191 (αCD8) (Cobbold, Jayasuriya et al. 1984), YTS 169 (αCD4) (Benjamin, Cobbold et al. 1986)). 72 hours after depletion mice were inoculated s.c. with a sub-optimal dose of MB49 cells (see Fig. 3.5, 2 x 10^4). It is anticipated that tumours should not develop in the majority of WT B6 female mice following this suboptimal dose inoculation.

Depletion of the CD4 or CD8 endogenous T cell compartment results in more rapid tumour growth (Fig. 3.8a) coupled with reduced survival (Fig. 3.8b) when compared to control mice that receive no treatment (Fig. 3.8). Interestingly there is no significant difference in mean survival time (MST) between CD4- (MST 34 days) and CD8-depleted mice (MST 33.8 days), indicating that both subsets are important for tumour rejection. Depletion of both the CD4 and CD8 T cell compartment resulted in a synergistic effect and MST was reduced to 29.3 days (Fig. 3.8b). Therefore these data show that both CD4 and CD8 T cells are required for controlling tumour development in WT B6 female mice inoculated with a suboptimal dose of MB49 cells.

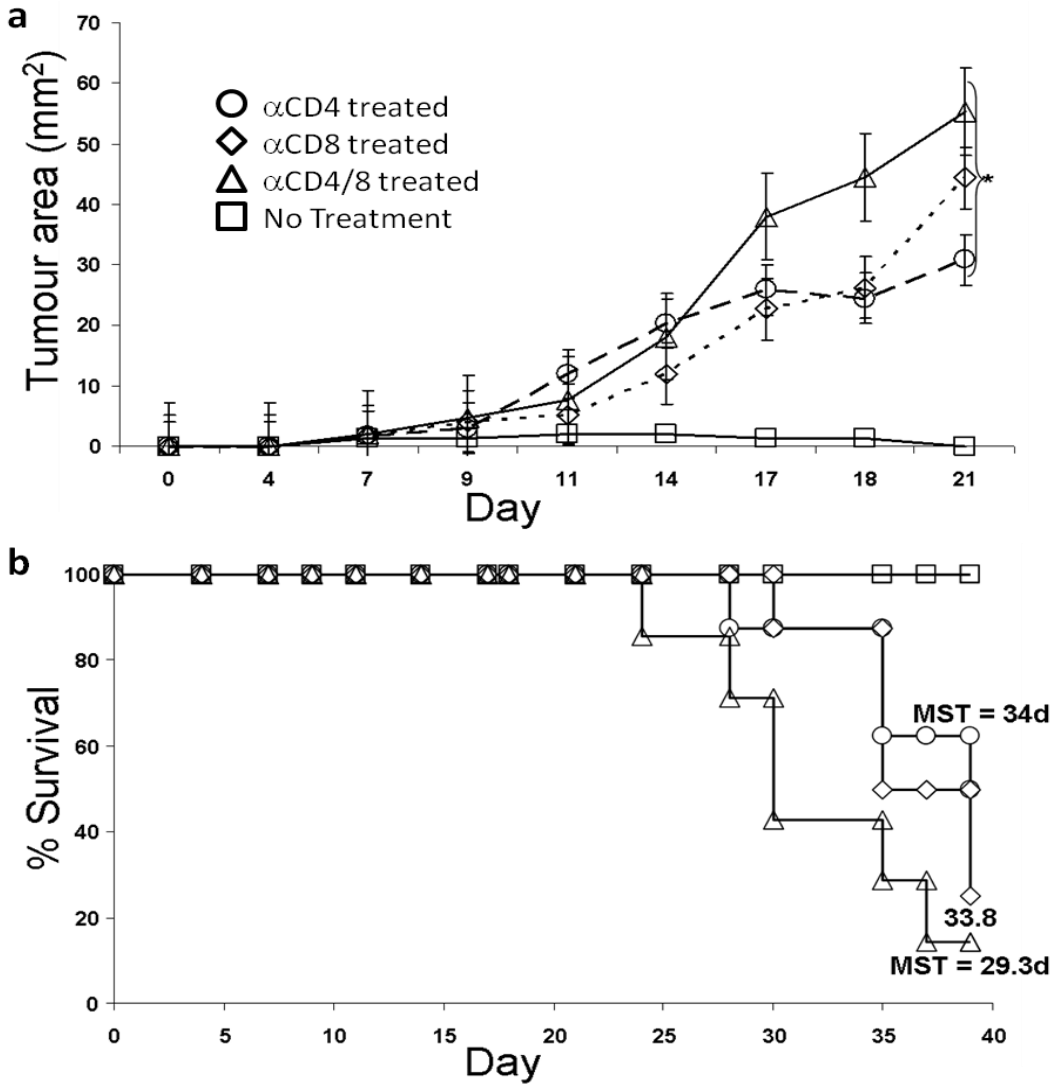


Figure 3.8. Impact of *in vivo* T cell depletion mediated by mAb treatment on tumour growth

a. 4 groups of B6 female mice were inoculated with 2 x 10^4 MB49 cells (s.c.). 3 days earlier, one group were treated with an αCD4 mAb (YTS 191) (n=7 open circles), one group with an αCD8 mAb (YTS 169) (n=7 open diamonds), one group treated with both (n=6 open triangles) (all doses 1mg i.p.) and one group received no treatment (open squares). Tumour growth was measured as in Fig. 3.5. (* P value <0.0001).

b. MB49 tumour progression was also represented as % survival of mice as described in Fig. 3.5. Error is represented as ±-standard error mean.

3.2.9: Endogenous regulatory T cell responses to MB49

The inhibitory role of regulatory T cells (Tregs) has been highlighted in many tumour systems (Curiel 2007). Studies using Treg depletion have demonstrated promising results in enhancing tumour rejection (Shimizu, Yamazaki et al. 1999; Ko, Yamazaki et al. 2005). The role of tumour antigen-specific Tregs will be addressed later in this study, however, polyclonal Treg depletion using mAb PC61 (αCD25) has shown promise in promoting tumour rejection (Onizuka, Tawara et al. 1999). To see if this is the case in the MB49 tumour model, WT female B6 mice were depleted of $CD4^+$ or $CD25^+$ T cells 3 days prior to inoculation with a sub-optimal dose of MB49 (2×10^4 (s.c)) (Fig. 3.9). Untreated mice rejected tumours quickly as in the previous experiment. Mice depleted of CD4 T cells showed a more rapid tumour development with a reduced MST than mice depleted of $CD25^+$ cells (34 days vs 37.3 days) (Fig. 3.9b). This is at odds with predictions of published results where $CD25^+$ depletion was shown to decrease tumour growth and increase survival compared to controls. It is likely in this model that other $CD25^+$ effector T cells may also be deleted. Despite this CD25 depletion induces increased survival and decreased tumour growth compared to CD4 depletion.

This result is supported by published results from our lab (Coe, Begom et al. 2010). In this study it was shown that removal of Tregs using DTA-1 anti-GITR mAb therapy can induce MB49 tumour regression in WT B6 females (5×10^5 optimal dose) while administration of PC61 is insufficient to prevent tumour growth.

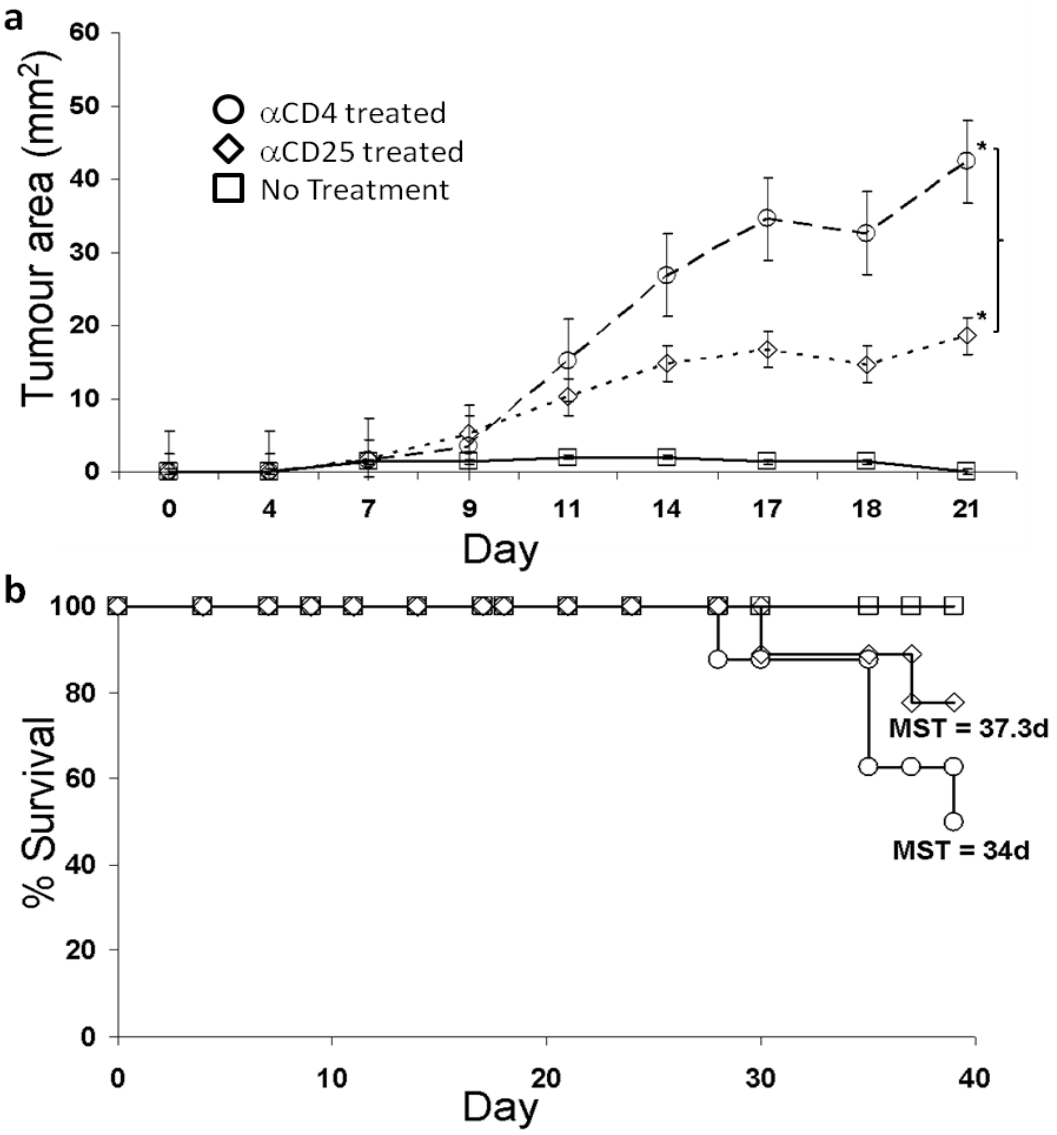


Figure 3.9. Impact of *in vivo* CD4$^+$ CD25$^+$ depletion on tumour growth
a. 3 groups of B6 female mice were inoculated with 2 x 10^4 MB49 (s.c.). 3 days previously, one group were treated with an αCD4 mAb (YTS 191) (n=7 open circles), one group with an αCD25 mAb (PC61) (n=7 open diamonds) (all doses 1mg i.p.) and one group received no treatment (n=6 open squares). Tumour growth was measured as in Fig. 3.5. (* P value <0.001).
b. MB49 tumour progression was also represented as % survival of mice as described in Fig. 3.5. Error is represented as ± standard error mean.

3.2.10: Local vs systemic endogenous T cell responses against MB49 tumour *in vivo*

T cells are required for the rejection of MB49 tumours and CD4 and CD8 endogenous populations synergise in their anti-tumour response (Fig. 3.8). To further quantify the endogenous tumour response, tumour dLN and ndLN (non-draining LN) were harvested from WT B6 female mice bearing MB49 tumours at different stages of progression (day -21, day -13 or day -7). pLN (peripheral LN) from a naive B6 female was used as a control. Lymph node samples were analysed for the proportion of lymphocytes, CD4, CD8 and Treg cells ($CD4^+FoxP3^+$) (Fig. 3.10e). Fig. 3.10 represents the mean absolute cell numbers in naive pLN (control), tumour dLN and ndLN for lymphocytes (Fig. 3.10a), CD8 T cells (3.10b), conventional CD4 T cells (3.10c) and Tregs (3.10d).

The absolute number of lymphocytes was significantly higher in the dLN compared to ndLN and naive pLN (control) at all time points tested. The number of lymphocytes in mice inoculated with MB49 cells on day -7 was on average three times higher in the dLN compared to ndLN, indicative of a local antigen dependent anti-tumour response (Fig. 3.10a). Although the number of CD4, CD8 and Tregs in the dLN tended to be higher than that in the naive pLN control and ndLN (Fig. 3.10b-d), the difference was not significant.

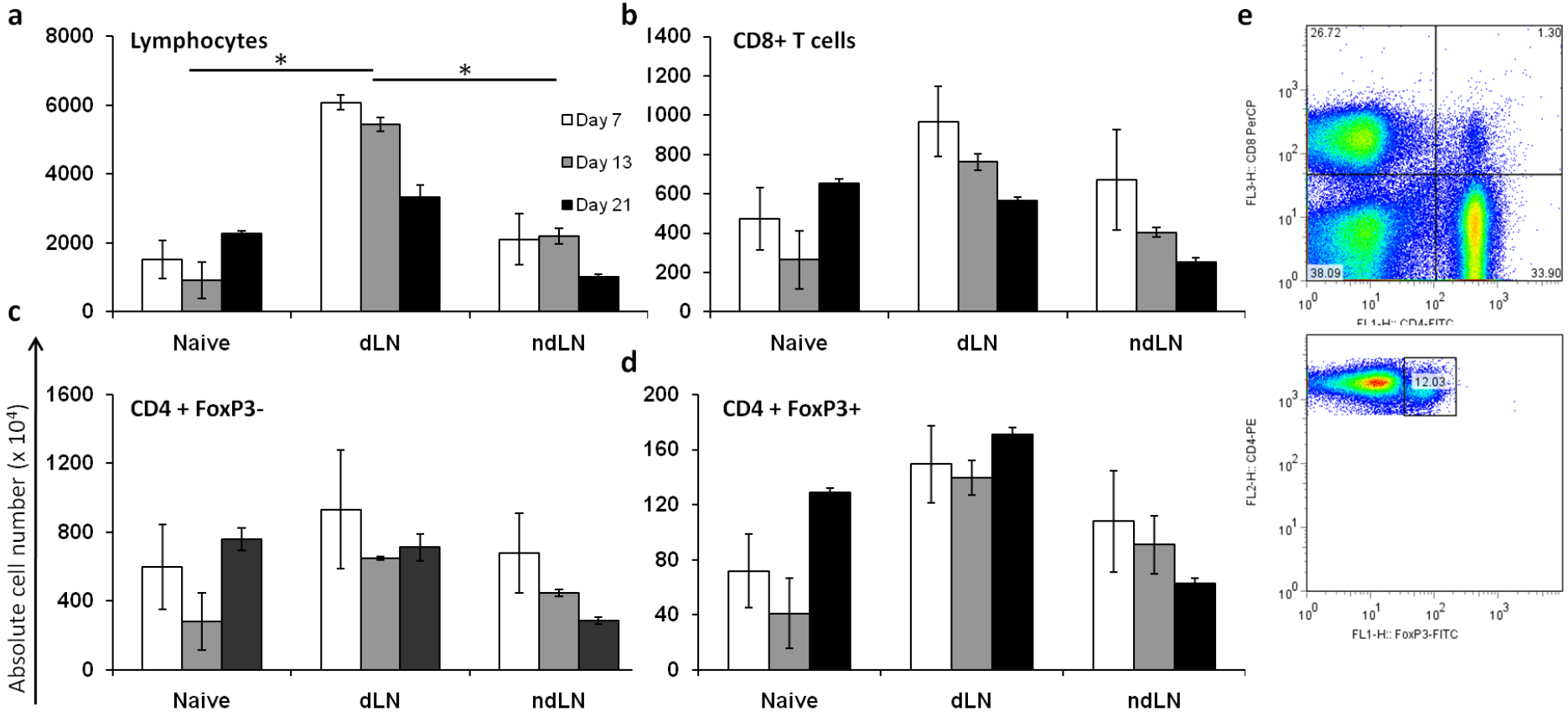

a
Lymphocytes
8000
6000
4000
2000
0
*
*
Day 7
Day 13
Day 21
Naive
dLN
ndLN
b
CD8+ T cells
1400
1200
1000
800
600
400
200
0
Naive
dLN
ndLN
c
Absolute cell number (x 10⁴)
CD4 + FoxP3-
1600
1200
800
400
0
Naive
dLN
ndLN
d
CD4 + FoxP3+
200
160
120
80
40
0
Naive
dLN
ndLN
e
26.72
1.30
38.09
33.90
FL3-H:: CD8 PerCP
FL1-H:: CD4-FITC
12.03
FL2-H:: CD4-PE
FL1-H:: FoxP3-FITC

Figure 3.10. Representation of T cell subsets in tumour dLN from MB49-bearing B6 females
3 groups of B6 female mice were inoculated with 5 x 10^5 MB49 (s.c.) on day -21 (black bars), day -13 (grey bars) and day -7 (white bars) (n=2/group). On day 0 mice were culled dLN and ndLN was harvested or pLN from a naive B6 female control. Cells were counted and stained with anti-$CD4^{FITC}$ and anti-TCR^{PE} for $CD4^+$ T cells, anti-$CD8^{PerCP}$ and anti-TCR^{PE} for CD8 T cells, and anti-$CD4^{PE}$ and anti-$FoxP3^{FITC}$ for Treg cells. Cells were analysed by FACS and gated for relevant populations. Absolute cell numbers were calculated (proportion of cell population x total cell number).
a. Absolute number of lymphocytes in naive pLN, tumour dLN and ndLN. (* P value <0.05).
b. Mean absolute number of CD8 T cells.
c. Mean absolute number of conventional CD4 T cells.
d. Mean absolute number of Tregs ($CD4^+$ $FoxP3^+$ T cells).
e. Representative FACS plots of gating strategy for CD4 and CD8 (top) and Tregs (bottom). Error is expressed as ± standard error mean.

3.2.11: Development of positive HY tetramer responses in MB49-bearing mice upon HY re-challenge

As MB49 tumours naturally express HY antigens and can induce HY-specific responses (Loskog, Ninalga et al. 2005; Perez-Diez, Joncker et al. 2007), it is of interest to measure anti-tumour CD8 T responses during the course of tumour progression. Female B6 mice were challenged with an optimal dose of MB49 (5 x 10^5 (s.c)) and tail bled on day 17 after tumour challenge when tumours were approximately 25mm^2 in size. To measure the frequency of HY-specific CD8 T cells in circulation, the peripheral blood lymphocytes (PBL) were stained using a HY-specific tetramer (Millrain, Chandler et al. 2001) (D^b-UTY-PE) along with CD8 (Fig. 3.11a). No detectable levels of $tetramer^+$ $CD8^+$ T cells were found in any of the mice tested (2 representative FACS plots shown), indicating that systemic HY-specific CD8 cell activation did not occur during the course of the primary response to the tumour.

In a second experiment mice that had previously rejected an MB49 tumour by day 35 were subjected to MB49 re-challenge at a high dose (1 x 10^6). Mice were again tail bled 7 days later and PBL stained as above (Fig. 3.11b). $Tetramer^+$ $CD8^+$ cells were detected in peripheral blood of all mice indicating that systemic HY-specific CD8 T cell responses are induced during the course of a secondary memory response. To further confirm these results another group of mice that had rejected

MB49 tumours by day 35 were boosted with an injection of male spleen cells in suspension (i.v), 7 days later mice were tail bled and tested for the presence of tetramer^{+}CD8^{+} T cells. This generated a very strong tetramer response in all mice tested (Fig. 3.11c) indicating a potent secondary response to HY antigens presented by male spleen cells and this response was markedly greater than that to MB49 re-challenge. The significantly greater tetramer positive response seen in mice boosted with male spleen is likely due to the non-immunoevasive environment under which HY is recognised.

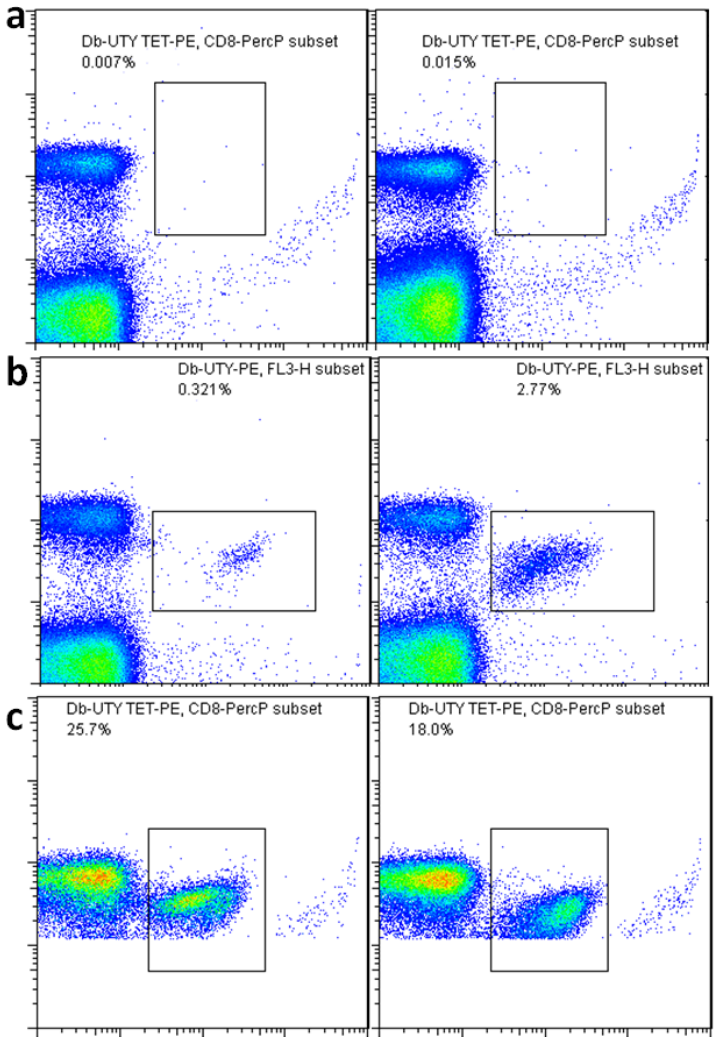


Figure 3.11. Measurement of the frequency of HY-specific CD8 T cells in response to primary and secondary tumour challenge by tetramer staining
a. B6 female mice were inoculated with 5 x 10^5 MB49 cells (s.c.) (n=4). 17 days later when tumour area was ~25mm^2 mice were tail bled. Red blood cells were lysed and the peripheral blood lymphocytes were stained with D^b-UTY^{PE} and then stained with anti-CD8^{PerCP}. Cells were analysed by FACS, 2 representative FACS plots shown.
b. 3 B6 female mice that had previously rejected MB49 tumours were subjected to secondary tumour re-challenge by injection (s.c.) of 1 x 10^6 MB49 cells. Mice were bled on day 7 and stained as above, 2 representative FACS plots shown.

c. 3 B6 female mice that had previously rejected MB49 tumours were boosted with B6 male SP cells (i.v.) with $10x10^6$ cells. Mice were bled on day 7 and stained as above, 2 representative FACS plots shown.

3.2.12: MB49 tumours are infiltrated and tumour cells killed *in vivo*

Evasive tumours can often grow by preventing host lymphocytes and leukocytes entering the tumour, thereby inhibiting tumour killing (Yu, Lee et al. 2003). Tumour-specific T cells that do infiltrate the tumour can be exposed to factors such as death ligands (e.g. PD-L1, FAS-L) that can potentially induce apoptosis in lymphocytes such as T cells. To test the cytotoxic activity within MB49 tumours, B6 female mice were injected with an optimal dose of MB49 (5×10^5) on both the left and right lateral flanks. Once the tumours were established into a large progressive solid state tumour >8mm in diameter (Fig. 3.12b) mice were culled and tumour tissues were analysed by Propidium Iodide (PI) staining (Fig. 3.12). PI is an intercalating agent that binds non-specifically to DNA and RNA, PI increases fluorescence 30 fold in the red spectrum (PE channel) upon DNA binding. This allows cell viability to be measured as PI can is not membrane permeable and therefore will only stain dead or dying cells (Lecoeur 2002).

Large tumours (Fig. 3.12b) and dLN cells were harvested and stained for CD45 and PI. $CD45^-$ cells were classified as MB49 and $CD45^+$ as lymphocytes. A large proportion of MB49 tumour cells stain PI^+ and are therefore actively undergoing cell death even in tumours that have escaped immune control (Fig. 3.12b). This is also true for lymphocyte cell death within the tumour (Fig. 3.12b) but not for lymphocytes in the local dLN where only a small fraction are dead or dying (Fig. 3.12a). When this was quantified from 24 individual tumours (12 mice) this showed a large proportion of tumour cells are dead or dying within the tumour with an average ratio of dead to live cells of 0.36:1. This was also true of lymphocyte compartment within the tumour which had an average ratio of 0.46:1 (Fig. 3.12c). This effect was not as profound in the local dLN where the average ratio was 0.04:1. These data indicate MB49 tumours are sites of active cell death of both tumour cells and host lymphocytes even in tumours that have escaped immune control.

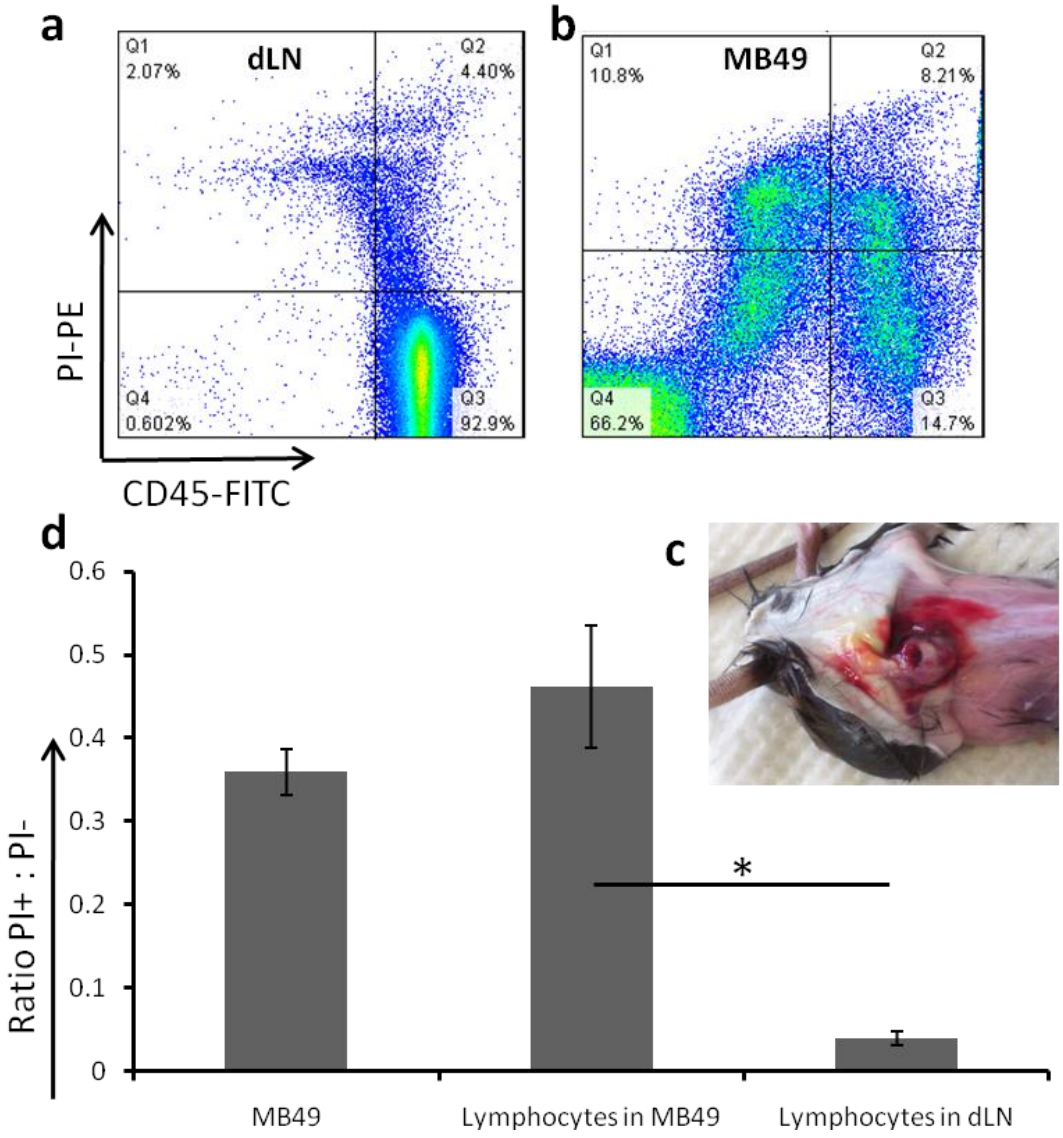


Figure 3.12. Both MB49 cells and lymphocytes stain PI^+ in large progressive tumours

B6 female mice were inoculated with 5 x 10^5 MB49 (s.c.) to the left and right flanks (n=12). 18 days later when tumour area was >60mm^2 mice were culled and tumour tissue and dLN excised and stained for $CD45^{FITC}$ and with PI.

a. Representative FACS plot of dLN stained as above.

b. Representative FACS plot of MB49 tumour stained as above.

c. Representative image of a large progressive tumour analysed in this experiment.

d. Ratio of live to dead cells in the tumour for MB49 gate and lymphocyte gate and for lymphocyte gate in the dLN (n=24 tumours and 24 dLN in 12 individual mice). Error is expressed as ± standard error mean. (* p value <0.05).

3.2.13: MB49 can express IL-10, TGF-β and IL-17 cytokine genes

Solid tumours often produce immunosuppressive and immunoevasive factors in the local microenvironment (Zitvogel, Tesniere et al. 2006). Tumours can induce lymphocyte cell death by direct binding through death receptors such as PD-1 and FAS as seen earlier, or by inducing an immunosuppressive environment driving Tregs and myeloid derived suppressor cells to negatively augment the anti-tumour response. Several studies including studies in this and other labs have linked the production of IL-10 and TGF-β in the local tumour microenvironment to poor tumour prognosis driven by Treg activation (Fukuyama, Ichiki et al. 2007; Coe, Addey et al. 2010; Coe, Begom et al. 2010). MB49 cells were isolated from B6 tumour-bearing females (WT B6, IL-10KO and IL-17KO) as described in Fig. 3.4 and compared with the MB49 *in vitro* cell line for expression of TGF-β (Fig. 3.13a), IL-10 (Fig. 3.13b) and IL-17 (Fig. 3.13c) using an RT-PCR Taqman® assay. *18sRNA* was used as the normaliser gene results are expresses as relative quantification (RQ) to this gene.

MB49 expresses TGF-β in both the cell line and the *in vivo* tumour, indicating the tumour is naturally immunomodulatory, presumably by inducing Treg activation and expansion (Fig. 3.13a). MB49 cell line however does not express high levels of IL-10, but upregulates IL-10 expression after forming tumours (Fig. 3.13b). Moreover IL-10 is upregulated further by inoculation of MB49 cells in IL-10 KO mice, indicating IL-10 is secreted in even higher amounts by the tumour cells if it is lacking exogenously in the *in vivo* setting. IL-17 production by MB49 *in vivo* displays a similar kinetic to IL-10. In IL-17 KO mice tumours express much higher levels of IL-17 again potentially compensating for lack of exogenous cytokine.

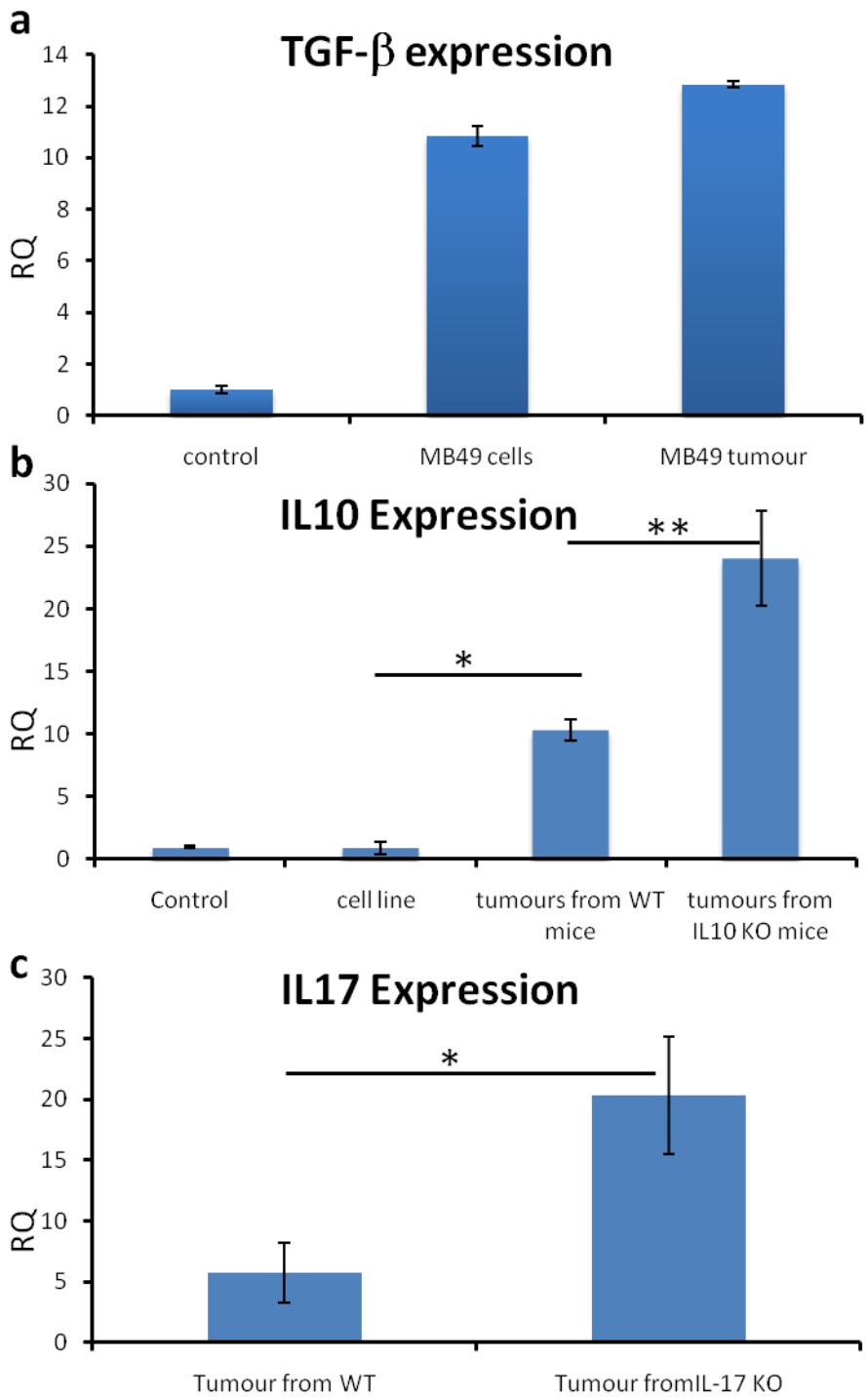


Figure 3.13. Comparison of cytokine gene expression profiles between various MB49 tumours and cell line

Ex vivo MB49 cells were harvested from a B6 tumour-bearing female (5 x 10^5 day 17) from either WT, IL-10KO or IL-17KO mice. MB49 cell line was also harvested and both populations were subjected to RNA extraction and cDNA conversion before running RT-PCR Taqman® assay in triplicate. Control represents expression of 18SRNA gene. Error is expressed as ± standard error mean (* P value <0.01, ** 0.0134).

a. TGF-β gene expression changes between cell line and tumour.

b. IL-10 gene expression changes between cell line, tumour from WT and IL-10KO.

c. IL-17 gene expression changes between tumour from WT and IL-17KO mice.

3.2.14: MB49 produces Indoleamine 2,3-dioxygenase (IDO) *in vivo*

IDO is involved in the activation and maintenance of Tregs. Due to the potent immunosuppressive effect of IDO (see section 1.6.3) produced by plasmacytoid DCs (pDC) in tumour dLN, it is of interest to evaluate the ability of the MB49 tumour itself to produce IDO. *Ex vivo* MB49 tumours express high levels of IDO, significantly higher than the cell line (Fig. 3.14a). IDO was also significantly higher in the dLN compared to ndLN in agreement with published data (Sharma, Hou et al. 2009).

In addition to tryptophan consumption, other essential amino acids have been identified that may be rate limiting to effector T cell activation and proliferation (Cobbold, Adams et al. 2009). One enzyme branched chain amino acid transaminase (Bcat1) has been shown to be upregulated in proinflammatory conditions of allogenic skin graft from lymphopenic CBA male to lymphopenic female mice while the enzyme histidine decarboxylase (Hdc) was shown to only be upregulated with both skin graft and Treg adoptive transfer (Cobbold, Adams et al. 2009). Bcat1 enzyme catalyzes the reversible transamination of branched-chain alpha keto acids to branched chain L amino acids essential for cell growth while Hdc metabolises histidine. MB49 tumours were also tested for the expression of Hdc (Fig. 3.14b) and Bcat1 (Fig. 3.14c), compared with the cell line. Both genes were upregulated in the tumour compared to the cell line, with Hdc demonstrating a significant change. These data in combination indicates essential amino acids in the local tumour microenvironment are consumed which may abrogate the T cell response and promote the activation of Tregs.

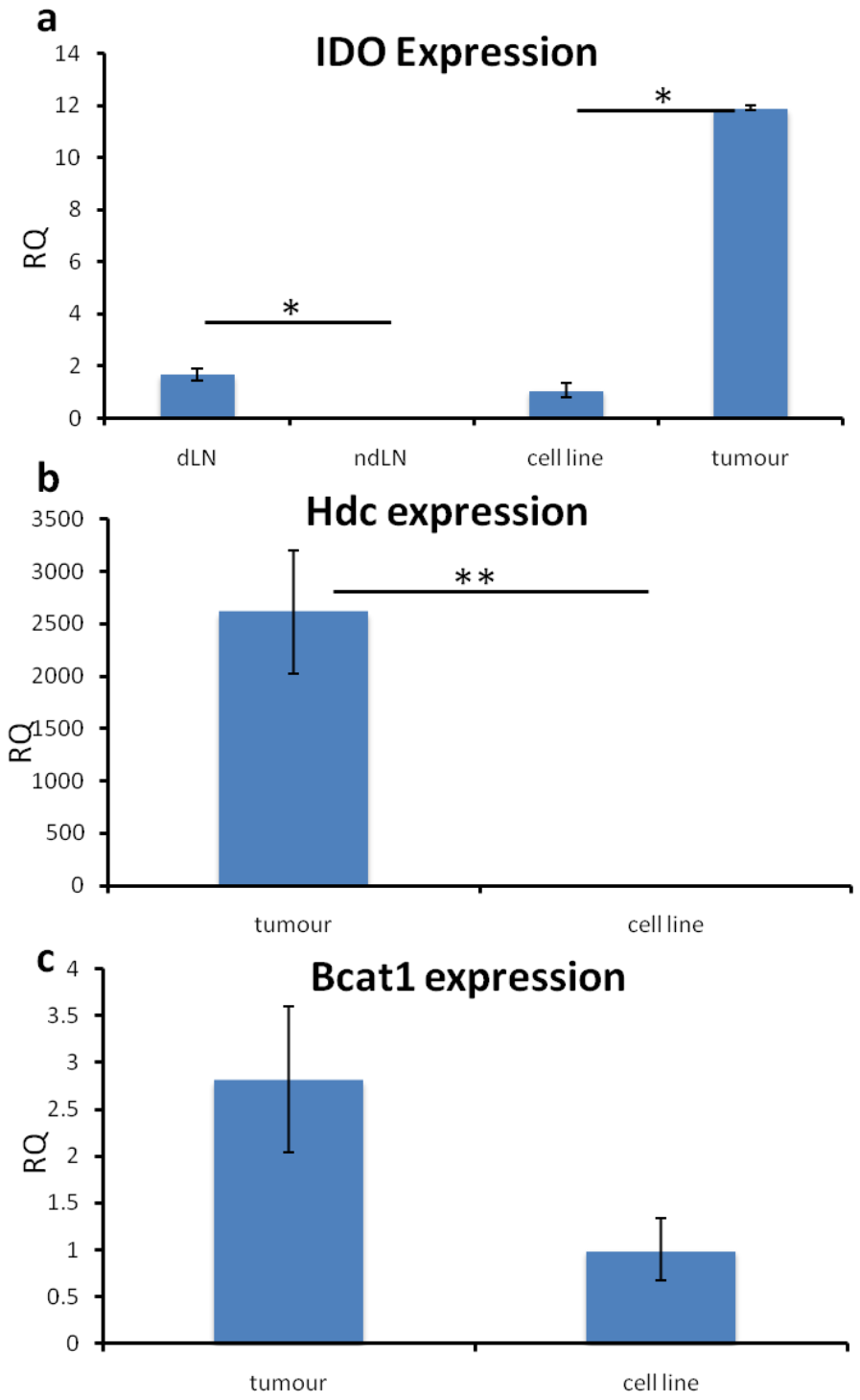


Figure 3.14. Upregulation of IDO, Hdc and Bcat1 by MB49 tumours
Ex vivo MB49 cells were harvested from a WT B6 tumour-bearing female (5 x 10^5 day 17), MB49 cell line was also harvested and both populations were subjected to RNA extraction and cDNA conversion before running RT-PCR Taqman® assay in triplicate. Error is expressed as ± standard error mean (* P value <0.005, ** 0.03)
a. IDO gene expression changes between cell line and tumour.
b. Hdc gene expression changes between cell line and tumour.
c. Bcat1 gene expression changes between cell line and tumour.

3.3: Discussion

To create a transplantable tumour model for adoptive cancer immunotherapy it was necessary to characterise the MB49 tumour model both in terms of kinetics and phenotype. Later in this study I will evaluate tumour antigen-specific responses by T effector cells and Tregs in an adoptive cell transfer model. Prior to conducting these later studies MB49 cells were characterised to give a fundamental phenotype around which future therapies could be designed. MB49 is a carcinoma cell line that expresses HY genes, derived from a B6 male mouse (Fig. 3.1), expression being verified by RT-PCR. MB49 expresses all 3 HY antigens (Fig. 3.1a) as well as the MHC restriction molecules $H2D^b$, $H2K^b$ (MHC-I) and $H2A^b$ (MHC-II), and co-stimulatory molecules (Fig. 3.1b and c). MB49 is therefore a good model for measuring responses to HY minor antigen as the tumour is able to present these physiological antigens in an immunological manner.

The ability to maintain MB49 as a stable *in vitro* cell line allows for consistent replicate experiments to be conducted. MB49 tumours however show significant changes in surface marker expression profile compared to the *in vitro* cell line (Fig. 3.1b and c). Experiments in which MB49 cells were cultured in the presence of IFNγ, a Th1 cytokine known to be highly upregulated during anti-tumour responses (Fig. 3.2) (Nishikawa, Kato et al. 2005), and others in which CD45 negative MB49 tumour cells were isolated *ex vivo* (Fig. 3.3) (Arendt and Ostergaard 1995), showed high levels of MHC-I and II expression as well as positive co-stimulatory molecules, indicating the tumour has immunogenic features. MB49 cells also expressed PD-L1 and PD-L2 (Fig. 3.2a and 3.3) and expressed Fas-L (Fig. 3.2b) indicating that it has the potential ability to directly bind PD-1 and Fas on activated T cells as a mechanism of immune evasion. Interestingly a series of other rather unexpected molecules emerged as surface markers, including leukocyte adhesion molecules CD44, CD49b and CD62L. The function of these molecules on MB49 tumours is not fully understood, but they may be involved in mediating tumour cell adhesion and extra cellular matrix modification (Sillanpaa, Anttila et al. 2003). The stem cell marker Sca-1 was also upregulated by MB49 cell line, a molecule often identified as an oncogene in many tumours (Bradfute, Graubert et al. 2005). Finally the chemokine CCR9 was shown to be expressed in MB49 tumour cells *ex vivo*. CCR9 is involved in gut homing and is often expressed in prostate and colon cancers

(Koizumi, Hojo et al. 2007), so perhaps expression by MB49 is unsurprising, as it originated as a bladder carcinoma (Fig. 3.2b and 3.3).

MB49 tumour cells from WT B6 female mice and the *in vitro* MB49 cell line were subjected to RNA extraction and conversion to cDNA (Fig. 3.4). They were compared for the expression of a range of cytokine, chemokine and receptor genes, significant and relevant gene expression by the MB49 tumour and cell line are shown in the table below.

Table 7: MB49 gene expression changes

Gene upregulated *in vivo*	**Immunological relevance**	**Reference**
IL-1β	Proinflammatory mediator expressed mainly by activated macrophages	(Fukuyama, Ichiki et al. 2007)
CCL3, 4 and 5	CC chemokines recruit lymphocytes in many cancer systems. CCL3 (MIP1α) and 4 (MIP1β) shown to recruit CTLs, CCL5 (RANTES) shown to recruit Tregs	(Luboshits, Shina et al. 1999)
CXCR3 and CXCL9	Activation marker of T cells, expressed highly on pDCs and binds CCL9	(Smit, Verdijk et al. 2003)
IL-3RA	Cell surface marker of pDCs and immature myeloid DCs	(Iwasaki and Medzhitov 2004)
KIT	Proto-oncogene implicated in the sequestration of myeloid derived suppressor cells by binding stem cell factor. MDSCs can promote tolerance and Treg recruitment.	(Bradfute, Graubert et al. 2005)
Gene downregulated *in vivo*		
IL-13RA	Originally believed to be redundant to IL-4, new studies have shown this factor can induce potent anti-tumour responses	(Kioi, Shimamura et al. 2009)
Genes expressed in cell line and *in vivo*		
TGF-β, IL10 and IL-4	Th2 Immunosuppressive cytokines associated with suppression of effector T cells and survival and expansion of Tregs	(O'Farrell, Liu et al. 1998; Coe, Addey et al. 2010)

Taken together with surface phenotyping data, this demonstrates MB49 tumours are immunogenic but crucially have the potential to expresses suppressive

factors on the cell surface or into the local tumour microenvironment, with potential to directly and indirectly abrogate tumour-specific T cell responses.

As MB49 is immunogenic *in vivo* and expresses HY minor antigens, the disparity in the response between male and female B6 mice is not surprising (Loskog, Ninalga et al. 2005). Female B6 mice are able to mount antigen-specific T cell responses to HY antigens *in vivo* whereas males are tolerant (Scott, Ehrmann et al. 1995), initially demonstrated through skin grafting experiments (Eichwald and Silmser 1955). MB49 cells were injected in a range of doses (1×10^4- 6×10^5) to WT B6 female and male mice (Fig. 3.5). At all doses a protective effect was seen for female mice with a significantly increased survival time. B6 females were in fact able to reject MB49 tumours at low doses whereas males could not, demonstrating the effect of the HY response could be immuno-dominant over TAA expressed by the tumour (Fig. 3.5a-d). Female B6 mice that received irradiated MB49 cells were able to reject a high dose of MB49 cells while only 60% of males were able to do so (Fig. 3.5e). MB49 tumours expresses several TAAs, such as prostate cancer stem cell antigen (PSCA), and therefore male mice do make an immune response to these TAA (Loskog, Ninalga et al. 2005). However this response is insufficient to protect at low doses of MB49 challenge or a high dose of MB49 re-challenge. HY is the major surrogate TAA and responses to this minor H antigen show a clear difference between male and female mice.

Female B6 mice were challenged with a range of MB49 doses and tumour growth measured to define an optimal dose for tumour formation in >90% of mice (Fig. 3.6). An optimal dose of between 3-6 x 10^5 MB49 cells to WT B6 female mice is able to induce a progressively growing solid tumour reaching approximately 64mm^2 by day 14 (Fig. 3.6b and c). Therefore from this point MB49 cells will be used at a dose of 5×10^5 to WT B6 female mice to produce rapidly growing solid tumours (Fig. 3.6d) for the evaluation of various therapeutic experiments.

Having established the phenotype of MB49 *in vivo* and its growth kinetics, it was necessary to assess the various aspects of the endogenous immune response to MB49. DCs ($CD11c^+$) in the local draining lymph node of MB49-bearing female mice were purified and co-cultured with Marilyn CD4 responder cells (Fig. 3.7a). Marilyn CD4 T cells are TCR transgenic and recognise $H2A^b$-Dby expressed by MB49 (Chen, Demir et al. 2003) (see chapter 4). Marilyn T cells responded to all APCs from tumour dLN (day -24, -17 or -10) with the highest response to DCs from

the draining lymph node of 17 day old tumours (Fig. 3.7b). Marilyn T cells showed the greatest proliferative response to DCs. MB49 is the only source of HY antigen in this system and therefore must be processed and presented in the local draining lymph node, and the most effective presentation is achieved by DCs.

T cell responses to MB49 are required to control the tumour (Perez-Diez, Joncker et al. 2007). B6 female mice were therefore depleted of CD4, CD8 T cells or both and subsequently challenged a sub-optimal dose of MB49 cells (Fig. 3.8) and in a separate experiment mice were depleted of $CD4^+$ or $CD25^+$ cells prior to tumour challenge (Fig. 3.9). Tumours grew quicker in mice depleted of CD4, CD8 or both but were controlled in mice that received no treatment, who showed 100% survival (Fig. 3.8). Tumour growth was further increased and mean survival time decreased in mice that were depleted of both CD4 and CD8 T cells, indicating that there is a synergistic effect between the two compartments (Fig. 3.8). Interestingly when $CD25^+$ cells were depleted this also had a negative impact on survival with faster tumour growth although these effects were not as profound those produced by CD4 depletion (Fig. 3.9). This result may be accounted for by the expression of CD25 not just on Tregs but also on anti-tumour effector cells. Subsequent studies by colleagues have revealed that more selective Treg depletion using other mAbs such as DTA-1 (anti-GITR mAb) in MB49-bearing B6 females can induce increased tumour survival (Coe, Begom et al. 2010).

Comparing lymphocyte numbers from the draining lymph node of tumour-bearing mice (day -21, 13 and -7) to non-draining lymph node or peripheral lymph node from a naive B6 shows draining lymph node has significantly higher absolute numbers of lymphocytes at all time points tested (Fig. 3.10a) with absolute numbers of CD8, CD4 and Tregs on average higher than non-draining lymph node or control peripheral lymph node (Fig. 3.10b-d). These results, combined with T cell depletion data and APC assays, indicate that MB49 induces a local antigen-driven T cell response. In support of this, HY tetramer data also shows that MB49 tumours can induce antigen-specific CD8 cells upon re-challenge (Fig. 3.11). MB49 induces a poor systemic T cell response upon primary challenge and consequently no tetramer positive cells are detected in peripheral blood samples (Fig. 3.11a). When female mice are re-challenged with a high dose of MB49 cells, tetramer positive CD8 T cells are detected (Fig. 3.11b). This demonstrates MB49 induced priming to HY is sufficient to induce a HY antigen-specific secondary response. This was confirmed

by vaccinating mice that had rejected MB49 following boosting with male spleen cells that induced a potent tetramer positive response 7 days later (Fig. 3.11c). The response to male spleen cells propagates systemically and does not elicit the immunoevasive responses that may be the reason for lower levels of MB49 tetramer^{+} response.

As many tumours evade the immune system by excluding immune effector components, MB49 tumours were excised for *ex vivo* analysis by staining with propidium iodide (PI) to show cell death within the MB49 tumour (Fig. 3.12). Lymphocytes show a very high proportion of dying or dead cells within the tumour compared to the local draining lymph node (0.46:1 vs 0.04:1) (Fig. 3.12a,b and d). Tumour cells themselves also show a high proportion of dead or dying cells (0.36:1) (Fig. 3.12b and d). MB49 tumours are therefore active sites of immunological relevance with a high proportion of cell turnover of both lymphocytes and tumour cells.

To fully elucidate the phenotype of a tumour *in vivo*, it is necessary to understand the tumour microenvironment and its effects on modulating the immune system. MB49 is clearly immunogenic (Fig. 3.1-4) and recognisable *in vivo* where potent T cell responses can be induced to its HY antigens (Fig. 3.5-12). However, at higher does (>2 x 10^4) MB49 cells can escape immune control and develop into a progressively growing solid tumour (Fig. 3.6d, 3.12c). One likely contributing factor is the modulation of the T cell anti-tumour response by secretion of factors into the local microenvironment. This can be seen by gene expression studies (Fig. 3.4) that indicate expression of genes that encode TGF-β, IL-10, IL-4, IL-17 amongst others. TGF-β, IL-10 and IL-17 have potent effects on the induction of Tregs, the suppression of Th1 responses and the induction of Th17 responses respectively. Excised *ex vivo* MB49 tumours were again subjected to RNA extraction and cDNA conversion in order to analyse the production of these factors (Fig. 3.13). As indicated by previous genotyping (Fig. 3.4), TGF-β is highly expressed in both the MB49 cell line and tumour (Fig. 3.13a) and seems to be a constitutively expressed factor involved in immune modulation by the induction of Tregs. Subsequent studies by colleagues have shown blocking TGF-β signalling on Tregs, through over expression of dominant negative form of TGF-β receptor type II, can abrogate Treg functionality and induce an improved tumour prognosis (Coe, Addey et al. 2010).

Expression of IL-10 and IL-17 is induced *in vivo* by MB49 tumours (Fig. 3.13b and c). However IL-10 and IL-17 expression is increased further in tumours from mice lacking exogenous cytokine (IL-10 KO and IL-17 KO mice). This demonstrates MB49 has the capacity to compensate for modifications to the local tumour microenvironment such as reduced cytokine availability. IL-10 is important for suppression of NF-κB induced Th1 responses by monocytes and tends to lead to poor tumour prognosis (de Waal Malefyt, Haanen et al. 1991). The function of IL-17 in tumour systems is controversial, with some groups reporting IL-17 as anti-tumour (Nakae, Komiyama et al. 2002) and others with pro-tumour effects (Wang, Yi et al. 2009) in the MB49 system. MB49 upregulates IL-10 and IL-17 expression *in vivo* and maintains expression of TGF-β into the local tumour microenvironment.

Another well documented mechanism for tumour evasion from immune responses is through the depletion of essential amino acids (EAAs), depriving T cells of these EAAs can prevent proliferation and activation in response to antigen (Munn, Sharma et al. 2004). This is well documented for enzymes such as IDO which has a potent role in controlling foetal immune responses in the placenta. MB49 was therefore tested as described previously for the production of enzymes involved in EAA depletion, namely IDO, Hdc and Bcat1 (Fig. 3.14). IDO, which catabolises tryptophan, was shown to be significantly upregulated both in the draining lymph node and tumour compared to non-draining lymph node and cell line respectively (Fig. 3.14a). Hdc an enzyme that metabolises histidine was shown to be significantly increased and Bcat1 was also expressed by tumours (Fig. 3.14b and c). MB49 therefore actively modifies the local microenvironment by the secretion of several immune-modulating factors that can have a significant impact on the local immune response.

MB49 can now be characterised as an aggressive, immunogenic solid tumour that can induce potent antigen-specific local T cell responses. MB49 is an actively growing tumour that is capable of overcoming the T cell response at higher doses. This is achieved through specific interactions with lymphocytes (e.g PD-L1-PD-1) and through the induction of local tolerance driven by the local milieu of the tumour microenvironment. By understanding the endogenous anti-tumour response I am now able to evaluate antigen-specific T cell responses in an adoptive transfer system.

Chapter 4: Distinct *in vivo* antigen-specific CD8 and CD4 T cell responses against normal and malignant tissue

4.1: Introduction

In the previous chapter endogenous T cell mediated responses against MB49 were evaluated. This elucidated a definitive disparity in anti-tumour responses between female and male mice, conferred by reactivity to HY in female mice (see Fig. 3.5 and 3.6). Previous data also shows endogenous responses in female mice are insufficient to mediate the rejection of MB49 at moderate to high doses (Fig. 3.6). Further to this MB49, despite being immunogenic, is also highly aggressive and displays several immunoevasive and immunosuppressive markers and factors (Fig. 3.1-4) which would potentiate direct killing of tumour infiltrating lymphocytes (Fig. 3.12). This coupled with the ability of MB49 to modulate the tumour microenvironment *in vivo* (Fig. 3.13-14), prevents tumour rejection in WT mice. In this chapter the antigen-specific CD4, CD8 T and Treg cells are evaluated for their anti-tumour responses. In addition, these anti-MB49 specific T cell responses will be compared to those to non-malignant tissues, to attempt to define the immunosuppressive effect induced in the tumour microenvironment.

4.1.1: HY-specific T cells

As discussed in chapter 3, the availability of CD4 (Marilyn) and CD8 (Matahari) transgenic mice allows for the generation of TCR transgenic monoclonal T cells on a C57BL/6 $H2^{b+}$ $Rag^{-/-}$ background that can be utilised for adoptive transfer (Lantz, Grandjean et al. 2000; Valujskikh, Lantz et al. 2002). The studies of T cell responses to HY minor antigens were initially developed in the context of transplant rejection (Scott, Ehrmann et al. 1995; Simpson, Scott et al. 1997; Simpson, Scott et al. 2001). Consequently HY antigens are one of the best characterised transplantation antigens (Simpson, Scott et al. 2001). Subsequent development of tetramers (Millrain, Chandler et al. 2001) and TCR transgenic mice (Valujskikh, Matesic et al. 1999; Lantz, Grandjean et al. 2000) has allowed for the detailed dissection of HY-specific T cell responses in the context of tolerance and

transplantation. More recently our lab, and others, has explored the role of HY as a surrogate tumour-associated antigen (Perez-Diez, Joncker et al. 2007). This approach takes advantage of HY-expressing tumour cell lines that can form transplantable solid progressive tumours (see chapter 3). As T cell mediated immunity is highly dependent on the environment in which they are primed, the study of HY-specific T cell responses in normal and malignant tissues provides an insight into the immunoevasive environments tumours can employ.

4.1.2: HY-expressing malignant and normal tissues

T cell mediated responses to allografts of normal tissues generally produce a strong productive response leading to tissue rejection (Auchincloss and Sultan 1996). Conversely T cell mediated responses to transplantable tumours are generally defective, leading to a failure to eliminate the tumour (Kim, Emi et al. 2006). The HY male antigen allows for studies to be conducted in situations where normal and tumour allografts share the same alloantigens. The distinct outcomes to these tissues can be at least in part attributed to the T cell response, giving two distinct ways of comparing antigen-driven responses. Comparison of antigen-specific T cell immunity to normal and tumour allografts can provide insights into the molecular and cellular mechanisms that underlie the failure to induce tumour rejection.

4.1.3: Antigen-specific allograft responses to minor H antigens

T cell responses against minor histocompatibility (H) antigens are responsible for the rejection of MHC matched transplants. These responses can elicit major clinical complications by inducing host versus graft (HvG) responses and graft versus host disease (GvHD) (Zheng, Matte-Martone et al. 2009). Therefore understanding the cellular and molecular mechanisms that underpin T cell responses to allografts has become crucial in clinical applications such as bone marrow transplantation in treating leukaemia patients.

Initial skin grafting experiments revealed a large number of minor H antigens at multiple loci scattered across the genome (Graff and Bailey 1973). The antigens are encoded by mitochondrial DNA, the Y chromosome and autosomal chromosomes (Rotzschke, Falk et al. 1990). Despite many minor H antigens having been characterised in humans and mice, relatively little is known about natural

regulation of immunity to minor H antigens (Simpson, Scott et al. 2001). CD8 T cells alone have been shown to be capable of rejecting allografts expressing multiple minor H antigens (Youssef, Otley et al. 2004), however, the identification of both MHC I and MHC II restricted epitopes indicates the requirement for both CD4 and CD8 responses to generate effective responses (Roopenian 1992). Indeed the depletion of either CD4 or CD8 T cell subsets can lead to a state of unresponsiveness to allografts (VanderVegt and Johnson 1993). Subsequent experiments have shown that while CD8 priming can occur in the absence of CD4 help, this results in a diminished capacity to differentiate into memory cells (Janssen, Lemmens et al. 2003). Most strategies used for the induction of tolerance have therefore focused on the CD4 T cell subset, mainly by the induction of Tregs from conventional CD4 T cells (Karim, Feng et al. 2005). It has also been demonstrated that nTregs can modulate immunity to minor H antigens in TCR transgenic mice (Benghiat, Graca et al. 2005). The specific role of antigen-specific Tregs in regulating allograft responses has not been fully explored but this population is clearly a powerful factor in the suppression of allograft responses (Chai, Coe et al. 2008; Verginis, McLaughlin et al. 2008).

HY antigen-specific CD4, CD8 and Treg responses to male allografts are evaluated in this chapter. Male skin graft can induce potent T cell responses in female mice when antigens are presented in the context of $H2^b$ but not other haplotypes (Robertson, Chai et al. 2007). Previous published data has shown both Matahari CD8 and Marilyn CD4 transgenic females are capable of rejecting male skin grafts through direct antigen presentation by target cells (Valujskikh, Lantz et al. 2002) and also indirectly through host APC interaction (Braun, Grandjean et al. 2001). These transgenic mice have therefore been used to evaluate the potency of the allograft response in comparison with that to a HY^+ tumour. HY-specific CD4 T cell responses in male lymphopenic mice can also induce GvHD when radiation pre-conditioned, this is dissected in Chapter 7.

4.1.4: Antigen-specific responses to minor H antigen in the context of tumour

The expression of minor H antigens in tumours is becoming increasingly recognised as a way to induce potent anti-tumour T cells responses in adoptive immunotherapy (Overwijk 2005). Directional approaches targeting T cells against

one specific minor H antigen is a promising new approach designed to elicit effective local anti-tumour responses. This has an obvious role in the eradication of hematopoietic malignancy using donor minor H antigen-specific lymphocytes (Hambach and Goulmy 2005). Mismatches in minor H antigens are currently exploited to induce rejection of malignant cells for a graft versus leukaemia (GvL) effect (Feng, Hui et al. 2008). These responses are also responsible for the induction of GvHD, depending on the distribution and expression level of the minor H antigens (Kaplan, Anderson et al. 2004). In chapter 7 I will also explore the role of Marilyn antigen-specific T cells in the induction of GvHD in male lymphopenic mice where the relevant minor H antigen is expressed ubiquitously.

Minor H antigen-specific therapies against solid state tumours are now becoming clinically relevant (Meunier, Delisle et al. 2005). The identification of T cell populations able to target malignant tissue without inducing GvHD is the key objective to this therapeutic approach. Some antigens are restricted to tumour tissue and often result from onco-related genes, however, like HY, most self antigens are universally expressed (den Haan, Meadows et al. 1998). In spite of ubiquitous expression, immunity to a single minor H antigen can induce regression of solid tumours without causing GvHD (Meunier, Delisle et al. 2005) (see chapter 7).

Many anti cancer vaccine approaches and adoptive transfer therapies have previously focused on methods to elicit primarily tumour-specific CD8 CTLs. CTLs have historically been shown to be capable of rapid allograft rejection and tumour killing *in vitro* (Lee, Wang et al. 1999), therefore it is reasonable to assume they should be potent anti-tumour effectors *in vivo*. T cell transgenic mouse models have generated similar results for CD8 anti-tumour responses, with some able to clear small tumours (Hanson, Donermeyer et al. 2000), but most do not do so without additional treatment (Blank, Brown et al. 2004; Antony, Piccirillo et al. 2005; Gattinoni, Klebanoff et al. 2005). CD4 anti-tumour responses have been generally less studied or if they have it is usually in the context of providing help to CD8 CTLs (Antony, Piccirillo et al. 2005). However reports have revealed that CD4 cells are capable of eradicating tumours completely independently of CD8 T cells (Corthay, Skovseth et al. 2005), It is therefore crucial to the understand the nature of CD4 and CD8 responses to antigen in the context of tumour in order to design new vaccines and therapeutic approaches that focus on both CD4 and CD8 responses *in vivo*.

As discussed previously, MB49 is an $H2^{b+}$ bladder carcinoma that expresses both MHC I and II restricted HY antigens as a model for TAA (see chapter 3). MB49 is immunogenic and recognised *in vivo* where these responses are mainly HY-specific (Fig. 3.11). However MB49 is able to overcome endogenous T cell responses to HY at moderate to high doses due to immunosuppressive mechanisms employed such as the production of IL-10, TGF-β and IDO in the local tumour microenvironment (Fig. 3.13-14). Previous studies by Perez-Diez *et al* have shown both Matahari and Marilyn $Rag^{-/-}$ transgenic T cells are capable of recognising and killing MB49 cells *in vitro*, however only Marilyn $Rag^{-/-}$ but not Matahari $Rag^{-/-}$ mice are able to reject MB49 *in vivo*. Furthermore, Marilyn CD4 but not Matahari CD8 are capable of causing tumour rejection following adoptive cell transfer to female $Rag^{-/-}$ MB49-bearing (10^5) mice (Perez-Diez, Joncker et al. 2007). In addition, HY-specific Marilyn Tregs have also been shown to cause a worse prognosis for tumours and a positive outcome for normal tissue allograft survival by our lab (Chai, Coe et al. 2008; Coe, Begom et al. 2010). This section attempts to understand the kinetics of these T cell responses and characterise the cellular and molecular mechanisms that underlie the different outcomes.

4.1.5: Allograft and tumour responses, opposite side of the same coin

In a clinical setting most transplants between MHC matched individuals have multiple mismatched minor H antigens and therefore powerful immunosuppressive treatments are often used to prevent T cell mediated rejection of donor tissue (Grinyo, Bestard et al. 2010). In order to prevent rejection in the long term without these drugs, T cell tolerance must be induced to the minor H antigens on donor tissue. Many techniques use the induction of Tregs to achieve this tolerance (Sakaguchi, Ono et al. 2006). In contrast, the primary approach to tumour immunotherapy is to overcome immune tolerance to minor H antigens in the tumour environment, and therefore approaches for cancer immunotherapy tend to be opposite to therapies designed to mediate transplant tolerance. Here, I have applied adoptive transfer of defined populations of HY-specific effector and regulatory T cells to compare responses in mice bearing tumours, male skin grafts or both. These experiments provide insight into the cellular and molecular mechanisms that

determine why T cell mediated immune responses can lead to the rejection of allografts but acceptance of tumours.

4.2: Results

4.2.1: Matahari mice are capable of rejecting skin graft but not tumour

Syngeneic B6 male skin (from tail skin) was grafted to either WT B6 or $Rag1^{+/-}$ Matahari females (Fig. 4.1a). 100% of Matahari mice are able to efficiently reject the skin graft with a mean survival time (MST) of 15 days while only 50% of B6 female mice had rejected the graft by day 70 with an MST of 60 days. This demonstrates that Matahari have an enhanced primary anti-HY response mediated by HY-specific CD8 T cells. In spite of this response, when Matahari females are challenged with MB49 cells at an optimal dose (5×10^5) (see chapter 3), tumours develop over a similar period to WT mice (Fig. 4.1b and c). Matahari CD8 T cells but not CD4 are capable of responding to cognate antigen *in vitro* (Fig. 4.1d). Matahari spleen and lymph node cells and sorted Matahari CD8 T cells respond similarly to antigen, while Matahari CD4 T cells are non-functional (Matahari mice are capable of producing a small fraction of non-functional CD4 T cells that are MHC I restricted $V\beta8.3^+$ TCR transgenic). Taken together, these results show that while the presence of high frequency functional HY-specific CD8 T cells alone is sufficient to mediate rejection of non-malignant transplants, they cannot mediate rejection of malignant tissues.

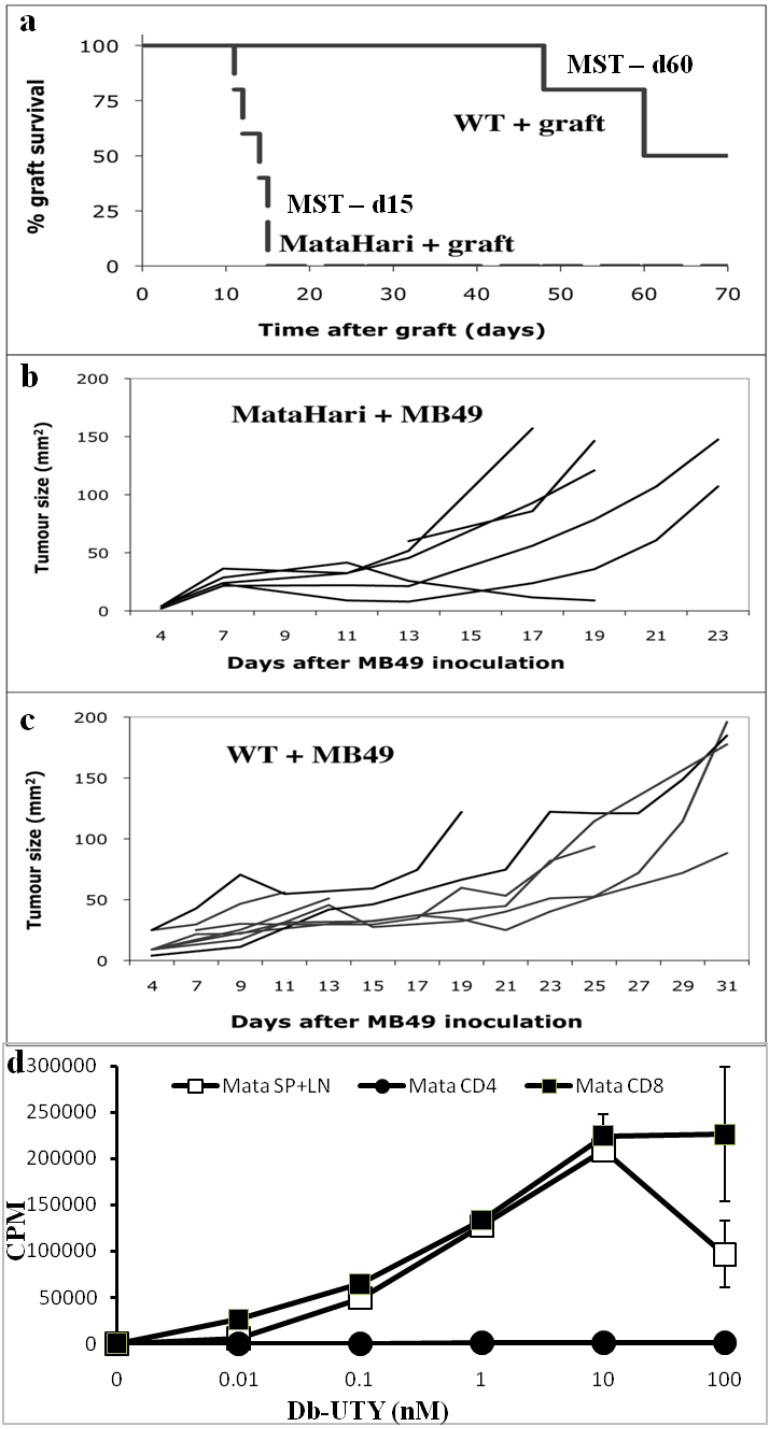


Figure 4.1. Rag1$^{+/-}$ Matahari mice can reject male skin grafts but not MB49 tumours

a. Kinetics of rejection of syngeneic B6 male skin grafts by Rag1$^{+/-}$ Matahari and WT B6 females. Tail skin from syngeneic B6 male mice was transplanted onto B6 WT (n=6, WT+graft) or Rag1$^{+/-}$ Matahari females (n=5, Matahari+graft). Grafts were assessed every other day and <10% healthy graft tissue was recorded as rejection.
b and c. MB49 tumour growth in Rag1$^{+/-}$ Matahari and WT B6 females. Rag1$^{+/-}$ Matahari females (n=6, B) and WT B6 (n=8, C) were inoculated s.c with 5x10^5 MB49 cells on day 0. MB49 tumour growth was monitored by measuring tumour size every other day.

d. CD4-expressing transgenic Vβ8.3 do not recognise cognate peptide - tritiated thymidine proliferation assay. Matahari $Rag^{+/-}$ SP+LN (2×10^5), FACS sorted $CD8^+V\beta8.3^+$ T cells (2×10^4) and $CD4^+V\beta8.3^+$ T cells (2×10^4) were plated out with cognate antigen D^b-UTY in tenfold dilution and cultured for 64 hours in the presence of APC (irradiated B6 female spleen cells, $5x10^5$ cells/well). 1μl thymidine (containing 1 μCi activity) was added/well. 8 hours later the plate was harvested and counted using a microbeta counter (Wallac). Assays were conducted in triplicate error is displayed as ± standard deviation.

4.2.2: Distinct Matahari CD8 responses to skin graft and tumour

To attempt to elucidate the cellular basis for the distinct outcomes to tumour and skin grafts, a series of CD8 adoptive transfer experiments were performed (Fig. 4.2). Purified Matahari CD8 T cells were CFSE labelled prior to infusion (i.v.) to groups of WT B6 female mice that were untreated (group 1, G1) or one day later were given either a male skin graft (G2) or MB49 tumour cells (G3) (Fig. 4.2a). On day 12 mice were culled and pLN of G1 or graft/tumour dLN of G2 and G3 were analysed for the presence of donor CD8 T cells. The absolute donor cell number recovered from the graft dLN was significantly higher than those recovered from the tumour dLN (Fig. 4.2b), indicating that the graft environment is more potent than tumour at the local priming of CD8 T cells. Similarly, the frequency of donor cells was higher in the graft ndLN compared to tumour and control pLN (Fig. 2a and b) suggesting that the graft environment is also more efficient for inducing systemic T cell activation.

Prior labelling with CFSE allows for T cell proliferation to be measured *in vivo* (Fig. 4.2c). Over 90% of donor T cells recovered from control pLN failed to divide, due to lack of antigen, while donor cells in the skin graft and tumour dLN did show proliferation. T cells in the tumour dLN underwent ~6 divisions with ~20% undivided while T cells in the graft dLN divided over 6 times with only 7% undivided (Fig. 4.2c). This increased division of donor T cells in graft dLN was also correlated with increased CD44 expression compared to those in tumour dLN (Fig. 4.2c (top)).

The expression level of TCR on donor T cells in pLN and dLN was also evaluated (Fig. 4.2d). Donor cells from control pLN maintained a high expression of Vβ8.3, while TCR downregulation was seen particularly in the tumour dLN and also in graft dLN to a lesser extent. Finally, CFSE dilution and CD44 expression was

measured for donor T cells within the tumour or skin grafts (Fig. 4.2e). Although donor cells were present in both tumours and grafts with a similar frequency (Fig. 4.2e left), they display distinct CD44 expression, with 96% of T cells in graft being CD44^{high} compared to only 11% in MB49 tumours (Fig. 4.2e right). Matahari CD8 T cells are therefore able to recognise and respond to both graft and tumour *in vivo*. However, this response is only sufficient to mediate skin graft not tumour rejection (Fig. 4.1).

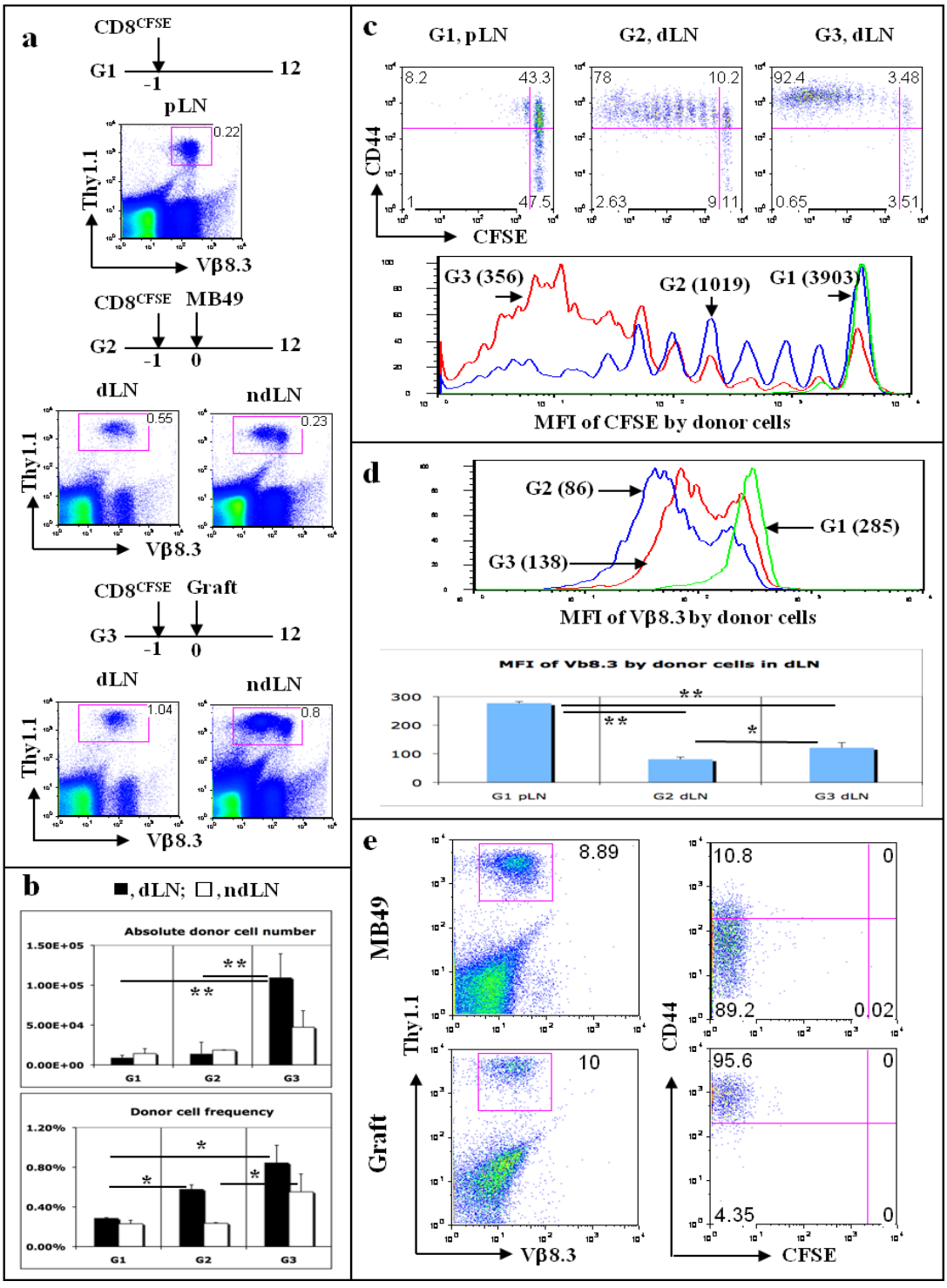


Figure 4.2. Distinct responses of Matahari CD8 T cells to tumours and male skin grafts

a. $5x10^6$ CFSE labelled Matahari CD8 cells (Thy1.1) were injected (i.v.) into 3 groups of Thy1.2 WT B6 females at day -1. On day 0, the mice in G1 were left untreated, those in G2 were inoculated with $5x10^5$ MB49 cells (s.c.) and those in G3 were transplanted with syngenic B6 male skin grafts. All mice were culled on day 12 and the pLN cells of G1 and dLN and ndLN cells of G2 and G3 were stained with anti-$V\beta8.3^{PE}$ and anti-$Thy1.1^{PerCP}$. Panels show the percentage of donor CD8 T cells identified by co-expression of Thy1.1 and Vβ8.3. One representative experiment of three is shown.

b. Comparison of absolute number and frequency of donor CD8 cells in pLN, dLN and ndLN. Absolute cell numbers (top) and frequency of donor CD8 cells (bottom) recovered from pLN of G1, dLN and ndLN of G2 and G3 are represented. For G2 and G3, black and white bars represent dLN and ndLN, respectively, for G1 bars represent inguinal and axillary LN. * $p < 0.05$, ** $p < 0.01$.

c. Top panel: Comparison of CFSE and CD44 expression by donor CD8 cells. pLN of G1, dLN cells of G2 and G3 were stained with anti-$V\beta8.3^{PE}$, anti-$Thy1.1^{PerCP}$ and anti-$CD44^{APC}$. Gated $Thy1.1^+V\beta8.3^+$ cells were further analysed for CFSE and CD44 expression. Bottom panel: Comparison of Mean Fluorescence Intensities (MFI) of CFSE by donor CD8 cells.

d. Donor CD8 cells in tumour dLN show greater downregulation of Vβ8.3 compared to those in graft-dLN. Top panel: pLN of G1, dLN cells of G2 and G3 were stained with anti-$V\beta8.3^{PE}$ and anti-$Thy1.1^{PerCP}$. MFI of Vβ8.3 by gated $Thy1.1^+V\beta8.3^+$ cells in each group is showed in histogram. Bottom panel: MFI of Vβ8.3 MFI of donor CD8 cells in each group are represented. * $p < 0.05$, ** $p < 0.01$.

e. Graft-infiltrating but not tumour-infiltrating donor CD8 cells express high levels of CD44. The cells from either MB49 tumour mass or male skin grafts were stained with anti-$V\beta8.3^{PE}$, anti-$Thy1.1^{PerCP}$ and anti-$CD44^{APC}$. Both frequency (left hand) and CD44 expression (right hand) of donor CD8 cells in tumour and skin graft were shown.

Error is represented as ± standard deviation and all data is representative of three independent experiments.

4.2.3: CD8 responses are inversely correlated with tumour progression

To dissect the kinetics of the Matahari T cell response to MB49, CFSE labelled T cells were infused into WT B6 female MB49-bearing mice inoculated with MB49 cells 1, 3 or 7 days prior to T cell adoptive transfer. Responses were then measured on days 4, 7 and 14 following transfer (Fig. 4.3) with analysis being conducted as in the previous section. The proportion of tumour infiltrating donor T cells was significantly higher in the most recently established tumours, with the maximum accumulation seen by day 14 (Fig. 4.3a). Donor T cells in the tumour dLN display a similar profile of CFSE dilution at each time point for all groups (Fig. 4.3b). It therefore seems to be a narrow window in which activated CD8 T cells are able to enter the tumour despite a similar level of proliferation and activation in the dLN. This limitation does not apply to male skin grafts where Matahari T cells are able to migrate into grafts that have already healed (not shown).

The efficiency of priming of Matahari CD8 T cells by MB49 challenge plus HY-Uty peptide and CFA vaccination was compared with that in an untreated control group (Fig. 4.3c). Donor Matahari CD8 T cells show a vigorous expansion (at least 4 fold increase in terms of donor CD8 frequency) and a complete CFSE (99%, and up to 9 divisions) dilution 4 days after vaccination, but they displayed no responses to MB49 challenge in the absence of peptide vaccination. Moreover, poor responses by donor CD8 T cells in MB49-bearing mice was not due to 'ignorance', because both transgenic Vβ8.3 on donor CD8 T cells and endogenous Vβ8.3 on host T cells in MB49 dLN showed a significantly reduced expression, revealed by decreased MFI (reduced from 124 to 90 for donor CD8, and from 513 to 382 for host T cells). In contrast, peptide vaccination induced upregulation of transgenic Vβ8.3 expression by donor CD8 T cells (from 124 to 178) while this had no influence on endogenous Vβ8.3 expression (from 513 to 494) (Fig. 4.3c).

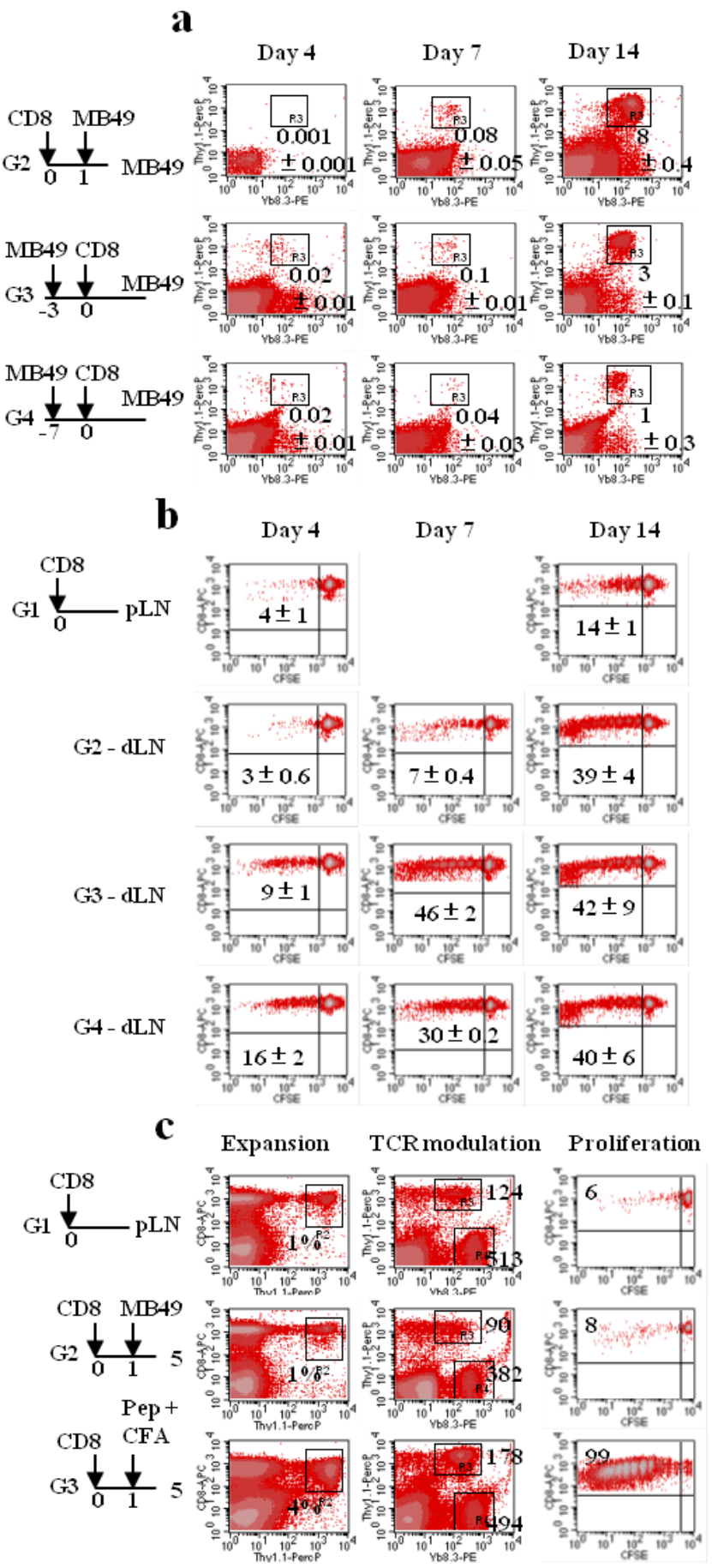

a
Day 4
Day 7
Day 14
CD8 MB49
G2
0 1
MB49
0.001 ± 0.001
0.08 ± 0.05
8 ± 0.4
MB49 CD8
G3
-3 0
MB49
0.02 ± 0.01
0.1 ± 0.01
3 ± 0.1
MB49 CD8
G4
-7 0
MB49
0.02 ± 0.01
0.04 ± 0.03
1 ± 0.3
b
Day 4
Day 7
Day 14
CD8
G1
0
pLN
4 ± 1
14 ± 1
G2 - dLN
3 ± 0.6
7 ± 0.4
39 ± 4
G3 - dLN
9 ± 1
46 ± 2
42 ± 9
G4 - dLN
16 ± 2
30 ± 0.2
40 ± 6
c
Expansion
TCR modulation
Proliferation
CD8
G1
0
pLN
1%
124
13
6
CD8 MB49
G2
0 1
5
1%
90
382
8
Pep + CFA
CD8
G3
0 1
5
4%
178
494
99

Figure 4.3. Kinetics of CD8 responses to MB49 tumours at different stages of progression

a and b. MB49 inoculation was performed on day -7, -3 or 1 and CD8 transfer was conducted at day 0, as illustrated in Figure 4.3. Analysis was carried out at day 4, 7 and 14 as described in Figure 4.2 except anti-$CD8^{APC}$ rather than anti-$CD44^{APC}$ was used. One representative experiment of three is shown.

c. Three groups of Thy1.2 B6 females (n=3) were adoptively transferred with CFSE-labelled Thy1.1 Matahari CD8 T cells ($4x10^6$ cells/mouse) by i.v. injection. On the next day mice were inoculated (s.c) with $5x10^5$ MB49 cells (G2), or inoculated with HY-Uty peptide (100μg) plus CFA (s.c.) (G3). Four days later, all mice were culled and pLN (G1) or dLN (G2 and G3) cells were analysed for the presence of donor CD8 T cells (R2, left hand panels), the MFI of Vβ8.3 by donor CD8 (R3, middle panels) and by endogenous T cells (R4, middle panels) and CFSE dilution profiles (right hand panels). In each panel, data is representative of two independent experiments.

4.2.4: Impact of concurrent male skin grafts on CD8 responses against tumours

Matahari CD8 T cells can mediate HY^+ skin graft rejection (Fig. 4.1) but cannot cause regression of HY^+ MB49 solid tumours (Fig. 4.1-3). In an attempt to investigate these different outcomes, a dual tumour and skin grafting experiment was conducted (Fig. 4.4). The potential for cross talk between MB49 tumours and male allografts was assessed by comparing CD8 T cell responses between mice either bearing MB49 tumour alone, allograft alone (Fig. 4.4a) or both (Fig. 4.4b). Skin allografts and MB49 tumour cell injections were conducted as described previously. The mice that received MB49 alone or allograft alone recapitulated the data shown in previous figures, causing rejection of the skin graft while being unable to mediate tumour rejection (Fig. 4.4a). Importantly, in the group that received both graft and tumour, the CD8 T cell response was significantly enhanced to both the graft and the tumour as evidenced by the significantly increased frequency of tumour and graft infiltrating donor CD8 T cells (Fig. 4.4b). This ability to cross talk suggests that a synergistic positive effect between graft and tumour may be able to overcome the suppressive factors induced in the tumour environment.

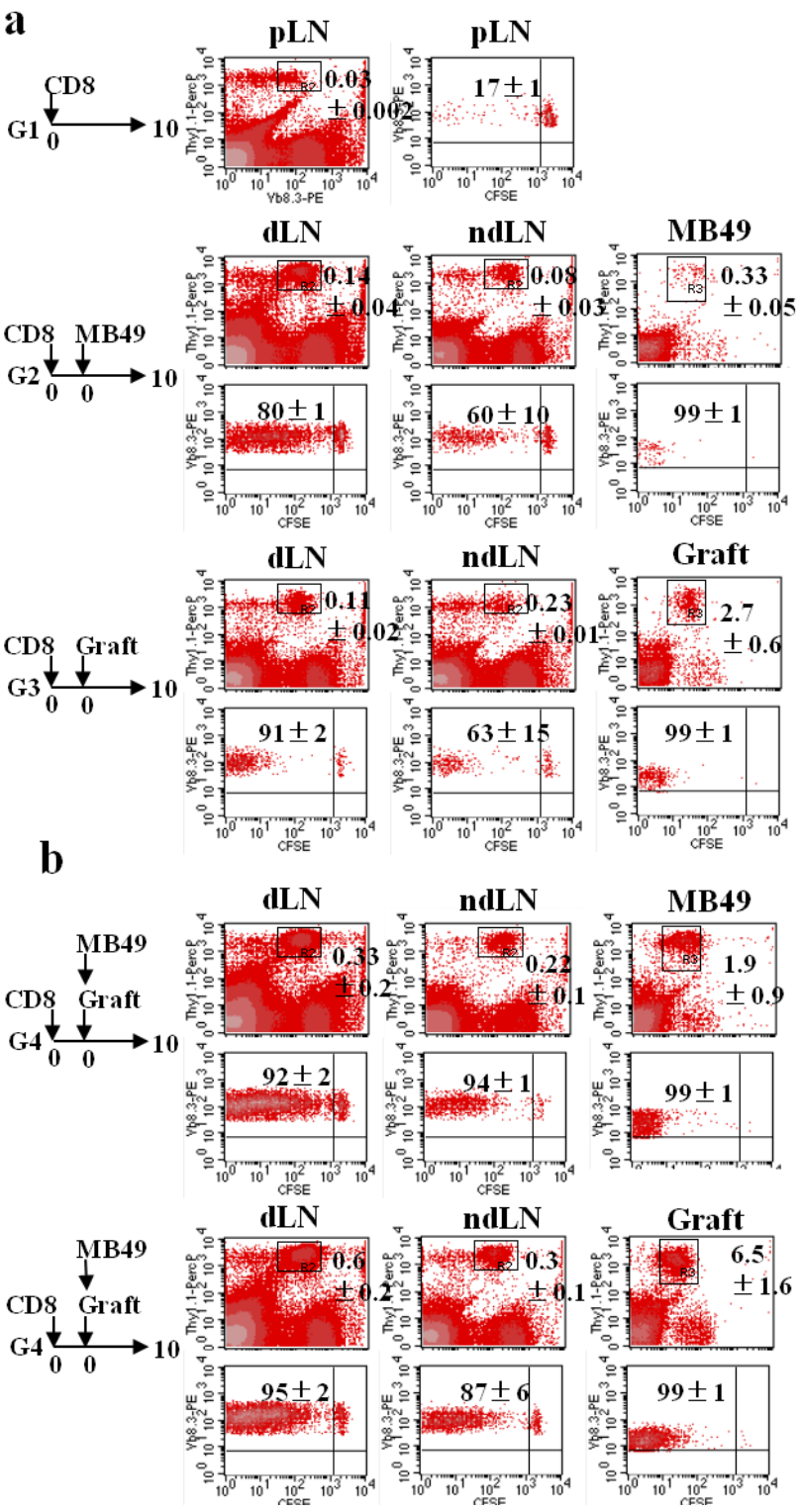


Figure 4.4. CD8 T cell responses to MB49 tumours in the absence or presence of male skin grafts

a and b. MB49 inoculation, skin grafting, CD8 transfer and analysis were conducted as described in Figure 4.2. One representative experiment of two is shown.

4.2.5: CD4 help in restoring of the diminished anti-MB49 CD8 responses

Matahari CD8 T cell responses are unable to control MB49 tumours even if inoculated on the same day as the tumour (Fig. 4.1b). To assess the requirement for CD4 help in the Matahari anti-tumour response, a co-adoptive transfer experiment was set up (Fig. 4.5). Four groups of WT Thy1.2 B6 females were either inoculated with MB49 (5×10^5) on day -7 (G2 and G4), or received no tumour (G1 and G3). All groups received CFSE labelled CD8 T cells purified from Thy1.1 $Rag^{+/-}$ Matahari mice ($4x10^6$ cells/mouse). Two groups of mice (G3 and G4) also received CFSE labelled CD4 T cells purified from Thy1.1 $Rag^{+/-}$ Marilyn mice ($1x10^6$ cells/mouse) (i.v.). On day 8, mice were analysed for the accumulation and CFSE dilution of donor Matahari CD8 T cells (Fig. 4.5).

Mice that received CD8 T cells alone failed to control the established tumour and showed reduced accumulation of donor CD8 T cells in ndLN and tumour compared to those that received both CD4 and CD8 T cells. Mice that received both CD4 and CD8 controlled the tumour and showed greater CFSE dilution in the donor CD8 compartment in dLN and ndLN (Fig. 4.5). Control mice that received CD8 T cells alone were similar in the levels of CD8 expansion and proliferation to control mice that received CD4 and CD8; demonstrating CD8 cells do not undergo additional expansion in response to CD4 alone. CD4 help is therefore capable of inducing an enhanced anti-tumour response in adoptively transferred Matahari CD8 T cells.

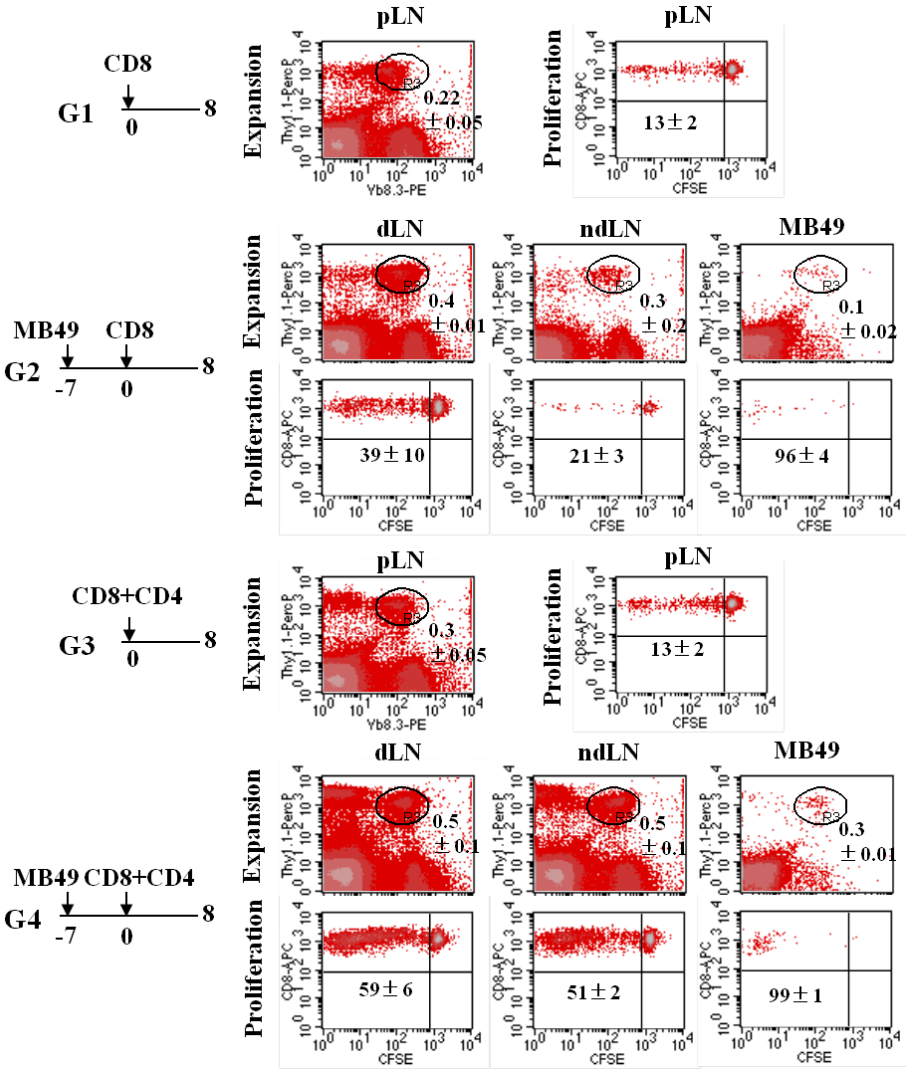


Figure 4.5. CD4 help enhances CD8 anti-tumour responses
Two groups of Thy1.2 B6 females (n=3/group) were inoculated with $5x10^5$ MB49 cells (s.c) on day -7 (G2 and G4) an additional two groups received no inoculation (G1 and G3). On day 0, all mice were adoptively transferred with CFSE labelled Thy1.1 Matahari CD8 T cells ($4x10^6$ cells/mouse) alone (G1 and G2) or co-transferred with Thy1.1 Marilyn CD4 T cells ($1x10^6$ cells/mouse, G3 and G4). On day 8 all mice were culled and pLN of G1 and G3, dLN, ndLN and MB49 tissue cells were analysed for the presence of donor CD8 (R3) and their CFSE profiles as described in Fig. 4.2. Data is representative of two independent experiments.

4.2.6: Matahari T cells appear to be self reactive and have a low TCR expression

To investigate the effects of this phenotype, $Rag^{-/-}$ Matahari and WT B6 mice were tail bled at various ages and tested for CD44 vs CD62L expression by $V\beta8.3^{+}$ CD8 T cells (Fig. 4.6a) ($CD62L^{high}$ $CD44^{low}$ = naive, $CD62L^{high}$ $CD44^{high}$ = central memory, $CD62^{low}$ $CD44^{high}$ = effector). Matahari mice have a high proportion of $CD44^{high}$ cells in the CD8 Vβ8.3 compartment while WT mice maintain a $CD44^{low}$ phenotype, this effect is also compounded with age (Fig. 4.6a). $Rag^{-/-}$ Matahari mice also have defective thymic development compared to WT and $Rag^{-/-}$ Marilyn mice, with only 1 $x10^{6}$ thymocytes (Fig. 4.6b). The phenotype of the T cells in these mice may be due to homeostatic expansion of Matahari CD8 transgenic T cells to fill the peripheral 'space' due to the low numbers of cells that enter the periphery from the thymus. Alternatively it may be caused by Matahari T cells reacting to other self antigens due to a self reactive TCR. Nevertheless Matahari T cells have a very good avidity and generate CTLs *in vivo* and *in vitro* and the rejection of skin grafts is efficient, as shown in Fig. 4.1.

Matahari $Rag^{+/-}$ mice have a more obvious self reactive phenotype, with young adult mice (>8 weeks) displaying splenomegaly and ongoing proliferation of transgenic T cells shown through Ki67 expression in the spleen of Matahari mice compared to WT B6 (Fig. 4.6c). To evaluate the effect of self reactivity on TCR expression, Matahari or Marilyn transgenic T cells were CFSE labelled and adoptively transferred to WT B6 females. 5 days later, pLN harvested and tested for proliferation of donor cells (Fig. 4.6d and e). Matahari CD8 transgenic T cells have a much lower expression of Vβ8.3 (MFI 124) compared to the host endogenous CD8 compartment (513) while Marilyn CD4 transgenic T cells have similar expression level of Vβ6 as the endogenous compartment of the host (Fig. 4.6d). In addition, $Rag1^{+/-}$ Matahari CD8 T cells gradually diluted CFSE after adoptive transfer in WT B6 females, whereas adoptively $Rag2^{+/-}$ Marilyn CD4 T cells did not undergo proliferation (Fig. 4.6e). Taken together, $Rag1^{+/-}$ Matahari CD8 T cells exhibit self-reactivity and express reduced level of transgenic Vβ8.3.

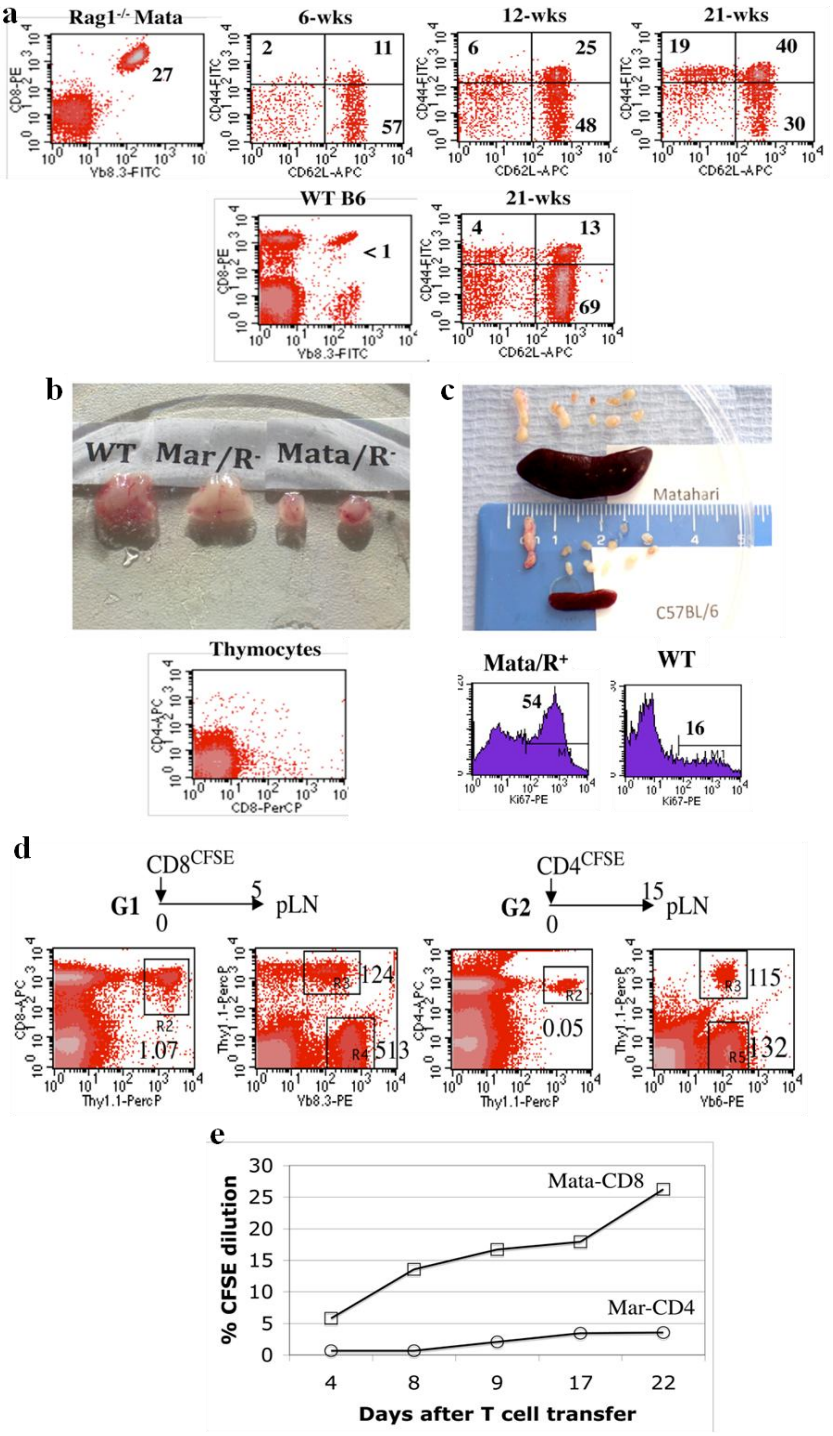


Figure 4.6. Perpetual reactivity and proliferation by Matahari CD8 T cells
a. PBL from Rag1$^{-/-}$ Matahari or WT B6 mice with indicated age were stained with anti-CD44^{FITC}, anti-Vβ8.3^{PE}, anti-CD8^{PerCP} and anti-CD62L^{APC}. Vβ8.3^{+}CD8^{+} cells were gated and analysed for the expression of CD44 vs. CD62L.
b. Image comparing the size of thymus from a WT, Rag2$^{-/-}$ Marilyn female and Rag1$^{-/-}$ Matahari female or male (all 12 weeks old). Thymocytes cells from Rag1$^{-/-}$ Matahari females were stained with anti-CD8^{PerCP} and anti-CD4^{APC}. In each panel, data are representative of ten independent experiments.
c. Image comparing the size of spleen and LN isolated from a Rag1$^{+/-}$ Matahari female with a WT B6 female mouse (20 weeks old). Spleen cells from Rag1$^{+/-}$

Matahari or WT B6 mice were surfaced stained with anti-Vβ8.3^{FITC}, anti-CD8^{PerCP} and then stained intracellulary for anti-Ki67^{PE}, Vβ8.3^{+}CD8^{+} cells were gated and analysed for the expression of Ki-67.
d and e. Two groups of Thy1.2 B6 females (n=3) were adoptively transferred with CFSE labelled Thy1.1 Matahari CD8 T cells (4x10^{6} cells/mouse, G1) or CFSE labelled Thy1.1 Marilyn CD4 T cells (1x10^{6} cells/mouse, G2) (i.v.). 5 days later, all mice were culled and pLN cells were analysed for the presence of donor CD8 or CD4 T cells (R2). The MFI of Vβ8.3 between donor CD8 (R3) and host T cells (R4), and the MFI of Vβ6 between donor CD4 (R3) and host T cells (R4) was compared. In each panel, data are representative of two independent experiments. At different time points, pLN cells from the three individual mice in each group were stained with PE-conjugated anti-Vβ8.3 or anti-Vβ6, anti-Thy1.1^{PerCP} and APC-conjugated anti-CD8 or anti-CD4 and analysed for the presence CFSE dilution amongst transgenic populations (**e**) data representative of two experiments.

4.2.7: Comparison of CD4 responses against MB49 tumours and male skin grafts

Matahari CD8 T cells are capable of rejecting syngeneic skin grafts but not HY^{+} tumours; these mice have defective thymic T cell development and display a self reactive phenotype in the periphery. Previous work had shown HY-specific Marilyn CD4 T cells are capable of controlling both skin graft rejection (Braun, Grandjean et al. 2001) and tumour growth following adoptive cell transfer (Perez-Diez, Joncker et al. 2007). The different responses to skin grafts and tumour tissue by Marilyn T cells were assessed using the same strategy as previously used to evaluate Matahari responses. Three groups of WT Thy1.2 B6 females received no treatment or were inoculated with MB49 cells or transplanted with syngeneic male skin grafts on day -7. All groups then received CFSE labelled Thy1.1^{+} Marilyn CD4 T cells on day 0, and analysis was conducted on day 5 (Fig. 4.7).

Donor Marilyn CD4 T cells accumulated in the graft dLN and ndLN to a significantly greater degree than in tumour dLN and ndLN (Fig. 4.7a and b) in a similar manner to Matahari T cells. Marilyn T cells also showed an accelerated progression of cell division in the graft dLN compared with tumour dLN (Fig. 4.7c) indicating Marilyn cells proliferated in the local dLN in response to antigen rather than just accumulating there. Activated donor cells were also present in the skin graft but were excluded from the tumour mass (Fig. 4.7d). These data indicate that Marilyn T cells are capable of responding to antigen with a more pronounced effect to skin grafts compared to tumours, as is seen with Matahari T cells.

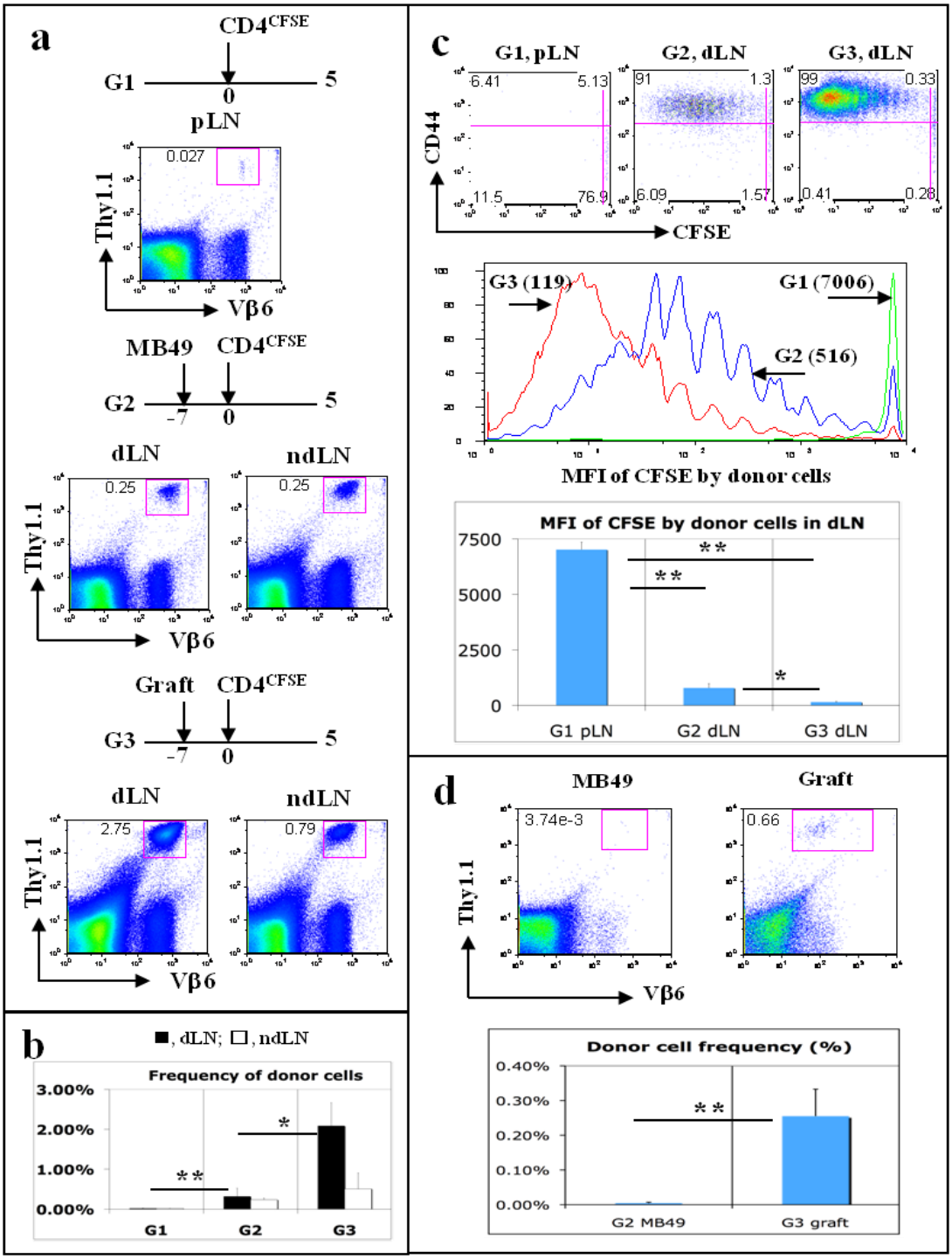


Figure 4.7. Differential response of Marilyn CD4 T cells to MB49 and skin grafts

a. On day -7, Thy1.2^{+} WT B6 females were split into 3 groups. G1 did not receive a HY challenge, those in G2 were inoculated with 5x10^{5} MB49 cells (s.c) and those in G3 were transplanted with male B6 skin grafts. On day 0, 1x10^{6} Thy1.1^{+} CFSE-labelled Marilyn CD4 T cells were injected (i.v.) into all recipient mice. On day 5 pLN cells of G1, dLN and ndLN cells of G2 and G3 were stained with anti-Vβ6^{PE} and anti-Thy1.1^{PerCP}. Panels show the frequency of donor CD4 cells identified by co-expression of Thy1.1 and Vβ6.

b. Comparison of the frequency of donor CD4 cells in pLN, dLN and ndLN.

c. Donor CD4 cells (Thy1.1^{+}Vβ6^{+}) present in pLN of G1, dLN of G2 and G3 mice were analysed for CFSE and CD44 expression. Data is representative of two independent experiments. Comparison CFSE MFI by donor CD4 cells detected in pLN of G1 and dLN of G2 and G3 (middle and bottom panels).
d. Donor cells in MB49 and grafts were identified by co-expression of Thy1.1 and Vβ6 (top). The frequency of donor CD4 cells is represented. Error is expressed as mean ± SD (bottom). Data is representative of two independent experiments. * $p < 0.05$, ** $p < 0.01$.

4.2.8: CD4 responses are inversely correlated to tumour progression

The previous figure shows Marilyn CD4 responses against an established tumour (day -7) at an early time point (day 5). The kinetics of the Marilyn T cell response to tumours established at different times was also evaluated, as with Matahari mice in Figure 4.3, and assessed at different time points (day 4, 8 and 15) (Fig. 4.8). Marilyn CD4 T cell infiltration into tumour tissue was significantly lower the longer the tumour has been established (Fig. 4.8a) demonstrating that the progression of tumours are inversely correlated with the level of tumour infiltration by donor CD4 T cells, as with Matahari CD8. CFSE profiles show that donor cell proliferation in the dLN of the most established tumours (day -7) were already in a state of arrest at the first time point (day 4) which is maintained throughout the other two time points (day 8 and 15) (Fig. 4.8b). As is the case for CD8 T cells there is a restricted window in which CD4 cells can enter and proliferate in the tumour mass.

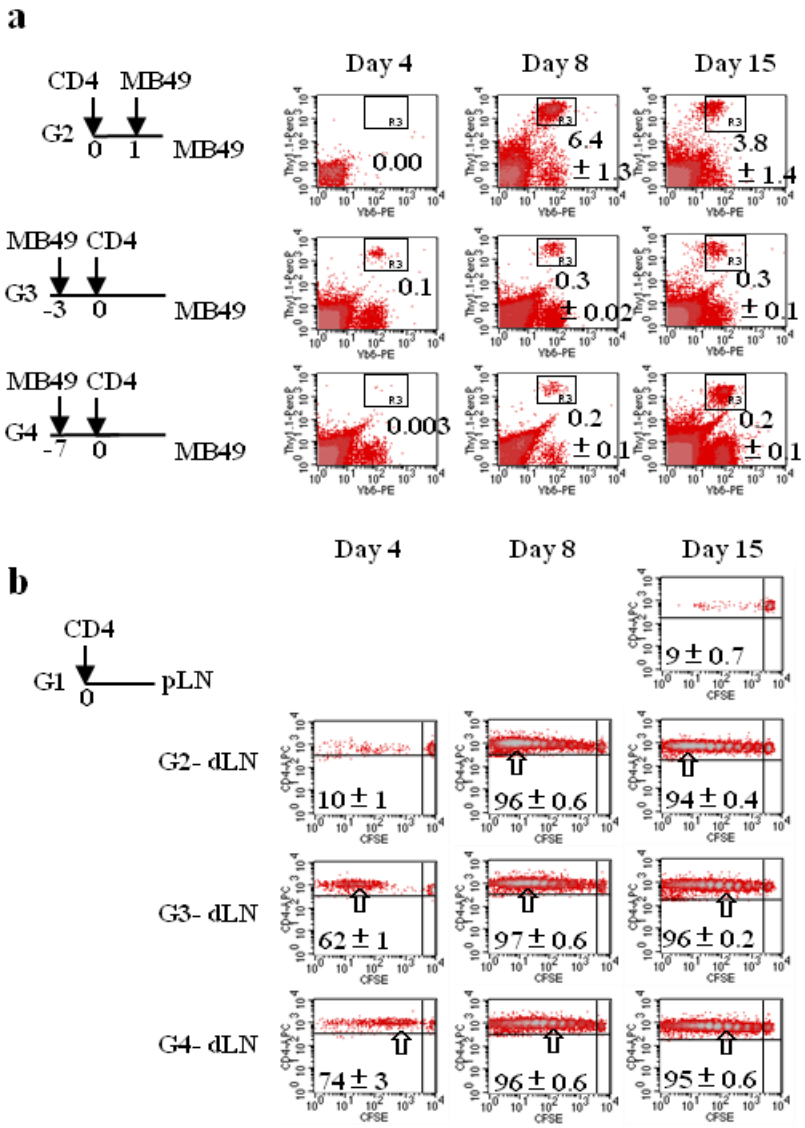


Figure 4.8. Kinetics of CD4 responses to MB49 tumours at different stages of progression

a and b. MB49 inoculation was performed at -7, -3 or 1 day and CD4 transfer was conducted at day 0, using the same dose as described in Figure 4.7. Analysis was carried out at day 4, 8 and 15 as described in Figure 4.7 except anti-CD4^{APC} rather than anti-CD44^{APC} was used. One representative experiment of two is shown.

4.2.9: The impact of male skin grafts on CD4 responses against tumours

The anti-tumour responses of Matahari T cells are increased in mice that receive both tumour and skin graft, demonstrating immune cross talk (Fig. 4.4). The ability of Marilyn CD4 T cells to cross talk was also assessed using the same procedure, with CFSE labelled Marilyn CD4 being infused in place of Matahari CD8 (Fig. 4.9). Mice that received MB49 alone or skin allograft alone recapitulated the data in previous figures (Fig. 4.7), mediating rejection of the graft and tumour.

Donor CD4 T cell infiltration was seen in both graft and tumour with a higher frequency of donor T cells in the local dLN compared to ndLN (Fig. 4.9a). Marilyn T cell responses against tumour were significantly enhanced in mice that received both graft and tumour as evidenced by an increased frequency of donor CD4 T cells in the tumour dLN, ndLN and to a lesser extent in the tumour itself (Fig. 4.9b). Anti-graft responses appeared to be inhibited in mice that received both graft and tumour, shown by a significantly decreased frequency of donor T cells in the graft dLN, ndLN and to a lesser extent the graft itself (Fig. 4.9b). Marilyn responses to tumour are therefore enhanced by the presence of an allograft but the allograft response itself is inhibited.

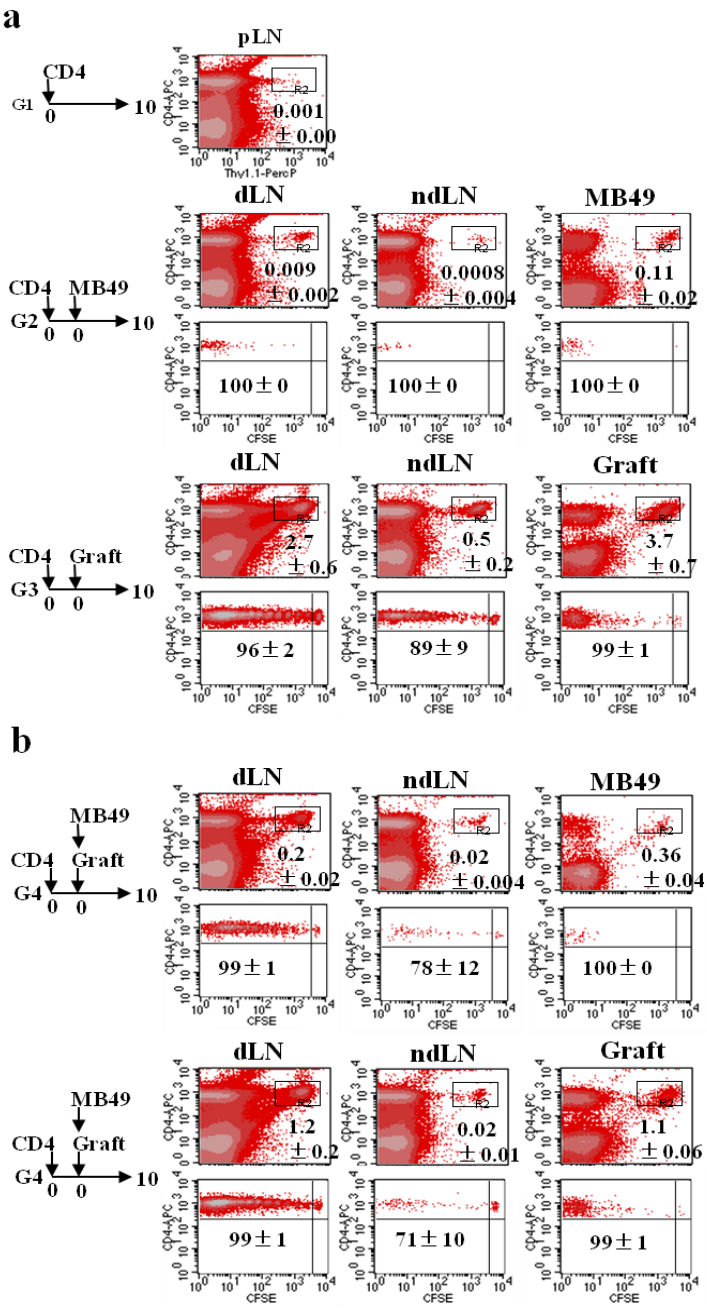


Figure 4.9. CD4 T cell response to MB49 tumours in the absence or presence of male skin grafts
a and b. MB49 inoculation, skin grafting, CD4 transfer and analysis were conducted as described in Figure 4.7. One representative experiment of two is shown.

4.2.10: HY antigens in the context of allograft but not tumour direct Marilyn CD4 T cells to differentiate towards a Th1-like effector state

Earlier in this section Marilyn CD4 T cell responses to skin allograft and established tumour were evaluated at an early stage (day 5) (Fig. 4.7). This comparison was extended to include functional analysis for later stage responses (day 16) (Fig. 4.10). Three groups of Thy1.2 B6 females received no treatment, inoculation with MB49 cells or transplant with syngeneic male skin grafts on day-1, on the following day all mice received Thy1.1 Marilyn CD4 T cells depleted of CD25 cells to minimise the impact of Tregs on subsequent *ex vivo* assays. On day 16, mice were analysed for the accumulation and phenotype of donor T cells by CD44 and CD62L expression in various tissues (Fig. 4.10a). In addition, donor T cells from pLN (G1) or dLN (G2 and 3) were assayed for *in vitro* proliferation against cognate antigen (A^b-Dby) (Fig. 4.10b) and for intracellular cytokine production (Fig. 4.10c).

Donor cells in the graft dLN showed a greater accumulation compared to tumour dLN and control pLN, in fact tumour dLN had a 60% and 85% reduction in frequency compared to control pLN (Fig. 4.10a). This suggests that anti-tumour CD4 responses are initiated but not sustained as demonstrated by previous data (Fig. 4.7 and 4.8). Donor cells in both tumour and graft dLN became $CD44^{high}$ while those in control pLN maintained a primarily naive phenotype in the absence of antigen (Fig. 4.10a). *Ex vivo* functional responses to antigen were measured by plating out pLN (G1) and dLN cells (G2 and G3) with A^b-Dby cognate antigen to measure total proliferation (Fig. 4.10b (left panels)) with responses also presented as CPM per donor CD4 cell (Fig. 4.10b (right panels). dLN cells from skin grafted mice (G3) showed 3 fold greater proliferation than pLN (G1) while dLN cells from tumour mice (G2) did not show a dose-dependent response to peptide but a high background proliferation in the absence of peptide. Similar results were seen when comparing proliferation per donor cell (Fig. 4.10b). IFNγ production by pLN or dLN cells was

evaluated following a 5 day *ex vivo* culture with male spleen cells (Fig. 4.10c). Donor cell frequency in dLN of G3 increased from 0.7 to 4% of which 92% produced IFNγ. Donor cells from tumour dLN (G2) failed to expand further and produced limited IFNγ upon re-stimulation (Fig. 4.10c). This demonstrates that donor cells from dLN of male skin graft can differentiate to a Th1-like phenotype while donor cells in tumour dLN largely fail to respond to re-stimulation suggesting these cells are in a state of anergy.

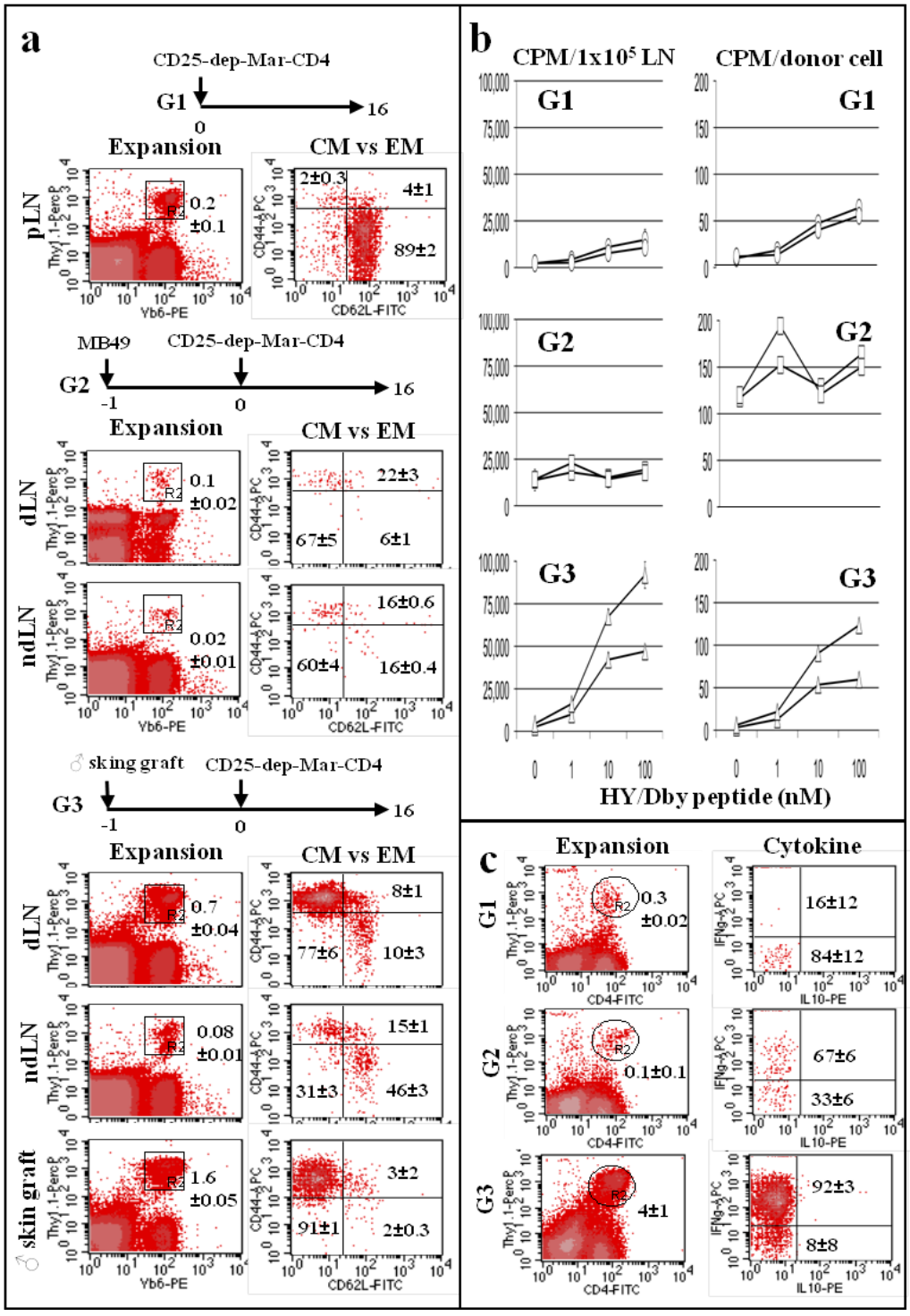


Figure 4.10. Anti-tumour and graft responses by Marilyn CD4 T cells
a. MB49 inoculation, skin grafting and CD4 transfer were performed as described in Figure 4.7. Analysis was conducted at day 16 by staining pLN, dLN, ndLN, MB49 or graft tissue cells with anti-$CD62L^{FITC}$, anti-$Vb6^{PE}$, anti-$Thy1.1^{PerCP}$ and anti-$CD44^{APC}$. Donor CD4 cells identified by $Thy1.1^{+}Vb6^{+}$ were further analysed for CM (central memory, $CD62L^{+}CD44^{+}$), EM (effector memory, $CD62L^{-}CD44^{+}$) or naïve ($CD62L^{+}CD44^{-}$) phenotypes.
b. pLN, MB49-dLN and graft-dLN cells (10^5/well) were cultured without or with HY/Dby peptide for 3 days. 1μl thymidine (equivalent to 1μCi) was added/well. 8 hours later the plate was harvested and counted using a microbeta counter (Wallac). Assays were conducted in triplicate.
c. Measurement of IFNγ and IL-10 production by intracellular staining. pLN, MB49-dLN, and graft-dLN cells ($1x10^6$/well) cultured with irradiated male B6 spleen cells ($4x10^6$/well) in 24 well plates for 5 days. Cells were then restimulated with PMA (50ng/ml) and ionomycin (250ng/ml) in the presence of Brefeldin A (1μg/ml) before staining for surface CD4 and Thy1.1 expression. Cells were fixed and stained for intracellular IL10 and IFNγ. Donor T cells identified by $Thy1.1^{+}CD4^{+}$ (R2) were further analysed for IFNγ vs. IL10. One representative experiment of two is shown.

4.2.11: Distinct levels of antigen presentation and immunomodulatory factors in graft vs tumour dLN

Donor CD4 cells from the MB49 dLN can proliferate in the absence of exogenous antigen (Fig. 4.10b). This effect may be explained due to a higher level of antigen presentation in MB49 dLN compared to the dLN of male skin graft. $CD11c^{+}$ DCs from graft and tumour dLN were compared for their ability to stimulate resting Marilyn T cells *in vitro* (Fig. 4.11a). $CD11c^{+}$ cells from MB49 dLN but not graft dLN were able to induce a suboptimal proliferative response suggesting that they display an elevated level of cross presentation for tumour.

In the previous chapter MB49 tumours were evaluated for their ability to express immunomodulatory factors IL-10, IDO and TGFβ (Fig. 3.13 and 3.14). MB49 tumours express TGFβ both *in vivo* and *in vitro*, IL-10 *in vivo* (confirmed in IL-10KO mice) and also express IDO mRNA *in vivo*. These results were compared to the ability of 4 individual male skin grafts to express the same modulatory factors (Fig, 4.11b-d). Male skin grafts expressed some TGFβ although not as much as *in vivo* tumour (Fig. 4.11b) and virtually no expression of IDO (Fig. 4.11c). Male skin grafts did however express relatively high levels of IL-10 mRNA (Fig. 4.11d). Male skin grafts are therefore less immunomodulatory than tumours but conversely have a lower level of antigen presentation by CD11c+ DCs.

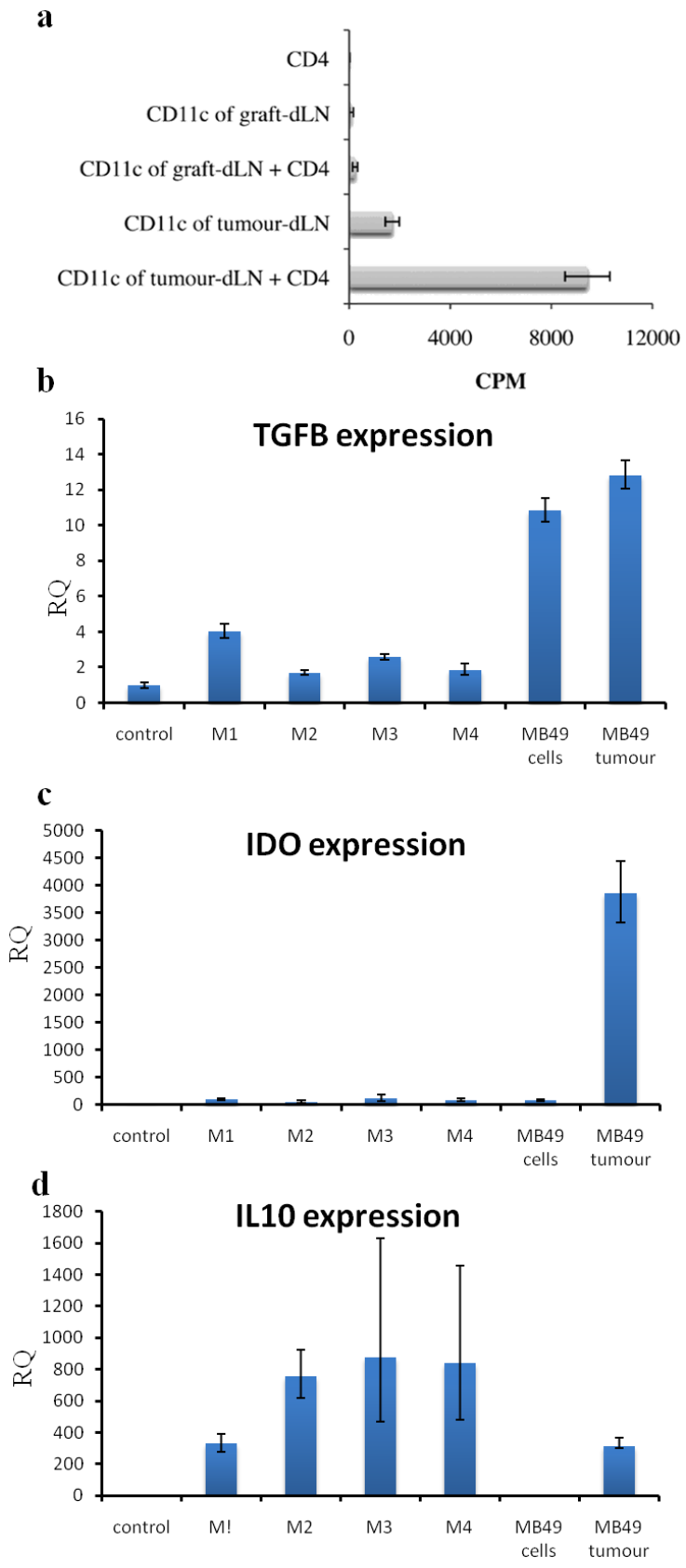


Figure 4.11. **Distinct microenvironments in tumour vs skin graft**
a. B6 females were transplanted with syngeneic male skin grafts or inoculated with MB49 tumour cells as described in Fig. 4.7. Two weeks later, $CD11c^+$ cells, purified from pooled dLN by MACS and cultured (from 1.5 $\times 10^4$/well to 12 $x10^4$/well) with

purified Marilyn CD4 T cells (2.5 $\times 10^5$/well) in 96-well round-bottomed plates for 3 days. 1μl thymidine (equivalent to 1μCi) was added to each well. 8 hours later the plate was harvested and counted using a microbeta counter (Wallac). Assays were conducted in triplicate.
b-d. *Ex vivo* MB49 cells and male skin graft cells were harvested from a B6 mice inoculated as described above. Samples were subjected to RNA extraction and cDNA conversion before running RT-PCR Taqman® assay in triplicate for the expression of TGFβ (**b**), IDO (**c**), and IL-10 (**d**). All data is expressed as relative quantification (RQ) to 18sRNA. An MB49 cell line was also harvested as well as male tail skin as a control. Error is expressed as ± standard deviation.
b. TGF-β gene expression changes between cell line and tumour and graft.
c. IDO gene expression changes between cell line, tumour and graft.
d. IL-10 gene expression changes between cell line, tumour and graft.

4.2.12: HY-specific Tregs in MB49-bearing versus male skin graft bearing mice

Tumours are known to promote the maintenance and expansion of Tregs *in vivo* (Curiel 2007). Marilyn $Rag^{+/-}$ mice produce $CD4^+$ $CD25^+$ $FoxP3^+$ regulatory T cells as ~5% of the CD4 T cell compartment, these Tregs are HY-specific (Coe, Addey et al. 2010). To evaluate the effect HY-specific Tregs would have in response to skin grafts and tumour, an adoptive transfer experiment was performed. As described previously, Thy1.2 WT B6 female mice were inoculated with MB49 cells or given syngeneic male skin graft or a matched female skin graft. The following day all mice received Thy1.1 Marilyn Tregs, on day 12 dLN, ndLN and the graft/tumour were harvested and assessed for the level of Treg expansion in these tissues (Fig. 4.12).

The proportion of Tregs recovered from male skin graft dLN was 4 fold higher than Tregs recovered from female grafts, demonstrating that Treg expansion *in vivo* is antigen dependent. No significant difference was seen in Treg frequencies between male skin graft dLN and tumour, indicating that both types of tissue are able to expand HY-specific Tregs. Interestingly MB49 dLN contained proportionally fewer host CD4 T cells (15±0.3%) than the MB49 ndLN (29±2%) whereas graft dLN and ndLN had similar proportions (30±2% and 31±2% respectively); this may indicate that growing MB49 tumours can inhibit local CD4 help. In support of these data, male skin grafts themselves accumulated over 3 fold more host CD4 T cells (37±4%) compared to tumours (10±2%). Conversely however the proportion of donor antigen-specific Tregs in the tumour was 4 fold higher (12%) in the tumour

compared to the male skin graft (3%). These distinct cell distributions are likely to contribute to the poor immunity to MB49 compared to skin graft. Finally Tregs that infiltrate into male skin grafts maintain FoxP3 expression while a significant fraction of tumour infiltrating Tregs became FoxP3 negative. This property is investigated in detail in Chapter 5 (Bluestone, Mackay et al. 2009; Addey, White et al. 2011).

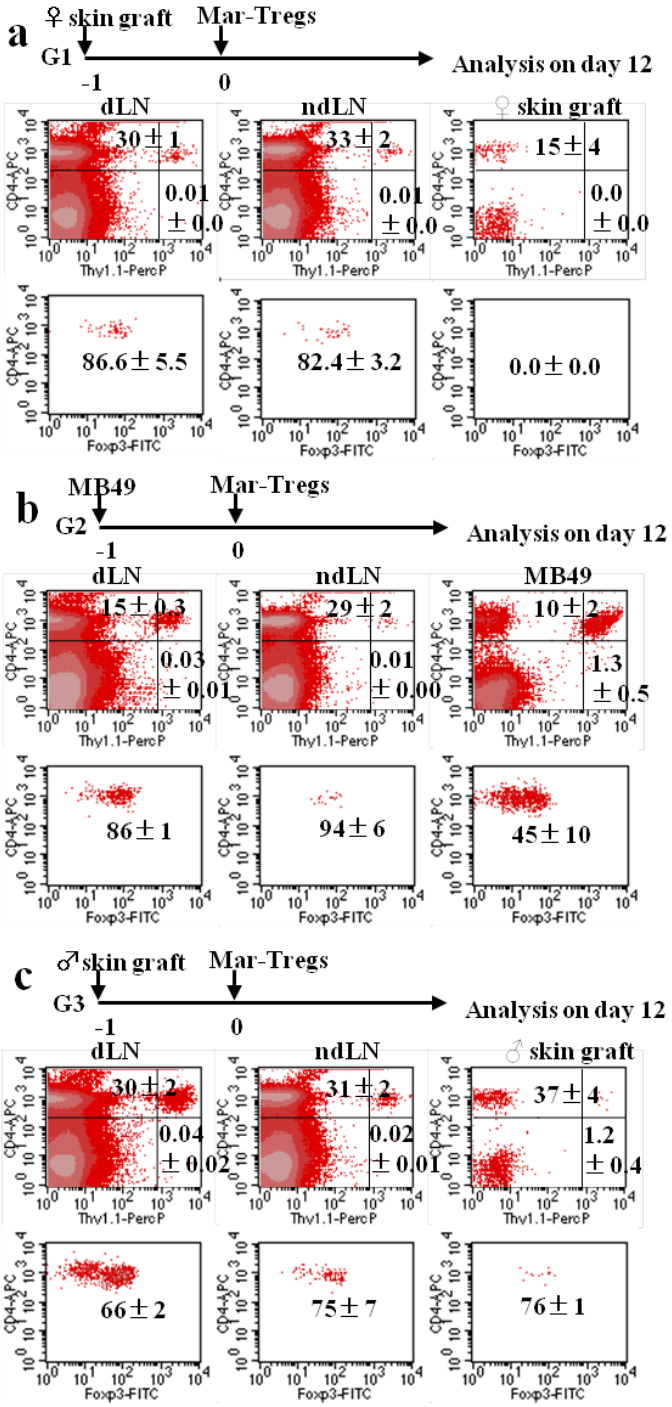


Figure 4.12. Distinct fates of adoptively transferred Marilyn Treg cells in MB49-bearing and male skin graft bearing mice

Three groups of Thy1.2 B6 females (n=3/group) were used. On day 0, mice in G2 were inoculated (s.c.) with $5x10^5$ MB49 cells (b), those in G1 and G3 were

transplanted with male and female skin grafts respectively (a and c). On the next day all mice were transferred with 10^5 FACS sorted Thy1.1 Marilyn Tregs. Analysis was performed at day 12 by surface staining of dLN, ndLN, MB49 or graft tissue cells with anti-Vβ6^{PE}, anti-Thy1.1^{PerCP} before intracellular staining with anti-FoxP3^{FITC}. Data is representative of four independent experiments. Error is represented as +/- standard deviation.

4.3: Discussion

Classically, anti-tumour responses *in vivo* are believed to be mediated directly by CD8 CTL due to the potency of *in vivo* killing and also by response to minor H antigens in the context of allograft and to tumour cells *in vitro* (Lee, Wang et al. 1999). In the MB49 model system Matahari transgenic CD8 T cells are capable of rejecting syngeneic male skin grafts (Fig. 4.1a) and recognising and responding to cognate antigen *in vitro* (Fig. 4.1d) but are incapable of mediating effective anti-MB49 responses *in vivo* (Fig. 4.2b). CD4 anti-tumour responses can mediate tumour and graft rejection independently of CD8 T cells (Corthay, Skovseth et al. 2005) and Marilyn CD4 transgenic T cells have been shown to be capable of rejecting both male syngeneic skin grafts (Valujskikh, Lantz et al. 2002) and MB49 tumours in the absence of CD8 T cells (Perez-Diez, Joncker et al. 2007).

Matahari CD8 T cells can respond to MB49 tumours upon adoptive cell transfer *in vivo* demonstrated by the expansion in local dLN and tumour (Fig. 4.2), however the functional state is poor within the tumour with most infiltrating cells remaining CD44^{low} (Fig. 4.2e). Matahari CD8 T cells in the male skin graft and graft dLN however expand to a significantly greater degree (Fig. 4.2) and graft infiltrating T cells remain in a functional state with a CD44^{high} phenotype (Fig. 4.2e). At this stage it was noted that Matahari CD8 T cells in the tumour dLN significantly down regulate TCR, and to a lesser extent in the graft dLN (Fig. 4.2d). Marilyn CD4 T cells respond to graft and tumour in a phenotypically similar manner (Fig. 4.7). Marilyn cells expand in response to both tumour and graft with significantly higher proliferation seen in the graft dLN compared to tumour dLN (Fig. 4.7b and c) and in the graft compared to tumour (Fig. 4.7d). Marilyn cells did not however demonstrate any significant downregulation of TCR.

Despite a similar T cell phenotype upon adoptive cell transfer, only Marilyn CD4 cells are sufficient to mediate tumour rejection. To attempt to elucidate this disparity, the kinetics of the tumour response was measured for Matahari (Fig. 4.3) and Marilyn (Fig. 4.8) adoptively transferred CFSE labelled T cells. Matahari and Marilyn T cell responses are inversely correlated to tumour progression. Matahari T cells show the highest infiltration into tumour mass by day 14 for all tumours with the highest infiltration for the most recently established tumours (day 1 post T cell transfer) (Fig. 4.3a), with Marilyn CD4 T cells peaking at day 8 again with highest infiltration for 1 day old tumours (Fig. 4.8a). Adoptively transferred Marilyn cells proliferated in the tumour dLN with over 96% CFSE dilution by day 8 in all groups (Fig. 4.7b). Matahari CD8 T cells did not proliferate to the same degree with on average 40% CFSE dilution in the local dLN by day 14 (Fig. 4.3b). This is not a failure of Matahari CD8 T cells to proliferate *in vivo* in response to cognate antigen, as shown through parallel vaccination experiment (Fig. 4.3c). Matahari and Marilyn T cells are both therefore capable of expanding and infiltrating the tumour mass in response to antigen, however Marilyn cells are capable of responding more robustly in the local dLN and are able to enter the tumour mass at an earlier time point. It is also of interest to note that unlike Matahari cells, Marilyn cells undergo less proliferation in homeostatic conditions (G1) where antigen is not present, which is consistent with the notion that Matahari TCR is self-reactive whereas Marilyn TCR is not.

It is generally unclear whether MB49 induces systemic or local immune suppression of T cell responses. To test this, an approach was used to measure the effect of cross talk between graft and tumour, by inoculation of tumour cells, skin grafting or both to mice that received adoptively transferred Matahari (Fig. 4.4) or Marilyn (Fig. 4.9) T cells at the same time. Data with skin graft or tumour alone recapitulates results described above for both Matahari and Marilyn (Fig. 4.4a and 4.9a). Mice that received Matahari T cells along with both a skin graft and tumour cells were able to mount a significantly enhanced response to both graft and tumour. In contrast, graft and tumour-bearing recipients of Marilyn CD4 T cells showed an increased response to tumour but a reduced response to graft. This is shown by an increased representation of both donor CD8 (Fig. 4.4b) and donor CD4 (Fig. 4.9b) cells in tumour dLN and ndLN.

Matahari responses to tumour were noticeably greater than Marilyn in the presence of the skin graft, thus, immunisation by male skin graft principally benefits the donor CD8 T cell mediated anti-MB49 response. On the other hand, when the reciprocal impact of MB49 tumours on the anti-graft T cell response was compared, it was found that Matahari CD8 cells and Marilyn CD4 T cells have distinctive susceptibility to MB49-derived suppression. Anti-graft CD8 responses were significantly enhanced by a contemporaneous MB49 tumour (Fig. 4.4) whereas anti-graft CD4 responses were reduced by at least 50% (Fig. 4.9). This effect is potentially due to the distinct mechanisms used by CD8 and CD4 cells in tumour rejection (Antony, Piccirillo et al. 2005).

CD8 T cells tend to kill tumours directly through cytotoxic mechanisms and therefore enhanced activation in response to antigen on the graft may be sufficient to induce tumour killing. CD4 on the other hand tend to kill tumours indirectly, usually by priming other cytotoxic mediators such as NK cells by IFNγ (Cohen, Peng et al. 2000; Perez-Diez, Joncker et al. 2007). It is likely tumour derived suppression could have a more potent effect on direct CD8 responses rather than modulating CD4 help. To further assess this hypothesis, a CFSE labelled Matahari and Marilyn T cells were co-transferred to mice bearing established tumours (day 7) (Fig. 4.5). The presence of Marilyn T cells significantly increases the Matahari response both in terms of accumulation in the dLN and tumour as well as increased proliferation in both (Fig. 4.5).

Matahari mice have a self reactive phenotype that may account for their impaired anti-MB49 abilities (Fig. 4.1 and 4.3). This is potentially due to several intrinsic factors pertaining to the development of the T cells. Firstly this is possibly due to self reactivity related to endogenous TCRα rearrangement in $Rag^{+/-}$ mice that could potentiate self reactive T cells into the periphery. These T cells in the periphery are in a constant state of division, this is also associated with splenomegaly in these mice (Fig. 4.6c). $Rag^{-/-}$ Matahari mice, that do not undergo TCRα rearrangement, however also display a self reactive phenotype with T cells progressively becoming $CD44^{high}$ with age (Fig. 4.6a). These mice also display visibly smaller thymus compared to female B6 and Marilyn $Rag^{-/-}$ with dramatically reduced $CD8^{+}$ thymocytes in the thymus (Fig. 4.6b). This phenotype may be due to increased clonal deletion to eliminate those cells with reactivity to self antigens

expressed in the thymus, but leaving others to populate the periphery where, as an oligoclonal population, they expand homeostatically. Overall this suggests Matahari mice have an altered TCR transgenic T cell development in both $Rag^{-/-}$ and $Rag^{+/-}$ mice.

In an adoptive therapy setting Matahari T cells reduce TCR (Vβ8.3) expression in response to antigen (Fig. 4.2d). TCR is further reduced in the tumour due to the inhibitory environment associated with MB49. This environment caused both donor and host CD8 Vβ8.3 to be downregulated (Fig. 4.3c). This effect is not seen with adoptively transferred Marilyn T cells (Fig. 4.7 and 4.8). Matahari $Rag^{+/-}$ low TCR expression is also seen in naive B6 female mice that receive T cells (Fig. 4.6d) and therefore is not solely a response to TCR/HY engagement. In this antigen free environment Matahari cells also show spontaneous proliferation (Fig. 4.6e) while Marilyn T cells do not. While it remains to be determined if this downregulation of TCR is also associated with reduced TCR signalling, there are significantly more CD44 expressing cells detected in grafts than in MB49 tumours (Fig. 4.2e).

Marilyn T cells have been found to be more effective than Matahari T cells at tumour rejection in five different HY-expressing tumour models (Perez-Diez, Joncker et al. 2007). Marilyn T cells are directed to a Th1-like phenotype in adoptive transfer to mice bearing male skin grafts but not in the tumour (Fig. 4.10). This effect is correlated with greater expansion and infiltration into the graft (Fig. 4.7, 4.9 and 4.10a), along with high levels of IFNγ production (Fig. 4.10c). When cultured *ex vivo* Marilyn cells from control pLN or graft dLN but not from tumour dLN respond to cognate antigen in a dose-dependent manner (Fig. 4.10b). However donor cells in the dLN of tumour showed a uniform proliferation at all doses of antigen and even in the absence of exogenous antigen. This effect was further highlighted when $CD11c^{+}$ DCs sorted from graft, tumour dLN and control pLN were plated out with Marilyn CD4 T cells in the absence of exogenous antigen (Fig. 4.11a). Proliferation was seen in response to CD11c cells from tumour dLN was far higher than the background while very little was seen in response to graft CD11c cells. DCs from tumour dLN may therefore have elevated levels of cross presentation of HY/Dby antigen to T cells that may mediate increased anti-MB49

responses. Although CD11c cells from graft dLN expressed lower levels of antigen complexes they are able to induce strong Th1 responses (Fig. 4.10).

Despite naturally occurring self reactivity, Matahari T cells can still mediate skin graft rejection but not rejection of MB49. This may be due to the immunosuppressive microenvironment in tumours but not accepted grafts that are known to upregulate immuno-regulatory factors such as IDO and TGF-β. Indeed knockout of IDO or TGF-β results in an enhanced tumour clearance in a number of murine models (Munn and Mellor 2007; Coe, Addey et al. 2010). As seen here and in the previous chapter, the MB49 tumour cell line constitutively express TGF-β genes and upregulate IDO and IL-10 expression following *in vivo* tumour formation (Fig. 3.14 and 4.11b-d) demonstrating that a highly modulatory environment by MB49 tumour. Expression of IDO and TGF-β was significantly lower in male skin grafts (Fig. 4.11b and c), although IL-10 expression was higher in ¾ grafts compared to that of the tumour (Fig. 4.11d). One major prospect for this effect is an increased induction and maintenance of Tregs in tumours mediated by IDO and TGF-β, previously shown to facilitate tumour growth (Coe, Addey et al. 2010; Coe, Begom et al. 2010).

Tregs have been shown to be powerful mediators in the induction of tolerance (Karim, Feng et al. 2005). Previous work in our group have shown that intranasal inhalation of HY-Dby peptide and injection (s.c) of DCs pulsed with HY-Uty (James, Scott et al. 2002) can cause transplantation tolerance through induction of Tregs. Marilyn HY-specific Tregs were therefore evaluated in tumour and, male and female, graft environments (Fig. 4.12). The frequency of Tregs in the MB49 tumour mass and dLN is significantly increased while the proportion of host CD4 T cells was reduced by up to 50% (Fig. 4.12b). While Treg infiltration into male grafts and dLN was significantly higher than female graft, this did not lead to a reduction of host CD4 frequency (Fig. 4.12c). Growing tumours are generally associated with increased Treg proportions compared to rejecting tumours (Curiel 2007). Therefore HY-specific Tregs are preferentially promoted in the MB49 tumour microenvironment, supported by previous published results from our lab (Chai, Coe et al. 2008). At this point it is of note that HY-specific Tregs lose FoxP3 upon antigen exposure (55% loss in tumour, 24% loss in male graft) and can even lose FoxP3 expression under non-antigenic inflammatory conditions (14% loss in female

graft dLN), this is supported by several other labs (Bluestone, Mackay et al. 2009). This effect and further HY-specific Treg responses are investigated in detail in chapter 5 (Addey, White et al. 2011).

A final consideration must be given to the distinct process of antigen presentation between graft and tumour. Skin contains three major populations of DC: Langerhans cells in the epidermis; and Langerin^{+} and Langerin- CD11c^{+} conventional DC populations in the dermis (Shortman and Liu 2002), which can mature and migrate to the dLN in response to inflammation caused by surgery. Consequently, these graft-derived professional APCs can participate in direct antigen presentation resulting in rapid T cell activation. In contrast, as MB49 cells are non-migrating, non-professional APCs, antigen presentation will be indirect and delayed until host DCs have infiltrated the developing tumour. In addition, the local inflammation associated with skin transplantation but not inoculation (s.c.) of cultured MB49 cells. Indeed vaccination using CFA and peptide is sufficient to mediate potent responses by Matahari T cells potentially due to increased inflammatory factors that may increase DC maturation, migration and antigen presentation (Fig. 4.3c).

This study provides an insight into antigen-specific CD4, CD8 and Treg responses to antigen presented in two dramatically different *in vivo* environments. Marilyn CD4 T cells are seen to be more efficient in mounting anti-MB49 responses compared to Matahari CD8 T cells. This effect is due to several factors including different mechanisms of tumour rejection, antigen presentation by APCs and the nature of Matahari TCR being self reactive. MB49 generates a local immuno-regulatory environment that prevents full T cell activation, accumulation and proliferation particularly for CD8 T cells. This effect can be abrogated when graft and tumour are given simultaneously creating cross talk sufficient to induce more robust anti-tumour responses for CD4 and CD8 T cells. Finally HY-specific Tregs are preferentially induced and maintained in tumour environments further modulating T cell responses. The role that antigen-specific T cells and Tregs play in both allografts and tumour environments in critical to the prognosis of transplant and tumour. By characterising and manipulating these responses it is possible to understand the factors that lead to graft tolerance and conversely tumour rejection.

Chapter 5: Tumour antigen-specific regulatory T cells lose FoxP3 expression following antigen recognition *in vivo*

5.1: Introduction

5.1.1: Regulatory T cells and tumours

Regulatory T cells (Tregs) are a subset of CD4 T cells with high avidity T cell receptors against a self antigen and they are characterised by their ablity to suppress immune responses against that antigen (see section 1.3.3) (Kim, Rasmussen et al. 2007). Tregs suppress self reactive peripheral T cells that escape thymic deletion, this was shown by studies using neonatal thymectomy, or by depletion experiments using anti-CD25 antibodies (Sakaguchi, Sakaguchi et al. 1995; Kohm, McMahon et al. 2006). Tregs are crucial components of the immune regulatory network and can potently inhibit immune responses to a wide range of cancers (Curiel 2007). Most tumour antigens are self antigens which are either over expressed for their cell type or altered in sequence or structure (see chapter 3) (Dranoff 2009). Tregs are primarily specific for self antigens and functionally suppress immune responses to these antigens, thus interaction between Tregs and tumours constitute a major evasive mechanism in cancer (see section 1.6.3) (Kim, Emi et al. 2006).

Despite recognition of the effect of suppressive $CD4^+$ cells on tumour immunity in the 1980s (Bursuker and North 1984) it was largely forgotten until the turn of this century. *In vivo* depletion of $CD25^+CD4^+$ cells led to increased endogenous tumour immunity (Shimizu, Yamazaki et al. 1999), which was also tumour antigen-specific (Tanaka, Tanaka et al. 2002). With interest in Tregs and cancer re-established, it was further noted that Tregs can inhibit both tumour-specific CD4 (Nishikawa, Jager et al. 2005) and CD8 effector responses (Chen, Pittet et al. 2005). Understanding of the mechanisms of Treg action on anti-tumour effector T cells is incomplete, although it is known to occur through cell-cell contact and the production of soluble cytokines such as IL-10 and TGF-β (Levings, Sangregorio et al. 2002) (Wang 2006).

Tregs are composed of two distinct subsets called natural (nTreg) and adaptive/induced Tregs (iTregs) (Thornton, Korty et al. 2010). nTregs arise in the thymus in a setting in which T cells with high affinity against thymically expressed self antigens are removed, in order to protect against autoimmunity (see section 1.3.3). Peripheral Tregs in lymph nodes and spleen contain both nTregs and iTregs (Thornton, Korty et al. 2010). iTregs arise in Proinflammatory situations in the periphery, such as in cancer, and suppress immunity by heterogeneous mechanisms (Bluestone and Abbas 2003) (see section 1.3.3). nTegs can be sorted directly from the CD8 depleted thymus while iTregs can be selected from conventional CD4 T cells by their capacity to express FoxP3. nTregs are believed to be the main regulatory cell type that infiltrates the tumour microenvironment, although the relative contribution of nTreg and iTreg is yet to be fully understood (Zhou and Levitsky 2007).

Tumours that secrete TGF-β have been shown to expand tumour-specific nTregs in an antigen dependent way (Ghiringhelli, Puig et al. 2005). MB49 and B16/HY tumours can induce expansion and migration of HY-specific nTregs, but HY-specific nTregs are inhibited in dN^{+}TGF-β mice (Ghiringhelli, Puig et al. 2005; Coe, Addey et al. 2010). Furthermore, *in vivo* depletion of nTreg improves tumour immune surveillance and enhances the efficacy of cancer vaccines (Shimizu, Yamazaki et al. 1999; Turk, Guevara-Patino et al. 2004), shown by using DTA-1 (αGITR) mAb depletion and adoptive transfer in models of B16 melanoma. Understanding and characterising the fate of tumour-specific Tregs *in vivo* is increasingly important when using tumour-specific immune-therapies.

5.1.2: FoxP3 transcription factor

The Forkhead box P3 (FoxP3) transcription factor is crucial in controlling development and maintaining the suppressive phenotype and function of Tregs (Fontenot, Gavin et al. ; Hori, Nomura et al. 2003; Khattri, Cox et al.). FoxP3 expression is regulated by a positive autocrine feedback loop (Gavin, Rasmussen et al. 2007; Williams and Rudensky 2007) and by epigenetic modification of the *FoxP3* locus (Floess, Freyer et al. 2007); nTregs display several features of a stable CD4 subset, for example nTregs retain high FoxP3 expression after adoptive transfer

to lymphoreplete hosts (Floess, Freyer et al. 2007; Gavin, Rasmussen et al. 2007; Williams and Rudensky 2007).

With a recent explosion in research involving Treg adoptive therapy in animal models (Fontenot, Gavin et al. 2003; Hori, Nomura et al. 2003; Fontenot, Rasmussen et al. 2005); T cells that express FoxP3 have become critical in the transference of self-tolerance. Induction or transfer of $FoxP3^{+}$ Tregs has led to significant improvements in disease severity in diabetes, asthma, inflammatory bowel disease, multiple sclerosis and renal disease models (Suri-Payer and Fritzsching 2006). Recent investigations however also noticed that the Treg lineage is more plastic than first thought, possibly inducing Th1 or Th17-like T effector cells under proinflammatory conditions (Zhou, Bailey-Bucktrout et al. 2009). This effect, unwelcome in autoimmune models, may be beneficial in the context of tumours.

Auto aggressive T cells arising from Tregs could be a serious consequence in adoptive immunotherapy, as use of Treg therapy is becoming a progressively favoured approach in autoimmune diseases such as diabetes. Recent results have shown that attenuation of the FoxP3 locus expression can lead to multi-organ autoimmunity but has no effect on thymic development, homeostatic expansion, or TGF-β production *in vivo* (Wan and Flavell 2007). Zhou *et al.* have also shown polyclonal Tregs that lose FoxP3 expression under proinflammatory conditions in autoimmune pancreatic tissue can cause autoimmune pathology and rapid onset of diabetes upon adoptive cell transfer (Zhou, Bailey-Bucktrout et al. 2009). Conversely inducing auto aggressive T cells from tumour-specific Tregs has the potential for future cancer immunotherapy strategies.

5.1.3: Functional plasticity of Tregs

Initially FoxP3 loss was noted in polyclonal Tregs from $FoxP3^{GFP}$ B6 mice following transfer into lymphopenic hosts. For example, green tagged Tregs, $CD4^{+}CD25^{+}GFP^{+}$ cells isolated from $FoxP3^{GFP}$ reporter mice (Fontenot, Rasmussen et al. 2005), eventually decrease GFP expression upon adoptive transfer into $Rag2^{-/-}$ B6 mice, indicating that Tregs can lose FoxP3 following homeostatic stimulation *in vivo*. Our lab also noticed that loss of FoxP3 could also occur *in vitro* (Chai, Coe et al. 2008). FACS sorted "green" Tregs (with >99% purity) from $FoxP3^{GFP}$ mice were expanded *in vitro* by CD3/CD28 beads plus IL-2, 7 days after the first round of

stimulation, expanded green Tregs were largely GFP-positive, suggesting that FoxP3 expression in expanded Tregs is well maintained. In contrast by day 14, 7 days after the second round of culture, the majority of expanded Tregs became GFP negative, indicating that repeated stimulation leads to the loss of FoxP3 in Tregs (Chai, Coe et al. 2008). Others have also shown that *in vitro* TCR stimulation in the presence of IL-6 led to down-regulation of FoxP3 in polyclonal green Tregs (Xu, Kitani et al. 2007; Yang, Nurieva et al. 2008).

The functional plasticity of peripheral Tregs has also been observed *in vivo*. Our lab has recently demonstrated that a significant fraction of tumour antigen-specific green Tregs lost FoxP3 expression after adoptive transfer to tumour-bearing WT B6 mice (see chapter 4, Fig. 4.12) (Coe, Addey et al. 2010). Other studies have also shown that a large proportion of polyclonal green Tregs became GFP-negative and differentiated to Th1-like effector cells or follicular helper T cells in $Rag^{-/-}$ (Duarte, Zelenay et al. 2009; Komatsu, Mariotti-Ferrandiz et al. 2009) and $CD3^{-/-}$ recipients (Tsuji, Komatsu et al. 2009), respectively. In addition, mice with attenuated expression of endogenous FoxP3 develop lympho-proliferative syndromes which are correlated with impaired suppressive function of Tregs and their conversion to effector cells (Wan and Flavell 2007). Bluestone and colleagues have used a well designed dual reporter mouse model to distinguish T cells that constitutively express or have ceased expressing FoxP3 at any time point *in vivo* (Zhou, Bailey-Bucktrout et al. 2009). They have found that a substantial fraction of peripheral Tregs can lose FoxP3 even under steady state conditions in un-manipulated healthy mice, and these "ex-Tregs" also had an activated memory phenotype. It is important to note that ex-Tregs are present at a significantly higher frequency in an autoimmune diabetic settings and these auto aggressive proinflammatory effector cells capable of transferring diabetes (Zhou, Bailey-Bucktrout et al. 2009).

The reprogramming of Tregs was also noted in an *in vivo* tumour model by Mellor *et al.* Previous studies by this group have demonstrated Tregs can be activated and maintained in tumour tissues through the expression of IDO by pDCs; many immunosuppressive tumours express IDO (see chapters 3 and 4). When IDO is blocked, Tregs in tumour-bearing hosts can differentiate towards a Th17-like linage (Sharma, Hou et al. 2009), however it is unclear whether these converted Tregs are functional. Mellor showed polyclonal Tregs reprogrammed in a standard

CPG-OVA vaccine model are functional in priming of tumour antigen-specific OT-1 (CD8 OVA transgenic) T cells. Treg reprogramming is driven by proinflammatory signals, and can provide helper function almost immediately without prior priming. This effect is believed to be IL-6 driven; IL-6 is expressed by pDCs in a negative feedback loop with IDO. Proinflammatory signals through pathogen recognition receptors are sufficient to inhibit IDO and induce IL-6 in this model (Sharma, Hou et al. 2010). Results here suggest Tregs could be acting as first responders providing help or rapid suppression, depending on the context of the immune response.

Conversely Rubtsov *et al.* suggest Tregs are a stable *in vivo* linage. This group use the same dual reporter system as Bluestone, whereby FoxP3$^+$ Tregs express GFP and YFP and ex-Tregs express YFP alone, providing an elegant system to track differentiation of Tregs. Results showed Tregs maintained FoxP3 expression over a period of 5 months under conditions including lymphopenia (6Gy treatment) and IL-2 blocking using an anti-IL-2 mAb (Rubtsov, Niec et al. 2010). It is important to note however that these results are generated in a non-antigenic homeostatic system not in a proinflammatory environment, where Treg conversion is seen in other studies.

Despite the development of ex-Tregs being studied in homeostatic (Duarte, Zelenay et al. 2009; Komatsu, Mariotti-Ferrandiz et al. 2009; Tsuji, Komatsu et al. 2009) and autoimmune models (Wan and Flavell 2007; Zhou, Bailey-Bucktrout et al. 2009), very little information is available in terms of their differentiation in the context of tumours (Sharma, Hou et al. 2009; Sharma, Hou et al. 2010). Adoptive transfer experiments have so far utilised polyclonal Tregs that have limited value in studying the role of antigen-driven responses, therefore not much is known in terms of the cellular and molecular mechanisms of ex-Treg generation and regulation (Zhou, Bailey-Bucktrout et al. 2009). A current controversial question asks whether the development of ex-Tregs is restricted to the CD25 negative subset of Tregs (Komatsu, Mariotti-Ferrandiz et al. 2009; Zhou, Bailey-Bucktrout et al. 2009) and whether thymic nTregs, like iTregs, could also be converted to ex-Tregs; neither been directly assessed.

Due to the preferential development of ex-Tregs in the lymphopenic environment (Duarte, Zelenay et al. 2009; Komatsu, Mariotti-Ferrandiz et al. 2009; Tsuji, Komatsu et al. 2009) a recently described *in vivo* model has been modified (Coe, Addey et al. 2010) to generate and characterise antigen-specific ex-Tregs, by

adoptively transferring HY-specific green Tregs (thymic or peripheral) to MB49 tumour-bearing $Rag^{-/-}$ B6 female recipients. To explore the cellular and molecular events that affect ex-Treg development, a second *in vivo* model has been developed in which green HY-specific peripheral Tregs were adoptively transferred to $Rag^{-/-}$ B6 male mice in which the HY antigen is expressed endogenously and systemically.

5.2: Results

5.2.1: Impact of HY-specific Treg cells on co-transferred HY-specific conventional CD4 T cells in response to MB49 tumours *in vivo*

To assess the role of HY-specific nTregs in regulation of anti-MB49 responses by conventional CD4 T cells with matched antigen-specificity *in vivo*, $CD4^+$ $V\beta6^+$ $Thy1.1^+$ T cells from HY-specific TCR Marilyn $Rag2^{+/-}$ female mice were either depleted of $CD25^+$ cells (Fig. 5.1a and b) or not (Fig. 5.1c and d). These cells were CFSE labelled and adoptively transferred to WT B6 females inoculated with MB49 on the following day, and 5 days later mice were analysed for donor cell expansion. MB49-bearing mice that received CD25 depleted Marilyn T cells showed a significant expansion (8 fold) in the dLN (Fig. 5.1a) compared to MB49 free recipients (Fig. 5.1b) (0.545 vs 0.065%, p-value <0.01) with a high accumulation of donor cells in the tumour (3.55%), this expansion was also correlated with an increased CD44 expression compared to the MB49 free control (86.7 vs 5.98%, p-value < 0.01). Mice that received donor cells without CD25 depletion showed a comparatively poor response to MB49 (Fig. 5.1c) in the dLN (0.01 vs 0.055%, p-value >0.05) and tumour itself (0.06%).

This effect is likely due to a failure of conventional CD4 T cells to respond to antigen in the presence of Tregs, either by not activating or through activation induced cell death. Marilyn nTregs therefore have a significant impact on the success of the conventional CD4 response even when transferred as a minority population (5-10%).

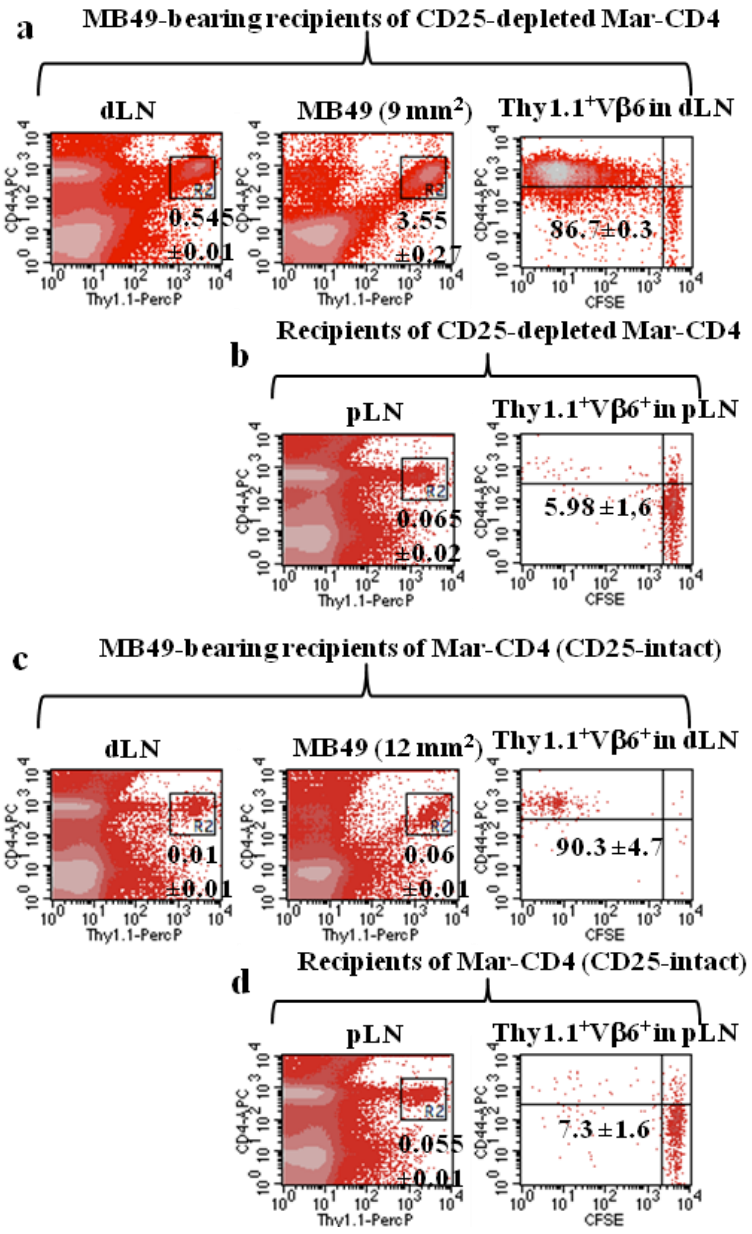


Figure 5.1. Anti-MB49 responses by CD25-depleted and CD25-intact Marilyn CD4 T cells

Four groups of Thy-1.2^{+} B6 females (n=3 or 4/group) were adoptively transferred with (1.5 x 10^{6}) CFSE labelled CD25-depleted (**a**, **b**) or CD25-intact Thy-1.1^{+} Marilyn CD4 T cells (**c**, **d**) (i.v.). On the next day two groups of mice were inoculated with MB49 cells (5 x 10^{5}) (s.c.) (**a**, **c**). Five days after MB49 inoculation, all groups were analysed by staining of dLN and MB49 (**a**, **c**) or pLN (**b**, **d**) with anti-Vβ6^{PE}, anti-Thy-1.1^{PerCP} and anti-CD4^{APC}. The donor cells LN and MB49 tissue were identified by co-expression of Thy-1.1 and CD4. In addition, LN samples were also stained with anti-Vβ6^{PE}, anti-Thy-1.1^{PerCP} and anti-CD44^{APC}. The donor cells were identified by co-expression of Thy-1.1 and Vβ6 and their CFSE profiles were presented as CFSE versus CD44. A total of 2 million events (no gate) were acquired for each sample. For MB49 samples, tumour size is also indicated. One representative experiment of three is shown.

5.2.2: Marilyn and B6 nTreg responses to MB49

To evaluate how HY-specific Marilyn nTregs inhibit the anti-tumour response by conventional CD4 T cells, an adoptive transfer experiment was set up to compare Marilyn Thy1.1^{+} nTregs with polyclonal Thy1.2^{+} nTregs from a B6 mouse. These Treg cells were transferred to MB49-bearing (inoculated on the next day) or MB49 free B6 female hosts (Thy1.2^{+}).

In the presence of MB49 tumours, Marilyn nTregs underwent an 8 fold expansion in the dLN (Fig. 5.2b) compared to MB49 free recipients (Fig. 5.2a) (0.0207 vs 0.0026, p-vlaue <0.05), this was profoundly reflected in the increase of absolute cell number (Fig. 5.2c). In recipients that received polyclonal nTregs, no expansion of the nTreg was seen in response to tumour (Fig. 5.2e) in the dLN or the tumour itself. This effect was true in terms of frequency (Fig. 5.2d and e) and absolute cell number (Fig. 5.2f). Therefore MB49 tumours cannot expand nTreg non-specifically, a crucial observation for the use of this model.

Importantly at this stage it was noticed that expanded donor Marilyn nTregs showed a significant loss of FoxP3 expression in the dLN of MB49-bearing (Fig. 5.2.b) compared with MB49 free mice (Fig. 5.2a) (84.83 vs 96.55%, p-value <0.01). This effect was not seen to the same degree in MB49-bearing (Fig. 5.2d) and MB49 free (Fig. 5.2e) mice that received polyclonal nTreg (91.74 vs 90.88%, p-value >0.05). Strikingly, in the tumour itself only 33.93% of transferred Marilyn nTregs maintained FoxP3 expression (Fig. 5.2b) whereas 89.12% polyclonal nTregs maintained FoxP3 expression in the tumour (Fig. 5.2e). MB49 clearly induced antigen-specific nTreg expansion and induction of FoxP3 loss. However the lack of a HY-negative MB49 variant does not allow for the role of HY to be assessed in terms of nTreg expansion. From here on Tregs that have lost FoxP3 will be referred to as “ex-Tregs”.

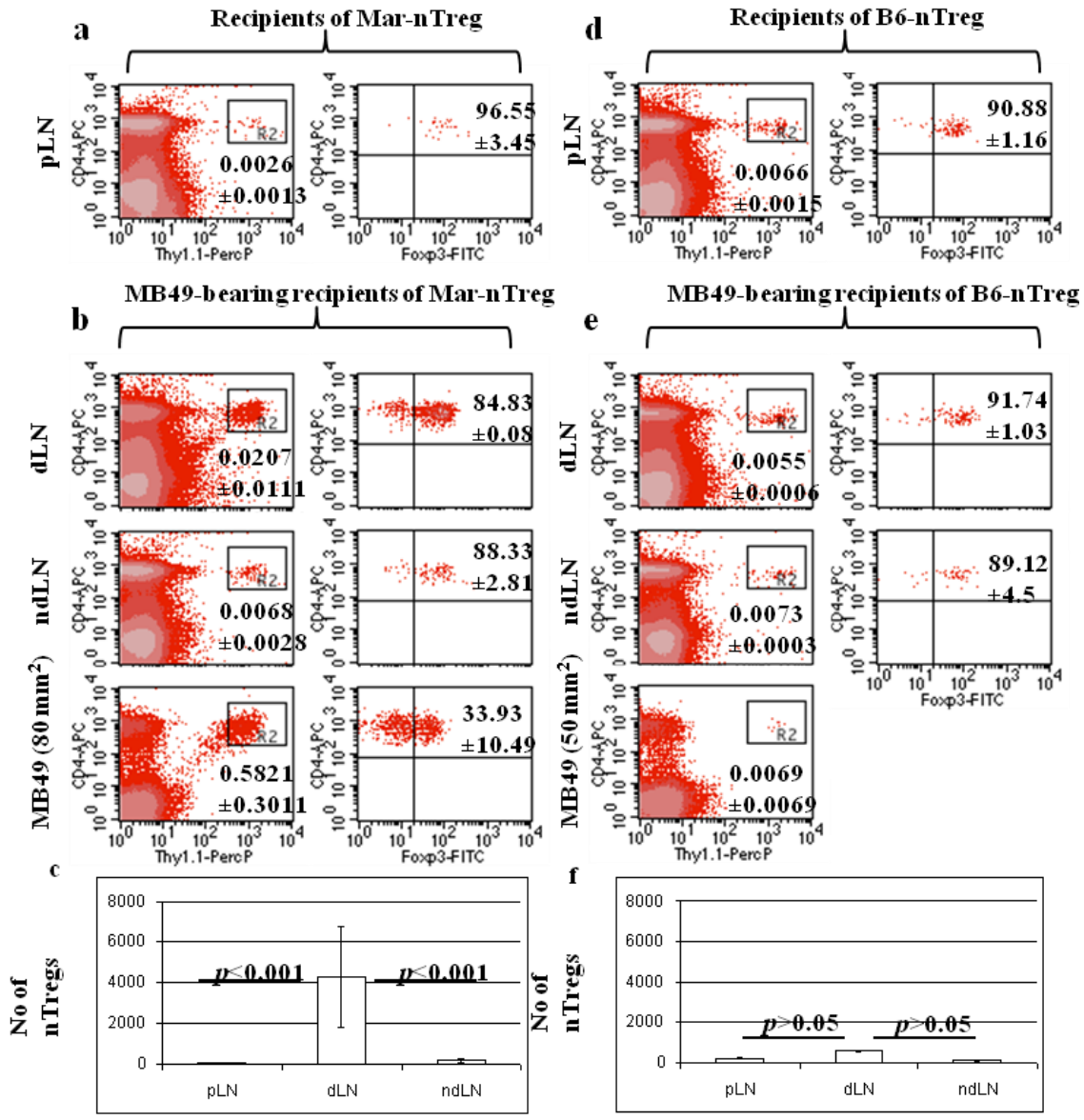


Figure 5.2. MB49 tumour expands Marilyn but not B6 nTreg cells *in vivo*
nTregs are derived from CD8 depleted thymocytes of a Marilyn-FoxP3^{GFP} Rag2$^{-/-}$ or B6-FoxP3^{GFP} donor and FACS sorted for CD4^{+} GFP^{+} CD25^{+} CD8^{-} cells. Four groups of Thy-1.2 B6 females (n=3/group) were transferred with 10^5 Thy-1.1^{+} Marilyn nTreg (**a**, **b**) or Thy-1.1^{+} B6 nTreg cells (**d**, **e**), of which two groups of mice were given MB49 cells the on next day (**b**, **e**) the other two groups were MB49 free (**a**, **d**). 11 days after MB49 inoculation, analysis was performed by staining cells (dLN, ndLN and MB49) with anti-Vβ6^{PE} or anti-TCR-β^{PE}, anti-Thy-1.1^{PerCP} and anti-CD4^{APC}, before intracellular staining with anti-FoxP3^{FITC}. The donor cells were identified by co-expression of Thy-1.1 and CD4 (R2), and their FoxP3 expression was presented as CD4 versus FoxP3. The absolute numbers of donor cells in various tissues, peripheral lymph nodes (pLN), dLN and ndLN, were calculated by total cell number, % of gated lymphocytes (R1) then by % of donor cells (R2) (**c**, **f**). One representative experiment of four is shown. A total of 2 million events were acquired for each sample. For the MB49 samples, tumour size is also indicated. All error is represented as ± standard error mean.

5.2.3: Marilyn HY-specific FoxP3-GFP$^+$ nTreg responses to B16 and B16/HY tumours

To evaluate the effect of the HY antigen for the expansion and loss of FoxP3 in Marilyn nTregs, a second B16 tumour model was used. Although B16 is a relatively non-immunogenic tumour, it is known to expand polyclonal nTregs in a TGF-β dependent manner (Ghiringhelli, Puig et al. 2005). B16 cells do not express HY antigens but this tumour cell line has been previously transduced with the HY gene *Dby* encoding CD4 T cell epitope (B16/HY). This system provides a model in which antigen-driven expansion of Tregs, by tumour, can be evaluated *in vivo*. To expose the property of antigen induced FoxP3 loss, green Marilyn Tregs ($CD4^+V\beta6^+CD25^+GFP^+$ cells) were FACS sorted from ($FoxP3^{GFP}$ B6 × Marilyn) F_1 females; these antigen-specific green nTreg cells ($Thy1.1^+$) were adoptively transferred into three groups of $Thy1.2^+$ B6 females, of which two groups were injected (s.c.) with either B16 or B16/HY cells on the next day. Donor Treg expansion and FoxP3 expression was measured 11 days after tumour inoculation.

As with MB49, HY-specific Tregs do not expand effectively without antigen (Fig. 5.3a and b) but are more capable of expanding in mice bearing B16/HY tumours (Fig. 5.3c). Following antigen recognition, expanded Tregs can also lose FoxP3 (Fig. 5.3c). In addition, it seems that loss of FoxP3 by tumour-infiltrating Treg cells is greater (77·68 ± 0·02%, Fig. 5.2c) than that by those in dLN (91·37 ± 0·08%, Fig. 5.2c).

To confirm the HY *Dby* expression by the B16/HY cell line, RT-PCR was performed (Fig. 5.3d), this showed that while *Dby* B16/HY is lower than the MB49 cell line, it was significantly higher than the B16 line ($p < 0.05$). Furthermore, B16/HY tumour cells *ex vivo* were analysed for *Dby* expression, which showed that it was maintained *in vivo* (Fig. 5.3e), and that the HY transgene is stable *in vivo*.

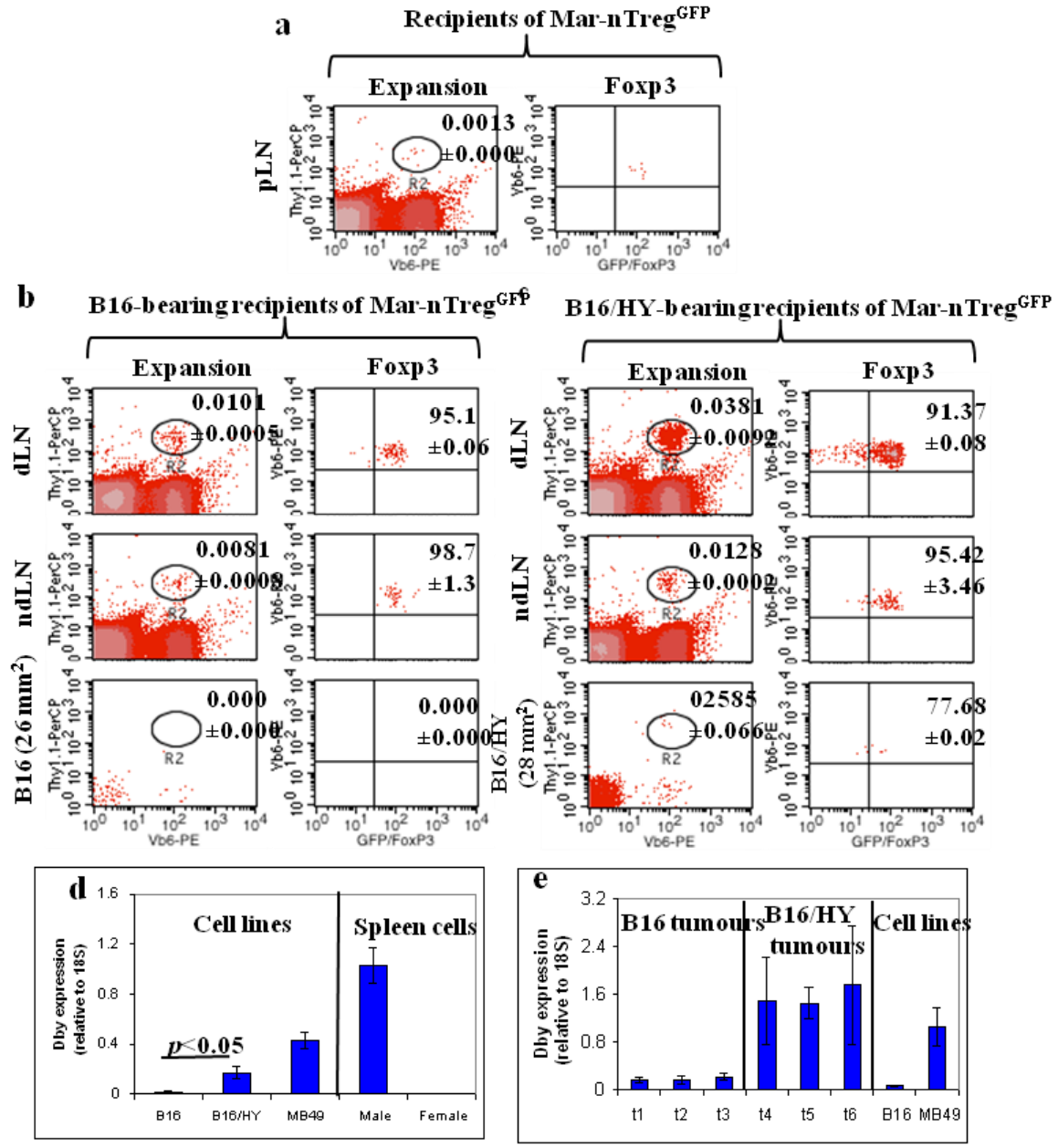


Figure 5.3. Antigen-specific downregulation of FoxP3 expression by expanded adoptively transferred Marilyn Tregs

Three groups of Thy1.2^{+} B6 females (n=3/group) were adoptively transferred with 10^5 Thy1.1^{+} Marilyn nTreg cells co-expressing FoxP3^{GFP} (Mar-nTreg^{GFP}) (i.v.) (**a-c**), of which two groups of mice were inoculated (s.c.) with B16 cells (**b**) or B16/HY cells (**c**) (5×10^5) on the next day. Eleven days after tumour inoculation, analysis was performed by staining the cells from various tissues with anti-Vβ6^{PE}, anti-Thy-1.1^{PerCP} and anti-CD4^{APC}. The donor cells were identified by co-expression of Thy-1.1 and Vβ6 and their FoxP3 expression was presented as Vβ6 versus GFP. One representative experiment of two is shown. A total of 2 million events were acquired for each sample. For the B16 and B16/HY samples, tumour size is also indicated.

a. Treg cells alone.

b. Treg cells with B16 inoculation.

c. Treg cells with B16-HY inoculation.

d. B16, B16/HY and MB49 cell lines were compared for *Dby* expression by RT-PCR with male or female B6 spleen cells were used as internal controls. One representative experiment of three is shown.
e. Two groups of B6 females (n=3 /group) were inoculated (s.c.) with 5 x 10^5 B16 or B16/HY cells, respectively. Two weeks later, B16 and B16/HY tumours from individual mice were compared for *Dby* expression by RT-PCR with B16 and MB49 cell lines were used as internal controls. One representative experiment of two is shown. All error is represented as ± standard error mean.

5.2.4: Ex-Treg development in MB49-bearing WT B6 female hosts

To confirm that the generation of HY-specific ex-Tregs is antigen-driven and not a product of other stimuli from the tumour environment, green Tregs (containing both nTreg and iTreg cells) were adoptively transferred to WT B6 female hosts (Fig. 5.4a and b) and MB49-bearing B6 female hosts (Fig. 5.4c-f). HY-specific Tregs maintained GFP expression (Fig. 5.4b) and failed to expand (Fig. 5.4a) in naive female mice, similar to the effect seen in female mice given nTreg (Fig. 5.2a), further confirming that HY-specific Tregs do not expand in a homeostatic manner in lymphoreplete hosts. In the presence of MB49 tumour, a significant expansion of green Tregs was observed (Fig. 5.4c and e) and a proportion of these became ex-Tregs (Fig. 5.4d and f) (14% dLN, 20% MB49).

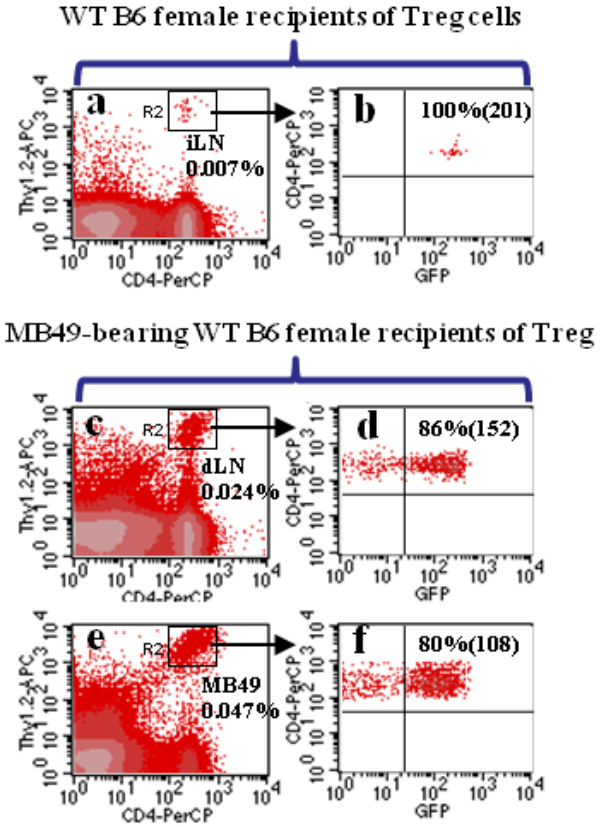


Figure 5.4. Development of ex-Tregs in MB49-bearing WT B6 female mice
Two groups of Thy1.1^{+} B6 females (n=3/group) were adoptively transferred with 10^5 Thy1.2^{+} Marilyn Treg cells co-expressing FoxP3^{GFP} (Mar-Treg^{GFP}) (i.v.) (**a,c**), of which one group of mice were inoculated (s.c.) with MB49 cells (**c**) (5×10^5) on the next day. Seven days after tumour inoculation, analysis was performed by staining the cells from iLN/dLN (**a,c**) and MB49 (**e**) with anti-Vβ6^{PE}, anti-CD4^{PerCP} and anti-Thy1.2^{APC}. The donor cells were identified by co-expression of Thy-1.2 and Vβ6 and their FoxP3 expression was presented as CD4 versus GFP. One representative experiment of two is shown. iLN, inguinal LN.

5.2.5: Generation of HY-specific ex-Tregs in lymphopenic hosts

In order to develop an *in vivo* model in which ex-Tregs can be generated in sufficient quantities, highly purified HY-specific peripheral green Treg (Fig. 5.5a) were transferred into MB49 free (Fig. 5.5b) or MB49-bearing Rag2$^{-/-}$ (lymphopenic) B6 females (Fig. 5.5c). A significant accumulation of donor Tregs progeny were found in tumour dLN (27%), spleen (0.6%) and tumour tissue (27%) on day 12 (Fig. 5.5c). The representation of donor Tregs accumulation in inguinal LN (iLN) and spleen in control mice was low due to lack of antigens (Fig. 5.5b) (0.3 and 0.05%). The difference in frequency (Fig. 5.5d) and absolute cell number (Fig. 5.5f) of donor Tregs between iLN and dLN was statistically significant.

In tumour-bearing mice A fraction of the donor Tregs in the dLN (23%), spleen (35%) and MB49 (8%) become GFP-negative (Fig. 5.5c), whereas the donor Tregs in the iLN and spleen of control mice remained 100% GFP-positive (Fig. 5.5b). Ex-Tregs in dLN have statistically significant increased frequency (Fig. 5.5e) and absolute cell number (Fig. 5.4g), in comparison to those in iLN.

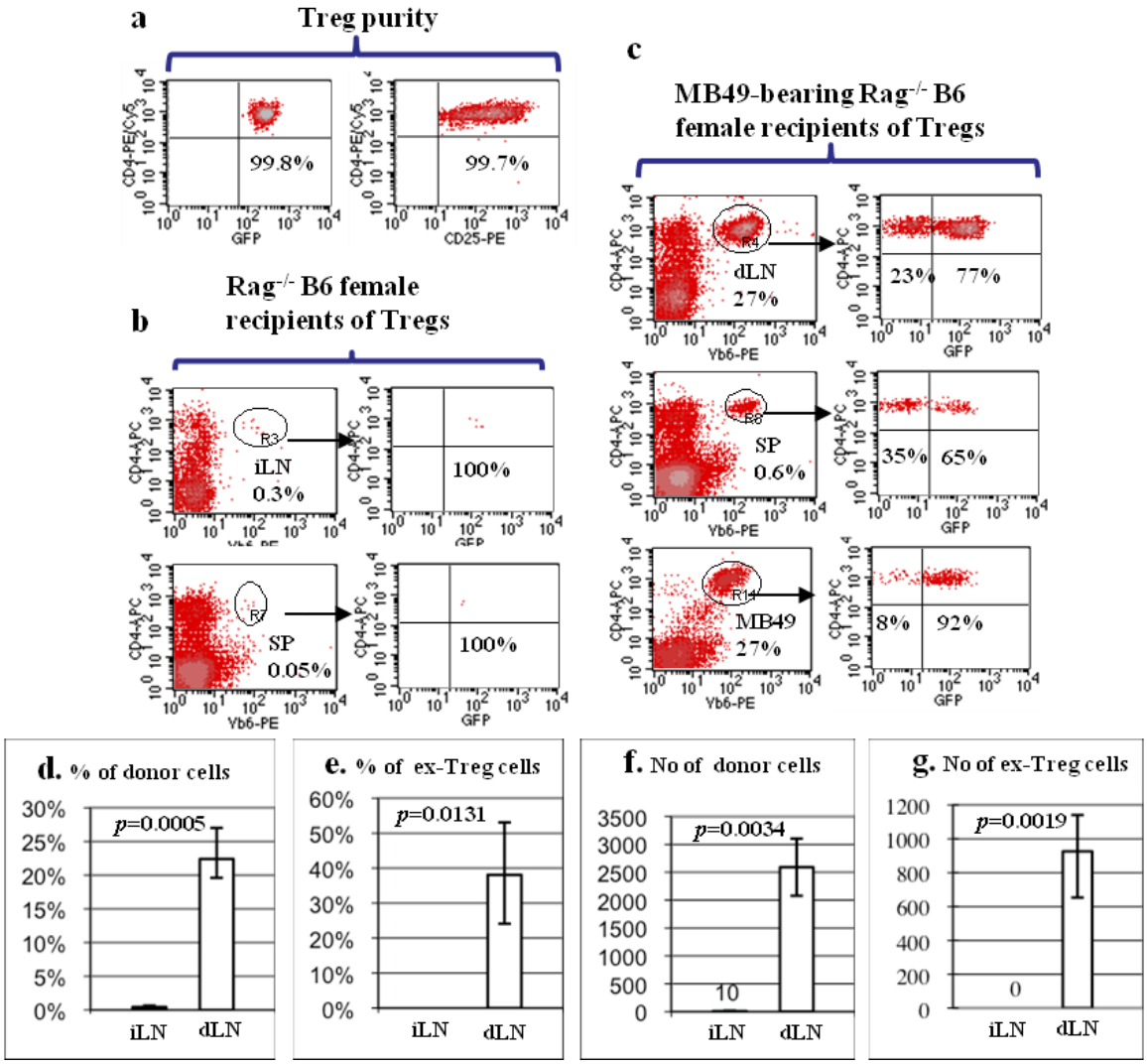


Figure 5.5. **Development of HY-specific ex-Tregs *in vivo***

a. The purity of HY-specific peripheral green Tregs, following FACS sorting, prior to injection.

b and c. Analysis of representation of ex-Tregs *in vivo*. HY-specific green Tregs ($2x10^4$/mouse) were adoptively transferred to MB49-free (**b**) or MB49-bearing Rag2$^{-/-}$ B6 females (**c**) (n=4/group). FACS analysis was conducted on day 12. dLN, spleen (SP) and MB49 tissue cells were stained with anti-Vβ6^{PE}, anti-Thy1.1^{PerCP} and anti-CD4^{APC}. The donor cells were identified by co-expression of Thy-1.1 CD4 and Vβ6 and their FoxP3 expression was presented as CD4 versus GFP. Dot-plots of one representative mouse of each group are shown. One representative experiment of three is shown.

d to g. The percentage (d and e) and absolute cell number (f and g) of donor Tregs (d and f) and donor ex-Tregs (e and g) in iLN of control (black bars) and dLN of MB49-bearing mice (white bars) are shown. All error is represented as ± standard error mean.

5.2.6: Generation of HY-specific ex-Tregs in MB49-bearing Rag$^{-/-}$ B6 female recipients of Marilyn nTregs

Peripheral Tregs isolated from the lymph node and spleen are composed of both nTregs and iTregs, whereas those from thymus are largely, if not exclusively, nTregs (Thornton, Korty et al. 2010). FACS sorted HY-specific thymic green Tregs (Fig. 5.6a) were transferred to MB49-bearing Rag2$^{-/-}$ B6 females. Ex-Treg cells were clearly present in dLN (41%), spleen (67%) and MB49 (23%) on day 12 (Fig. 5.6b), therefore nTregs can be converted into ex-Tregs following *in vivo* activation, recapitulating the behaviour of peripheral Tregs.

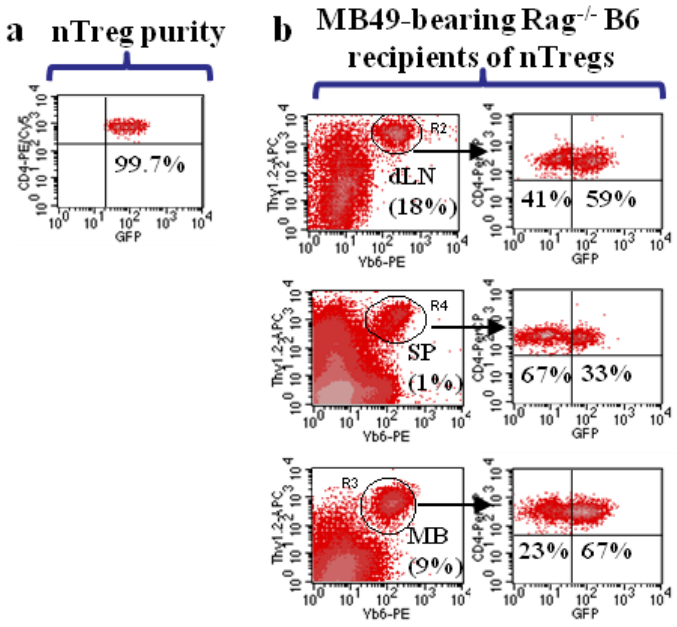


Figure 5.6. Development of HY-specific ex-Tregs from nTregs in MB49-bearing Rag$^{-/-}$ B6 females
a. The purity of HY-specific thymic green nTregs (developed as in Fig. 5.2), following FACS sorting, prior to injection.
b. Analysis of the representation of ex-Treg cells *in vivo*. HY-specific green nTregs (1x10^4/mouse) were transferred to MB49-bearing Rag2$^{-/-}$ B6 females (n=4). Analysis was conducted on day 12 dLN, spleen (SP) and MB49 tissue cells were stained with anti-Vβ6^{PE}, anti-CD4^{PerCP} and anti-Thy1.2^{APC}. The donor cells were identified by co-expression of Thy-1.2 CD4 and Vβ6 and their FoxP3 expression was presented as CD4 versus GFP. FACS dot-plots of one representative mouse of four are shown. One representative experiment of three is shown.

5.2.7: GFP negative Ex-Tregs are FoxP3 negative cells

Lymphoreplete Marilyn female mice are used to generate sufficient quantities of green Tregs (Fig. 5.7a), since they have many more of this population compared to

lymphopenic Marilyn female mice. To confirm ex-Tregs are Tregs that have lost FoxP3 expression, both GFP^+ and GFP^- cells, as shown in Fig. 5.5c, were re-isolated by FACS sorting before staining with anti-$FoxP3^{FITC}$ (Fig. 5.7b). Ex-Treg cells are FoxP3 negative in cells from both types of donor.

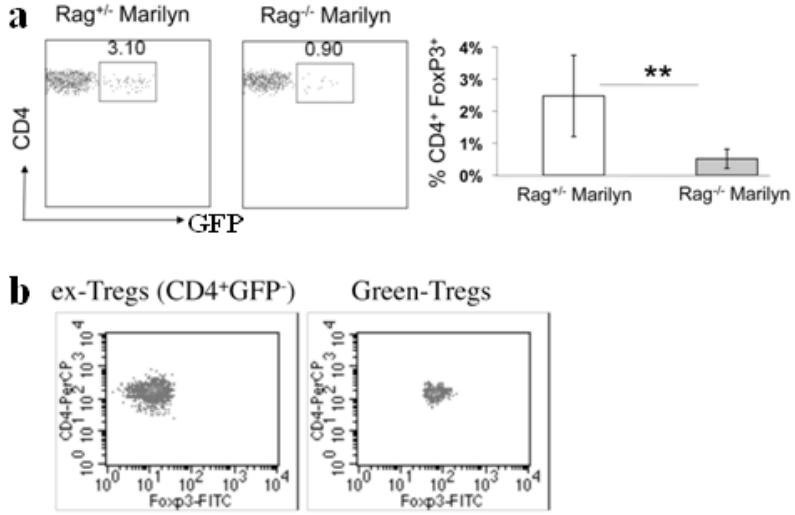


Figure 5.7. Anti-FoxP3 mAb does not produce positive staining for Ex-Tregs
a. PBL from Marilyn females co-expressing $FoxP3^{GFP}$ as a fusion protein on Rag sufficient ($Rag^{+/-}$ Marilyn, n=>40) or Rag deficient background ($Rag^{-/-}$ Marilyn, n=8) were stained with anti-$V\beta6^{PE}$, anti-$CD4^{PerCP}$ and anti-$B220^{APC}$. The frequency of Treg cells is represented by the percentage of GFP^+ cells within gated $CD4^+V\beta6^+$ population. Absence of both $B220^+$ and $CD4^+$ cells indicates the Rag-deficiency and presence of a large fraction of $CD4^+V\beta6^+$ suggesting the phenotype is TCR transgenic. **, $p<0.01$. All error is represented as ± standard error mean.
b. The development and identification HY-specific ex-Treg and unconverted Treg cells were described in Fig. 5.5. After re-isolation by FACS sorting, ex-Treg (CD4+GFP-) (left hand panel) and unconverted green Treg cells (right hand panel) were subsequently subjected to intracellular FoxP3 staining using anti-$FoxP3^{FITC}$ mAb (e-Biosciences) (e-Biosciences fixation buffer causes an abolition of GFP expression).

5.2.8: Development of ex-Tregs is not due to over-expansion of GFP^{-ve} contaminants in the input donor cell population

A contamination experiment was designed to rule out the possibility that the generation of ex-Tregs is caused by selective expansion by contaminating GFP negative conventional CD4 T cells. 1,000 $Thy1.1^+$ HY-specific naïve CD4 T cells were transferred alone or co-transferred with 19,000 $Thy1.2^+$ HY-specific green Tregs to two groups of MB49-bearing $Rag2^{-/-}$ B6 females.

In the group that received conventional CD4 alone, a significant accumulation of donor naïve CD4 T cell progeny was found in the dLN (11%), spleen (2.6%) and MB49 (5.4%) after 12 days, despite the limited number of input cells (Fig. 5.8a). In the presence of co-transferred Tregs however expansion of donor naive CD4 T cells was dramatically impaired, with a 93-99% reduced accumulation in the dLN (0.8%), spleen (0.08%) and MB49 (0.01%) (Fig. 5.8b). A significant reduction was also revealed by quantitative analysis of frequency (Fig. 5.8c) and absolute number (Fig. 5.8d) of donor naïve cells in the dLN of mice that received both naive and Tregs. Naïve CD4 T cells completely failed to expand when co-transferred with excess Tregs, from this evidence it is clear that the emergence of ex-Tregs represents differentiation from donor Treg cells rather than over-expansion of FoxP3 negative contaminants.

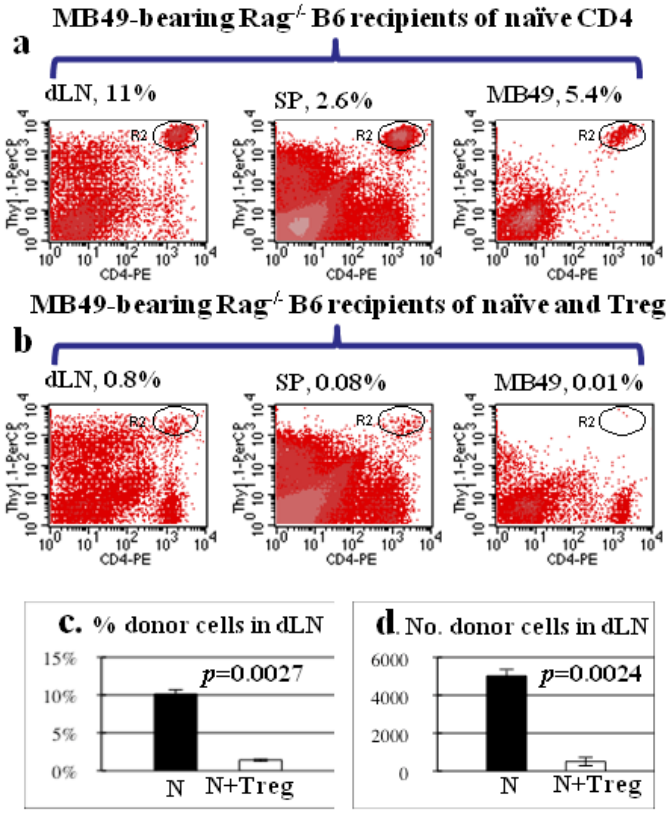


Figure 5.8. Development of ex-Tregs is not due to over-expansion of GFP negative contaminants

a and b. Analysis of donor naïve CD4 T cells *in vivo*. Thy1.1^{+} HY-specific naive CD4 T cells alone (1x10^{3}/mouse), or mixed with Thy1.2^{+} HY-specific green Tregs (19x10^{3}/mouse) were transferred to two groups of MB49-bearing Rag2$^{-/-}$ B6 females (n=4/group). Analysis was conducted on day 12 as described in Figure 5.5. FACS dot-plots of one representative mouse of four are shown. One representative experiment of two is shown.

c and d. The percentage (c) and absolute number (d) of donor naive CD4 T cells in the dLN were compared for both groups. N, naïve alone; N+Treg, co-transfer. All error is represented as ± standard error mean.

5.2.9: Ex-Tregs expand more potently than Tregs in Rag2$^{-/-}$ B6 male mice

A secondary adoptive transfer was performed to compare the function of ex-Tregs and Tregs which retained FoxP3. Tregs and ex-Tregs, generated as described in Fig. 5.5, were re-isolated from MB49-bearing Rag$^{-/-}$ B6 females by FACS sorting with >99% purity (Fig. 5.9a and 5.9b) and separately transferred to two groups of Rag2$^{-/-}$ B6 males. The frequency of donor cells in recipients of ex-Tregs (Fig. 5.9d) was much greater than that in recipients of Tregs on day 28 (Fig. 5.9c). These differences were statistically significant in dLN, mesenteric LN (mLN) and spleen (Fig. 5.9e to 5.9g). In addition, the progeny of donor ex-Tregs showed a higher frequency in mLN but lower in pLN (Fig. 5.9d). Interestingly, a significant fraction of donor ex-Tregs in spleen but not in mLN or pLN re-expressed GFP following the secondary transfer (Fig. 5.9h). These differences were also statistically significant (Fig. 5.9i) elucidating the functional plasticity of these ex-Tregs.

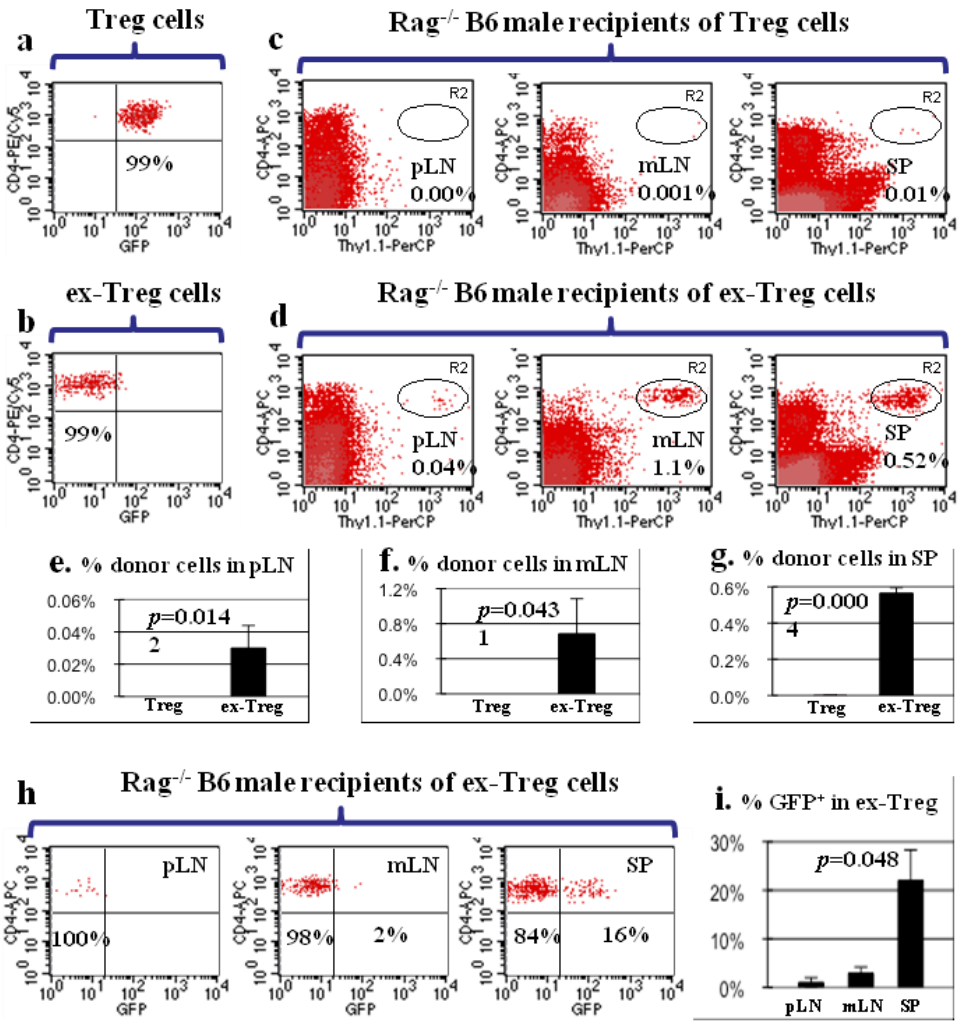


Figure 5.9. **Ex-Tregs are functional effector cells *in vivo***
a and b. Purity of input donor cell populations. Tregs (**a**) and ex-Tregs (**b**) were FACS sorted from spleen and pooled dLN of MB49-bearing Rag2$^{-/-}$ B6 female recipients of HY-specific Tregs at day 12 as described in Fig. 5.5.
c and d. ex-Tregs but not Tregs are responsive to endogenous HY antigens. Tregs and ex-Tregs ($2x10^3$/mouse) were transferred into two groups of Rag2$^{-/-}$ B6 males (3 mice/group). Analysis was conducted at day 28. FACS dot-plots of one representative mouse of three are shown. One representative experiment of two is shown.
e to g. Comparison of the frequency of donor Tregs and donor ex-Tregs in pLN (**e**), mLN (**f**) and spleen (**g**).
h. Re-expression of GFP by ex-Tregs *in vivo*. FACS dot-plots of one representative mouse of three are shown. One representative experiment of two is shown.
i. Comparison of the frequency of donor ex-Tregs which re-gained GFP expression in pLN, mLN and SP. Error is represented as ± Standard error mean.

5.2.10: Ex-Tregs downregulate Treg specific genes and upregulate Th1-like genes

To further characterise the phenotype and function of ex-Tregs, gene expression profiles between ex-Tregs and Tregs were compared. Tregs and ex-Tregs generated as described in Fig. 5.5, from MB49-bearing $Rag^{-/-}$ B6 females, were re-isolated by FACS sorting before analysing gene expression profiles. As expected, *FoxP3* expression was reduced by 10 fold in ex-Tregs which also showed a 116 fold decrease of *Gpr83* (G protein-coupled receptor 83), a Treg-specific surface marker (Sugimoto, Oida et al. 2006; Lu, Gavin et al. 2007). Other Treg-associated genes including *Ctla4* (CTLA4) (Wing, Onishi et al. 2008), *Folr4* (FR4) (Yamaguchi, Hirota et al. 2007), *Nt5e* (CD73) (Deaglio, Dwyer et al. 2007), and *Ccr7* (CCR7) (Menning, Hopken et al. 2007) were also downregulated (Fig. 5.10a). Conversely, ex-Treg cells displayed a significant increase of *Ifnγ* (IFNγ), *Cd80* (CD80), *Gzmb* (Granzyme B) and *Gzma* (Granzyme A) (Fig. 5.10b). To a lesser extent, Th1 cytokine genes including *Csf2* (GM-CSF), *Tnfα* (TNFα) and *IL-2* (IL-2) were also moderately but significantly upregulated in ex-Treg cells. Several Treg-related genes including *Runx1* (Kitoh, Ono et al. 2009; Klunker, Chong et al. 2009) and *Gal1* (Galectin 1) (Garin, Chu et al. 2007) were maintained in ex-Treg cells, indicating that re-differentiation was incomplete and possibly reversible.

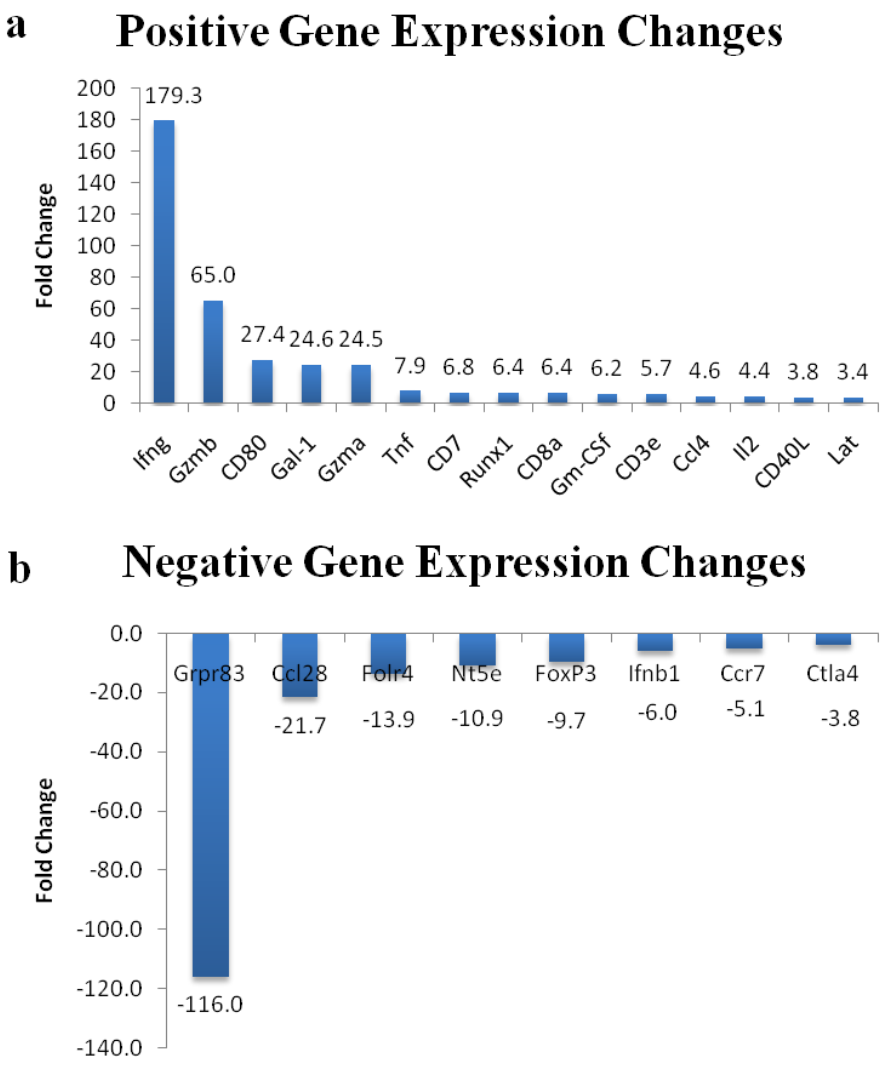


Figure 5.10. **Comparison of gene expression profiles between Tregs and ex-Tregs**

a and b. Tregs and ex-Treg cells, sorted from pooled dLN and SP cells of MB49-bearing Rag2$^{-/-}$ B6 female recipients of HY-specific green Tregs at day 12, were subjected to RNA extraction and cDNA conversion before running qPCR array assay using mouse Treg Phenotyping 383 StellARray^{TM} plates. 8 genes were downregulated (**b**) and 15 genes up-regulated (**a**) in ex-Tregs compared with Tregs.

5.2.11: Validation of qPCR data by FACs analysis

To confirm if ex-Tregs express more IFNγ than Tregs with sustained FoxP3 at protein level, an intracellular staining approach was used. MB49-bearing Rag2$^{-/-}$ B6 female recipients of HY-specific green Treg cells were prepared as described in Fig. 5.5. On day12, dLN cells were subjected to a standard intracellular IFNγ staining procedure following a brief *in vitro* re-stimulation (Fig. 5.11a). The

frequency of IFNγ-producing cells in ex-Tregs was significantly higher than that in Tregs. The mean fluorescence intensity (MFI) of IFNγ in ex-Tregs (304) was also higher than that in Tregs (173), thus overall, ex-Tregs produced more IFNγ than Tregs. Neither IL-4 nor IL-17 was detectable in this experiment (Fig. 5.11b), indicating that Tregs differentiated into Th1-like but not Th2 or Th17-like cells. Using FACS analysis, it was also confirmed that expression of both FR4 and CCR7 was significantly reduced on ex-Tregs (Fig. 5.11b), compared with Tregs.

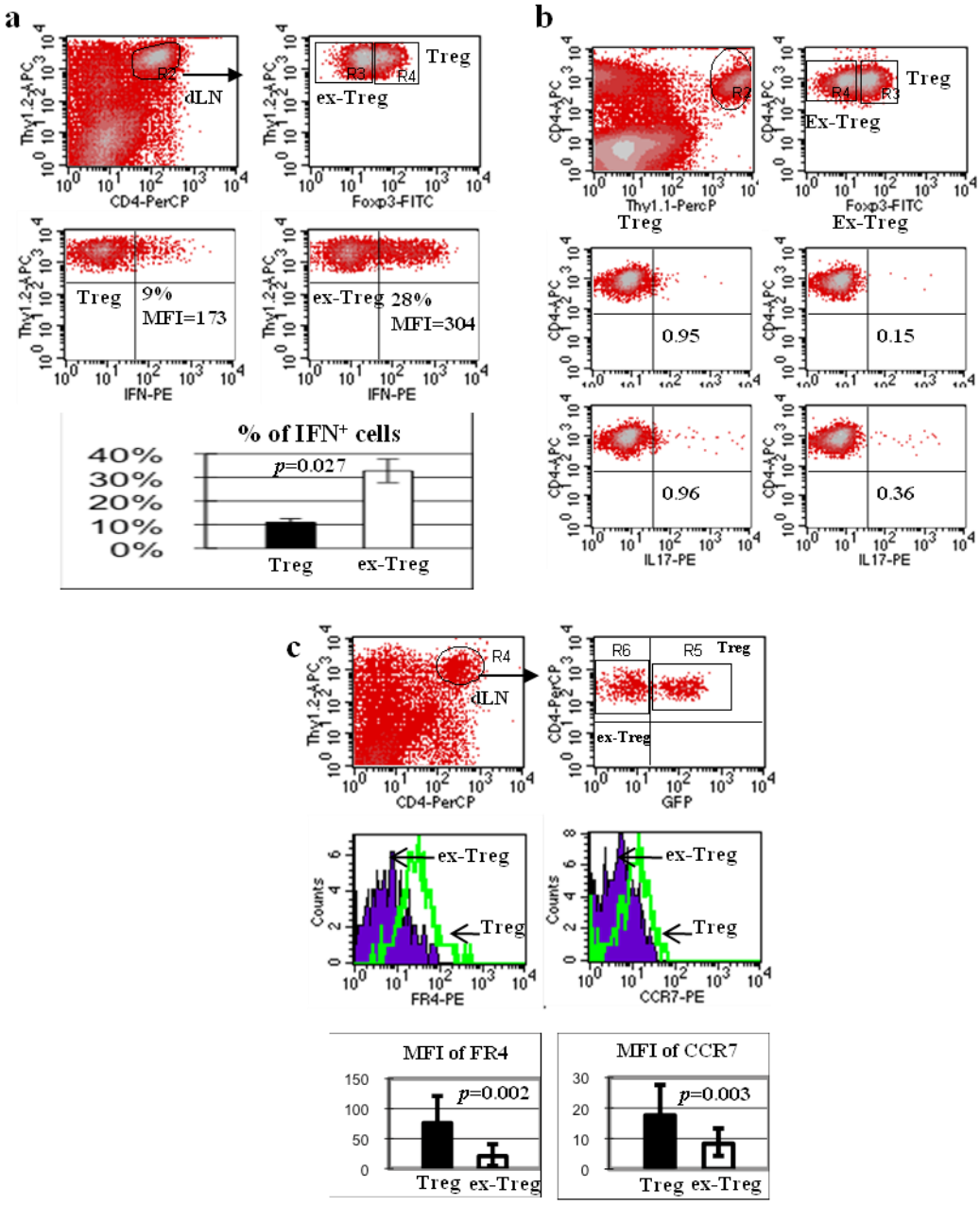


Figure 5.11. Comparison of IFNγ, FR4 and CCR7 expression between Tregs and ex-Tregs by FACS analysis

a. HY-specific green Tregs were transferred to MB49-bearing Rag2$^{-/-}$ B6 females (n=4). 12 days later, dLN cells were briefly stimulated with PMA (50ng/ml) and

Ionomycin (250ng/ml) in the presence of Brefeldin A (1μg/ml) before surface staining for CD4 and Thy1.2. After fixation and permeabilisation, the cells were stained for FoxP3 and IFNγ. Donor cells were further divided into Tregs (R4) and ex-Tregs (R3). Both the frequency and MFI of IFNγ-positive cells in Tregs (black bars) and ex-Tregs (white bars) were measured. FACS dot-plots of one representative mouse of four are shown. One representative experiment of two is shown.
b. Staining conducted as described in 'a', except that two additional anti-IL4^{PE} and anti-IL17^{PE} were also utilized for intracellular staining.
c. HY-specific green Tregs (Thy1.2) were transferred to MB49-bearing Rag2$^{-/-}$ B6 females (n=3). 12 days later, dLN cells were stained with anti-FR4^{PE} (or anti-CCR7^{PE}), anti-CD4^{PercP} and Thy1.2^{APC}. Donor Treg cells identified by CD4^{+}Thy1.2^{+} were distinguished as Treg (R5) or ex-Tregs (R6) based on GFP expression. Expression of FR4 and CCR7 by Tregs (open area) and ex-Tregs (closed area) are shown as histograms. FACS profiles of one representative mouse of three are shown. One representative experiment of three is shown. MFI of FR4 and CCR7 by Tregs (black bars) and ex-Tregs (white bars) were measured. Error is represented as ± Standard error mean.

5.2.12: HY-specific green Tregs preferentially differentiate into ex-Tregs in the mesenteric lymph nodes of Rag$^{-/-}$ B6 male mice

The mechanisms of the differentiation of ex-Tregs were examined in the second model in which HY-specific green Tregs were adoptively transferred into Rag2$^{-/-}$ B6 males, i.e. hosts expressing the cognate antigen endogenously on all somatic tissues. Surprisingly, a complete ex-Treg conversion was seen in mLN (98%) but not in pLN (24%) or spleen (25%) on day 14 (Fig. 5.12a and 5.12b). When mLN cells were compared with pLN for cytokine/chemokine/receptor gene expression, 7 genes showed significant up-regulation (Fig. 5.12c), of which IL6 mRNA displayed an over 300 fold increase. A moderate increase of IL-1 receptor antagonist, CXCL10, IL-10, CXCR4 and IL-21 was also observed.

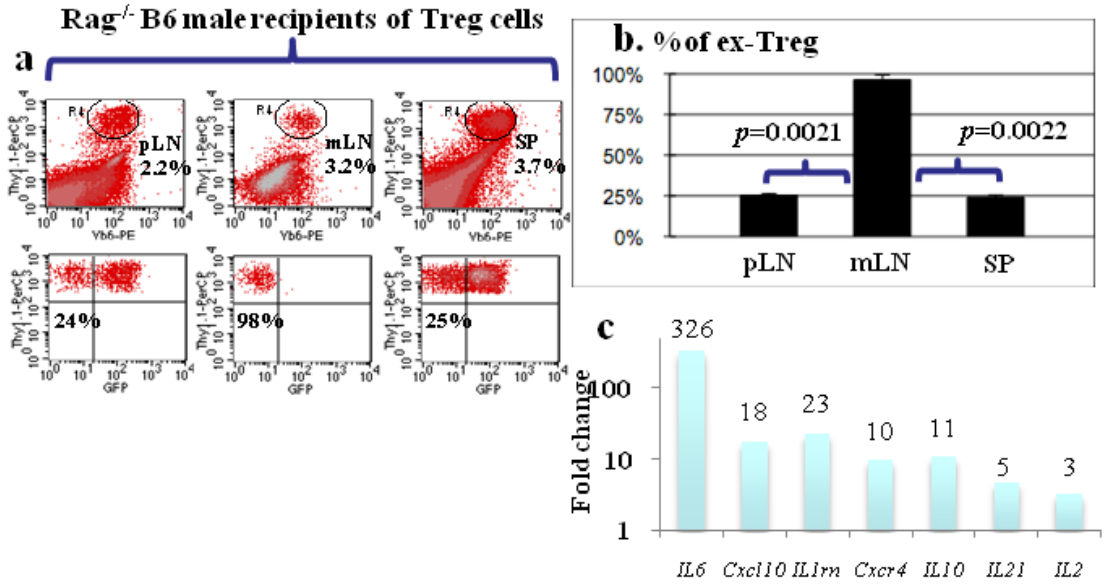


Figure 5.12. Microenvironment of mLN in Rag2$^{-/-}$ B6 male mice favours ex-Treg development

a. HY-specific green Tregs (Thy1.1) (1 × 10^4/mouse) were transferred to Rag2$^{-/-}$ B6 male mice (n = 4). Analysis was conducted on day 14 by staining of pLN, mLN, or spleen cells with anti-Vβ6^{PE}, anti-Thy1.1^{PerCP}, and anti-CD4^{APC}. Donor Tregs identified as Vβ6$^+$ Thy1.1$^+$ and analysed for FoxP3 expression. FACS profiles of one representative mouse of four are shown. One representative experiment of three is shown.

b. Comparison of the frequency of ex-Tregs in various tissues. Error is represented as ± Standard error mean.

c. Comparison of gene expression profiles between pLN and mLN. pLN and mLN cells from a Rag2$^{-/-}$ B6 male mouse were subjected to RNA extraction and cDNA conversion before running qPCR array assay using mouse cytokine/ chemokine/ receptor 383 StellARray plates. One representative experiment of two is shown.

5.2.13: Regulation of ex-Treg differentiation by naive CD4 T cells

To investigate whether HY-specific naïve CD4 T cells could influence HY-specific ex-Treg development in Rag2$^{-/-}$ B6 male recipients, a co-transfer experiment was performed. As shown in Fig. 5.13a and 5.13b, the co-transfer of naive CD4 T cells led to a striking reduction (~80%) of ex-Treg frequency in the mLN. A modest but significant decrease (~40%) of ex-Treg frequency was also seen in pLN but not in the spleen. Feedback from naïve CD4 T cells seems to prevent the loss of FoxP3 by Tregs. The presence of co-transferred HY-specific naïve CD4 T cells did not increase the frequency of donor Tregs, indicating that the promotion of Treg expansion does not represent the major feedback pathway.

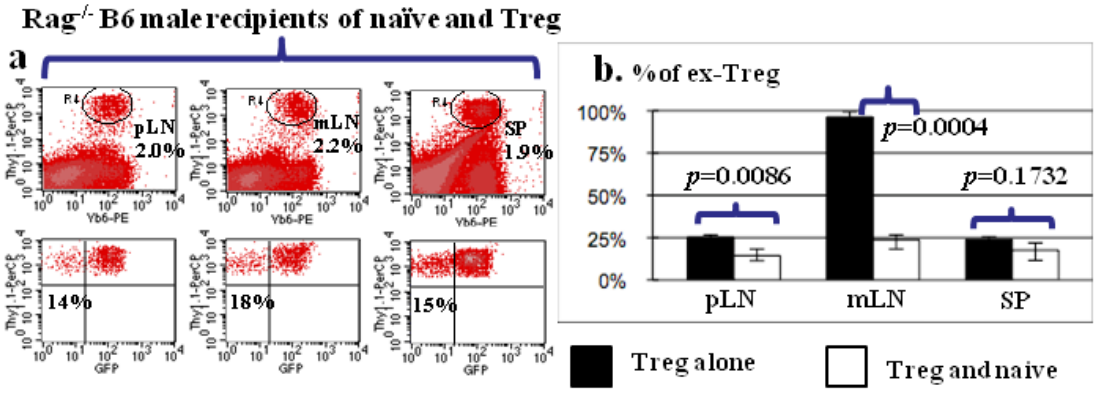


Figure 5.13. Co-transfer of naive CD4 inhibits FoxP3 loss and ex-Treg generation

a. Inhibition of ex-Treg differentiation by co-transferred naive CD4 cells. HY-specific green Tregs (Thy1.1) (1×10^4/mouse) mixed with an equal number of HY-specific naive CD4 T cells (Thy1.2) were transferred to Rag2$^{-/-}$ B6 male mice ($n = 4$). Analysis was conducted on day 14, as described in Fig. 5.9a. FACS profiles of one representative mouse of four are shown. One representative experiment of three is shown.

b. Comparison of the frequency of ex-Tregs in the absence (black bars) or presence of co-transferred naive CD4 T cells (white bars). Error is represented as ± Standard error mean.

5.2.14: Tregs regulate HY-specific naive CD4 expansion

To evaluate the suppressive function of HY-specific Tregs in this second model of ubiquitous somatic cell antigen expression, a co-transfer experiment was set up to measure naive CD4 cell expansion in the presence of Tregs in a 1:1 ratio (Fig. 5.14). In the absence of HY-specific Tregs, naive CD4 T cells expanded in a typical manner until day 15 before contracting (Fig. 5.14a). However in the presence of Tregs, this response was suppressed, with reduced expansion of naive CD4 cells (Fig. 5.14a). In parallel experiment, pLN, mLN and spleen were analysed for the representation of conventional donor CD4 T cells on day 14. In all peripheral lymphoid organs tested, the expansion of naive CD4 T cells was reduced when Tregs were co-transferred (Fig. 5.14b), although in the mLN this impaired expansion was not significant, possibly due to reduced suppression induced by the increased generation of ex-Tregs (Fig. 5.12). This result suggest that while the presence of naïve CD4 T cells prevents loss of FoxP3 by Tregs, and co-presence of Tregs also reduces the ability of naive CD4 T cells to expand in respond to antigen.

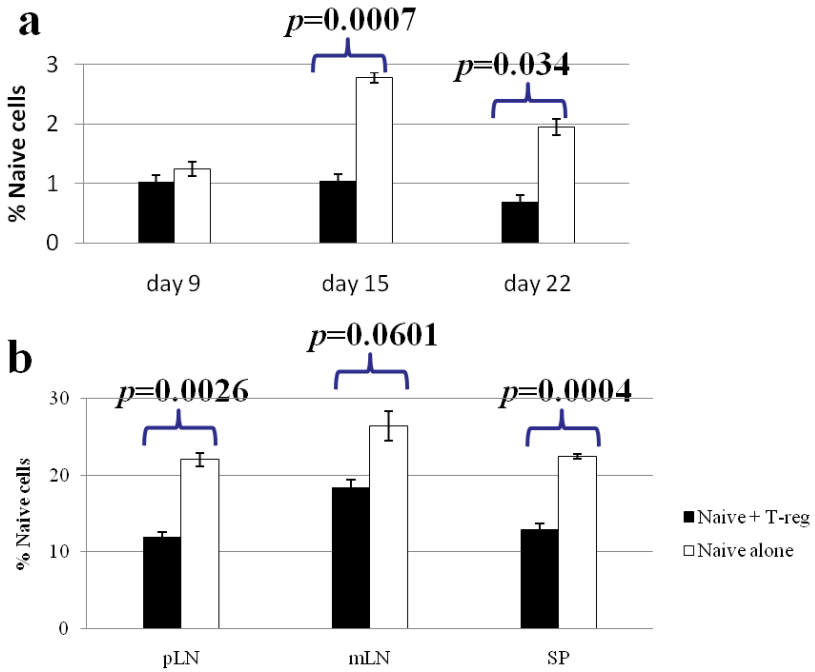


Figure 5.14. Co-transferred Tregs inhibit antigen-specific naive CD4 T cell responses *in vivo*

Comparison of the frequency of naïve CD4 T cells in the absence or presence of co-transferred Tregs.

a. HY-specific green Treg cells (Thy1.1) ($1x10^4$/mouse) alone or co-transfer with HY-specific naive CD4 T cells were transferred to $Rag2^{-/-}$ B6 males (n=3/group) (naive alone white bars, co-transfer black bars). Analysis was conducted by PBL day 9, 15 and 22. Cells were lysed and stained using anti-$CD4^{PE}$, anti-$Thy1.1^{PerCP}$ and anti-$Thy1.2^{APC}$. Naive cells were identified as GFP- CD4+ Thy1.2+, Tregs as GFP+ CD4+ Thy1.2-. Error is represented as ± Standard error mean.

b. In a parallel experiment pLN, mLN, or SP were analysed on day 14 (n=3/group).

5.2.15: Modulation of ex-Treg development by co-transferred naïve CD4 T cells in MB49-bearing $Rag2^{-/-}$ female hosts

To explore whether the conclusions obtained from experiments using $Rag2^{-/-}$ B6 male recipients would also be applicable to other HY models, HY-specific green Tregs (Thy1.2) were transferred with or without HY-specific naive CD4 T cells (Thy1.1) to MB49-bearing $Rag2^{-/-}$ B6 females. In the absence of naïve CD4 T cells, Tregs expanded and some of them became ex-Tregs during the course of antigen recognition (Fig. 5.15a), thus confirming previous data as shown in Fig. 5.5c. In the presence of naive CD4 T cells, the accumulation of Tregs in the dLN and tumour mass was reduced (Fig. 5.15b), indicating that naive CD4 T cells do not modulate Tregs by promoting their expansion. Conversely, the development of ex-Tregs in MB49 tissues was significantly impaired when naive CD4 T cells co-existed,

suggesting that naive CD4 T cells can regulate Tregs by preventing the loss of FoxP3. Taken together, data from two different models demonstrate that naive CD4 T cells can protect Tregs with the same antigen-specificity from losing FoxP3 *in vivo*, defining a previously unrecognised feedback pathway which acts to maintain a functional Treg population.

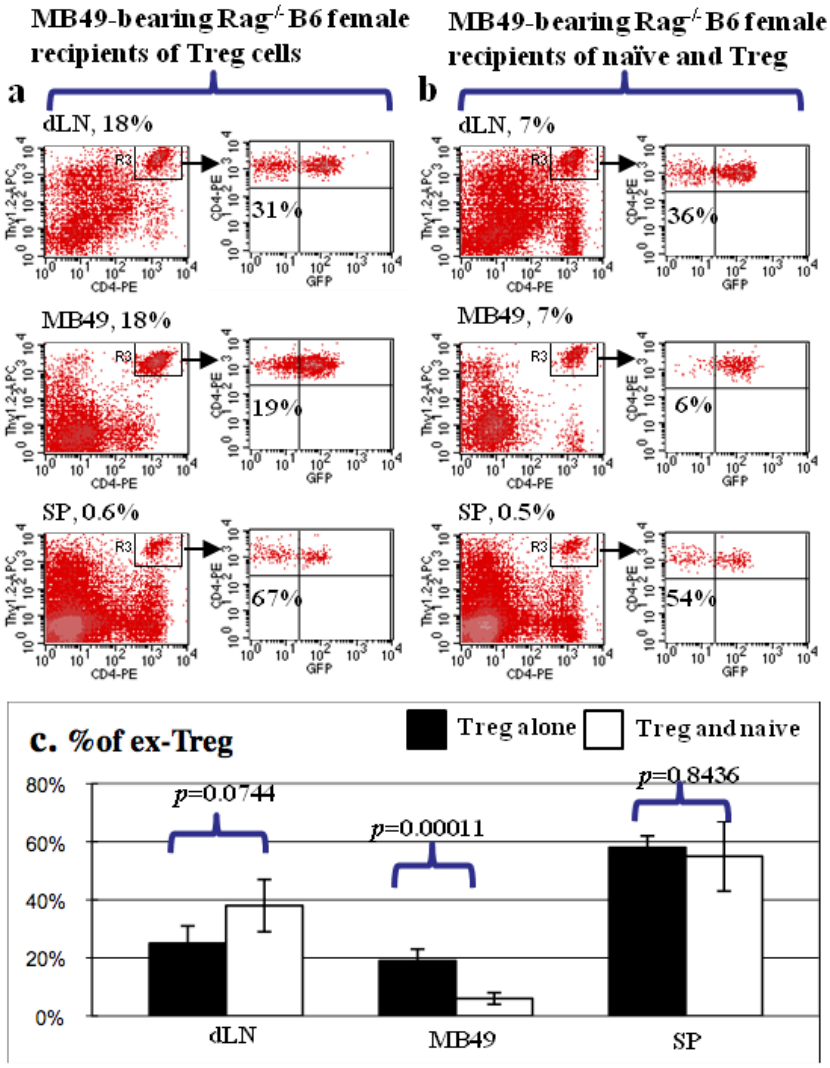


Figure 5.15. Development of ex-Tregs in MB49-bearing female Rag2$^{-/-}$ mice was impaired by co-transferred naïve CD4 T cells

a and b. HY-specific green Treg (Thy1.2) (1x10^4/mouse) alone (a) or mixed with an equal number of HY-specific naive CD4 T cells (Thy1.1) (b) were transferred to two groups of MB49-bearing Rag2$^{-/-}$ B6 females (n=4). Analysis was conducted on day 12 by staining of dLN, MB49, or SP cells with anti-CD4^{PE}, anti-Thy1.1^{PerCP} and anti-Thy1.2^{APC}. Donor Treg cells identified as CD4$^+$Thy1.2$^+$ and were analysed for FoxP3. FACS profiles of one representative mouse of four are shown. One representative experiment of three is shown.

c. Comparison of the frequency of ex-Treg cells in the absence (black bars) or presence of naive CD4 T cells (white bars). Error is represented as ± Standard error mean.

5.3: Discussion

This chapter describes the establishment of an *in vivo* model in which the fate of tumour antigen-specific adoptively transferred Tregs in tumour-bearing mice can be studied. $CD25^{+}$ Tregs from HY-specific TCR transgenic Marilyn females were found to prevent expansion of conventional Marilyn CD4 T cells in response to MB49 tumours (Fig. 5.1). How could this minor population of Treg cells (5%) have such a huge impact on a large population of conventional CD4 T cells (95%)? One possible explanation is that Treg cells undergo a rapid expansion in response to cognate antigen released from tumour. Indeed, polyclonal Tregs from a WT B6 female did not expand in response to MB49 (Fig. 5.2e) while Marilyn Tregs showed a significant expansion in MB49-bearing females, both in terms of frequency (Fig. 5.2b) and absolute cell number (Fig. 5.2b). Unlike other reports that rely on viral vaccination to detect Treg in tumour (Zhou, Drake et al. 2006; Zhou and Levitsky 2007; Sharma, Hou et al. 2010), in this model, Tregs were readily detected without any boost in dLN and tumour (Fig. 5.2). The use of endogenous (MB49) and transfected (B16/HY) HY antigen expressing tumour lines has demonstrated that HY-specific Tregs can be induced to proliferate in dLN and subsequently migrate into tumours *in vivo* (Fig. 5.3).

In the course of characterising the Treg cellular response to tumour, it was noticed that a proportion of proliferating Treg cells lost FoxP3 (Fig. 4.12, 5.2 and 5.3). To expand this model, Marilyn Treg cells were sorted and used for an adoptive transfer experiment to MB49-bearing B6 female recipients (Fig. 5.4). This showed significant loss of $FoxP3^{GFP}$ expression in tumour-bearing mice. However weak expansion of Treg in WT hosts (0.024% of dLN) is not particularly sensitive. Therefore in order to derive sufficient quantities of ex-Tregs lymphopenic hosts were used for the remainder of the study.

The downregulation of FoxP3 in polyclonal Treg subsets *in vivo* has been observed previously (Zhou, Bailey-Bucktrout et al. 2009; Sharma, Hou et al. 2010) and was also noted by our group in an *in vitro* culture system in which $FoxP3^{GFP}$ polyclonal Tregs lost GFP expression upon repeated *in vitro* expansion (Chai, Coe et al. 2008). For the purposes of this study, $Rag^{-/-}$ B6 mice were used as recipients of HY-specific green Tregs, which were purified from F1 (B6 $FoxP3^{GFP}$ x Marilyn) female mice. This is in contrast with a study by Rubtsov *et al.* (Rubtsov, Niec et al.

2010), in which FoxP3$^{GFP\text{-}Cre\text{-}ERP2}$ x ROSA26^{YFP} mice were used as a model of Treg differentiation, this group demonstrated FoxP3 stability over 5 months. The different conclusions with regard to FoxP3 stability are likely to be due to the use of a non-inflammatory homeostatic system.

FoxP3 expression by ex-Tregs was examined by intracellular staining (FACS sorted from MB49-bearing mice, based on the loss of GFP expression), before the secondary transfer (as shown in Fig. 5.9) and qRT-PCR analysis (as shown in Fig. 5.10). These GFP negative ex-Tregs had lost mRNA FoxP3 expression and staining with an anti-FoxP3 mAb was also negative, whereas positive FoxP3 staining was seen in the sorted GFP$^+$ population (Fig. 5.7b). Therefore, it can be confirmed that Tregs which lose GFP are indeed FoxP3 negative, consistent with RT-PCR analysis showing a significant downregulation (10-fold) of FoxP3 mRNA expression in ex-Tregs compared with that in unconverted green Tregs (Fig. 5.10a).

In the first part of this investigation HY-specific ex-Tregs (Fig. 5.5-5.7) that have developed in an *in vivo* tumour model (Fig. 5.5) were characterised. The possibility that these represent over-expansion of contaminating conventional CD4 cells (Fig. 5.8) was excluded. This work has several novel features: This is the first study to explore the plasticity of antigen-specific Tregs in the context of tumour (Fig. 5.5), extending previous studies using polyclonal Tregs in either homeostatic (Chai, Coe et al. 2008; Yang, Nurieva et al. 2008; Coe, Begom et al. 2010) or autoimmune settings (Chai, Coe et al. 2008; Tsuji, Komatsu et al. 2009). Secondly, analysis of gene expression is more comprehensive than in previous studies in which only a very limited number of genes were considered (Fig. 5.10) (Chai, Coe et al. 2008; Coe, Addey et al. 2010). Thirdly, this is the first report showing that HY-specific green Tregs sorted from thymus (Fig. 5.2, 5.3, 5.6) are also cable of differentiating into ex-Tregs in response to HY-expressing MB49 tumours, thus providing direct evidence that nTregs can be the precursors of ex-Tregs. Finally, novel mechanisms were found that are involved in the regulation of ex-Treg development *in vivo*.

HY-specific green Tregs (CD4$^+$Vβ6$^+$CD25$^+$GFP$^+$) are capable of differentiating into ex-Tregs in both MB49-bearing Rag$^{-/-}$ B6 females (Fig. 5.5c and Fig. 5.15a) and Rag$^{-/-}$ B6 males (Fig. 5.12a), demonstrating that FoxP3 expression in CD25$^+$ Treg subset is unstable after *in vivo* activation. These results are consistent with another report showing that a fraction of islet auto antigen-specific CD4$^+$GITR^{hi}GFP$^+$ cells (of which a vast majority are CD25$^+$) lose FoxP3 after

transfer to TCR$\alpha^{-/-}$ NOD mice (Zhou, Bailey-Bucktrout et al. 2009), but appears to conflict with another study suggesting that the plasticity of Tregs is exclusive to the CD25 negative Treg subset (Komatsu, Mariotti-Ferrandiz et al. 2009). Failure to detect ex-Tregs in $Rag^{-/-}$ B6 recipients of $CD25^+$ Treg cells by Komatsu et al (Komatsu, Mariotti-Ferrandiz et al. 2009) could be because the test was carried out only at an early time point (day 5), and presumably only pLN (whose environment is less inflammatory than that in mLN) was analysed. At the later time point on day 28, 90% of donor $CD4^+FoxP3^+$ Tregs (of which a vast majority are $CD25^+$) became FoxP3 negative when recovered from epithelial lymphocytes (Duarte, Zelenay et al. 2009) and Peyer's patches (Tsuji, Komatsu et al. 2009). The cytokine milieu in a Proinflammatory environment can promote ex-Treg development. Given IL-2 is critical to maintain stable expression of FoxP3 in Tregs (Fontenot, Rasmussen et al. 2005), it is understandable why the CD25 negative Treg subset is more susceptible to FoxP3 loss (Komatsu, Mariotti-Ferrandiz et al. 2009), however, it does not necessarily indicate that CD25 negative Treg subset is the sole population from which ex-Tregs are generated. Upon homeostatic expansion in lymphopenic mice, $CD25^+$ Tregs lose CD25 expression (Gavin, Clarke et al. 2002) and conversely $CD25^-$ Tregs regain CD25 expression *in vivo* (Zelenay, Lopes-Carvalho et al. 2005).

Here nTregs are shown to be able to differentiate to ex-Tregs (Fig. 5.2, 5.3 and 5.6). Previous studies have exclusively used peripheral Tregs (Yang, Nurieva et al. 2008; Duarte, Zelenay et al. 2009; Komatsu, Mariotti-Ferrandiz et al. 2009; Tsuji, Komatsu et al. 2009; Zhou, Bailey-Bucktrout et al. 2009), containing both nTregs and iTregs, which have differential Helios expression (Thornton, Korty et al. 2010). As a transcription factor, Helios has limited value in separating the two subsets of peripheral Tregs. The use of thymic green Tregs as an alternative strategy provides direct evidence that *in vivo*, nTregs can be the precursors of ex-Tregs.

In this model, ex-Tregs have characteristics of Th1 rather than Th17 or Th2 cells (Fig. 5.11a and c). This result is supported in a study by Zhou et al. (Zhou, Bailey-Bucktrout et al. 2009) showing that polyclonal ex-Tregs in the lymph node produced IFN-γ but not IL-17, whereas those in Peyer's patches made both cytokines. The cytokine profile of ex-Tregs therefore depends on the location and environment in which they were generated. The ex-Treg population appears to be heterogeneous. qPCR analysis has shown that expression of some Treg related genes, including *Runx1* (Kitoh, Ono et al. 2009; Klunker, Chong et al. 2009) and

galectin 1 (Garin, Chu et al. 2007), are maintained in ex-Tregs (Fig. 5.10). Upon secondary transfer to $Rag2^{-/-}$ B6 male mice, some ex-Tregs in spleen but not lymph node became $FoxP3^{+}$ (Fig. 5.9h), suggesting that re-expression of FoxP3 also depends on the local tissue environment. FoxP3 re-expression by polyclonal ex-Tregs has been reported (Komatsu, Mariotti-Ferrandiz et al. 2009), although it is unclear whether ex-Tregs in the spleen are more likely than those in lymph node to regain FoxP3 expression.

Treg conversion in lymphoreplete hosts appears to be less efficient than that in lymphopenic hosts (Fig. 5.2 and 5.4) (Duarte, Zelenay et al. 2009; Komatsu, Mariotti-Ferrandiz et al. 2009; Zhou, Bailey-Bucktrout et al. 2009). This raises concerns over the biological significance of the conversion of Tregs *in vivo*; nevertheless, Tregs can be converted to ex-Tregs under non-lymphopenic conditions. Interestingly, this conversion takes place mainly in inflamed tissues expressing antigen, including the MB49 tumour (Fig. 5.2 and 5.4) (Coe, Addey et al. 2010) and the pancreas of prediabetic NOD mice (Zhou, Bailey-Bucktrout et al. 2009). Conversion of Tregs takes place in the MB49 microenvironment despite immunosuppressive gene expression (Fig. 3.4, 3.13) with production of IDO and TGF-β into the local microenvironment (Fig. 4.11). From such studies, it has been proposed that the conversion of Tregs under physiological conditions could contribute to the development of autoimmune diseases and cancer surveillance. In addition, the generation of effector like ex-Tregs at sites of infected tissue might help initiate viral clearance (Zhou, Bailey-Bucktrout et al. 2009). Exploration of the biological relevance of Treg conversion in WT mice requires robust model systems and novel approaches. A commonly used strategy is to perform a secondary adoptive transfer of ex-Tregs to lymphopenic hosts, which has been used by us in the current study and by others (Komatsu, Mariotti-Ferrandiz et al. 2009; Tsuji, Komatsu et al. 2009; Zhou, Bailey-Bucktrout et al. 2009).

The second part of this study explores the cellular events controlling the development of ex-Tregs. There are three key observations. The first is that the microenvironment of the mesenteric but not peripheral lymph node favours the generation of ex-Tregs (Fig. 5.12a and b). A subsequent comparison of gene expression profiles revealed that *IL6* mRNA shows a significant increase (>300-fold) in the mesenteric lymph node compared with peripheral lymph node (Fig. 5.12c). IL-6 alone can induce downregulation of FoxP3 expression by TCR-stimulated

Tregs, which can be further promoted by the presence of IL-1 and IL-21 (Xu, Kitani et al. 2007; Yang, Nurieva et al. 2008; Sharma, Hou et al. 2009; Tsuji, Komatsu et al. 2009). IL-6 induces re-methylation of the FoxP3 nonintronic upstream CpG-rich island, closing the chromatin structure of the FoxP3 locus (Lal, Zhang et al. 2009). Higher IL-6 expression in the mesenteric lymph node provides a mechanistic basis for increased development of ex-Tregs in this tissue, which extends previous studies showing that inflammatory environments such as gut-associated tissues (Duarte, Zelenay et al. 2009; Tsuji, Komatsu et al. 2009) and islets of NOD mice (Zhou, Bailey-Bucktrout et al. 2009; Zhou, Bailey-Bucktrout et al. 2009) can facilitate the development of ex-Tregs.

It is of interest to test whether ex-Tregs that resulted from *in vivo* conversion would have pathological consequences, such as the development of colitis. However, there is a concern that the activities of ex-Tregs would be limited by the coexistence of non-converted Tregs, especially in the peripheral lymph node where the Treg conversion was incomplete. Another concern is that the use of HY-specific Tregs is the main focus in the current investigation, whereas the development of colitis in B6 mice appears to require a polyclonal CD4 T cell response. To explore whether ex-Tregs can induce a pathological response *in vivo*, unconverted Tregs would need to be removed as described earlier. The effector potential of ex-Tregs can be assessed by their anti-MB49 immunity or the development of graft-versus-host disease (GvHD) after transfer to irradiated male recipients, which has been seen after adoptive transfer of conventional Marilyn CD4 T cells (See chapter 7). It is also of interest to use $Rag^{-/-}$ IL6 KO mice to examine whether these hosts fail to convert the Tregs.

The second novel observation is that HY-specific naive CD4 T cells can partially prevent ex-Treg development *in vivo* (Fig. 5.13 and 5.15). Similar results have been reported by others using polyclonal Tregs (Almeida, Zaragoza et al. 2006; Duarte, Zelenay et al. 2009). Although the precise molecular mechanisms for regulating FoxP3 expression in Tregs by co-transferred naive CD4 cells are still elusive, involvement of cytokines including IL-2 and TGF-β have been suggested. Duarte *et al.* showed that lymphopenia induced loss of FoxP3 by polyclonal Tregs was accelerated by anti–IL-2 treatment but prevented by IL-2 administration (Duarte, Zelenay et al. 2009). Similarly, IL-2 treatment can restore CD25 expression and Treg function in inflamed islet of NOD mice (Tang, Adams et al. 2008). TGF-β

produced by Tregs is required for maintaining FoxP3 expression and protection from apoptosis (Li, Sanjabi et al. 2006).

Finally, in a converse situation, HY-specific Treg cells can partially inhibit HY-specific naive CD4 expansion upon co-transfer to $Rag^{-/-}$ B6 male mice (Fig. 5.14). This result indicates a cyclical feedback pathway between antigen-specific Tregs and conventional CD4 cells preventing outgrowth of CD4 cells. These results are mirrored in the development of GvHD in irradiated FoxP3-DTR (diphtheria toxin receptor) male mice that receive 5Gy, conventional CD4 cells and diphtheria toxin to remove Tregs. Without Treg removal GvHD is inhibited, however, removal of Tregs can induce lethal GvHD (see chapter 7 Figure 7.11)

What are the implications of this study? Firstly, some features of Tregs revealed by previous studies might warrant alternative interpretations due to the instability of FoxP3 expression. For example, in a vaccination model, Tregs act as effector cells in lymphopenic hosts (Vendetti, Davidson et al. 2010); Tregs secrete granzyme B and/or perforin to kill DC, CD8, and NK cells in tumour dLN (Boissonnas, Scholer-Dahirel et al. 2010) or in tumour tissues (Cao, Cai et al. 2007); and Tregs preferentially expand in response to cancer vaccines (Zhou, Drake et al. 2006). Secondly, there is concern over the use of Treg adoptive therapy in autoimmune diseases (Zhou, Bailey-Bucktrout et al. 2009; Zhou, Bailey-Bucktrout et al. 2009). Because IL-6 plays a key role in converting Tregs to ex-Tregs (Xu, Kitani et al. 2007; Yang, Nurieva et al. 2008; Sharma, Hou et al. 2009), the efficiency of adoptive Treg therapy may be enhanced by blockade of IL-6 signalling pathways. Finally, the promotion of ex-Treg development in the context of tumours can be of therapeutic benefit.

Chapter 6: Genetic modification of MB49 with recombinant human IL-2 as a novel *in vivo* vaccine approach

6.1: Introduction

6.1.1: IL-2 and T cells

Interleukin-2 as a soluble factor was known to immunology many years before its use for tumour therapy: IL-2 was one of the first endogenous mitogenic factors for lymphocytes described. In 1965 two reports described a 'blastogenic factor' in the culture supernatant of stimulated lymphocytes that induced their proliferation (Gordon and MacLean 1965; Kasakura and Lowenstein 1965). Over the next 15 years many reports described mitogenic factors in lymphocyte conditioned media. A quantitative assay was developed based on the ability for this T cell Growth factor to promote the long-term survival and proliferation of T cells in culture (Gillis, Ferm et al. 1978; Robb, Munck et al. 1981; Robb and Smith 1981). In 1981, Kendall Smith and colleagues used immunoaffinity chromatography to attribute this to one molecule, IL-2 (Robb and Smith 1981; Smith, Favata et al. 1983). IL-2 was the first cytokine discovered to mediate its effects through a receptor (IL-2R, later named CD25/CD122) (Robb, Munck et al. 1981) and the first to be cloned from a cDNA library (Taniguchi, Matsui et al. 1983). Despite being discovered earlier, IL-2 was called number two as IL-1 released by macrophages was initially believed to induce IL-2 in T cells (Smith, Gilbride et al. 1980; Smith, Lachman et al. 1980).

IL-2 is now known to be released as part of an adaptive immune response (Cantrell and Smith 1984; Smith 1988). Antigen binding to the TCR induces the secretion of IL-2, and stimulates the expression of IL-2 receptors. The IL-2/IL-2R interaction can then induce the proliferation and survival of antigen-specific cytotoxic lymphocytes (CTLs) causing the upregulation of genes characteristic of activation such as Granzyme B (Stern and Smith 1986; Beadling, Johnson et al. 1993; Beadling and Smith 2002). IL-2 is also necessary for the development of T cell memory and is required both for the development of Tregs in the thymus and their maintenance in the periphery (Sakaguchi, Sakaguchi et al. 1995). Tregs can

prevent Th1 cells producing IL-2 and therefore act as regulators of CTLs; this is believed to be a cause of suppression of anti-tumour responses (Khattar, Chen et al. 2009).

IL-2 is a 15 kDa α–helical cytokine produced mainly by activated T cells and to some extent by activated DCs, NK and NKT cells (Granucci, Vizzardelli et al. 2001; Yui, Sharp et al. 2004). IL-2 mRNA production is induced transiently by TCR engagement to peptide-MHC-II with subsequent formation of a DNA binding protein (NF-AT/AP-1). The mRNA is then stabilised through a second DNA binding protein induced by the additional engagement of co-stimulatory molecules such as CD28 on the cell surface. IL-2 then undergoes transcriptional silencing in activated T cells through a poorly understood mechanism believed to be influenced by binding of T-bet to the IL-2 promoter (Jain, Loh et al. 1995; Hwang, Hong et al. 2005). IL-2 works in an autocrine fashion, upregulating its receptor and downregulating its own production in T cells by induction of the Stat5 transcription factor (Villarino, Tato et al. 2007). IL-2 also sensitises activated T cells to undergo apoptosis upon TCR re-stimulation (Su, Cousens et al. 1998) and conversely induces effector T cell development *in vitro* (Leung, Morefield et al. 2000). Therefore IL-2 has roles in the contraction of the immune response and the promotion of effector cell development.

IL-2 is also crucial in the maintenance and expansion of Tregs *in vivo,* signalling through the highly expressed CD25 (IL-2Rα) (Fontenot, Rasmussen et al. 2005) this can result in much of the IL-2 in a local environment being used by Tregs. Therefore IL-2 is now suspected to have a dual role *in vivo* being necessary for the induction of Th1 immune responses but also inducing tolerance through Treg activation in the local environment. Indeed IL-2 knockout mice have a lympho-proliferative disorder and autoimmunity rather than immunodeficiency (Sadlack, Lohler et al. 1995; Klebb, Autenrieth et al. 1996), since many functions of IL-2 are redundant. This has influenced the use of IL-2 therapy in cancer due to the tolerogenic effects produced in certain tumour models. It is therefore important to understand how IL-2 is used and made available to different T cell populations in order to induce therapeutic anti-tumour responses.

6.1.2: IL-2 and immunotherapy

Immunotherapy was initially discovered when William Coley noticed that, rarely, tumours can spontaneously regress and these patients had commonly suffered from recent bacterial infections. He took this further by injecting live *Streptococcus pyogenes* directly into inoperable tumours, causing some tumour regression (Hoption Cann, van Netten et al. 2003). In the 1970s, Thomas and Burnet had developed the "cancer immunosurveillance" hypothesis suggesting that tumours only develop when circulating lymphocytes fail to either survey or destroy tumourigenic cells (Burnet 1970). Subsequent experiments using knockout mice showed that $Rag2^{-/-}$ mice had an increase in spontaneously arising and chemically induced tumours (Shinkai, Rathbun et al. 1992). Spurred by these results, investigators began to modulate the immune system searching for target molecules to boost tumour regression. Combined with the timely discovery of IL-2 in 1976 (Morgan, Ruscetti et al. 1976) this signals the beginning of cytokine based immunotherapy.

Recombinant human IL-2 has been used for many years in the treatment of cancers and chronic viral infections (Gratama, Bruin et al. 1993; Simpson, Heys et al. 1995). The primary aim of these treatments is to overcome immune suppression caused by chronic antigen exposure or immunoevasive mechanisms which often lead to CTL death or anergy. Initially the belief that IL-2 had a central role in promoting T cell growth and differentiation drove its use as a systemic infusion, following reports of significant anti-tumour effects in mice (Rosenberg, Mule et al. 1985). IL-2 is now clinically used as a treatment of renal cell carcinoma (Fyfe, Fisher et al. 1995) and several forms of malignant melanoma (Parkinson, Abrams et al. 1990). Despite this initial enthusiasm, optimal clinical responses to IL-2 therapy are only seen for a limited number of patients demonstrating a clear lack of efficacy. Combined with this, IL-2 was found to have significant toxicity when administered systemically in humans (White, Schwartzentruber et al. 1994)

The positive therapeutic results of using IL-2 for viral infections has not been mirrored in treatment of cancer as the mechanisms by which IL-2 induces cancer regression are not fully understood. Indeed, as discussed above, IL-2 can support tumour growth by inducing immune suppression. New approaches are beginning to reveal that IL-2 may have differing physiological effects at different doses. At a high dose IL-2 is believed to stimulate Treg development and homeostasis (Thornton, Donovan et al. 2004) while at lower doses IL-2 may preferentially stimulate effector

T cell expansion/contraction and function. Such findings have led to the use of low dose IL-2 in cancer treatment (Dudley and Rosenberg 2003; Dudley and Rosenberg 2007).

6.1.3: IL-2 and gene therapy

Despite the mixed results of IL-2 administration, research into the use of cytokine gene therapy has continued unabated. This has many advantages since the gene of interest can be delivered locally at high concentrations, mirroring a natural immune response while also providing sustained paracrine effects supporting immune activation. For established tumours *in vivo* this can be achieved through local injection of viral carriers containing the gene targeted to tumour cells and/or specific immune cells (Dummer, Hassel et al. 2004; Dummer, Rochlitz et al. 2008). A wide range of vectors are also now available for *in vivo* or *ex vivo* modification of the immune system, including lentivirus (Poeschla, Wong-Staal et al. 1998), adenovirus (Bramson, Hitt et al. 1996), retrovirus (Miller, Miller et al. 1993), herpes simplex virus (Rees, McArdle et al. 2002), vaccinia (Carroll, Overwijk et al. 1998), fowlpox (Kim, Cormier et al. 1998) etc. For example, this method has been used, with therapeutic effect, to transduce several cytokine genes into prostate cancers using canarypox viral carriers (Griffith, Kawakita et al. 2001). Some studies are even using inert vectors as carriers of DNA to avoid safety concerns with recombinant viruses. A recent study has demonstrated experimental treatment of murine melanoma using IL-2 cDNA loaded onto inert nanoparticles (Yao, Ng et al. 2011).

The first IL-2 experimentally expressing tumours were developed over 20 years ago by Fearon *et al.* (Fearon, Pardoll et al. 1990). This group transduced a poorly immunogenic murine colon cell line (CT26) and the B16/F10 melanoma cell line with a murine IL-2 gene under a CMV promoter. A strong CTL response was induced to these tumours even in the absence of CD4 T cells, however, anti-tumour responses were abrogated when CD8 T cells were depleted. These data, along with other studies, suggest intra-tumoural IL-2 may act in a paracine fashion bypassing the requirement for T cell help (Fearon, Pardoll et al. 1990; Guarini, Riera et al. 1997). Indeed, a study by Alosco *et al.* using an IL-2 transduced murine fibrosarcoma cell line (CMS-5) showed that this tumour could be rejected in SCID

mice in an NK dependent manner without the need for T cells (Alosco, Croy et al. 1993). Other groups have reported that endogenous IL-2-producing renal carcinomas can be rejected in lymphopenic mice depleted of NK cells, suggesting rejection by non-specific inflammatory responses alone (Hara, Hotta et al. 1996).

IL-2 transduced tumours have also been used in vaccination experiments due to the potent CTL induction seen in these early experiments. The use of whole cell vaccines provides a major advantage to single target vaccines due to the potential for induction of polyvalent responses to multiple factors associated with the immunising tumour. In a model relevant to this study, a vaccine of murine bladder transitional carcinoma cells (MBT-2) modified to express IL-2 and irradiated, was able to cure mice bearing MBT-2 tumours and also protect against second challenge with a high dose of unmodified MBT-2 tumour cells inoculated to the bladder wall (Connor, Bannerji et al. 1993). Similar effects were also shown in a model of retrovirally modified human bladder carcinoma vaccine (Milella, Jacobelli et al. 1999). The protective effect of IL-2 retroviral modification introduced directly into tumours as a vaccine has also been noted in human cancers of multiple origin, such as melanoma (Gansbacher, Zier et al. 1992), renal cell carcinoma (Gastl, Finstad et al. 1992), myeloid and lymphoid leukaemic cells (Cignetti, Guarini et al. 1994), colon carcinoma (Stein, Walther et al. 1996) and lung carcinoma (Guarini, Riera et al. 1996). T cells from tumour-bearing hosts are often defective in signalling molecules required for their complete activation (Mizoguchi, O'Shea et al. 1992). The transduction of IL-2 into tumour cells as a vaccine is principally designed to compensate for defective cytokine signalling from Th cells (Fearon, Pardoll et al. 1990; Gansbacher, Zier et al. 1990).

Many groups, including our own, are now using lymphocytes genetically modified to express cytokines to increase the efficacy of adoptive cell transfer in cancer immunotherapy (Murphy, Westwood et al. 2005). These studies have had varied results, perhaps attributable to factors such as too few of the transferred lymphocytes being able to infiltrate the tumour to produce a significant local effect of the cytokine. Modification of tumour cells with immunomodulatory cytokines is an effective way of enhancing their immunogenicity *in vivo* without the need for complex vaccination protocols (Gansbacher, Zier et al. 1990). This is a feature afforded to the MB49 system since cell lines maintained *in vitro* can be modified before standard administration *in vivo* in this tumour model. In this study, MB49 is

modified by retroviral transduction with a retroviral rhIL-2 construct (provided by Dr Istvan Bartok) to model tumour development with local production of IL-2 by compared with wild type MB49. This is a study of direct *ex vivo* tumour modification, designed to elucidate the *in vivo* effects of local IL-2 production on tumour growth and anti-tumour immune responses aimed at providing insights into the cellular mechanisms of tumour rejection.

6.2: Results

6.2.1: Genetic modification of the MB49 cell line with rhIL-2 gene

To develop an *in vivo* model in which IL-2 could be produced in the local tumour microenvironment, an IL-2 expressing MB49 cell line was made. The Phoenix packaging cell line was transfected with a MIGR1 retroviral vector containing a recombinant human IL-2 (rhIL-2) cDNA gene (Fig. 6.1b) or with the empty MIGR1 vector containing a GFP reporter gene alone (Fig. 6.1a). Viral supernatant was harvested on day 3 and Phoenix cells tested for transfection efficiency (GFP expression) by FACS (Fig. 6.1a and b). MB49 cells were then transduced using a spin infection method. MB49 cells transduced with MIGR1 containing IL-2 (MB/IL-2) (Fig. 6.1c) and with an empty MIGR1 (MB/GFP) were tested for transduction efficiency by FACS. GFP^+ cells were then FACS sorted and placed into culture, cells were then tested for GFP expression two weeks later (Fig. 6.1b).

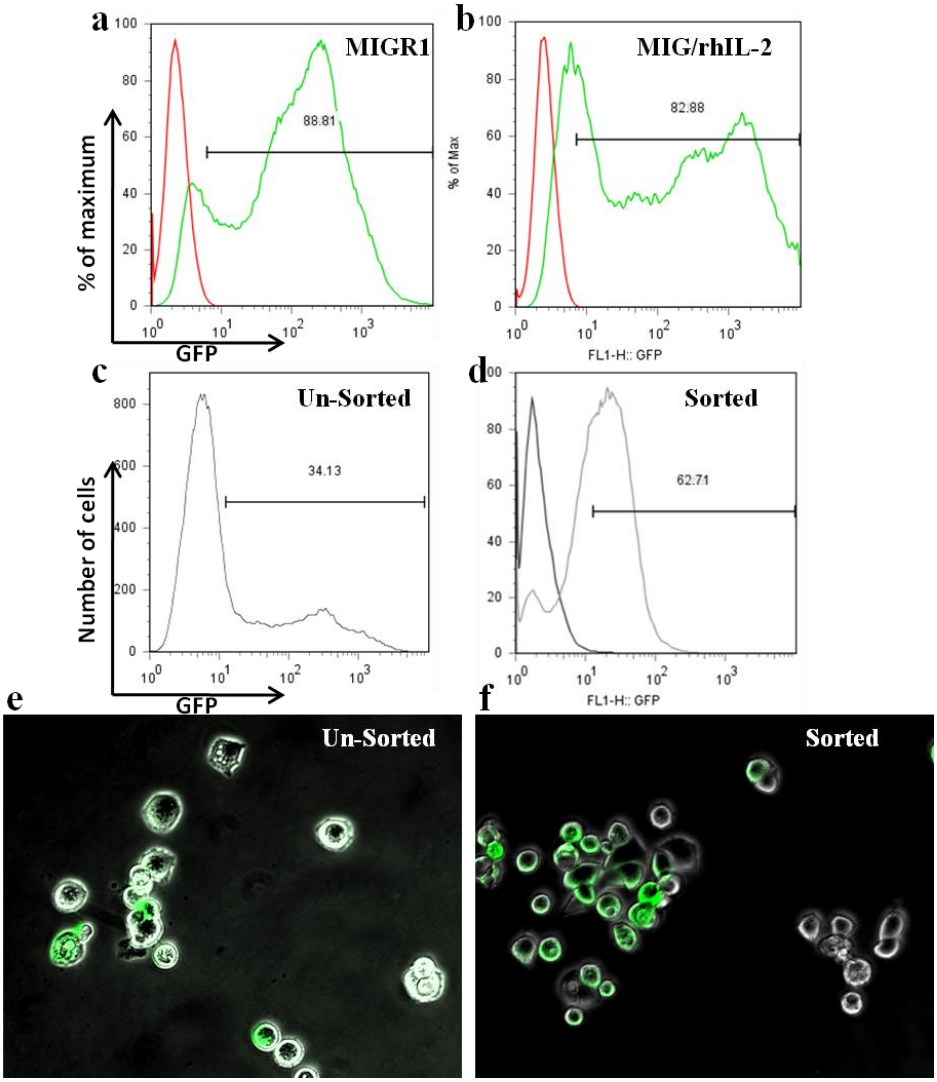


Figure 6.1. Retroviral transduction of the MB49 cell line with MIGR1/rhIL-2
Phoenix cells were transfected using the lipofectamine^{TM} 2000 method with either MIGR1 alone (**a**) or MIGR1 containing a rhIL-2 cDNA (**b**). MB49 cells were plated out at 10^5 cells/well for 48 hours and transduced using spin infection (2500 rpm, 120mins) with 1ml of viral supernatant from the transfected Phoenix culture.
a and b. Phoenix transfection efficiency for MIGR1 (**a**) and MIGR1/rhIL-2 (**b**). FACS profiles of phoenix cells were analysed for GFP expression by transfected cells (green histogram) and untransfected control (red histogram).
c and d. MB49-MIGR1/rhIL-2 transduction efficiency. GFP expression by transduced MB49 cells before (**c**) and 2 weeks after FACS sorting (**d**).
e and f. Deconvolution microscopy of MB49-MIGR1/rhIL-2 unsorted cells (**e**) and 2 weeks after sorting (**f**). Images display FITC channel overlaid onto standard visible light image.

6.2.2: Development of monoclonal MB/IL-2 and MB/GFP populations

To obtain uniform MB/IL-2 and MB/GFP cell lines, a single cell cloning approach was used. MB49 cells were plated out individually in culture and selected

using deconvolution microscopy and FACS for proliferation and GFP reporter gene expression. GFP positive single cell clones were selected (Fig. 6.2a and b) for the highest fluorescence of GFP (Fig. 6.2a) and best proliferation (Fig. 6.2b). Selected GFP expressing MB49 clones were found to proliferate *in vitro* with similar kinetics to untransduced MB49. Selected clones were maintained under the same conditions as un-transduced MB49 cell line *in vitro*.

The progeny of single cell cloned populations can lose transgene expression over time (Fig. 6.2c), as reported by others (Krueger, Jorgensen et al. 1999). This is important when considering long-term experiments using an *in vitro* cell line. The MB/IL-2 cell line was maintained in culture for 6 months and retested for GFP expression by FACS (Fig. 6.2c) and imaged using deconvolution microscopy (Fig. 6.2d). Despite the clone losing GFP expression over this time (~30%) it was considered to be sufficiently stable for *in vitro* and *in vivo* use (~5% GFP loss each month). Frozen stocks of both MB/IL-2 and MB/GFP clones were made at ~60% GFP^+ expression. All experiments from this point forward use MB/IL-2 or MB/GFP from these frozen stocks at between 50-60% GFP^+ expression. The *in vitro* cell line was refreshed every 4-6 weeks from frozen stocks.

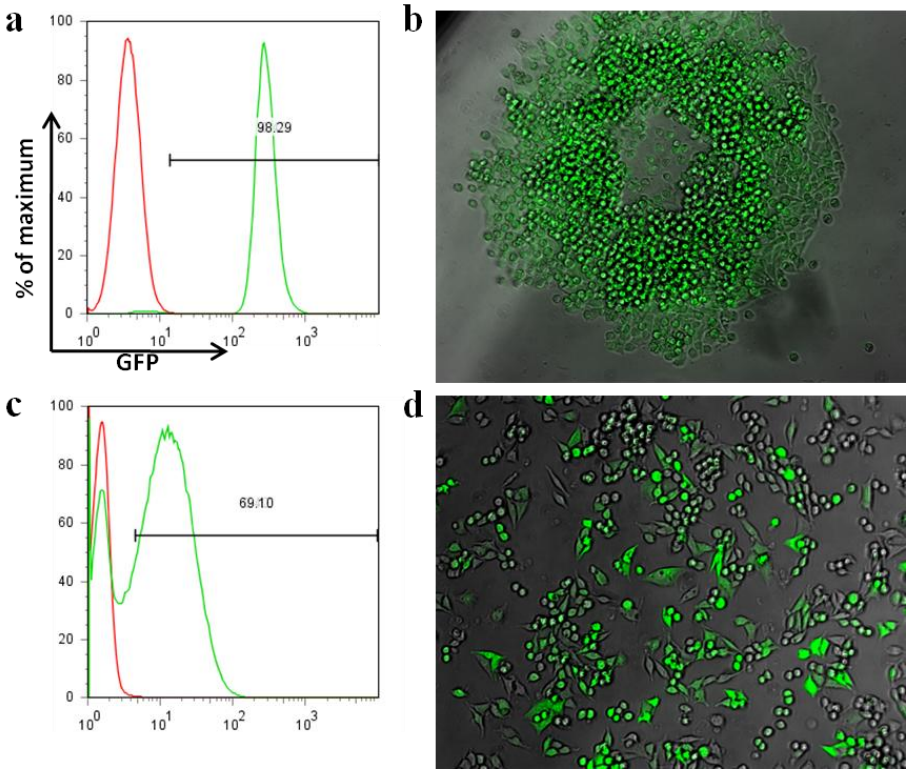


Figure 6.2. Single cell cloning and GFP loss by MB/GFP and MB/IL-2
After FACS sorting MB/GFP and MB/IL-2 were plated in 96 flat bottom plate at ~1 cell/3 wells and cultured for 14 days. Colonies were then selected by deconvolution microscopy based on GFP expression and proliferation.
a. FACS profile of MB/IL-2 single cell clone. GFP expression by transduced (green histogram) and untransduced control (red histogram) was analysed.
b. Deconvolution microscopy image of GFP^+ single cell clone of MB/IL-2.
c. FACS profile of an MB/IL-2 clone after 6 months continuous culture. GFP expression by transduced clone which has been maintained for 6 months (green histogram) and untransduced control (red histogram) was analysed.
d. Deconvolution microscopy image of GFP^+ single cell clone of MB/IL-2 after 6 months culture.

6.2.3: Confirmation of the rhIL-2 transgene activity

CTLL-2 $CD8^+$ T cells were derived from C57BL/6 mice as an IL-2 dependent T cell line that constitutively expresses the IL-2R on their surface. Following long-term culture, CTLL2 cells have lost their original antigen-specificity and CTL activity, but retain IL-2 dependency, and thus the activity of IL-2 in biological samples can be determined by measuring their proliferation using tritiated thymidine incorporation assays. Both human and murine IL-2 can support the *in vitro* proliferation of CTLL-2 cells. In order to confirm the rhIL-2 transgene was active in transduced cells, a second transduction into CTLL-2 cells was conducted and expression of IL-2 assessed by comparison with an unmodified CTLL-2 T cell clone.

Phoenix cells were transfected with a MIGR1/rhIL-2 construct as described previously, and transfection efficiency determined by FACS (Fig. 6.3a). CTLL-2 cells were then transduced and tested for transduction efficiency by FACS (Fig. 6.3b). The transduction efficiency was 11% and this CTLL-2/IL-2 cell line was maintained as bulk culture for 7 days *in vitro* with 20IU exogenous IL-2. CTLL-2 cells were starved of exogenous IL-2 for 24 hours before being plated out for a tritiated thymidine assay (Fig. 6.3c). Untransduced CTLL-2 cells failed to proliferate in the absence of exogenous IL-2 whereas those transduced with the rhIL-2 transgene were able to proliferate in media alone (without exogenous IL-2), suggesting that the rhIL-2 produced endogenously as a transgene is sufficient to maintain the growth of CTLL-2 cells in this bioassay, showing that product of transgene is biologically functional.

To confirm the production of rhIL-2 by the MB/IL-2 cell line and to evaluate whether MB49 produces IL-2, a second CTLL2 proliferation assay was performed

(Fig. 6.3d). MB49 or MB/IL-2 cells were plated out in serial dilutions and left to grow for 24 hours then irradiated (50Gy) to prevent further proliferation. Supernatants were filtered and plated out with the addition of CTLL-2 cells in a proliferation assay (Fig. 6.3d). CTLL-2 cells were also plated out with exogenous IL-2 to provide a reference (black line). As expected, CTLL-2 cells proliferated in a standard dose response curve to exogenous IL-2 (black line). CTLL-2 cells plated out with supernatant of MB/IL-2 also proliferated in a dose-dependent manner suggesting MB/IL-2 produces IL-2 in culture at approximately 20IU/ml/0.5M cells/day (red line). CTLL-2 cells plated out with MB49 supernatant, however, displayed no significant proliferation, indicating MB49 does not produce IL-2.

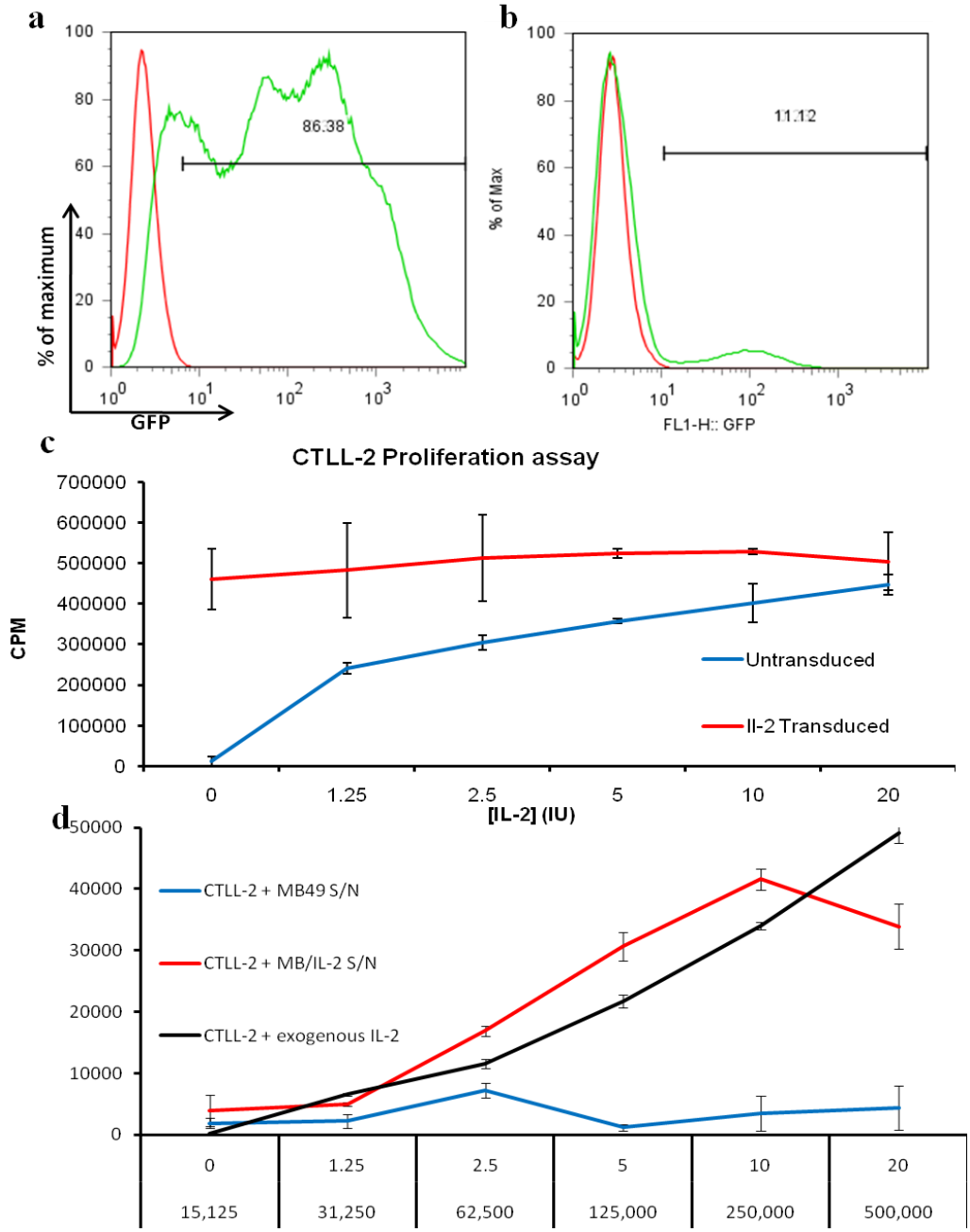


Figure 6.3. Functional verification of rhIL-2 transgene
a. Transfection efficiency of Phoenix by MIGR1/rhIL-2 vector. Phoenix cells were transfected as in Fig. 6.1. CTLL-2 cells were plated out for 48 hours at 10^5 cells/well and transduced as in Fig. 6.1. FACS profile shows GFP expression by transfected Phoenix cells (green histogram) and untransfected control (red histogram).
b. CTLL-2 transduction efficiency with MIGR1/rhIL-2. FACS profile shows GFP expression by transduced CTLL2 (green histogram) untransduced control (red histogram).
c. Thymidine proliferation assay. Transduced (red) or untransduced (blue) CTLL-2 cells plated out at 10^4 cells/well in a 96U bottom plate (BD Falcon) with or without exogenous rhIL-2. 48 hours later 1μl thymidine (containing 1 μCi activity) was added/well. 8 hours later the plate was harvested and counted using a microbeta counter (Wallac). Assays were conducted in triplicate and data is represented as mean ± standard deviation.
d. Thymidine proliferation assay. MB49 or MB/IL-2 cells were plated out in 200μl media on a 96U bottom plate in serial dilution from 5 x 10^5 to 15.125 x 10^4 cells/well. 24 hours later the plate was irradiated (50Gy) and 100μl supernatant was taken from each well and added to a new 96U plate. CTLL-2 cells which had been starved of IL-2 for 24 hours, were plated out at 10^4 cells /well in 96 U bottom plate containing with 100μl supernatant from either MB49 (blue line) or MB/IL-2 cells (red line). As a positive control, CTLL2 cells were also cultured with serially diluted exogenous rhIL-2 (black line) (IU/ml top row X axis). 48 hours later 1μl thymidine (containing 1 μCi activity) was added/well. 8 hours later the plate was harvested and counted using a microbeta counter (Wallac). Assays were conducted in triplicate and data is represented as mean ± standard deviation.

6.2.4: MB/IL-2 tumours fail to grow out in lymphoreplete hosts

At an innoculum of 5 x 10^5, MB49 establish tumours which are not rejected by lymphoreplete B6 mice (see chapter 3). To compare growth profiles of the transduced MB/IL-2 cell line with MB49, MB/IL-2 cells were injected into WT B6 female (Fig. 6.4a) and male (Fig. 6.4b) mice at a dose of 5 x10^5 cells (s.c.). In both female and male mice the MB/IL-2 tumour grew into a visible mass and then regressed. 100% of female B6 mice became tumour free (Fig. 6.4a) by 17 days and 100% of male B6 mice became tumour free after 19 days (Fig. 6.4b). To determine if the rejection of MB/IL-2 was mediated by an immune response to the tumour the same mice were re-challenged with a high dose of 1 x 10^6 MB49 cells to the same site on day 21. All mice, both male and female, failed to develop tumour upon re-challenge, suggesting that MB/IL-2 is immunogenic, inducing an immunological memory recall response to MB49.

Retrovirally transduced genes can be lost readily *in vivo* (Krueger, Jorgensen et al. 1999). To confirm the MB/IL-2 tumour maintained transgene expression *in vivo* one female B6 mouse challenged with MB/IL-2 was culled on day 10 and its tumour analysed by FACS for GFP expression (Fig. 6.4c). >50% of the cells displayed GFP expression indicating the MB/IL-2 tumour is stable *in vivo* for the course of tumour growth experiments.

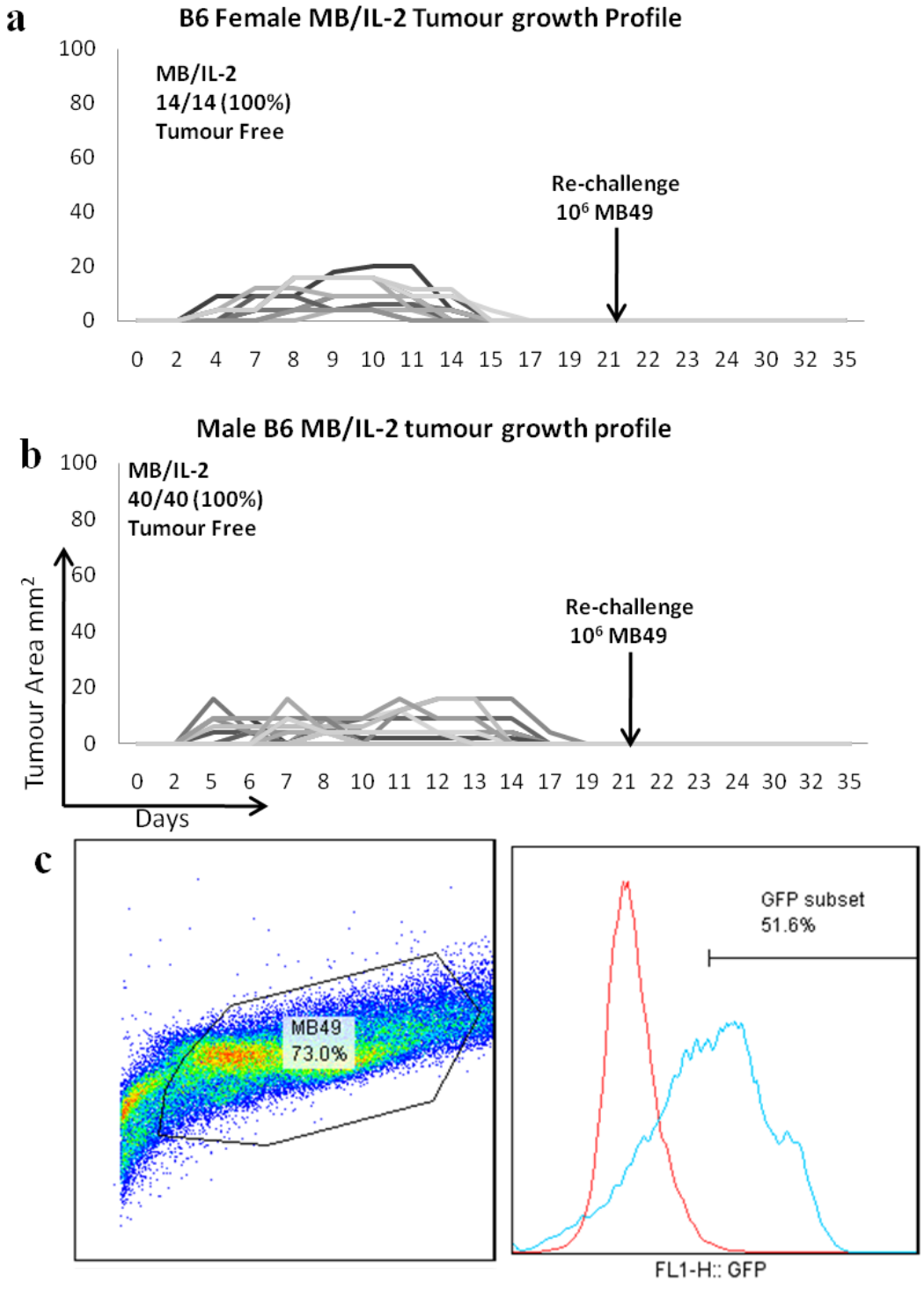


Figure 6.4. Spontaneous regression of MB/IL-2 in lymphoreplete recipients
a. MB/IL-2 was injected at 5 x 10^5 cells (s.c.) to the right flank of B6 female mice (n=14) tumour growth was measured on consecutive days using digital callipers and tumour area calculated (length x width (mm^2)). On day 21, mice were re-challenged with 10^6 MB49 cells (s.c.).
b. MB/IL-2 was injected at 5 x 10^5 cells (s.c.) to the right flank of B6 male mice (n=40) tumour growth was measured as above. On day 21 mice were re-challenged with10^6 MB49 cells (s.c.).
c. MB/IL-2 or MB49 were injected at 5 x 10^5 cells (s.c.) to the right flank of B6 female mice, tumour growth was measured as above. On day 10 tumours were excised and tested for GFP expression *ex vivo*. FACS profile shows GFP expression by MB/IL-2 (blue) or MB49 control (red).

6.2.5: MB/IL-2 tumours have a distinct appearance in lymphoreplete hosts

MB/IL-2 tumours regress in lymphoreplete hosts, therefore these mice cannot be killed at the humane endpoint used for MB49 for tumour analysis. Therefore two groups of female B6 mice were injected with $5x10^5$ of either MB49 or MB/IL-2 cells and culled on day 21 and day 10 respectively (Fig. 6.5). MB49 tumours grew in a typical fashion with a large tumour mass reaching the humane endpoint at day 21 (at least one dimension being 14mm): the mouse shown was culled and photographed (Fig. 6.5a, c and e). MB/IL-2 tumour-bearing mice (one shown in Fig. 6.5b, d and f) were culled on day 10, as previous data suggested this was the point of maximal tumour growth in WT B6 female mice (Fig. 6.5b). MB/IL-2 showed a small solid tumour mass.

The outer layer of skin was removed and tumour was photographed again. MB49 displays a clear tumour mass with angiogenesis and enlarged dLN (Fig. 6.5c) whereas MB/IL-2 tumours have limited angiogenesis and smaller dLN (Fig. 6.5d). Tumours were cut using a scalpel to examine the internal structure. MB49 tumours form an internal cavity with a high blood volume that creates a metastatic pressure leading to bleeding when the tumour is cut (Fig. 6.5e). MB/IL-2 tumours did not show an internal cavity or significant blood pooling (Fig. 6.5c); this resembles the features of rejected tumours.

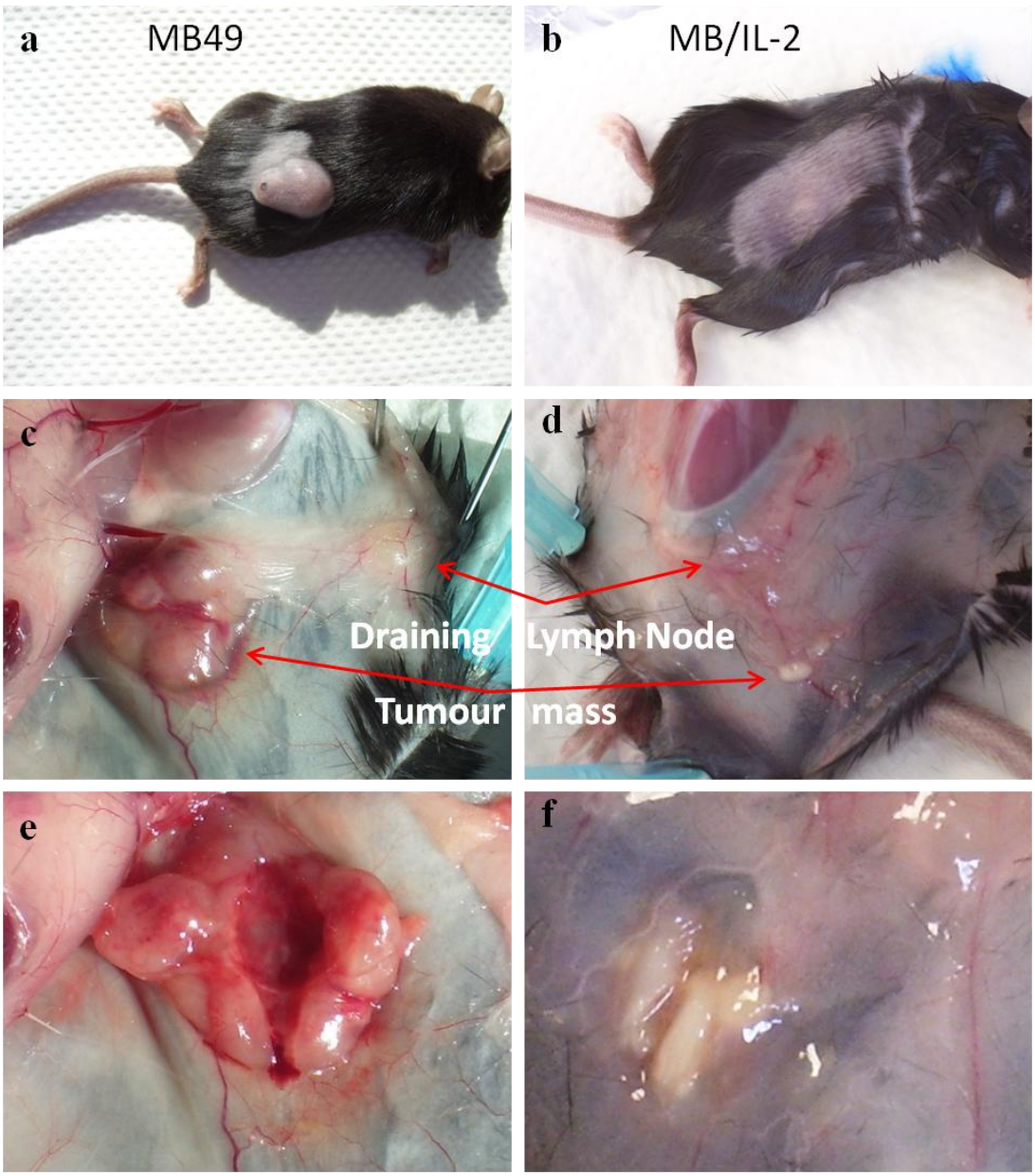


Figure 6.5. MB/IL-2 and MB49 have distinct tumour structure in lymphoreplete recipients
MB49 or MB/IL-2 cells were injected at 5 x 10^5 cells (s.c.) to the right flank of B6 female mice (n=2). Tumours were imaged on day 21 (MB49) (**a,c,e**) and day 10 (MB/IL-2) (**b,d,f**). Tumours were also cut using a 22 grade scalpel (**e** and **f**).

6.2.6: MB/GFP cells grow into tumours normally in lymphoreplete hosts

It is possible that retroviral modification of the MB49 tumour cell line leads to expression of additional antigens deriving from the MIGR1 retroviral vector, including the GFP reporter. This might lead to a more robust immune response contributing to elimination of the tumour in lymphoreplete hosts.

To minimise this possibility, the MB/GFP cells (Fig. 6.1, 6.2) were injected in both the right and left lateral flanks of B6 male mice (Fig. 6.6). The MB/GFP cell line had >50% GFP expression at the time of injection (Fig. 6.6b). 100% of mice developed tumours and reached the humane endpoint (Fig. 6.6a) indicating that the retrogenic MB/GFP tumour is viable and not rejected through antigenic contributions from MIGR1 or GFP. Tumour-bearing mice were also photographed on day 14 (Fig. 6.6b) (this mouse was culled as the humane endpoint had been reached due to ulceration). MB/GFP displays similar structure to MB49 *in vivo* developing angiogenesis and blood pooling (Fig. 6.6c).

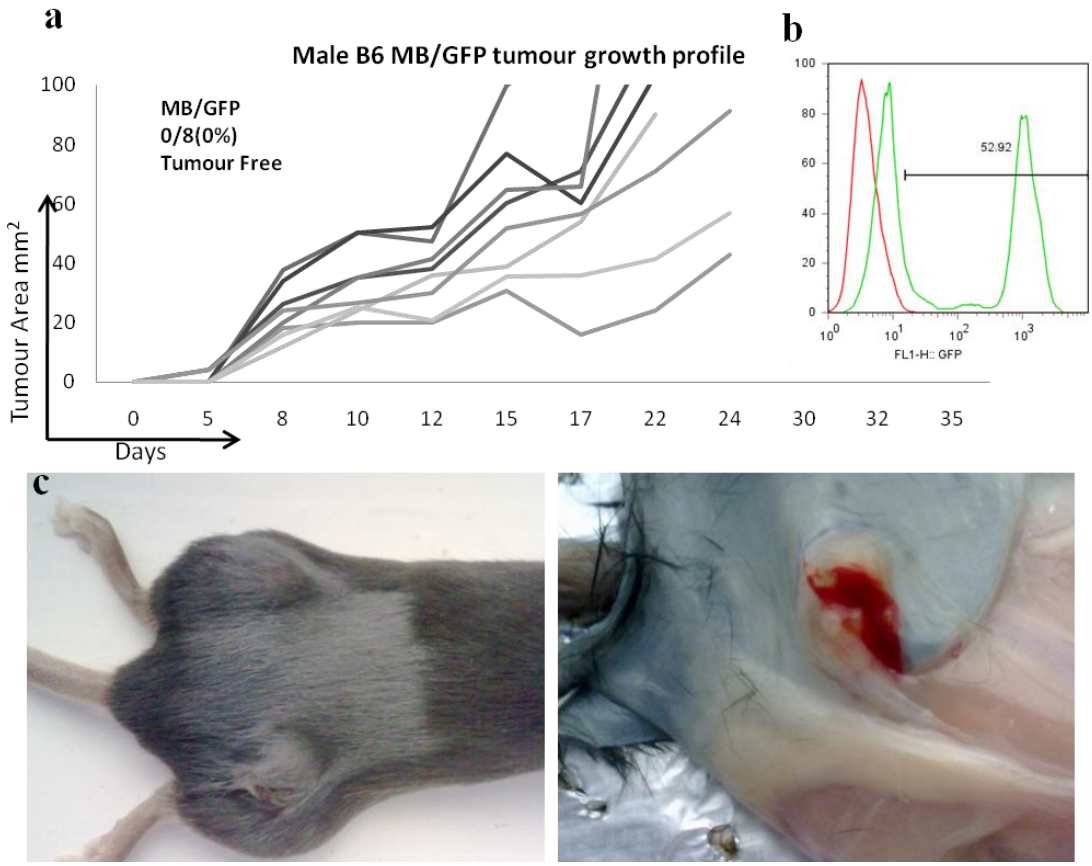


Figure 6.6. MB/GFP displays normal growth in lymphoreplete recipients
a. MB/GFP was injected at 5 x 10^5 cells (s.c.) to the right flank of B6 male mice (n=8) tumour growth was measured as in Fig. 6.4.
b. Expression of GFP by MB/GFP cells at time of injection. FACS profile shows GFP expression by transduced MB/GFP (green histogram) and untransduced control (red histogram).
c. MB/GFP was injected at 5 x 10^5 cells (s.c.) to the right flank of B6 female mice. Tumours were imaged on day 14.

6.2.7: MB/IL-2 cells can develop into tumours in lymphopenic recipients

There is a possibility that MB/IL-2 may be non-viable or the rhIL-2 produced by the tumour may cause non-specific toxicity to the tumour itself. To explore this further, lymphopenic recipients were injected with MB/IL-2 or MB49 cells at a standard dose of 1 x 10^5 cells, as suggested by others (Perez-Diez, Joncker et al. 2007). In male B6 Rag2$^{-/-}$ mice given MB/IL-2 cells 100% (33/33) developed tumour reaching the humane end point (Fig. 6.7a). 100% of female B6 Rag2$^{-/-}$ mice (8/8) developed MB49 tumours to the same end point at a similar rate (Fig. 6.7b), indicating that the MB/IL-2 is viable *in vivo* and has similar growth characteristics to MB49.

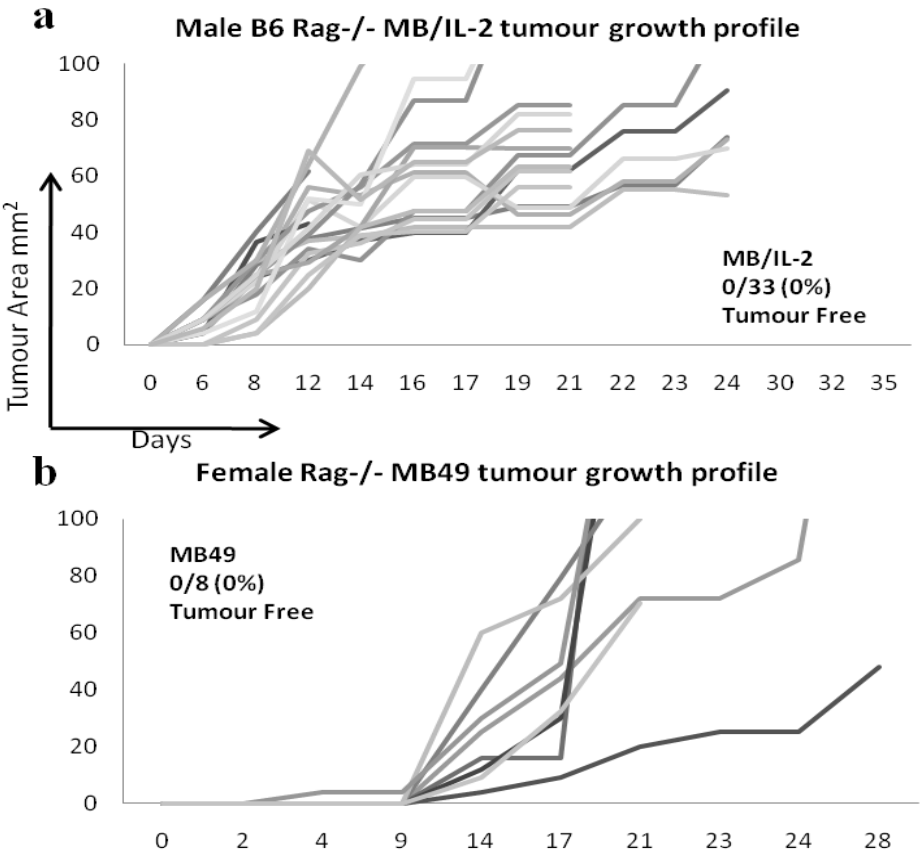


Figure 6.7. MB/IL-2 cells develop into tumours in lymphopenic recipients
a. MB/IL-2 was injected at 1 x 10^5 cells (s.c.) to the right flank of Rag$^{-/-}$ B6 male mice (n=33) tumour growth was measured as in Fig. 6.4.
b. MB49 was injected at 1 x 10^5 cells (s.c.) to the right flank of Rag$^{-/-}$ B6 female mice (n=8) tumour growth was measured as in Fig. 6.4.

6.2.8: MB/IL-2 has a similar appearance to MB49 in lymphopenic recipients

MB/IL-2 and MB49 tumours from lymphopenic hosts (From Fig. 6.7) were compared with respect to appearance and structure. Mice were culled at the relevant

humane end points and tumour structure was photographed for MB49 (Fig. 6.8a) and MB/IL-2 (Fig. 6.8b) tumours. In lymphopenic recipients both tumour types showed significant tumour mass (Fig. 6.8a and b), angiogenesis (Fig. 6.8c and d) and blood pooling (Fig. 6.8e and f), indicating that the MB/IL-2 tumour can grow with the same characteristics as MB49 in the absence of an adaptive immune system.

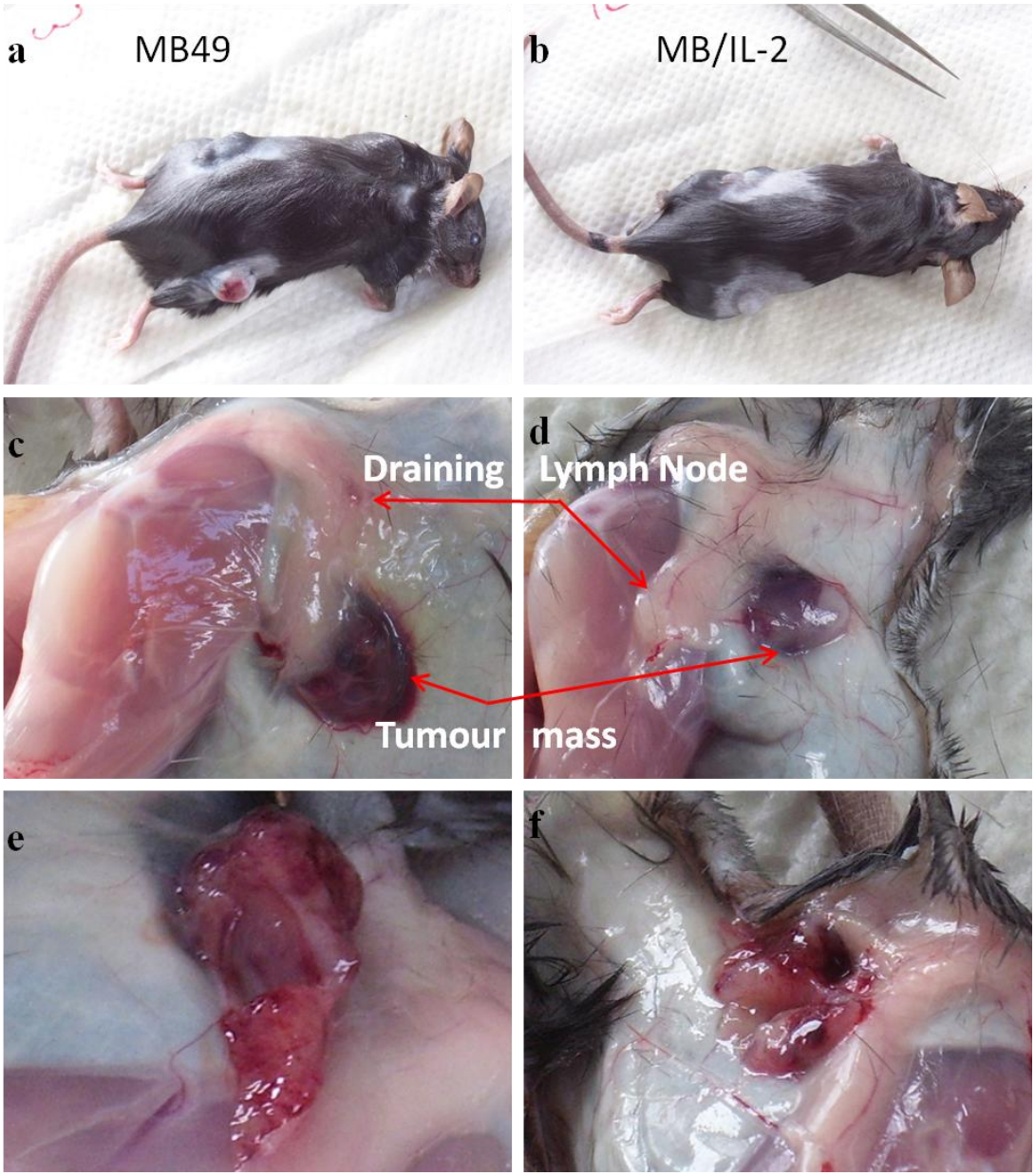


Figure 6.8. MB/IL-2 and MB49 have similar tumour structure in lymphopenic recipients
MB49 or MB/IL-2 was injected at 1 x 10^5 cells (s.c.) to the right and left flank of B6 $Rag2^{-/-}$ male mice. Tumours were imaged at the humane end point for MB49 (**a,c,e**) and MB/IL-2 (**b,d,f**). Tumours were also cut using a scalpel (**e** and **f**).

6.2.9: MB/IL-2 primarily induces a local immune response

MB/IL-2 is rejected in WT recipients and can induce a potent secondary response to MB49 re-challenge (Fig. 6.1). To explore whether MB/IL-2 induces a systemic or local primary response, a dual inoculation experiment was conducted using two groups of mice. MB49 was injected subcutaneously with a high dose of 5 x 10^5 cells into each of the left and right flanks of B6 female mice (n=4) (G1). Another group of 4 recipient B6 females received 5 x 10^5 MB49 cells in the left flank and 5 x 10^5 MB/IL-2 cells in the right flank (G2). All MB49 inoculations grew at a similar rate whether injected alongside MB/IL-2 or not (Fig. 6.9). Only MB/IL-2 tumours regressed, as described previously (Fig. 6.3) with no effect on growth of MB49 on the left flank of the mouse (Fig. 6.9 MB49 G2 left). These data indicate the MB/IL-2 induces a sufficient local immune response to cause primary rejection but insufficient systemic immunity to prevent MB49 tumour growth when injected at the same time point.

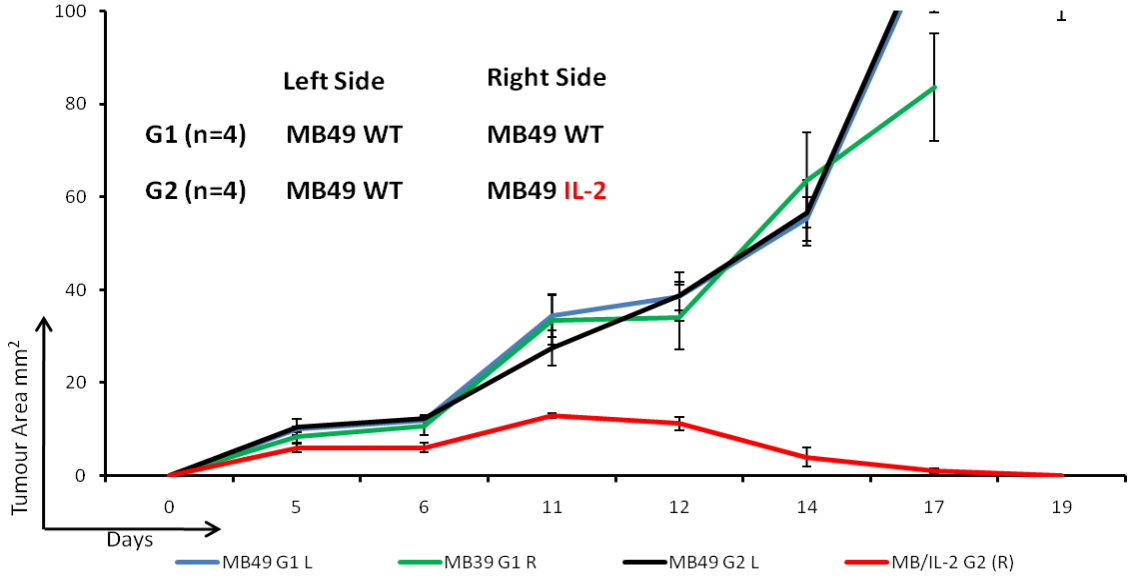


Figure 6.9. MB/IL-2 does not provide systemic protection to MB49 when injected at the same time
G1 - MB49 were injected at 5 x 10^5 cells (s.c.) to the left and right flank of B6 female mice (n=4).
G2 - MB49 were injected at 5 x 10^5 cells (s.c.) to the left flank and MB/IL-2 were injected at 5 x 10^5 cells (s.c.) to the right flank of B6 female mice (n=4), tumour growth was measured was in Fig. 6.4. Error is displayed as ± standard error mean.

6.2.10: MB/IL-2 is rejected even when mixed with MB49

The most likely cause of MB/IL-2 rejection is through a local immune response that is sufficient to induce systemic immunological memory (Fig. 6.1) but insufficient to cause simultaneous systemic immunity when co-inoculated with MB49 (Fig. 6.9). To examine the potency of the local immune response induced by MB/IL-2, a mixing experiment was set up. MB/IL-2 cells were mixed with MB49 cells in various ratios (50:50, 25:75, 10:90, 5:95, 1:99), and a total number of 5 x 10^5 tumour cells injected (s.c.) (Fig. 6.10). Mice that received tumours at ratios of MB/IL-2:MB49 at 50:50, 25:75 and 10:90 all failed to develop as large tumours; tumours in these mice were less than 20mm^2 and regressed by day 20 with 100% of mice becoming tumour free (not shown). Mice that received mixed tumour cells with a 5:95 MB/IL-2:MB49 ratio also showed tumour regression with 100% mice becoming tumour free, whereas those that received tumours at the ratio of 1:99 were 75% tumour free (Fig. 6.10). These data sets indicate that even a small number of MB/IL-2 cells (equal to or less than 5%) is sufficient to cause rejection of tumours incorporating those cells along with WT or homologous non-IL-2 expressing tumour cells.

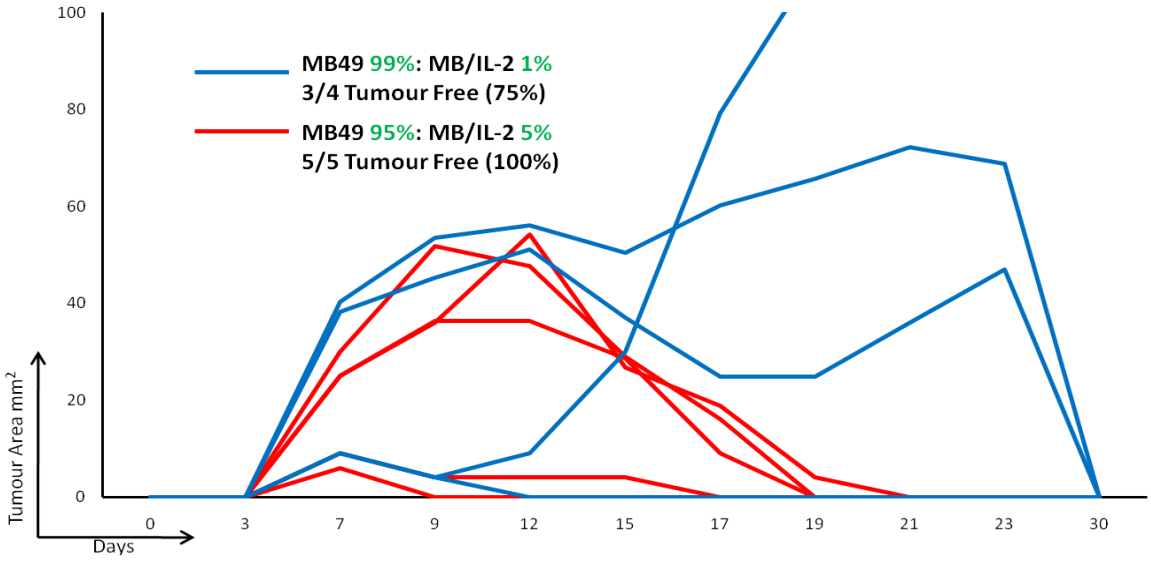


Figure 6.10. The presence of a small fraction of MB/IL-2 cells in mixture with MB49 cells can induce regression
MB49 cells mixed with MB/IL-2 cells at a ratio of 95:5 (n=5) and 99:1 (n=4). The MB49 mixtures were injected at 5 x 10^5 cells (s.c.) to the right flank of B6 female mice and tumour growth was measured as in Fig. 6.4.

6.2.11: MB/IL-2 rejection requires CD8 T cells

In the second part of this study an attempt was made to understand the cellular and molecular mechanisms underlying MB/IL-2 tumour rejection. MB49 is primarily recognised and killed by T cells (Perez-Diez, Joncker et al. 2007). To determine the relative contribution of different T cell subpopulations to protection, depleting monoclonal antibodies for CD4 and CD8 were used to remove either or both compartments, in B6 female mice before MB/IL-2 inoculation. Mice were injected with depleting antibody (i.p.) 1 day before MB/IL-2 cells and PBL tested on the day of inoculation to confirm depletion of the relevant T cells. Mice were injected with MB/IL-2 s.c. as described previously. All mice from the untreated control group and the CD4 depleted group showed typical MB/IL-2 growth with a small tumour that quickly regressed by day 20, with 100% of mice tumour free. Mice that received CD8 antibody alone or in conjunction with the CD4 antibody all developed tumours (0% tumour free), and no significant difference was seen between the CD8 alone and CD4/8 double depleted group (Fig. 6.11). These experiments strongly indicate the need for a CD8 T cell compartment in order to mediate rejection of MB/IL-2 in WT B6 recipients.

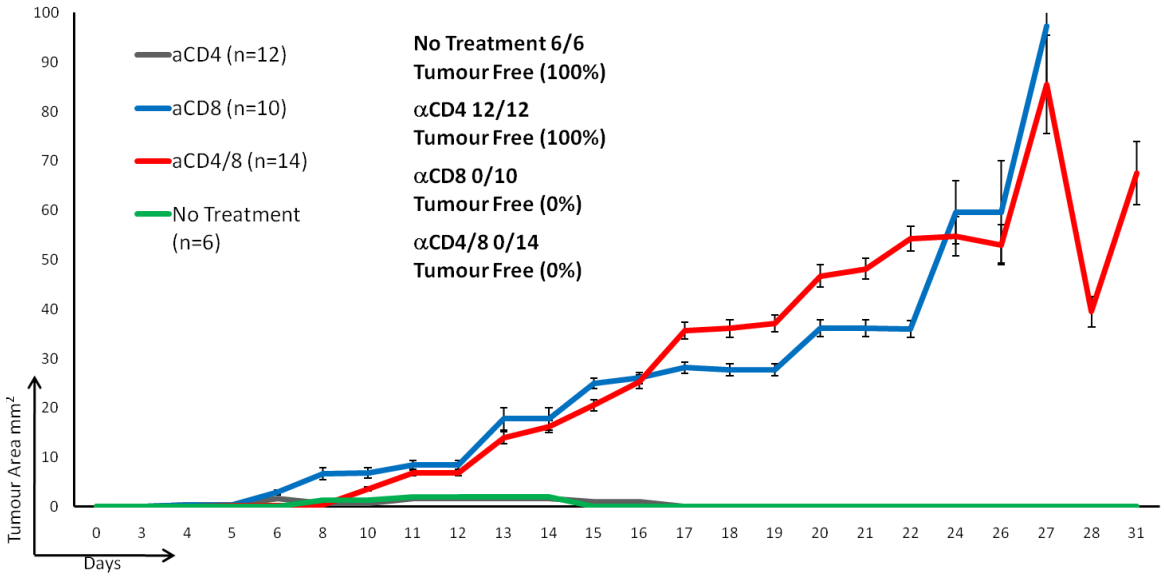


Figure 6.11. MB/IL-2 tumours can be controlled by CD8 but not CD4 T cells
MB/IL-2 cells were injected at 5 x 10^5 cells (s.c.) to the right flank of B6 female mice (n=42) and tumour growth was measured as in Fig. 6.4. Data displayed as average tumour growth for each group, Error is displayed as ± standard error mean.
G1 – mice received anti-CD4 depleting antibody (YTS191) 0.2ml (i.p.) 1 day prior to tumour injection (n=12).
G2 - mice received anti-CD8 depleting antibody (YTS169.4) 0.2ml (i.p.) 1 day prior to tumour injection (n=10).

G3 - mice received anti-CD4 and anti-CD8 depleting antibody (i.p.) 1 day prior to tumour injection (n=14).
G4 – mice received no treatment (n=6).

6.2.12: MB/IL-2 is rejected in Matahari Rag$^{-/-}$ mice

Based on the requirement for an intact CD8 T cell compartment to mediate rejection of MB/IL-2 in WT mice, an experiment was set up to explore if anti-HY CD8 TCR transgenic Matahari Rag deficient mice were capable of rejecting MB/IL-2. In Matahari Rag$^{+/-}$ female mice ~50% CD8 T cells are HY-specific while in Matahari Rag$^{-/-}$ females all CD8 T cells are HY-specific (Fig. 6.12a). Female Matahari Rag$^{-/-}$ mice were inoculated with MB49 or MB/IL-2. 79% of mice that received MB/IL-2 were tumour free by day 35 while all mice that received MB49 developed tumour by day 15 (Fig. 6.12b). All mice that received MB/IL-2 failed to reach the humane end point and survived to day 35 while all mice that received MB49 reached humane endpoint by day 30 and were culled (Fig. 6.12c). Based on the Matahari Rag$^{-/-}$ T cell phenotype (Fig. 6.12a) rejection of MB/IL-2 is most likely mediated solely by the TCR transgenic HY restricted monoclonal CD8 population.

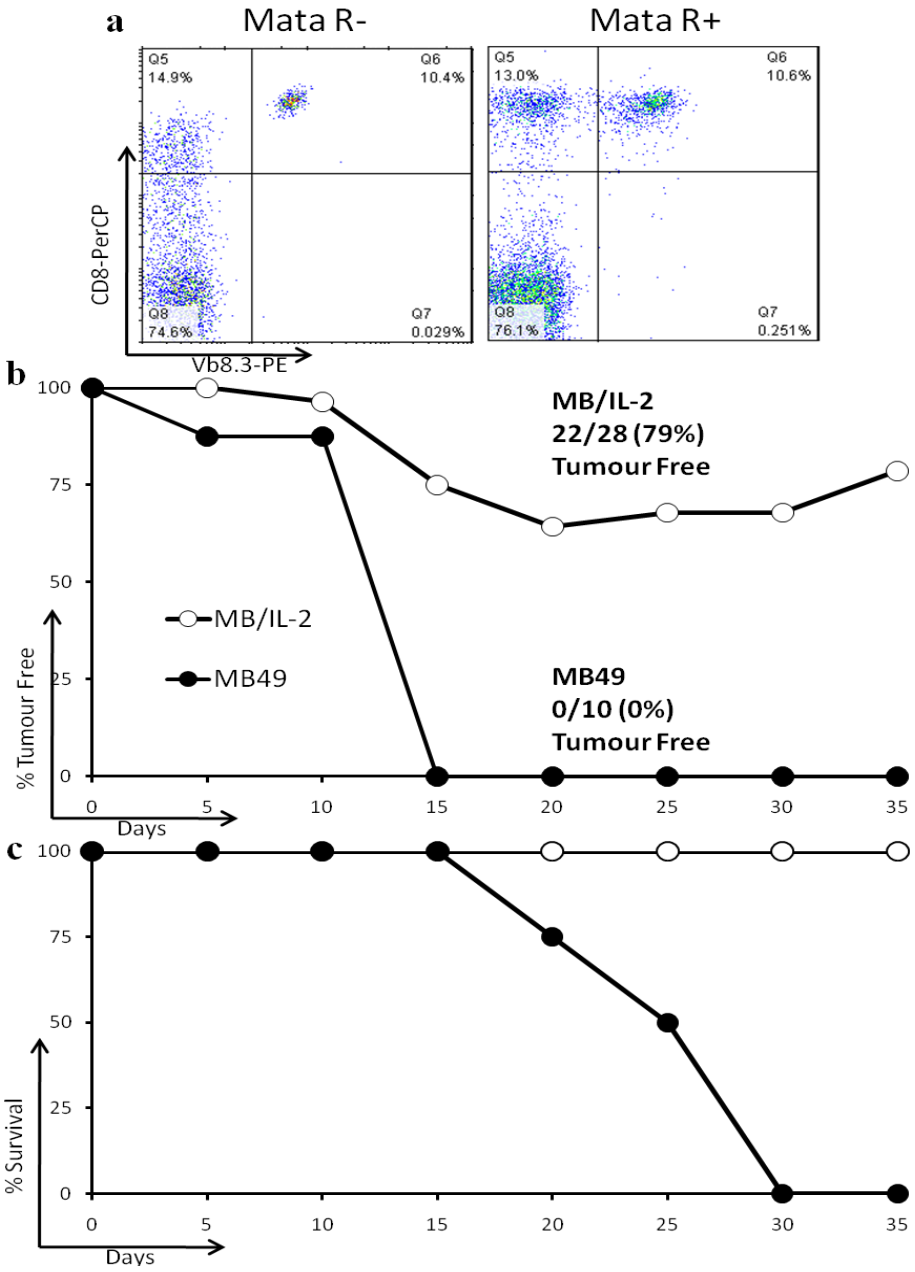


Figure 6.12. Matahari Rag deficient mice can reject MB/IL-2 tumours
a. Matahari Rag$^{-/-}$ and Matahari Rag$^{+/-}$ PBL, cells stained for anti-Vβ8.3^{PE} and anti-CD8^{PerCP}. FACS plots of lymphocytes gate then CD8 vs Vβ8.3.
b. MB/IL-2 (n=28) or MB49 (n=10) was injected (s.c.) to the right lateral flank of Matahari Rag$^{-/-}$ females at 1 x 10^5 cells. Tumour growth was measured as described previously. Data displays % tumour free mice at various time points.
c. % Survival for each group as described above.

6.2.13: Adoptively transferred Matahari and WT CD8 T cells can control MB/IL-2 but not WT MB49 in lymphopenic recipients

To further explore the role of CD8 T cells in tumour rejection, adoptive cell transfers (ACT) of naïve WT CD8 T cells and monoclonal Matahari T cells were

made to MB49 or MB/IL-2 bearing lymphopenic B6 female recipients. WT CD8 T cells were purified from a B6 female donor and Matahari CD8 T cells from a Matahari Rag$^{+/-}$ donor using MACS and FACS sorting. All recipients of both types of donor cells (WT CD8 T cells (grey line n= 2) or Matahari T cells (red line n=9) (Fig. 6.13a)), developed tumours following MB49 injection, reaching the humane end point by day 17 for the WT CD8 T cell group and day 25 for the Matahari group (Fig. 6.13b). On the other hand, mice that received MB/IL-2 were able to control the tumour in the presence of either WT CD8 (green line n=5) or Matahari CD8 T cells (blue line n=9) (Fig. 6.13a). All mice in both MB/IL-2 groups survived to day 25 (Fig. 6.13b). These data further demonstrate that CD8 T cells are both necessary and sufficient to induce rejection of MB/IL-2 but not MB49.

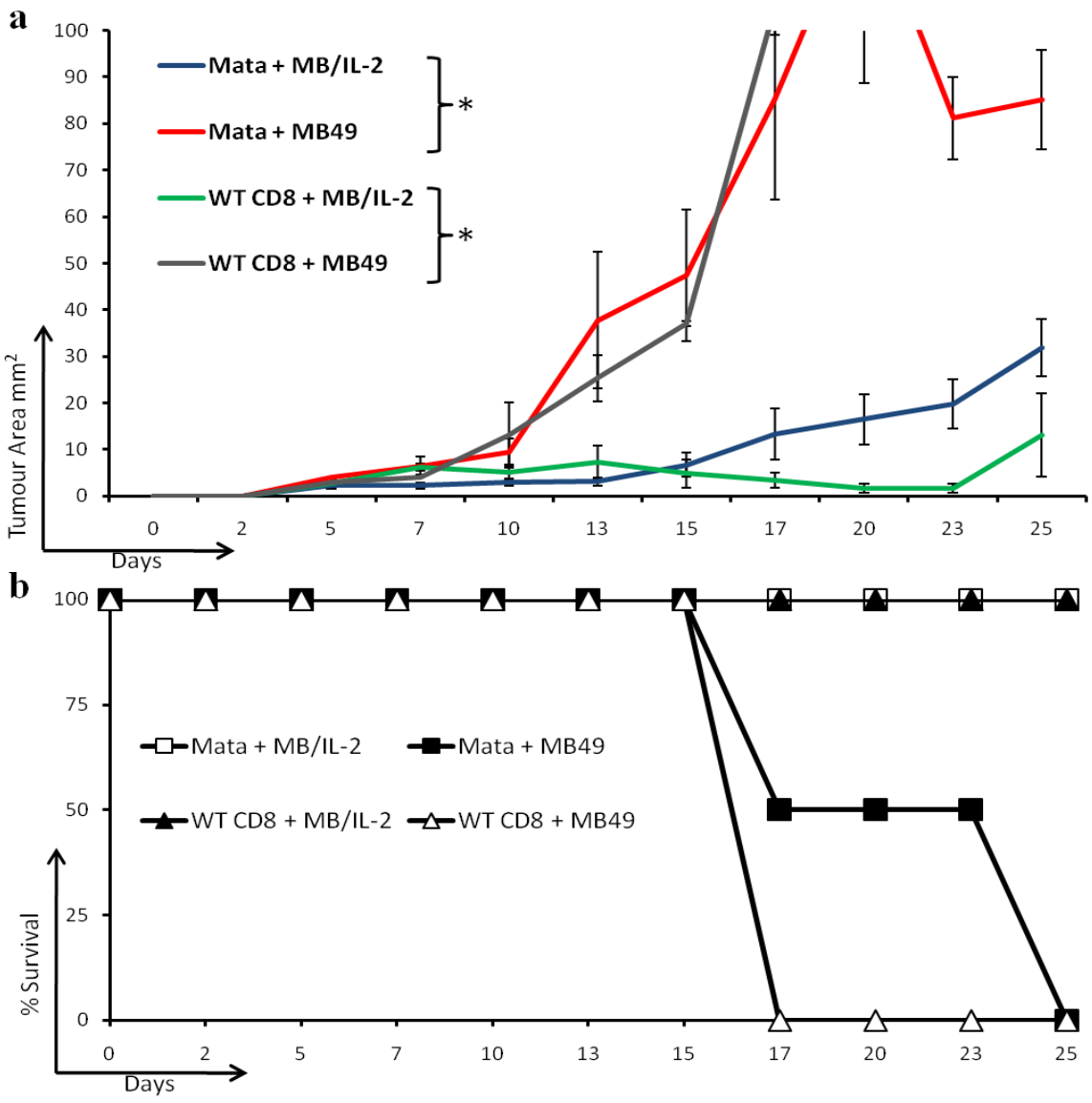


Figure 6.13. Matahari and WT CD8 can control MB/IL-2 but not MB49 tumour upon adoptive transfer to lymphopenic mice
a. Matahari T cells were purified from a Matahari $Rag^{+/-}$ donor by MACS enrichment of Vβ8.3 cells by anti-$V\beta8.3^{PE}$ staining and PE microbeads and further FACS sorting of CD8, Vβ8.3, Thy1.1 triple positive population (Vβ8.3-enriched cells were stained with anti-$CD8^{PE/Cy5}$ and anti-$Thy1.1^{PerCP}$ before sorting). WT CD8 T cells were purified using anti-CD8 MACS beads followed by further FACS sorting by staining for anti-$CD8^{PE/Cy5}$ and anti-$CD4^{APC}$ and sorted for CD8, single positive population. B6 female $Rag^{-/-}$ mice were challenged with either MB49 (n=6) or MB/IL-2 cells (n=14) at $1x10^5$ (s.c.). MB49-bearing (n=4 red line) and MB/IL-2 bearing mice (n=9 blue line) were given 1 x 10^6 Matahari T cells (i.v.). MB49-bearing (n=2 grey line) and MB/IL-2 bearing mice (n=5 green line) were given 1 x 10^6 WT CD8 T cells (i.v.). Tumour growth was measured as described previously. Error is displayed as ± standard deviation. P-value <0.01).
b. % Survival for each group as described above.

6.2.14: Matahari CD8 T cells proliferate more in dLN in response to MB/IL-2 compared with MB49

To elucidate the mechanism underlying CD8 T cell mediated rejection stimulated by MB/IL-2 in adoptive transfer, Matahari cells were labelled with CFSE for an *in vivo* proliferation assay. Labelled Matahari CD8 T cells (Thy1.2) were injected into control (G1), MB49-bearing (G2) or MB/IL-2 bearing (G3) B6 female (Thy1.1) recipients. Mice were culled on day 9 and donor T cell populations were analysed in the dLN and ndLN (Fig. 6.14). In mice that received MB/IL-2 tumour, donor T cells showed the highest expansion in the tumour dLN (0.096% of lymphocyte gate) compared to MB49 (0.026%) and T cell alone control (0.014%) (Fig. 6.14a). Donor cells in dLN of the MB/IL-2 bearing group also showed the highest CFSE dilution with 88.8% cells dividing compared to 38.3% for the MB49-bearing group (10.2% for control) (Fig. 6.14b). This was also reflected in the mean MFI of CFSE (Fig. 6.14c and d) which was statistically significant. These data show Matahari CD8 T cells proliferate to a greater extent in response to the IL-2 replete MB/IL-2 environment compared to MB49.

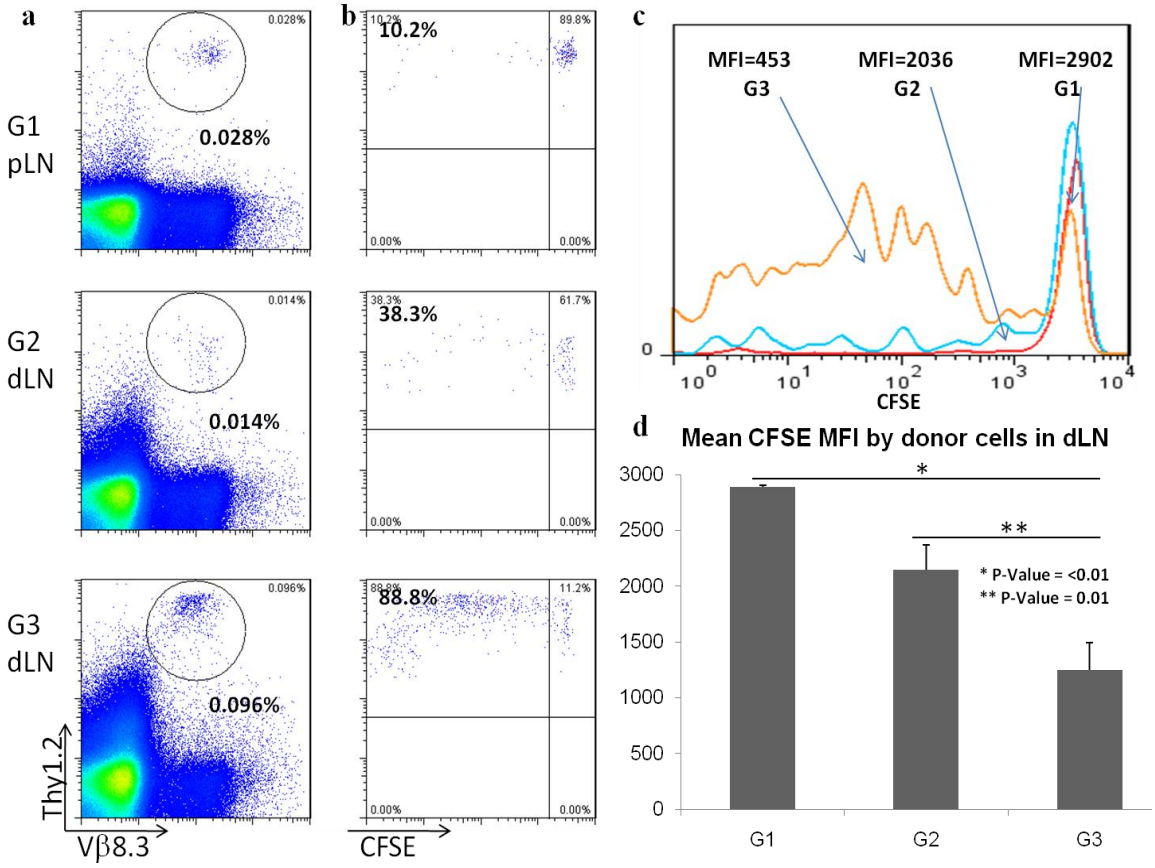


Figure 6.14. Adoptively transferred Matahari T cells are capable of proliferating in response to MB/IL-2

Matahari T cells were purified form Thy1.2 Matahari Rag$^{+/-}$ female mice by MACS sorting using anti-Vβ8.3^{PE} staining and PE microbeads. T cells were then FACS sorted (as in Fig. 6.13 with anti-Thy1.2^{APC} in place of anti-Thy1.1^{PerCP}) and then labelled with CFSE and injected at 4 x 10^6 /mouse (n= 10). Mice were then also challenged with a high dose (5 x 10^5 cells) with MB49 (G2) (n= 4) or MB/IL-2 (G3) (n=4) or no tumour challenge (G1) (n= 2). Mice were culled on day 9 and peripheral lymphoid organs analysed for donor cells.

a. pLN of G1 or dLN of G2 and G3 were harvested and stained using anti-Vβ8.3^{PE}, anti-CD8^{PerCP}, anti-Thy1.2^{APC}. Cells were analysed by FACS and gated using lymphocyte gate, then CD8$^+$ Vβ8.3$^+$ before final donor population FACS plot displayed as Thy1.2 vs Vβ8.3. One representative FACS plot of 3 is shown.

b. Cells were gated as in (a) FACS plot for Thy1.2^{APC} vs CFSE .

c. Histogram plot for MFI of CFSE for cells from gate described in (a).

d. Graph of mean MFI of CFSE for G1-3. Error displayed as ± standard error mean.

6.2.15: Matahari CD8 T cells accumulate in the dLN but not ndLN in response MB/IL-2 tumour

From the same experiment in Fig. 6.14, dLN and ndLN were compared to evaluate the systemic versus local responses of adoptively transferred Matahari CD8 T cells (Fig. 6.15). For both tumours the highest levels of donor cell expansion was seen in the local dLN compared to the ndLN (Fig. 6.15a). The level of donor cell expansion in the dLN of MB/IL-2 bearing mice was statistically higher than that of MB49-bearing mice and control mice both in terms of frequency and absolute cell number (Fig. 6.15b). MB49 tumours induced a poor donor cell expansion in dLN, with no statistically significant difference between MB49 tumour-bearing mice and control mice. Finally, donor cells in all mice showed similar levels of expansion in ndLN in terms of frequency and absolute cell number (Fig. 6.15c), indicating that adoptively transferred Matahari T cells expand in response to MB/IL-2 in the local dLN but not systemically in ndLNs.

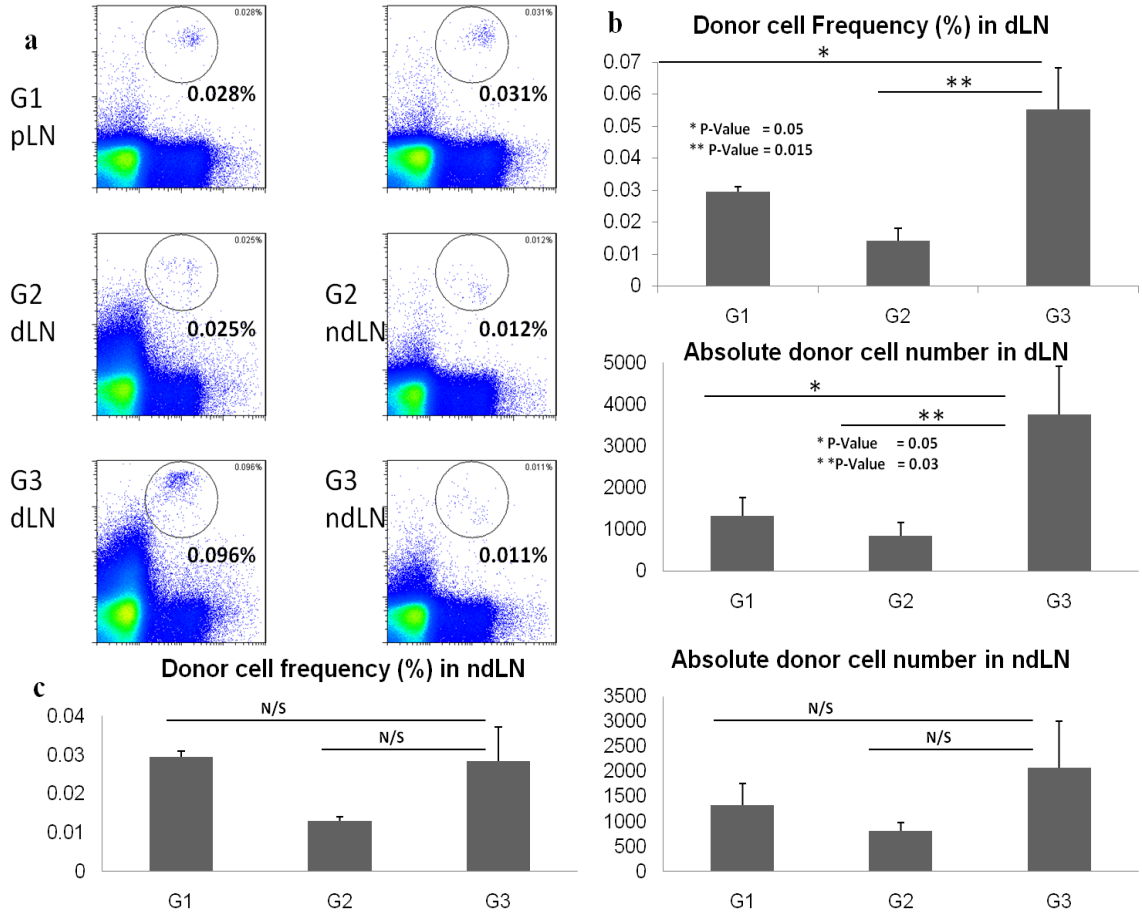


Figure 6.15. Adoptively transferred Matahari T cells are proliferate more extensively in the dLN in response to MB/IL-2 tumour
Experimental set up as in Fig. 6.14.

a. pLN of G1 or dLN of G2 and G3 were harvested and stained using anti-Vβ8.3^{PE}, anti-CD8^{PerCP}, anti-Thy1.2^{APC}. Cells were analysed by FACS and gated using lymphocyte gate, then CD8^{+} Vβ8.3^{+} before final donor population FACS plot displayed as Thy1.2 vs Vβ8.3. One representative FACS plot of 3 is shown.
b. Mean donor cell frequency in local dLN/pLN (top) and absolute donor cell number (bottom).
c. Mean donor cell frequency in local ndLN/pLN (left) and absolute donor cell number (right) Error displayed as ± standard error mean.

6.2.16: Matahari T cells infiltrate MB/IL-2 more effectively than MB49 tumours

In a parallel experiment (without CFSE staining) the representation of Matahari CD8 T cells infiltrating the MB49 and MB/IL-2 tumours were examined. Matahari CD8^{+} T cells (Thy1.2) were injected to MB49-bearing or MB/IL-2 bearing B6 female (Thy1.1) recipients. Mice were culled on day 9 and donor T cell populations were analysed in the dLN and ndLN. Tumours were harvested and stained for the presence of Matahari donor T cells as described in section 6.14. Tumour infiltrating donor Matahari T cells were gated and analysed for their activation status according to expression of CD62L and CD44 (Fig. 6.16a). Matahari CD8 T cells accumulated to a higher level in MB/IL-2 compared to MB49 tumours (Fig. 6.16b). This was also reflected in average CD44 MFI which was significantly higher in Matahari T cells from MB/IL-2 compared with those in MB49 tumours (Fig. 6.16c) consistent with an effector phenotype. Donor cells in the local dLN were also stained for CD44 and CD62L to give a readout of their activation status. Donor CD8s in the MB/IL-2 dLN showed a greater skew towards an effector memory phenotype (CD44^{high}, CD62L^{low}) compared to those in MB49 tumours (Fig. 6.16d). Together, these data suggest Matahari CD8 T cells are more capable of expanding in response to MB/IL-2, express a stronger effector phenotype and are more effective at entering the tumour itself compared to MB49.

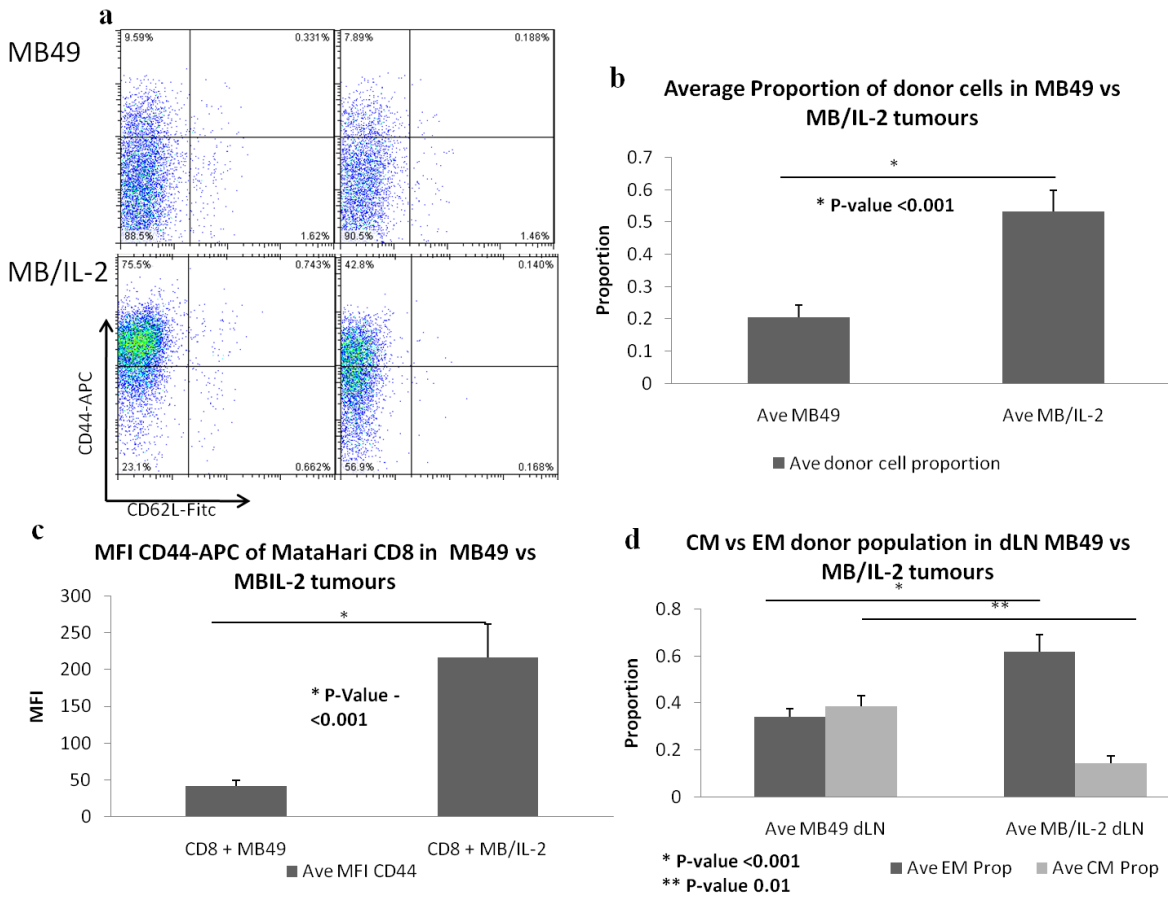


Figure 6.16. Adoptively transferred Matahari T cells infiltrate MB/IL-2 more effectively than MB49

Experimental set up as in Fig. 6.14, except CFSE staining was not performed.

a. Tumours were harvested and stained using anti-CD62L^{FITC}, anti-Vβ8.3^{PE}, anti-CD8^{PerCP} and anti-CD44^{APC}. Cells were analysed by FACS and gated using lymphocyte gate, then CD8^{+} Vb8.3^{+} before final donor population FACS plot displayed as CD44 vs CD62L. Four representative FACS plot is shown of four independent tumours.

b. Mean donor cell frequency in tumours (n=4/group).

c. Mean MFI of CD44 for donor cells tumours (n=4/group).

d. dLNs were harvested and stained using anti-CD62L^{FITC}, anti-Vβ8.3^{PE}, anti-CD8^{PerCP}, anti-CD44^{APC}. Cells were analysed by FACS and gated using lymphocyte gate, then CD8^{+} Vβ8.3^{+} before final donor populations were analysed for CD44 vs CD62L expression. Average donor populations of CM vs EM donor cells are shown (n=4/group). Error is displayed as ± standard error mean.

6.2.17: MB/IL-2 tumours induce a stronger systemic activation of donor Matahari and WT CD8 T cells compared to MB49

In the experiment described in Fig. 6.13, $Rag^{-/-}$ female B6 mice received either FACS sorted Matahari or WT CD8 T cells and were challenged with either MB49 or MB/IL-2. Both groups of mice controlled or cleared MB/IL-2 tumours but not MB49 tumours (see Fig. 6.13). On day 20, 2 mice from each group were sacrificed and analysed for the ability of donor T cells to produce the proinflammatory Th1 cytokines TNFα and IFNγ (Fig. 6.17). Spleen and tumour tissue cells were harvested and plated out with PMA and Ionomyin in the presence of Brefeldin A for 4 hours and stained for intracellular TNFα (Fig. 6.17a and b) and IFNγ (Fig. 6.17c and d). Both Matahari and WT CD8 T cells produced more TNFα and IFNγ in the spleen and tumour samples of mice injected with MB/IL-2 compared to those bearing MB49 tumours. This also was true in terms of MFI (Fig. 6.17e) and the proportion of cells expressing each cytokine (Fig. 6.17f). All comparisons of cytokine production by T cells from MB/IL-2 tumour-bearing mice show statistically greater expression than those from their MB49 counterparts. Therefore donor T cells from mice that were injected with MB/IL-2 are able to induce a stronger systemic Tc1 cytokine response than those given MB49. This is reflected by the ability for both types of CD8 T cells to control MB/IL-2 but not MB49 (Fig. 6.13).

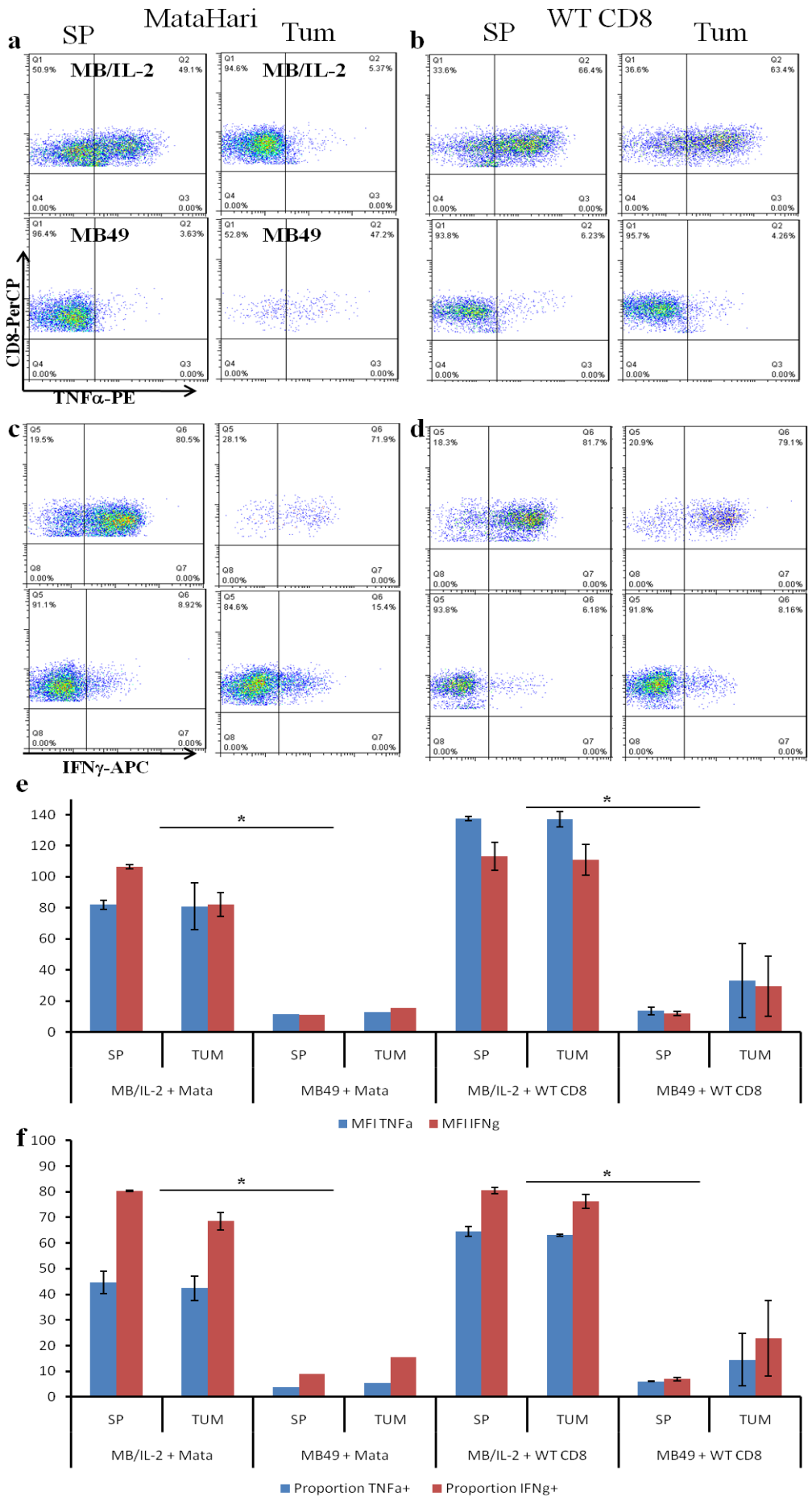

a
MataHari
SP
Tum
MB/IL-2
MB49
CD8-PerCP
TNFα-PE
b
WT CD8
SP
Tum
c
IFNγ-APC
d
e
MB/IL-2 + Mata
MB49 + Mata
MB/IL-2 + WT CD8
MB49 + WT CD8
MFI TNFa
MFI IFNg
f
Proportion TNFa+
Proportion IFNg+

Figure 6.17. Matahari and WT CD8 from MB/IL-2 bearing mice produce a stronger Tc1 response *ex vivo*

Experimental set up as in Fig. 6.13. On day 20, 2 mice from each group were culled (1 mouse for Mata + MB49 group) and SP and tumour were harvested and cell suspension plated out *in vitro* with PMA (50ng/ml) + Ionomycin (250ng/ml) + Brefeldin A (1μg/ml) for 4 hours. Cells were harvested and stained for anti-$TCR\beta^{FITC}$ (WT CD8) or $V\beta8.3^{FITC}$ (Matahari) and anti-$CD8^{PerCP}$ and then intracellular for anti-$TNF\alpha^{PE}$ and anti-$IFN\gamma^{APC}$. Cells were analysed by FACS and gated on lymphocytes and then $CD8^+$ $TCR\beta^+/V\beta8.3^+$ cells. (* P-value <0.01).

a and b. FACS plots of CD8 vs TNFα for Matahari ACT mice (a) and WT CD8 ACT mice (b). One representative FACS plot/sample shown.

c and d. FACS plots of CD8 vs IFNγ for Matahari ACT mice (c) and WT CD8 ACT mice (d). One representative FACS plot/sample is shown.

e. Average MFI of $TNF\alpha^{PE}$ (blue) and $IFN\gamma^{APC}$ (red) for each condition as described above.

f. Average proportion of TNFα (blue) and IFNγ (red) producing cells for each condition as described above. Error is displayed as ± standard error mean.

6.2.18: E*x vivo* adoptively transferred Matahari CD8 T cells from MB/IL-2 tumour dLN maintain a stronger Tc1 response after *in vitro* HY re-stimulation compared to those from MB49 dLN

Donor Matahari T cells from the dLN of experimental mice in Fig. 6.16 were isolated *ex vivo* and plated out in an *in vitro* assay to explore how donor T cells from MB/IL-2 and MB49-bearing mice respond to secondary antigen re-stimulation. dLN cells from MB49-bearing and MBIL-2 bearing mice, as well as control mice (CD8 cell transfer into non-tumour-bearing mice) were harvested and plated out with irradiated male spleen for 5 days *in vitro*. On day 5, cells were harvested and analysed for expression of Tc1 cytokines IFNγ and TNFα (Fig. 6.18a and b). Matahari donor cells from the dLN of MB/IL-2 and MB49-bearing mice showed a higher level of INF-γ (Fig. 6.18a) and TNFα (Fig. 6.18b) expression compared to the T cell alone control. However donor cells from the dLN of mice injected with MB/IL-2 showed higher expression levels of INF-γ (Fig. 6.18a) and TNFα (Fig. 6.18b) compared to those from MB49 tumour-bearing mice. These data indicate that donor T cells from mice injected with MB/IL-2 cells are more capable of maintaining a Tc1 phenotype *ex vivo* in response to male spleen compared to those from MB49-bearing hosts.

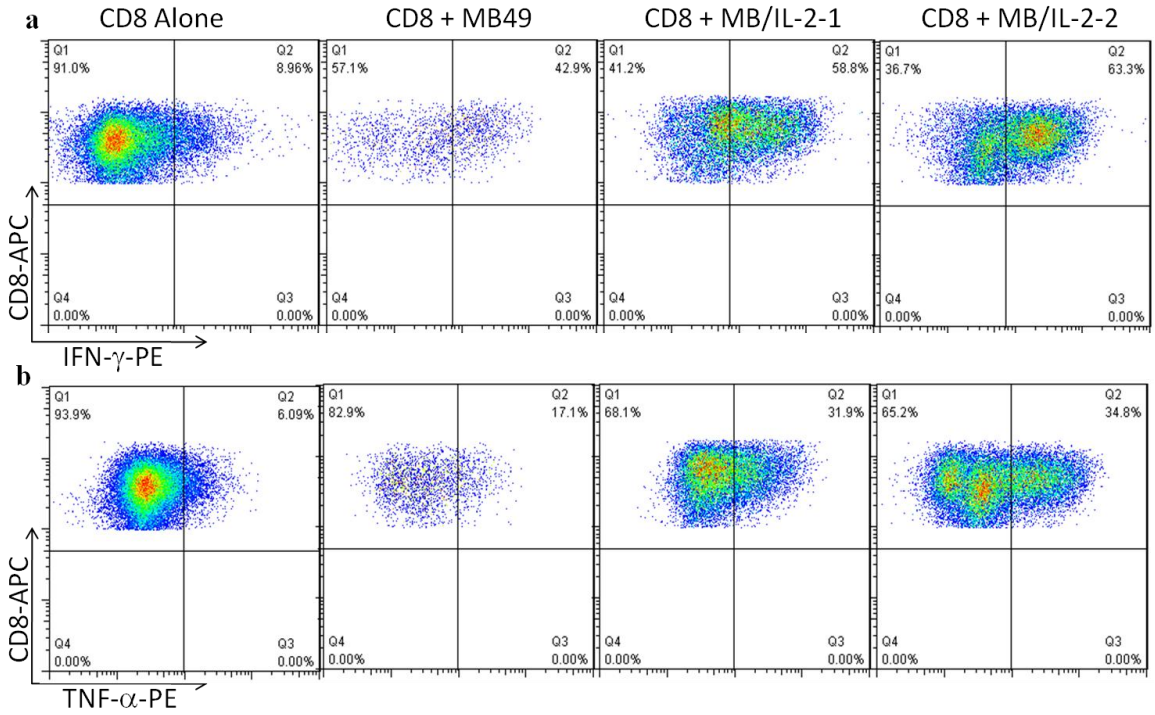


Figure 6.18. Adoptively transferred *ex vivo* Matahari *in vitro* Th1 responses to male spleen

Experimental set up as in Fig. 6.16. Mice were culled on day 9 and peripheral lymphoid organs were harvested and plated out with irradiated (30Gy) Male SP cells at 5 x 10^6/well in a 24 well plate and cultured for 5 days *in vitro* (G1 n=1, G2 n=1, G3 n=2). Cells were harvested and stained using anti-Vβ8.3^{FITC}, anti-CD8^{PerCP} and anti-Thy1.2^{APC}. Cells were then fixed and stained intracellular.

a. Cells were stained using anti-IFN-γ^{PE} and analysed by FACS, cells were gated as Vβ8.3, CD8 and Thy1.2 triple positive and then expressed as CD8 vs IFN-γ.

b. Cells were stained using anti-TNFα ^{PE} and analysed by FACS, cells were gated as Vβ8.3, CD8 and Thy1.2 triple positive and then expressed as CD8 vs TNFα.

6.2.19: MB/IL-2 displays a very similar surface phenotype to MB49

To assess the impact of genetic modification on the surface expression profile of the tumour cell lines, MB49 or MB/IL-2 cells were plated out for 4 days in the presence of IFN-γ. This method is used in the study to characterise natural MB49 responses (see chapter 3). MB49 cells are gated as a single population while MB/IL-2 cells are gated on MB49 then GFP^+ cells to ensure a transgenic population is assessed (Fig. 6.19a), surface expression on the cells were then measured for a range of molecules. MB49 typically upregulates MHC class I (D^b and K^b), Class II (A^b) as well as immunoevasive molecules such as PD-L1 and CTLA-4 upon exposure to IFN-γ (Fig. 6.19b). This is also shown by the MB/IL-2 cell line (Fig. 6.19c). MB49 and MB/IL-2 upregulate immunogenic molecules such as GITR/GITRL which might induce T cell co-stimulation and block suppression (Fig. 6.19b and c), while also expressing adhesion molecules such as CD49b and CD44. Both cell lines also upregulate stem cell marker Sca-1, as well as the CCR9 gut homing receptor (Fig. 6.19 b and c). These data indicate that IL-2 gene transfer to MB49 cell line by a retroviral approach does not have a significant influence on phenotype of the tumour cell, at least for the molecules analysed. In response to IFNγ, MB/IL-2 modulates gene expression with a similar phenotype to MB49 cells, adding further evidence for the direct role of rhIL-2 in stimulating CD8 T cells to induce rejection of this tumour line.

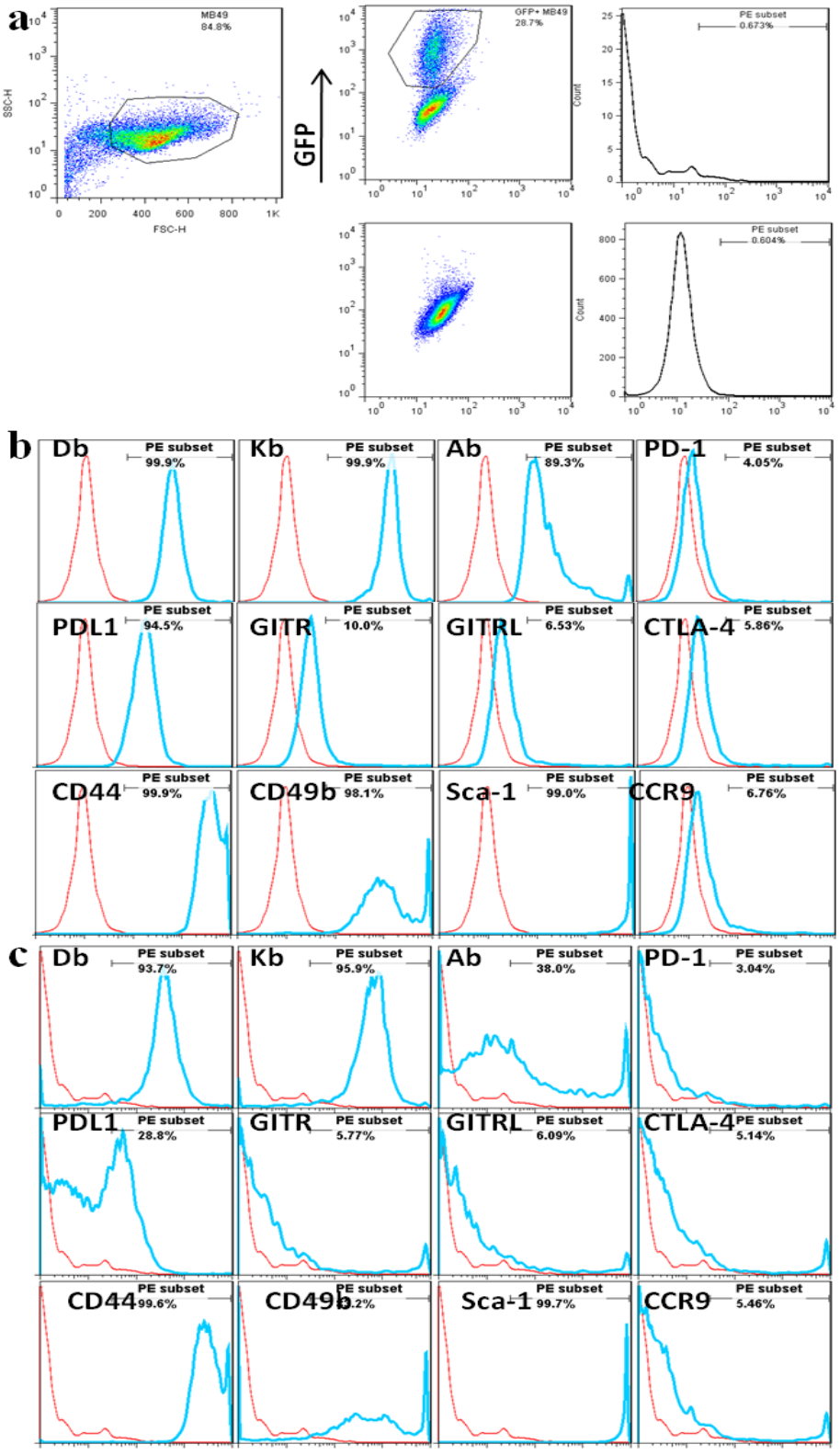


Figure 6.19. MBIL-2 and MB49 cells are phenotypically similar
MB49 and MBIL-2 cells were plated out at 5 x 10^5 cells for 4 days in the presence of IFNγ (10ng/ml). Cells were harvested and aliquoted in equal amounts for surface staining using a range of PE-conjugated mAbs and analysed using FACS.
a. MB49 cells were gated as a single population; MB/IL-2 cells were further gated using GFP^+ fraction.

b. MB49 surface expression of D^b, K^b, A^b, PD-1, PD-L1, GITR, GITRL, CTLA-4, CD44, CD49b, Sca-1, CCR9 (blue line) and isotype^{PE} control (red line).
c. MB/IL-2 surface expression of D^b, K^b, A^b, PD-1, PD-L1, GITR, GITRL, CTLA-4, CD44, CD49b, Sca-1, CCR9 (blue line) and isotype^{PE} control (red line).

6.3: Discussion

In this section, a model of IL-2 mediated tumour rejection is established. Previous studies using systemic IL-2 therapy in cancer therapy have yielded mixed results especially in patients showing non-specific IL-2 toxicity (Rosenberg, Packard et al. 1988; Siegel and Puri 1991). Despite this limitation, several types of cancer are currently treated using variable IL-2 dose regiments, including renal cancer (Fisher, Rosenberg et al. 2000), colorectal cancers (Simpson, Heys et al. 1995) and melanoma (Guminski and Thompson 2009; Powell and Dudek 2009)**.** However the efficacy of these treatments remains an issue with some patients experiencing complete regression and others having no protective response following systemic IL-2 treatment (Guirguis, Yang et al. 2002).

To overcome some of these problems, IL-2 gene therapy using recombinant viral vectors has been employed in treatment of a number of cancer types to deliver IL-2 locally. This has yielded some therapeutic responses particularly in prostate cancers (Pantuck and Belldegrun 2001) and other solid tumours such as melanomas (Bubenik 1993; Tan, Xu et al. 1996; Lichtor, Glick et al. 2002). Despite the reduced toxicity and increased efficacy, there remain many questions for a full understanding of the effects or IL-2 in contributing to cancer regression. For most tumours, IL-2 genes cannot be effectively delivered *in vivo,* resulting in poor IL-2 production. Several groups have transduced tumours with cytokine genes such as IL-2 *ex vivo* to act as a whole cell vaccine in murine (Connor, Bannerji et al. 1993) and human cancers (Milella, Jacobelli et al. 1999). While these approaches can be effective in inducing protective immunity, they have not provided insight into mechanisms of rejection.

The use of IL-2 transduced tumours to induce a potent anti-tumour immune response is not new (Fearon, Pardoll et al. 1990). Studies by Fearon *et al.* and others have demonstrated that a paracine effect of intra-tumoural IL-2 capable of inducing

strong CTL responses and protective immunity (Gansbacher, Zier et al. 1990; Cignetti, Guarini et al. 1994). Despite these initial findings, little is known regarding the cellular and molecular mechanisms that allow recognition and rejection of these tumours while overcoming immunosuppressive factors. Our current study allows the effects of local IL-2 to be studied in a murine solid carcinoma model which is not spontaneously rejected in WT hosts. The design of this system is an attempt to understand the cellular and molecular effects of IL-2 in overcoming tolerogenic mechanisms employed by MB49 tumours to prevent killing by the adaptive immune system.

In order to generate a model by which IL-2 would be released into the local tumour microenvironment, the MB49 cell line was directly transduced with MIGR1 retrovirus containing a rhIL-2 gene (Fig. 6.1). The tumour line was FACS sorted (Fig. 6.1c) and selected by single cell cloning (Fig. 6.2a) to generate a monoclonal cell line (called MB/IL-2). MB49 was also transduced with MIGR1 empty vector to generate a 'green' tumour cell line with no additional transgene, which acts as a control (called MB/GFP) to evaluate the effects of rhIL-2 (Fig. 6.1). MB/IL-2 and MB/GFP cells were maintained as an *in vitro* cell line, and it was noted that the retrovirally transduced transgene was subject to loss over time (Fig. 6.2b), as has been reported in several situations in which transgenes can 'jump out' of the genome (Krueger, Jorgensen et al. 1999). In order to ensure consistency in the level of rhIL-2 production, MB/IL-2 frozen stocks were used for all subsequent experiments between with a transduction efficiency of between 50-60% (as measured by GFP expression).

To confirm functional activity of the rhIL-2 construct, MIGR1/rhIL-2 retrovirus was generated (Fig. 6.3a) and used to transduce a CTLL-2 murine cytotoxic T cell line (Fig. 6.3b). As CTLL-2 is strictly dependent on exogenous IL-2 for survival and maintenance *in vitro*, this is an appropriate system to test the bioactivity of IL-2 and is often used as the industry standard IL-2 activity assay (Weston, Geczy et al. 1998). CTLL-2 cells transduced with rhIL-2 (11% GFP^+) were able to proliferate in the absence of exogenous IL-2 with the same level at all IL-2 doses tested, indicating that the functional rhIL-2 proteins are secreted by transduced cells (Fig. 6.3c). To confirm the transduced MB/IL-2 tumour secreted functional rhIL-2, supernatant from MB/IL-2 cells were examined for the presence of IL-2 by a standard CTLL2 bioassay. Supernatant from MB/IL-2 cells can support

CTLL2 to grow *in vitro* whereas those from MB49 cells cannot. In addition, based on this standard bioassay, it was estimated that rhIL-2 production by MB/IL-2 cells is approximate 20IU/0.5M cells/day *in vitro* (Weston, Geczy et al. 1998) (Fig. 6.3d) characterising MB/IL-2 as a low IL-2 producer (Yang, Topalian et al. 1994).

The MB49 bladder carcinoma is an aggressive immunogenic tumour (Perez-Diez, Joncker et al. 2007) which can induce rapidly growing tumours in WT B6 female mice (see chapter 3). MB/IL-2 tumours fail to grow in lymphoreplete hosts but are able to induce a protective immunity to MB49 re-challenge in both males and females (Fig. 6.4). This suggests that MB/IL-2 is immunogenic and capable of inducing a polyclonal immunological effector memory response directed against additional tumour-specific transplantation antigens, as male B6 mice do not generate T cells or B cells reactive to HY genes expressed by MB49 (Scott, Ehrmann et al. 1995) (Fig. 6.4b). This result is concordant with those of several studies showing protective immunity against IL-2 transduced tumours in lymphoreplete mice (Fearon, Pardoll et al. 1990; Cignetti, Guarini et al. 1994) capable of inducing long standing immunological memory. These data indicate that rejection of MB/IL-2 is at least not wholly dependent on HY-specific responses but more likely induces polyclonal immune response to additional antigens. Tumours from male mice were harvested on day 10 and analysed for GFP expression (Fig. 6.4c), and showed >50% GFP^+ expression demonstrating that the transgene is stable *in vivo* for the duration of tumour growth. MB/IL-2 tumours also display a distinct appearance in lymphoreplete hosts prior to complete rejection, with small tumours (~3mm maximum size), compact internal structure with no angiogenesis or blood pooling (Fig. 6.5).

To exclude the possibility that tumour rejection is mediated by additional antigenic components from the MIGR1 vector itself provides (such as GFP), tumourigenicity of transduced tumours was tested using the MB/GFP cell line (MIGR1 transduced alone (Fig. 6.6b)) inoculated to WT male recipients (Fig. 6.6). All mice developed tumours (Fig. 6.6a) which grew with similar kinetics to untransduced MB49 cells. MB/GFP tumours also showed significant mass, angiogenesis and blood pooling (Fig. 6.6c). This result indicates that retroviral modification itself does not induce tumour regression and any antigenic contribution of additional proteins is minimal.

Spontaneous rejection of MB/IL-2 tumours could also be attributed to non-specific toxicity of the transgene to tumour cells. If this was the case, one might anticipate that MB/IL-2 cells would not grow as progressive tumours in lymphopenic recipients in which T and B cells are absent. To test this possibility, lymphopenic recipients were inoculated with MBIL-2 (Fig. 6.7a) or MB49 cells (Fig. 6.7b): all tumours grew to reach the humane end point at very similar rates. In lymphopenic recipients MB/IL-2 showed significant tumour mass (Fig. 6.8b), angiogenesis (Fig. 6.8d) and blood pooling (Fig. 6.8c) similar to MB49 (Fig. 6.8a,c,e). The ability of MB/IL-2 to grow into tumours in lymphopenic hosts illustrates that the presence of an intact adaptive immune system is required for protection; IL-2 is therefore likely to be acting on specific adaptive immune responses to induce tumour killing rather than on innate responses alone. This result is consistent with several other examples of IL-2 transduced tumours (Fearon, Pardoll et al. 1990; Gansbacher, Zier et al. 1990; Cignetti, Guarini et al. 1994). In contrast, Alosco *et al*. have reported that the IL-2 transduced murine fibrosarcoma cell line (CMS-5) is rejected in SCID mice in an innate NK dependent manner (Alosco, Croy et al. 1993). And Hara *et al*. have even shown an IL-2 producing renal carcinoma is rejected in NK depleted athymic Balb/c mice by a non-specific inflammatory mechanism. These two models however used IL-2-high producing tumours, and high levels of IL-2 have been shown to independently activate NK, neutrophils and granulocytes that may mediate rejection. In addition, high levels of IL-2 from a therapeutic standpoint would be most likely toxic in humans (Assier, Jullien et al. 2004), while mice seem to be tolerant to high doses of IL-2.

Mice that have rejected MB/IL-2 are capable of rejecting large doses of MB49 (Fig. 6.4) indicating a potent memory immune response has been induced. Mice injected with MB/IL-2 and MB49 cells in the separate sites were investigated to determine if they could reject both tumours (Fig. 6.9). This experimental approach gives each tumour its own microenvironment and local dLN. Under these conditions MB/IL-2 was rejected as shown previously while MB49 on the opposite flank grew normally, with no difference in MB49 growth between those receiving two site injections of MB49 and those receiving MB49 and MB/IL-2. MB/IL-2 therefore is capable of inducing a potent primary local response but this is insufficient to provide systemic protection to MB49 on the same hosts when

challenged at the same time. To use MB/IL-2 as a therapeutic vaccine for established tumours it is likely that inoculation must therefore be applied locally.

In a parallel experiment to assess the local response, MB49 and MB/IL-2 cells were mixed in a range of ratios and inoculated to WT B6 female mice (Fig. 6.10). Mixtures of tumour cells were rejected in 100% of mice in ratios of MB49:MB/IL-2 as low as 95:5 and 75% rejection in 99:1 ratio (Fig. 6.10). This indicates that the level of IL-2 production required to induce rejection is relatively low, with less than 5% of cells needed to produce rhIL-2 to cause rejection of the whole tumour. This effect is even more pronounced when it is considered that MB/IL-2 is only ~60% GFP^+ at the time of injection. This result is consistent with those of Gansbacher *et al.* who showed that co-injection of IL-2 producing CMS-5 cells with unmodified tumour cells inhibited tumour formation even when highly tumourigenic doses of CMS-5 cells were used (Gansbacher, Zier et al. 1990).

Having elucidated the growth characteristics of MB/IL-2 cells, the second part of this study focused on the exploration of the mechanisms of rejection. From previous studies injecting MB49 cells *in vivo*, it is clear that T cells are required in order to induce rejection, although CD8 T cells alone are insufficient (see chapters 3 and 4) (Perez-Diez, Joncker et al. 2007). Previous evidence however has shown the role of CD8 effectors is crucial in the rejection of IL-2 producing tumours (Fearon, Pardoll et al. 1990; Gansbacher, Zier et al. 1990). Also, IL-2 is a potent regulator of CD4 and CD8 effector T cells and memory development, as well as Treg maintenance and expansion, (Fontenot, Rasmussen et al. 2005) and intratumoural cytokine vaccination has been shown to increase recruitment of CD4 and CD8 effectors to certain tumours (Kianmanesh, Hackett et al. 2001). To evaluate the effects of T cells in this model a series of experiments were performed.

B6 male mice were depleted of CD4 T cells, CD8 T cells or both using *in vivo* depleting antibodies before tumour inoculation (Fig. 6.11). Only mice that received anti-CD8 treatment, either alone or with anti-CD4, had their protection abrogated, leading to tumour outgrowth in all mice. This result indicates that CD8 T cells play a potent role in the rejection of MB/IL-2. No significant difference was seen in tumour growth between CD8 depleted and CD4/8 double depleted groups suggesting that CD4 T cells are not required for rejection. This conclusion was also in line with the observation that CD4 depleted mice are able to clear MB/IL-2 tumours as efficiently as mice in the untreated group. Fearon *et al.* performed

similar depleting experiments of CD8 and CD4 T cell with similar results (Fearon, Pardoll et al. 1990).

Several studies have highlighted the idea that intra-tumoural IL-2 may act in a non-specific or polyvalent manner, bypassing direct responses to TAAs (Alosco, Croy et al. 1993; Hara, Hotta et al. 1996). So far other studies have only evaluated polyclonal CTLs in the rejection of IL-2 producing tumours. Therefore CD8 mediated MB/IL-2 rejection was assessed further using HY-specific Matahari CD8 T cells (Fig. 6.12-19). Initially $Rag^{-/-}$ Matahari mice were challenged with MB/IL-2 tumour (Fig. 6.12) as these mice produce only TCR transgenic monoclonal CD8 T cells reactive to HY-*Uty*/D^b complexes expressed by MB49 (and MB/IL-2) (Fig. 6.12a). 79% of Matahari mice rejected MB/IL-2 with 100% survival to day 35 compared to complete tumour development and 0% survival in the MB49 group. This result suggests that antigen-specific CD8 T cells alone can be sufficient to control MB/IL-2 without any other components of the adaptive immune system.

Following the results using female Matahari $Rag^{-/-}$ mice themselves as, adoptive transfer experiments were designed to assess the role of Matahari CD8 T cells and WT CD8 T cells in MB/IL-2 and MB49-bearing $Rag^{-/-}$ B6 hosts (Fig. 6.13). Lymphopenic mice that received adoptive transfer of either WT CD8 or HY-specific Matahari CD8 T cells were able to control MB/IL-2 tumours but not those arising from MB49 cells (Fig. 6.13b). Importantly in this experiment, although the lymphopenic recipients were able to limit outgrowth of MB/IL-2 tumours, adoptive transfer did not induce a durable cure; this is most likely due insufficient reconstitution of CD8 T cells in the periphery, compared to the use of lymphoreplete WT (Fig. 6.9) or Matahari mice as recipients (Fig. 6.12). Collectively, these data indicate both WT and antigen-specific CD8 T cells can provide protection against an MB/IL-2 tumour in WT and transgenic mice as well as upon adoptive transfer to lymphopenic tumour-bearing recipients. CD8 T cells are therefore both necessary and sufficient to induce MB/IL-2 rejection *in vivo*.

In a separate experiment, Matahari CD8 T cells were purified, CFSE labelled and injected alone (G1) to MB49-bearing mice (G2) or to MB/IL-2 bearing (G3) WT B6 mice (Fig. 6.14, 6.15). Donor T cells expanded significantly more in MB/IL-2 bearing mice both in terms of frequency and absolute cell number (Fig. 6.14a and Fig. 6.15a and b). This was also reflected in the dilution of CFSE showing almost all donor cells in dLN of G3 underwent an extensive division while those in mice from

G1 and G2 did not (Fig. 6.14b,c and d). Interestingly, there was no significant difference in levels of expansion for each of the three groups with respect to the ndLN (Fig. 6.15c), again indicating that the primary CD8 response against MB/IL-2 is restricted locally.

In a parallel experiment (as in Fig. 6.14, except no CFSE staining of donor T cells) tumours and dLN were harvested and analysed for donor Matahari T cell tumour infiltration and their activation status (Fig. 6.16). Donor Matahari CD8 T cells again accumulated more efficiently in MB/IL-2 compared to MB49 tumours (Fig. 6.16b) and showed a significantly higher level of activation in terms of CD44 expression (Fig. 6.16a and c). The dLN of MB/IL-2 bearing mice was shown to have a significantly higher level of effector memory CD8 T cells and a significantly lower level of central memory CD8 T cells (Fig. 6.16d), effector memory CD8 T cells have been shown to be more effective than central memory T cells at tumour killing *in vivo* (Klebanoff, Gattinoni et al. 2005). A higher state of activation may partially explain the enhanced CTL response seen against IL-2 transduced tumours (Fearon, Pardoll et al. 1990; Gansbacher, Zier et al. 1992).

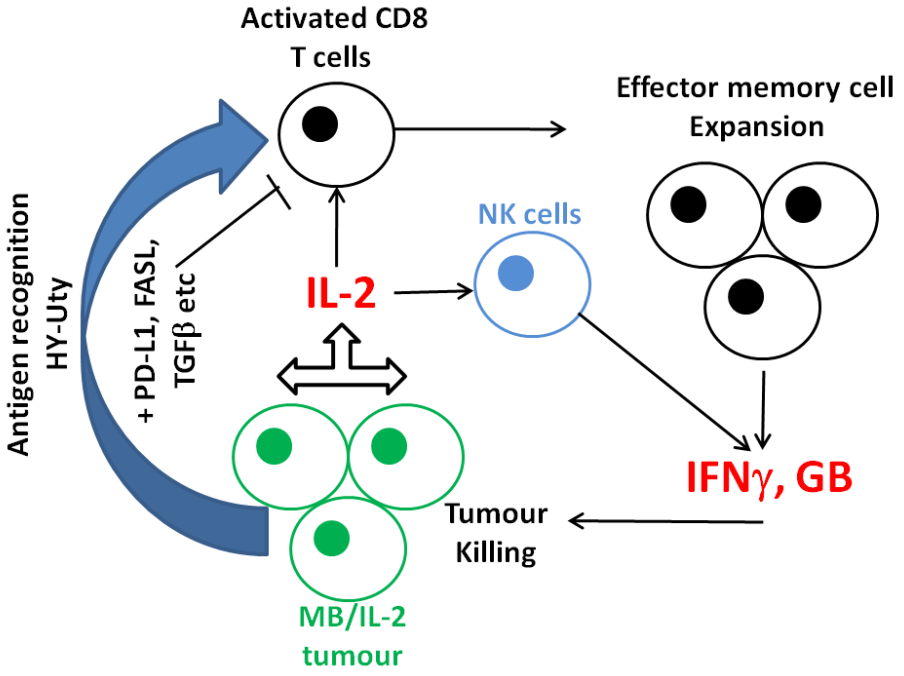
Due to the enhanced CD8 activation seen in MB/IL-2 bearing mice, HY-specific donor cells were also evaluated for their Tc1 cytokine response following HY re-stimulation (Fig. 6.18). Donor cells from MB/IL-2 bearing recipient mice showed the highest levels of IFN-γ and TNF-α production. These data suggest the donor CD8 T cells have been induced to differentiate to Tc1-like cells by the MB/IL-2 tumour. Taken together, these data clearly show a requirement for CD8 T cells for rejection of MB/IL-2. MB/IL-2 is capable of expanding and activating antigen-specific CD8 T cells, while MB49 does not. This suggests a role for rhIL-2 in the local tumour microenvironment that prevents the induction of tolerance in tumour-specific CD8 T cells.

HY-specific and WT CD8 responses to MB49 and MB/IL-2 were also directly compared *ex vivo* by measuring the ability of donor cells from mice depicted in Fig. 6.13 to produce Tc1 cytokine upon *in vitro* stimulation (Fig. 6.17). Both Matahari and WT CD8 T cells produced more IFNγ and TNFα from mice bearing MB/IL-2 tumours compared to those bearing MB49 tumours, with similar levels of cytokine production (by percentage and MFI) for both WT and antigen-specific CD8 T cells. This was true both systemically in the spleen and locally in the tumour itself. This shows MB/IL-2 tumours are able to induce a strong Tc1 proinflammatory

phenotype in CD8 T cells even in lymphopenic mice where no additional adaptive responses are present. This also shows little discernable difference in the potency of the WT and Matahari HY-specific CD8 response. This result is also profound when considering the naturally self-reactive phenotype usually seen in Matahari CD8 T cells (See chapter 4, Fig. 4.6).

A final consideration of this model is the phenotype of the tumour itself, as rhIL-2 may induce changes in surface marker expression that may indirectly increase the immunogenicity of the tumour (Fig. 6.19). MB/IL-2 shows a very similar pattern of surface molecule expression *in vitro* to MB49, crucially upregulating MHC Class I, Class II and PD-L1 in response to IFNγ exposure. rhIL-2 is therefore most likely to have a direct effect on T cells rather than changing the immunogenicity of the tumour itself. A model of MB/IL-2 based on these data is shown in the table below.

Table 8: Model of MB/IL-2 rejection



This study adds to and supports current knowledge regarding the role of intra-tumoural IL-2 in anti-tumour immunity (Fearon, Pardoll et al. 1990; Gansbacher, Zier et al. 1990; Gansbacher, Zier et al. 1992; Gastl, Finstad et al. 1992; Cignetti, Guarini et al. 1994; Milella, Jacobelli et al. 1999). Here it is shown that low level intra-tumoural IL-2 secretion is capable of inducing MB49 rejection in a CD8 dependent manner and that this response can be mediated by both WT and antigen-specific monoclonal CD8 T cell compartments. I also show that low IL-2 producing MB/IL-2 is not rejected by innate immune responses in lymphopenic hosts, as has

been reported with IL-2 high tumours (Alosco, Croy et al. 1993; Hara, Hotta et al. 1996). rhIL-2 production is sufficient to overcome tumour-induced tolerance (See chapter 3) providing a mechanism to break CD8 T cell adaptation seen with MB49 tumours (see chapters 4) and other antigenic environments (see chapter 7). MB/IL-2 rejection is also mediated locally, potentially avoiding the toxic systemic effects of IL-2 observed in humans.

The principal aim of this study was to investigate the kinetics of MB/IL-2 rejection, using these data to attempt to model mechanisms of primary rejection. The potential of IL-2 to provoke therapeutic responses in recipients bearing established tumours has not been addressed in this study. The capability of IL-2 transduced tumours to act as an *in vivo* vaccine boost for established tumours has been confirmed with several reports in mice (Fearon, Pardoll et al. 1990; Gansbacher, Zier et al. 1990; Alosco, Croy et al. 1993) and humans (Gansbacher, Zier et al. 1992; Cignetti, Guarini et al. 1994; Milella, Jacobelli et al. 1999). Having elucidated the potential mechanisms of rejection further investigation can now take place into the ability of this modified tumour to act as a therapeutic vaccine of established MB49 tumours.

Chapter 7: Acute Graft vs Host Disease (GvHD) can be induced in male mice by adoptive transfer of HY-specific CD4 but not CD8 T cells

7.1: Introduction

7.1.1: GvHD

Graft versus host disease (GvHD) is characterised by hematopoietic stem cell (HSC) transplantation and is produced by donor T cells attacking host tissues though recognition of either mis-matched MHC or minor H antigens. GvHD was originally observed when irradiated mice were infused with allogenic spleen cells and bone marrow cells. Mice recovered from the acute affects of irradiation and haematopoietic aplasia but died of a subsequent disease which caused weight loss, skin rash, diarrhoea and appetite loss (Barnes, Loutit et al. 1962), later described by Simonsen as GvHD (Simonsen 1965). The three requirements for the development of GvHD were formulated soon after by Billingham (Billingham 1966) who demonstrated that firstly, immunologically competent cells are required and that the level of GvHD severity is directly associated with donor mature T cells (Kernan, Collins et al. 1986; Korngold and Sprent 1987). Secondly, recipients must be immuno-compromised and therefore incapable of rejecting the donor cells, a conditioning induced by chemo/radio therapy (Welniak, Blazar et al. 2007). Thirdly, the recipient must express antigens not present in the original donor, this observation was a major step in the discovery of the mouse and human MHC loci (Petersdorf and Malkki 2006) (Krensky, Weiss et al. 1990).

7.1.2: Acute GvHD and T cells

GvHD has two major classifications, chronic and acute GvHD. Chronic GvHD occurs typically from 3 months post-transplant and is induced by T cells resulting from engrafted bone marrow. It is associated with T cells that escape negative thymic selection (Whedon and Wujcik 1997) and it is analogous to systemic Lupus Erythematosis in terms of symptoms. In contrast, acute GvHD represents a

normal but exaggerated inflammatory response, shortly after transplantation, in an environment where the effect is undesirable. Donor lymphocytes, usually infused to treat leukaemia, encounter tissues that have been damaged by conditioning radiation. Donor cells not only respond to a foreign environment but to one that has been altered to a proinflammatory phenotype promoting activation and proliferation. Therefore acute GvHD is an exaggerated version of a normal inflammatory immune response (Reddy and Ferrara 2003) and is characterised by three phases.

The first stage of acute GvHD is the activation of APCs. This can be triggered by total body irradiation therapy prior to the infusion of allogenic donor bone marrow (Clift, Buckner et al. 1990) leading to changes in the local inflammatory environment whereby damaged tissues release factors such as IL-1 and TNFα, in what is known as a cytokine storm (Hill and Ferrara 2000). This leads to increased expression of adhesion molecules, co-stimulatory molecules, MHC and chemokines that can recruit infused donor lymphocytes (Hill and Ferrara 2000). Furthermore, damage to certain tissues such as the gastrointestinal tract can lead to systemic translocation of microbial compounds such as LPS inducing a Th1 pattern APC activation in the local environment, which may be the source of donor T cell priming of activated APCs (Paris, Fuks et al. 2001; Murai, Yoneyama et al. 2003). The relationship between the initial radiation intensity and the resultant GvHD is strongly correlated with higher levels leading to increased cytokine production, tissue damage and APC activation, as shown by elegant murine studies (Hill, Crawford et al. 1997; Paris, Fuks et al. 2001). Amongst APCs, DCs are the most important in inducing GvHD which can be regulated by qualitatively or quantitatively modulating DC subsets (Duffner, Maeda et al. 2004; Chorny, Gonzalez-Rey et al. 2006), with DC ablation prior to irradiation resulting in reduced GvHD severity (Auffermann-Gretzinger, Lossos et al. 2002).

The second phase of acute GvHD is caused by infused donor T cells that interact with primed APCs in secondary lymphoid organs where they are primed and activated for subsequent proliferation and migration (Beilhack, Schulz et al. 2005). T cells can interact with either host (direct presentation) or donor APCs (indirect presentation) (Shlomchik 2003). For direct presentation T cells will bind either directly to MHC (in the case of a MHC mismatch) or to peptide bound to MHC (minor H mismatch) while indirect presentation results from the degradation of antigens for presentation by donor APCs in the context of self-MHC (Lechler, Ng et

al. 2001). It is direct rather than indirect presentation that is critical in inducing GvHD to minor H antigens (Shlomchik 2003). T cell activation is further induced by binding co-stimulation molecules on host APCs that have been upregulated in response to the cytokine storm induced by irradiation. The B7 and TNF family receptors play a crucial role in GvHD (Greenwald, Freeman et al. 2005), and their blockade can reduce murine GvHD (Blazar, Sharpe et al. 2003). On the other hand, blockade of co-inhibitory receptors such as PD-1 and CTLA-4 on T cells can increase the severity of GvHD (Perez, Van Parijs et al. 1997; Blazar, Carreno et al. 2003).

Following co-stimulation and TCR engagement, T cells are induced to transcribe Th1 cytokines such as IFN-γ, IL-2 and TNFα, all of which have been implicated in GvHD (Liu, Siegel et al. 2007). Anti-IL-2 therapy has been used to treat GvHD in many studies (e.g., cyclosporine therapy) although the role of IL-2 in inducing and maintaining Tregs seems to have a reverse role requiring IL-2 for the development of long-term tolerance (Gavin, Rasmussen et al. 2007). IFNγ and other Th1 cytokines play a role dependent on the level of mismatch and the initial radiation dose while regulatory cytokines such as IL-10 and TGF-β have a crucial role in suppressing experimental GvHD (Lin, Storer et al. 2003) (Banovic, MacDonald et al. 2005). Following activation in lymphoid organs, donor T cells traffic to target organs directed by chemokine gradients induced by the initial cytokine storm where they induce tissue damage (Wysocki, Panoskaltsis-Mortari et al. 2005). T cells can then cause further recruitment of leukocytes for example by the release of macrophage inflammatory protein 1 alpha (MIP-1α) which has been shown to increase CD8 T cell migration to liver, lung and spleen (Serody, Burkett et al. 2000).

The final and most damaging phase of acute GvHD is the effector phase. This can be grouped into cellular effectors such as CTLs and NK cells and inflammatory effectors such as cytokines and chemokines, a combination of which is a likely determinant of the severity of GvHD (Sallusto, Mackay et al. 2000). CTLs and NK cells are the main effectors of GvHD, mediating their effect through Fas-FasL, perforin and TNFR apoptosis-inducing ligands such as TRAIL (van den Brink and Burakoff 2002). Infusion of perforin deficient T cells can significantly delay the onset of GvHD against minor H antigens but does not abrogate the disease (van den Brink and Burakoff 2002). The perforin pathway seems to be the main mechanism

of CD8 T cell-induced GvHD, while CD4 effectors preferentially use the Fas-FasL pathway (Jaksch and Mattsson 2005). FasL deficient CD4 T cells effect significantly reduced GvHD in the liver, skin and lymphoid organs (van den Brink and Burakoff 2002), while treatment with anti-FasL can significantly reduce liver damage in GvHD (Hattori, Hirano et al. 1998). GvHD is not completely prevented where donor T cells are deficient for both perforin and Fas suggesting a role for other effector molecules and/or cells such as NK cells (Jiang, Podack et al. 2001).

The production of inflammatory mediators often synergises with CTL recruitment in GvHD. Macrophages primed with IFN-γ released by the donor T cells can produce TNFα and IL-1 upon secondary stimulation through Toll-like receptors. This can be caused by microbial compounds such as LPS which can be increased due to gastrointestinal tract damage during radiation conditioning (Hill and Ferrara 2000; Iwasaki and Medzhitov 2004). The severity of GvHD appears directly related to immune cell priming which is conversely related to the level of TNFα, IL-1 and nitric oxide produced (Teshima, Ordemann et al. 2002). A role for TNFα in GvHD was identified over 20 years ago when mice that developed GvHD following infusion of donor T cells showed high levels of TNFα mRNA in damaged tissues (Piguet, Grau et al. 1987). Anti-TNFα therapy can also inhibit organ damage (Hill, Crawford et al. 1997). TNFα can induce maturation of DCs enhancing antigen presentation, recruitment of T cells, recruitment of monocytes and neutrophilis to target tissues through the induction of chemokines, thus causing direct tissue damage by inducing apoptosis in target tissues. Phase II clinical trials using TNFα antagonists have demonstrated reduced GvHD severity (Uberti, Ayash et al. 2005). IL-1 has also shown similar increases in mRNA levels correlating with GvHD severity and therapy using soluble IL-1Rα reduces GvHD severity (Abhyankar, Gilliland et al. 1993; Antin, Weisdorf et al. 2002) although this is not as profound as for TNFα.

7.1.3: T cell response to persistent systemic minor H antigen in cancer models

T cells respond to minor H antigens on grafts or tumours in order to induce rejection of these tissues (see chapters 3 and 4) (Feng, Hui et al. 2008). However, expression patterns of minor H antigens are not generally different between normal and tumour cells. This broad expression of antigen can have the effect of inducing

chronic stimulation that can affect the activation threshold for T cells (Singh and Schwartz 2003). However, over exposure to antigen can lead to an unresponsiveness of T cells, characterised by reduced cytokine production and poor proliferation in response to subsequent exposure to cognate antigen. This is often characterised as T cell anergy; in some responses to minor H antigens this is actually T cell adaptive tolerance (Chiodetti, Choi et al. 2006). The major feature of such T cell adaptation is their requirement for constant exposure to cognate antigen to remain unresponsive: this is reversible if T cells are removed from the antigen bearing host (Tanchot, Barber et al. 2001). Adaptation therefore allows self antigen reactive T cells to persist which have obvious potential use against malignancies.

Several reports have recently demonstrated an ability to revive the function of antigen-specific CD4 T cells adapted to persistent systemic antigen stimulation (Rivas, Weatherly et al. 2009). In this study, Marilyn CD4 T cells are unable to fully respond to chronic antigen exposure in lymphopenic male mice. Marilyn T cells become quickly adapted, expressing high levels of negative co-stimulatory molecules. Following negative co-stimulation blockade (αPD-L1), a dramatic expansion of Marilyn cells was seen and a severe T cell mediated wasting disease was caused. If combined with a sub-lethal dose of LPS, this could cause acute GvHD. From these results it is clear that adapted T cells can be revived to function in proinflammatory environments where negative co-stimulation is blocked. Other studies have found blockade of CTLA-4 can have a similar effect (Tivol, Borriello et al. 1995; Perez, Van Parijs et al. 1997).

Kitamura et al (Kitamura, Sedlik et al. 2010) used adoptively transferred Marilyn CD4 T cells to treat methylcholanthrene induced HY positive (MCA-HY) transplantable tumour-bearing WT or $Rag^{-/-}$ female B6 mice. In these experiments vaccination using long chain peptides (containing HY-Dby (class II) and HY-Uty (class I) sequences) induced activation and proliferation of the adoptively transferred CD4 cells to an extent that caused death of recipients within hours of vaccination. This was associated with a dramatic increase in IFN-γ, IL-2 and TNFα causing a cytokine storm that correlated with increased APC activation and priming. This study implicates a role for both Th1 and Th17 responses in the induction of GvHD. Improvements in the specificity of T cells and priming strategies in anti-tumour treatments means caution must be taken when pre-conditioning or boosting as this can result in an acute, potentially lethal GvHD like syndrome.

7.1.4: GvHD models

The availability of well characterised inbred mouse strains has led to the development of several mouse models of GvH responses. GvHD caused by MHC disparity is well understood, CD4 and CD8 T cells mediate an additive pathology leading to a 'cytokine storm' capable of inducing GvHD in organs and tissues by direct recognition of non-self MHC (Teshima, Ordemann et al. 2002; Reddy, Negrin et al. 2008). Many mouse models are available for the study of minor H antigens in GvHD with CD4 or CD8 T cells having variable roles depending on genetic disparity and the experimental system.

In the case of HY disparity T cells are activated by recognition of HY peptides expressed in the context of MHC. These peptides are produced endogenously by male tissues, including the MB49 tumour (Scott, Ehrmann et al. 1995) (see chapters 3 and 4). In a series of experiments performed to assess MB49 tumour growth profile in lymphopenic male mice, it was noted that adoptively transferred HY-specific Marilyn T cells became 'adapted' in response to this chronic antigen exposure. However if mice were pre-conditioned with sub-lethal irradiation (5Gy) the same T cells induced acute GvHD leading to death. Based on these results it was decided to perform a study to evaluate the functional differences in T cell responses in lymphopenic male mice either pre-conditioned or not with radiation. This novel model allows dissection of the roles of minor H antigen-specific monoclonal HY-specific T cells in inducing GvHD

7.2: Results

7.2.1: Impact of adoptive transferred Marilyn T CD4 cells on the growth of MB49 tumours in lymphopenicB6 mice with or without pre-conditioning (5Gy)

Marilyn CD4 T cells are capable of rejecting MB49 tumours upon adoptive transfer to lymphopenic B6 female mice whereas Matahari CD8 T cells cannot do so (Perez-Diez, Joncker et al. 2007) (see chapter 4). Following adoptive Marilyn cell transfer to MB49-bearing $Rag^{-/-}$ B6 mice, all female recipients showed regression of MB49 tumours and survived as tumour free by day 30, whereas males developed tumours reaching humane end points by day 26 (Fig. 7.1a). Additional groups of females (Fig. 7.1b) and males (Fig. 7.1c) were sub-lethally irradiated (5Gy). Female mice developed tumours at the same rate whether irradiated or not, with no statistical difference between the groups (Fig. 7.1b). 3 out of 5 female mice were culled due to ulceration, most likely caused by irradiation damage, however, the 2 mice that did not ulcerate showed tumour regression by day 30, similar to the non-irradiated group given Marilyn cells (Fig. 7.1.b). Male mice that received irradiation and Marilyn cells developed tumours at the same rate as the non-irradiated male recipients of Marilyn cells, with no statistical difference in growth rate. However, these irradiated male recipients became very sick with weight loss, skin problems, hunched posture and low body temperature, requiring them to be culled by day 12. These observations suggested that the donor Marilyn cells were inducing an acute GvHD in male mice when infused with T cells after pre-conditioning with radiation.

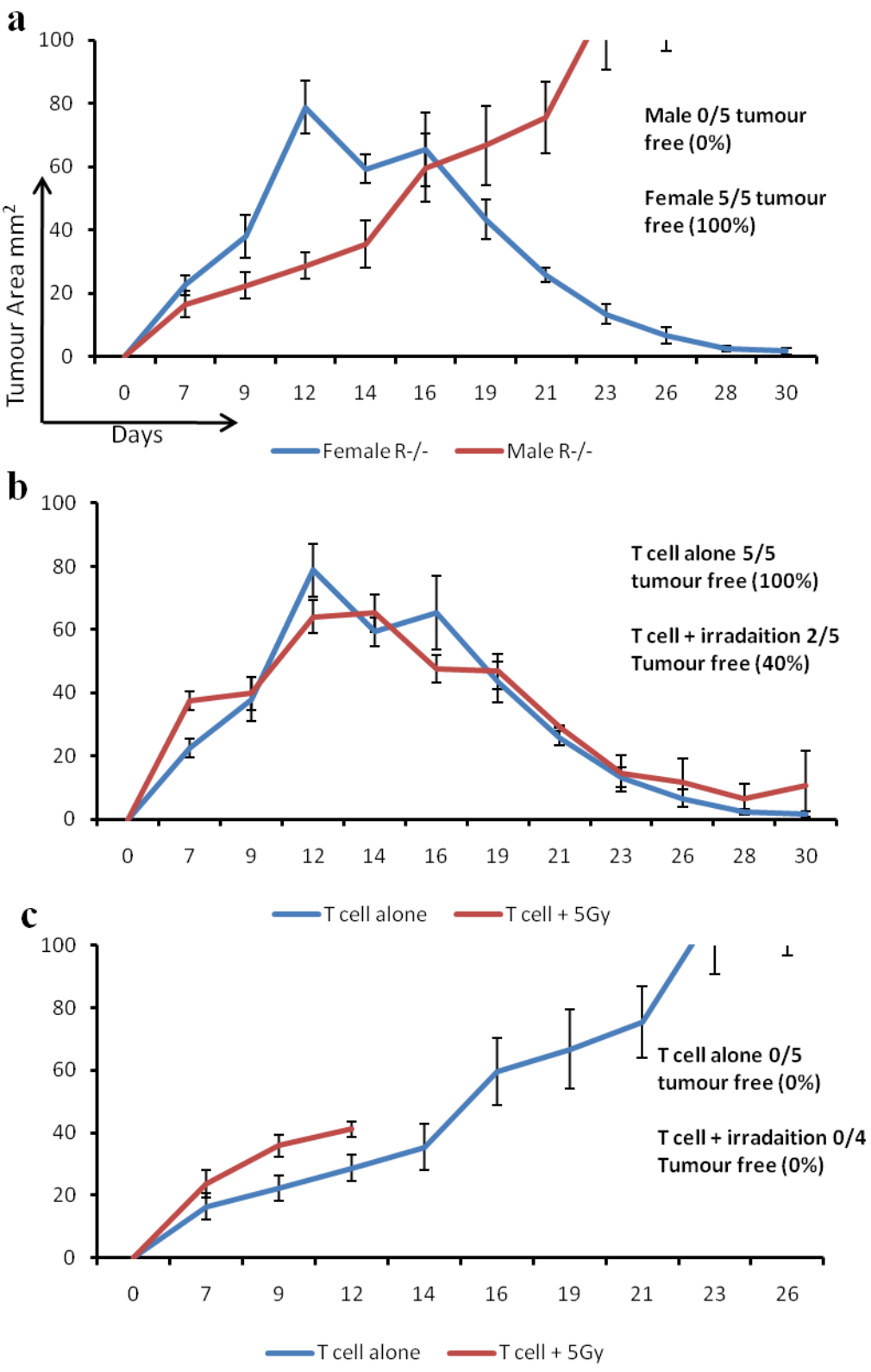


Figure 7.1. Marilyn cells can induce GvHD in B6 Rag$^{-/-}$ male mice following irradiation (5Gy)

MB49 cells (1 x10^5 cells/mouse) were injected (s.c) to the right lateral flank of female (n=10) and male (n=9) B6 Rag$^{-/-}$ mice on day 0. On day 7, 4 male and 5 female mice were irradiated with 5Gy. Also on day 7, SP+LN were harvested from a Marilyn Rag1$^{-/-}$ donor female mouse and injected at 1 x 10^7 (i.v.).

a. Average tumour growth profile of male (red line) and female (blue line) B6 $Rag^{-/-}$ mice that received Marilyn cells alone. Tumour growth was measured on consecutive days using digital callipers and tumour area calculated (length x width (mm^2)).
b. Average tumour growth profile of female mice that received either Marilyn cells alone (blue line) or with 5Gy irradiation (red line).
c. Average tumour growth profile of male mice that received either Marilyn cells alone (blue line) or with 5Gy irradiation (red line).
Results are an average of each group. Error is expressed as ± standard error mean.

7.2.2: Marilyn cell transfer induces weight loss in male mice but pre-conditioning (5Gy) is required to cause acute GvHD

Marilyn CD4 T cell transfer alone does not induce lethal GvHD upon adoptive transfer to male lymphopenic hosts, although this can cause a transient weight loss with recovery (Rivas, Weatherly et al. 2009). In an initial experiment, male $Rag^{-/-}$ B6 mice were either irradiated (5Gy) alone, subjected to Marilyn cell transfer alone or both, while female $Rag^{-/-}$ B6 mice were subjected to both irradiation and cell transfer (Fig. 7.2). Weight was measured at regular intervals up to day 6 when mice were sacrificed for analysis of donor populations (See Fig. 7.4). Weight change is plotted as percentage of weight at day 0, prior to irradiation or T cell transfer.

Non-irradiated male mice (open squares) showed very little weight change, similar to irradiated female recipients that received Marilyn cells (open triangles) (Fig. 7.2) suggesting that irradiation with adoptive transfer of Marilyn cells does not directly induce weight loss in the absence of HY antigens. Male mice that received 5Gy alone displayed some weight loss (closed circles) although this was not significant compared to the untreated group (open squares). Non-irradiated male mice that received Marilyn cells lost weight from day 3 following T cell transfer (open circles) and this weight loss was significant compared to male mice given irradiation alone group and female mice that received irradiation and Marilyn cells. These data indicate that Marilyn cell transfer alone in male lymphopenic mice does not cuase GvHD as these recipients remain healthy in appearance. Male mice that received Marilyn cell transfer and irradiation (closed triangles) showed the most dramatic weight loss from day 1, which was more profound than Marilyn cell transfer alone. Irradiated male mice given Marilyn cells also displayed signs of

clinical acute GvHD with hunched posture, reduced activity, piloerection, skin problems and low body temperature to the point where mice they either died or reached the humane end point by day 6.

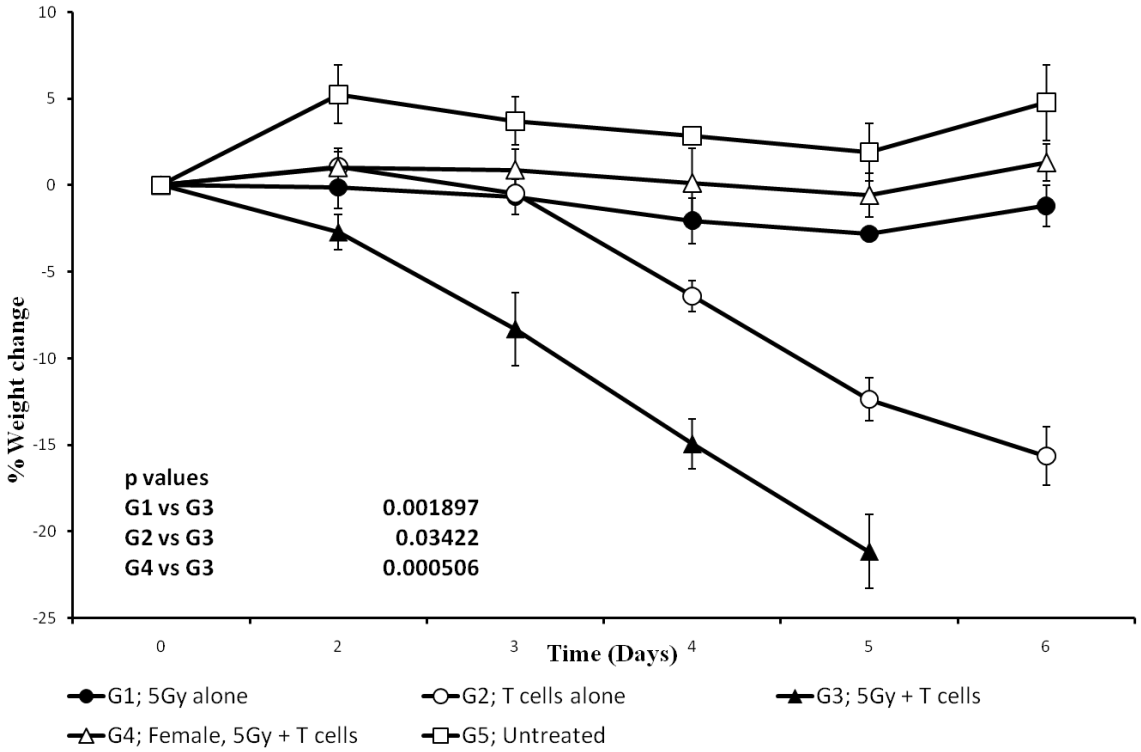


Figure 7.2. Marilyn cells can induce GvHD in male mice following radiation pre-conditioning

SP and pLNs were harvested from Marilyn Rag2$^{-/-}$ female mice and injected (i.v.) into 9 Rag$^{-/-}$ B6 mice at 1 x 10^{7}/mouse (6 male and 3 female). One group (n=3) of each sex was sub-lethally irradiated (5Gy) prior to Marilyn cell transfer. Weight was measured at regular intervals and plotted as % weight change. G1 - male with 5Gy alone (closed circles), G2 - male with Marilyn cells alone (open circles), G3 - male with 5Gy and Marilyn cells (closed triangles), G4 - female with 5Gy and Marilyn cells (open triangles), G5 - untreated male mice. n=3 for all groups. Error is expressed as ± standard error mean.

7.2.3: Marilyn CD4 T cell transfer together with radiation pre-conditioning (5Gy) induces lethal GvHD

Lymphopenic male mice that receive Marilyn cells lose a significant amount of weight without developing GvHD. To confirm weight loss is dependent on Marilyn T cells and not other cells present in the donor suspension of spleen and lymph node cells (SP+LN), the experiments were repeated using four groups of $Rag^{-/-}$ B6 male recipients. Male mice were given 5Gy alone (G1) with purified Marilyn T cells (G2) or Marilyn spleen and lymph node cell suspension (G3). In addition, one group of male mice were given purified Marilyn T cells alone (G4).

As shown in Fig. 7.3a, mice that received 5Gy alone (G1) lost some weight but this was fully recovered. All mice in G2 and G3 lost weight rapidly reaching the humane end point by day 8. Weight loss was accompanied by acute GvHD with hunched posture, low body temperature, piloerection, skin problems and low activity (Fig. 7.3b). Interestingly, the use of purified CD4 T cells (G2) did not accelerate the onset of GvHD compared to that of spleen and lymph node cell suspension (G3), with no significant difference in weight loss between the two groups. Importantly non-irradiated males that received enriched T cells alone (G4) did show a significant amount of weight loss compared to the 5Gy alone group (G1), and showed no signs of clinical GvHD, with 100% survival (Fig. 7.3b). These mice however also showed significantly reduced weight loss compared to G2 and G3 but recovered to within 5% of their starting weight by day 12. These data confirm that it is Marilyn CD4 cells that induce GvHD, as purified Marilyn CD4 T cells and Marilyn spleen and lymph node cells (containing both T cells and APC) have similar capacity to trigger GvHD in male mice after 5Gy.

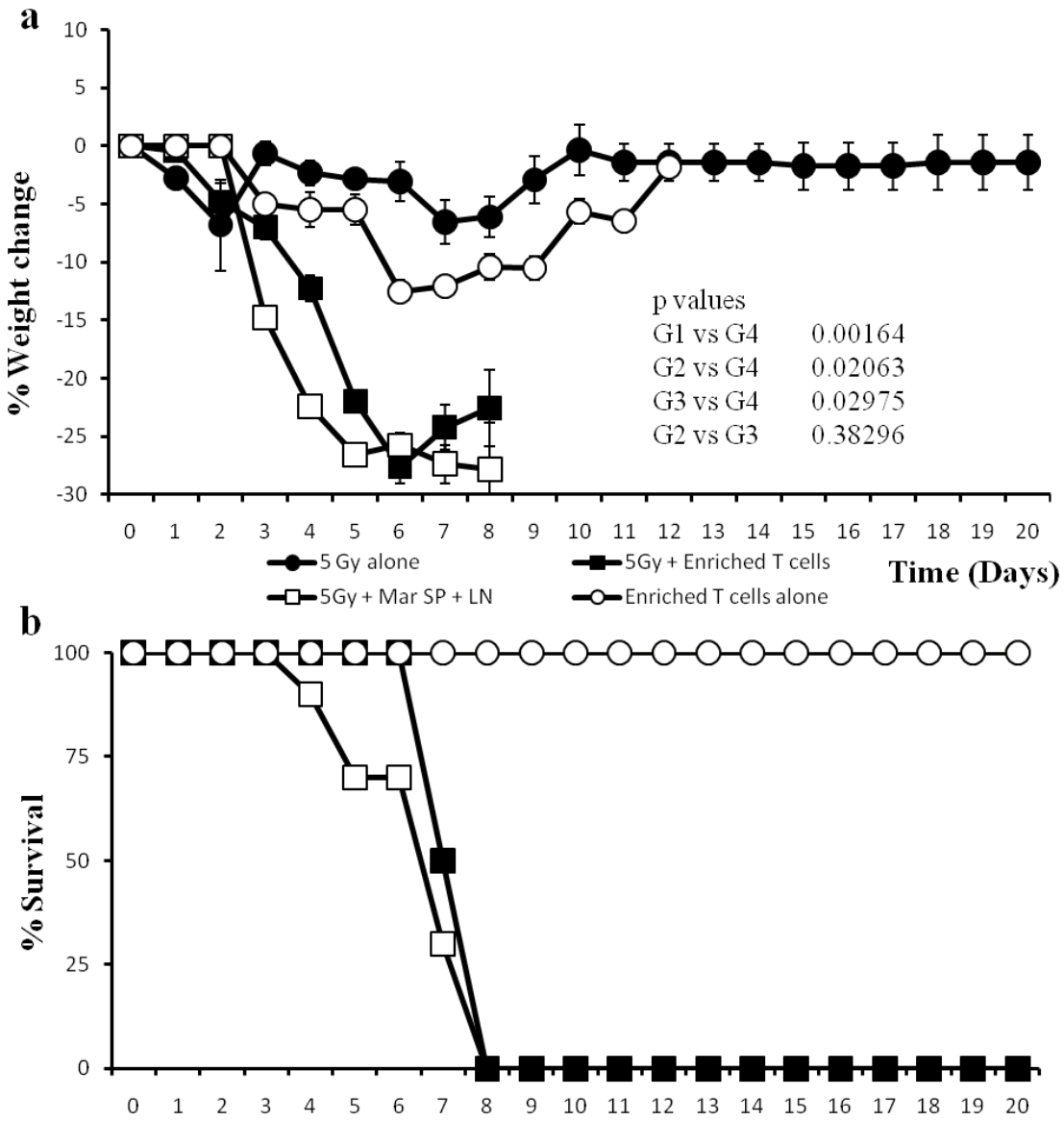


Figure 7.3. GvHD in irradiated male mice is induced by Marilyn CD4 T cells
Spleen and pLNs were harvested from two Marilyn Rag2$^{-/-}$ female mice and were injected at 1 x 10^{7} (i.v.) to sub-lethally irradiated (5Gy) Rag$^{-/-}$ B6 male mice (n=10, G3 open squares). One group of Rag$^{-/-}$ male mice (n=7) received sub-lethal irradiation (5Gy) alone (G1 closed circles). SP+LNs from two Marilyn Rag2$^{-/-}$ female mice were used to purify CD4 T cells by MACS which were injected (4 x 10^{6} cells; i.v.) into sub-lethally irradiated (5Gy) Rag$^{-/-}$ B6 male mice (n=6) (G2 closed squares) or to non-irradiated Rag$^{-/-}$ mice (n=6) (G4 open circles).
a. Weight was measured at regular intervals and plotted as % weight change. Error is expressed as ±-standard error mean.
b. Percentage survival for each group as described above.

7.2.4: Irradiation does not influence Marilyn T cell expansion in Rag$^{-/-}$ B6 male recipients

Marilyn CD4 T cells may be able to proliferate more robustly after irradiation in lymphopenic hosts which may lead to increased pathological T cell responses. To evaluate the expansion of adoptively transferred T cells, male and female Rag$^{-/-}$ B6 mice were adoptively transferred with Marilyn spleen and lymph node cells, with or without 5Gy pre-conditioning (Fig. 7.2 and Fig. 7.4a). On day 6, mice were analysed for the frequency of donor cells in peripheral lymphoid organs. As expected, donor cells transferred to males expanded significantly more than those in female mice, due to the combination of HY driven and non-specific homeostatic proliferation. Surprisingly, there was no indication that donor T cells underwent greater expansion in male mice given 5Gy compared with non-irradiated male mice, with no difference in donor T cell frequency in various lymph nodes between the groups (Fig. 7.4a). However, at day 6 when the mice were culled for the analysis, male mice that received 5Gy and T cells showed signs of weight loss, skin problems, low body temperature and a hunched appearance whereas male mice that received T cells alone showed some weight loss but appeared to be generally healthy. As shown previously, female mice that received both T cells and irradiation were also healthy and showed no signs of weight loss.

In a separate experiment, male and female Rag$^{-/-}$ B6 mice received enriched (MACS purified) Marilyn CD4 T cells with or without irradiation (5Gy); all mice were culled at day 8 for analysis. Absolute donor cell numbers were calculated in the organs specified in Fig. 7.4b. Donor T cells underwent significant expansion only in the male 0Gy and 5Gy groups. In addition, male mice showed donor T cell infiltration into non-lymphoid organs while females did not (Fig. 7.4b). However, there was no significant difference between absolute donor cell numbers recovered from various tissues between irradiated and non-irradiated male recipients (Fig. 7.4b). Tissues were also weighed, allowing absolute cell number per mg of tissue to be calculated (Fig. 7.4c). Again no significant difference was seen in absolute cell number between irradiated and non-irradiated male recipients (Fig. 7.4c). These data show that enhanced expansion of the donor T cell population is not the sole mechanism responsible for the development of GvHD in this model.

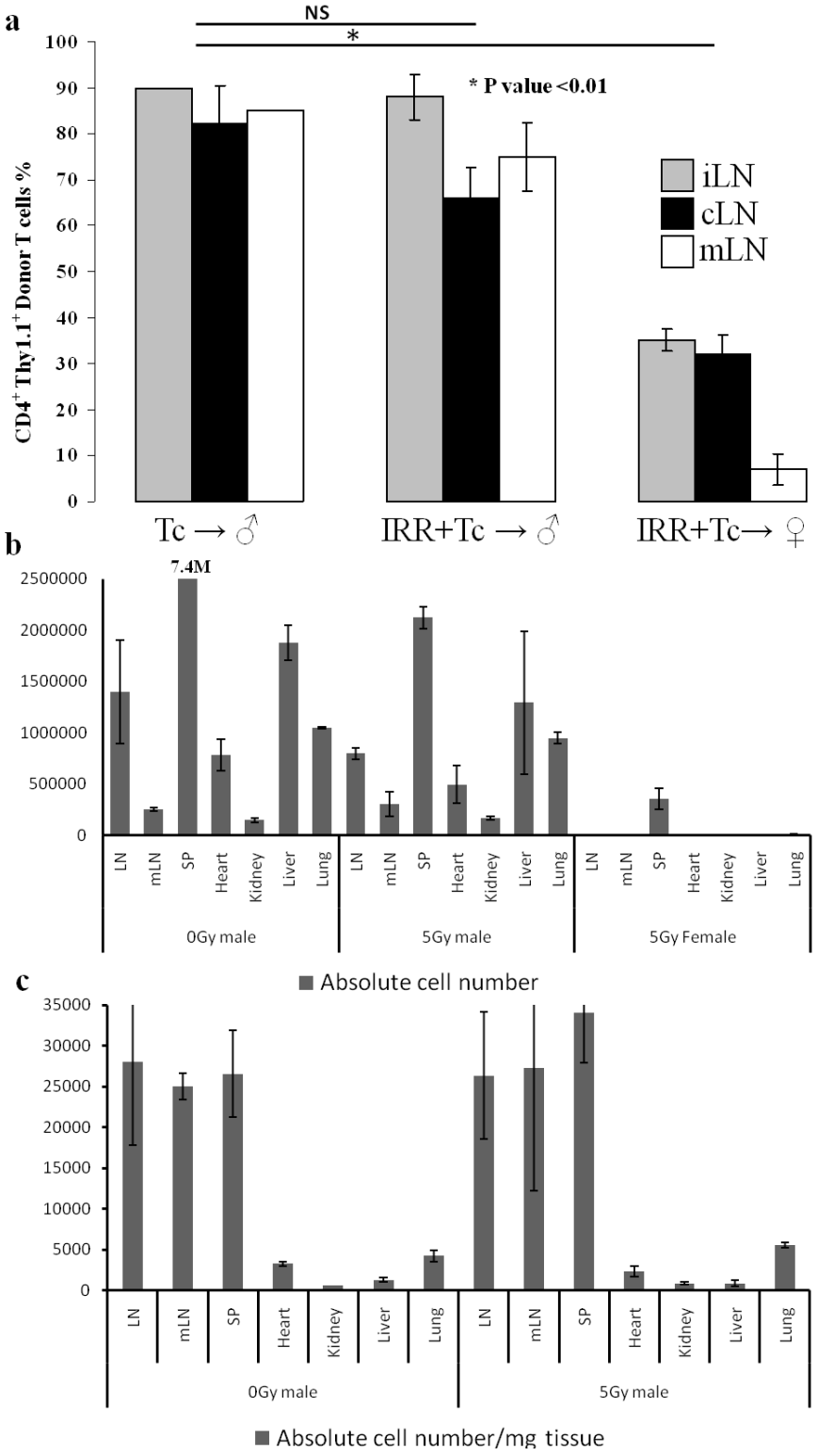


Figure 7.4. Comparison of Marilyn CD4 T cell expansion in Rag$^{-/-}$ B6 male mice with or without 5Gy pre-conditioning

a. SP and pLNs were harvested from Marilyn Rag2$^{-/-}$ female mice and injected (i.v.) into 9 Rag$^{-/-}$ B6 mice at 1 x 10^7/mouse (6 male and 3 female). One group (n=3) of each sex was sub-lethally irradiated (5Gy) prior to Marilyn cell transfer. On day 6, all mice were sacrificed and pLN including iLN (inguinal) cLN (cerviacal) and mLN (mesenteric) were harvested and stained using anti-Vβ6^{FITC}, anti-CD4^{PE} and anti-

Thy1.1^{PerCP} and analysed by FACS. Donor cells are represented as % of total lymphocytes. N=3 for each group.
b. Marilyn CD4 T cells were purified from SP +LN cells by MACS and injected (i.v.) at 4 x 10^6 cells/mouse to irradiated (5Gy) male (n=4) and female (n=2) Rag$^{-/-}$ B6 mice and one group of non-irradiated male Rag$^{-/-}$ B6 mice (n=4). On day 8 mice were sacrificed and peripheral lymphoid (pLN, mLN, SP) and non-lymphoid organs (heart, kidney, liver, and lung) were harvested and cells were counted. Cells were stained as above and analysed by FACs and absolute donor cells numbers in each organ was calculated using proportion of donor cells x total cells.
c. Tissues were weighed and data presented as absolute cell donor cell number per mg tissue. All Error is expressed as ± standard error mean.

7.2.5: Marilyn T cells transferred to male lymphopenic mice become activated

Donor T cells in the same experiment as shown in Fig. 7.4b were also examined for their phenotype. Donor T cells in lymphoid and non-lymphoid organs were gated for a further analysis of T cell activation markers CD44 and CD62L (Fig. 7.5). In addition, Rag2$^{-/-}$ Marilyn female mice whose T cells display a resting phenotype and 'double' transgenic Marilyn TCR$^+$/dominant negative TGFβ receptor type II$^+$ (dnTGFβRII$^+$) [F1(dnTGFβRII$^+$ x Rag2$^{-/-}$ Marilyn$^+$) female)], whose CD4 T cells are expected to display a chronic activation phenotype were included for comparison (Gorelik and Flavell 2001) (Fig. 7.5c).

All donor T cells recovered from lymphoid tissues in male mice with (Fig. 7.5b) or without (Fig. 7.5a) irradiation, were predominantly CD44 high, indicating an activated phenotype. Similarly, non-lymphoid organs from both groups of male mice were also significantly infiltrated (Fig. 7.5a and b) with donor CD4 T cells, which expressed of CD44. As expected, CD4 T cells from the naïve Rag2$^{-/-}$ Marilyn female mouse showed no signs of activation, with most cells being predominantly CD44 low and CD62L high, a naive phenotype, in both lymphoid and non-lymphoid organs (Fig. 7.5d). On the other hand, CD4 T cells from the Marilyn double transgenic mouse (Fig. 7.5c) showed a higher state of activation, with many CD4$^+$ T cells becoming CD44 high, particularly in non-lymphoid organs. Finally, female Rag$^{-/-}$ B6 mice that received irradiation and T cell transfer showed little T cell expansion (Fig. 7.4b) and displayed a naive phenotype (Fig. 7.5e), similar to those from Marilyn Rag$^{-/-}$ female mice (Fig. 7.5d). Only cells isolated from peripheral lymph nodes, spleen and liver are represented for female recipients as other organs did not have sufficient cells for analysis. These data indicate that Marilyn CD4$^+$ T cells

become activated upon transfer to lymphopenic male mice infiltrate into non-lymphoid organs. Donor CD4 T cells in female recipient mice show poor expansion, activation and organ infiltration, indicating that the induction of GvHD requires antigen dependent organ infiltration along with sub-lethal irradiation. This is supported by the phenotype of Marilyn double transgenic mice which have activated organ infiltrating T cells and a spontaneous autoimmune phenotype with wasting disease syndromes (loss of weight).

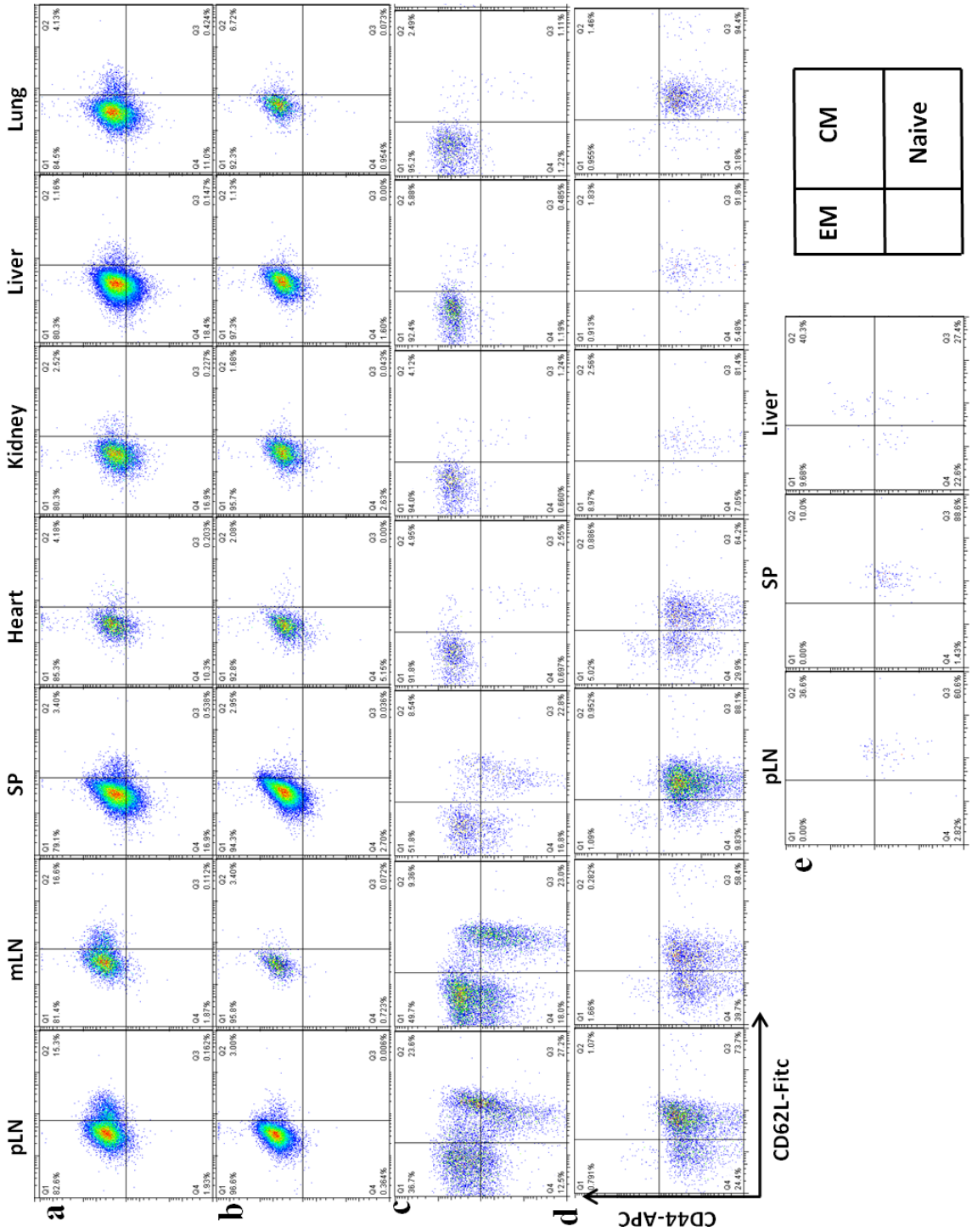


Figure 7.5. Marilyn CD4 T cells become activated in lymphopenic male mice
Donor Marilyn spleen and pLNs were harvested, purified by MACS and injected (i.v.) at 4 x 10^6 cells/mouse to irradiated (5Gy) male (n=4) and female (n=2) $Rag^{-/-}$ B6 mice and one group of non-irradiated male $Rag^{-/-}$ B6 mice (n=4), as described in Fig. 7.4 On day 8, two mice from each group were sacrificed along with a naive Marilyn $Rag2^{-/-}$ female mouse and a naïve double transgenic mouse. Lymphoid tissues (pLN, mLN, SP) and non-lymphoid organs (heart, kidney, liver and lung) were harvested and cells were stained with anti-$CD62L^{FITC}$, anti-$V\beta6^{PE}$, anti-$CD4^{PerCP}$ and anti-$CD44^{APC}$. Donor CD4 T cells were gated on lymphocytes and then $V\beta6^+$ $CD4^+$ cells, FACS plots represent CD44 and CD62L expression by gated donor T cells.
a. Male $Rag^{-/-}$ B6 mice received T cell alone (n=4) (one representative FACS plot shown).
b. Male $Rag^{-/-}$ B6 mice received T cells and 5Gy radiation pre-conditioning (n=4) (one representative FACS plot shown).
c. Double transgenic female mouse.
d. Marilyn $Rag2^{-/-}$ female mouse.
e. Female $Rag^{-/-}$ B6 mice received T cells and 5Gy radiation pre-conditioning (n=2) (one representative FACS plot shown).

7.2.6: Donor CD4 T cells in irradiated male mice harbour more effector memory and less central memory T cells

The data acquired from the peripheral lymph node suspensions in Fig. 7.5 was quantitatively analysed to reveal any differences in memory T cell subsets between lymphopenic male mice that receive T cells alone and those that received T cells and 5Gy (Fig. 7.6). Naive T cells are defined as $CD44^{low}CD62L^{high}$, effector memory (EM) cells are defined as $CD44^{high}$ $CD62L^{low}$ and central memory (CM) cells are defined as $CD44^{high}CD62L^{high}$. EM T cells are associated with cytotoxic activity and mediate killing through Granzyme B and IFNγ. CM cells act as a stem cell like memory pool that primarily home to lymphoid organs and produce IL-2 but not IFN-γ or Granzyme B (Okada, Kondo et al. 2008).

As expected, T cells in peripheral lymph nodes of naïve $Rag^{-/-}$ Marilyn female mice have a primarily naive phenotype while those from double transgenic mice have significantly higher levels of EM and CM T cells (Fig. 7.6), reflecting their spontaneous autoimmune phenotype. Donor CD4 T cells isolated from female $Rag^{-/-}$ B6 recipients that received 5Gy and Marilyn T cells maintained a mainly naïve T cell phenotype with ~20% becoming CM cells and ~1% EM cells. Male $Rag^{-/-}$ B6 mice that received T cells with or without 5Gy had converted >99% of donor T cells to an

EM or CM activated phenotype. There was a clear difference between the phenotype of donor cells recovered from male and female recipient mice and also between male mice and two the strains of control mice (i.e., single transgenic and double transgenic mice). Male mice that received 5Gy and T cells have significantly more EM cells and significantly fewer CM cells compared to the 0Gy and T cell group (Fig. 7.6). These mice have similar absolute donor cell numbers in lymphoid and non-lymphoid organs (Fig. 7.5b and c). Collectively, these results demonstrate that donor Marilyn T cells expand in male but not female mice (Fig. 7.4). In addition, activated T cells in males migrate into non-lymphoid organs, displaying a $CD44^{high}$ phenotype (Fig. 7.5). Male mice have a higher level of donor cell activation compared to female mice and control mice (Fig. 7.6) but similar absolute numbers of donor cells (Fig. 7.5b and c), male mice receiving 5Gy in addition to T cells preferentially expand EM T cells compared to 0Gy mice (Fig. 7.6).

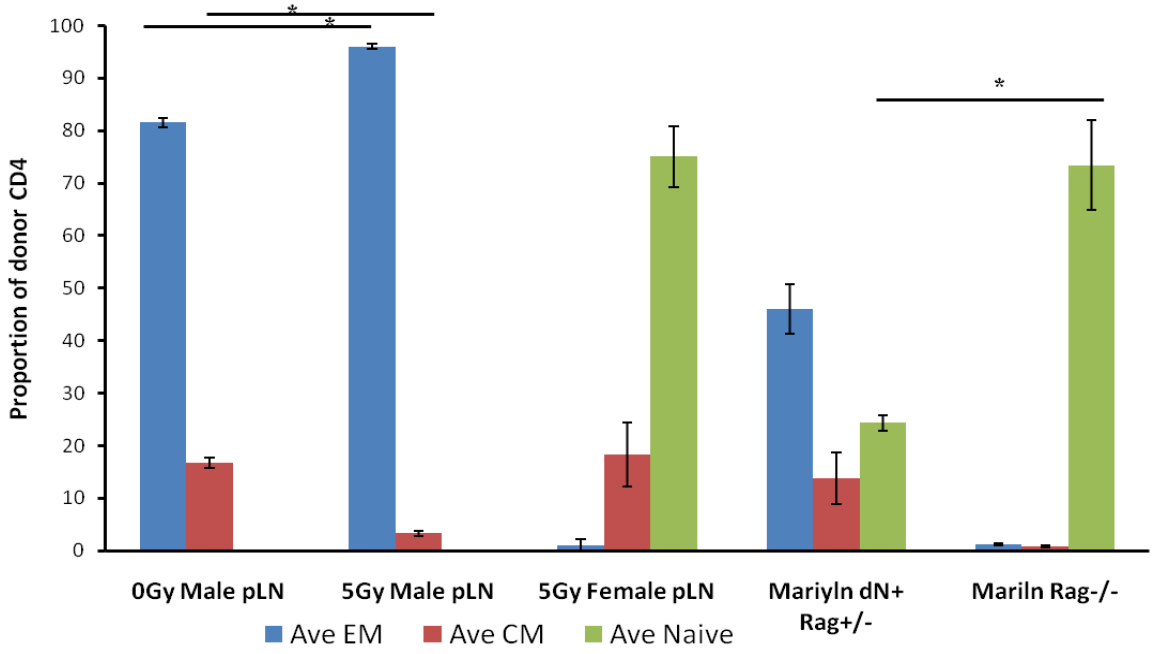


Figure 7.6. Adoptive transfer to male mice induces donor T cells to become EM
Donor Marilyn cells were harvested, purified by MACS and injected (i.v.) at 4 x 10^6 cells/mouse to irradiated (5Gy) male (n=4) and female (n=2) $Rag^{-/-}$ B6 mice and one group of non-irradiated male $Rag^{-/-}$ B6 mice (n=4), as described in Fig. 7.4. On day 8, 2 mice from each group were sacrificed along with a naive Marilyn $Rag2^{-/-}$ female mouse and a naïve double-transgenic mouse. Peripheral LNs were harvested and cells were stained using anti-$CD62L^{FITC}$, anti-$V\beta6^{PE}$, anti-$CD4^{PerCP}$ and anti-$CD44^{APC}$. Cells were gated on lymphocytes and then $V\beta6^+$ $CD4^+$ cells and analysed by FACS for CD62L and CD44 expression (Fig. 7.5). Mean % of naive ($CD62L^+$ $CD44^-$) (green), effector memory ($CD62L^-$ $CD44^+$) (blue) and central memory ($CD62L^+$ $CD44^+$) (red) T cells were calculated. Error is expressed as ± standard error mean.

7.2.7: Donor cells recovered from male recipient mice with or without 5Gy have similar cytokine production profiles

The cytokine production profiles of donor Marilyn cells in each group were analysed as for the experiment described in Fig. 7.5. Cells were stained *ex vivo* for T-bet and were also stimulated *in vitro* and stained for INFγ, TNFα, IL-17 and IL-10 expression. The Th1 transcription factor T-bet is induced upon activation of T cells, particularity CD4 T cells, and its expression is required for expression of IFNγ (Szabo, Kim et al. 2000). Marilyn donor T cells isolated from male mice that received T cells showed a very high proportion of cells expressing T-bet and IFN-γ (Fig. 7.7a and b), indicative of the strong activation phenotype (Fig. 7.5 and 7.6), although no significant difference was seen between recipients of 0Gy and 5Gy. This phenotype is also reflected in the mean fluorescence intensities (MFI) of T-bet and IFN-γ (Fig. 7.7c). Conversely, donor cells recovered from female mice that received 5Gy and T cells from Marilyn $Rag^{-/-}$ mice showed little expression of T-bet or IFN-γ (Fig. 7.7a) either by proportion or MFI (Fig. 7.7c). Donor CD4 T cells isolated from double transgenic mice displayed an intermediate phenotype in the lymphoid organs with a moderate upregulation of both T-bet and IFN-γ (Fig. 7.7 b and c)

Unlike IFNγ, TNFα production by donor T cells from male mice was low with no significant difference between male and control groups (Fig. 7.7a) either by proportion (Fig. 7.7b) or MFI (Fig. 7.7c). Female mice receiving 5Gy showed high expression of TNFα both as a proportion (Fig. 7.7b) and MFI (Fig. 7.7c) although this result is not conclusive due to the very low cell recovery (Fig. 7.7a). Importantly, neither expression of IL-17 nor IL-10 by donor CD4 T cells was seen in male recipient mice that received T cells, suggesting that donor T cells are driven towards a Th1 rather than a Th17 phenotype (Fig. 7.7a).

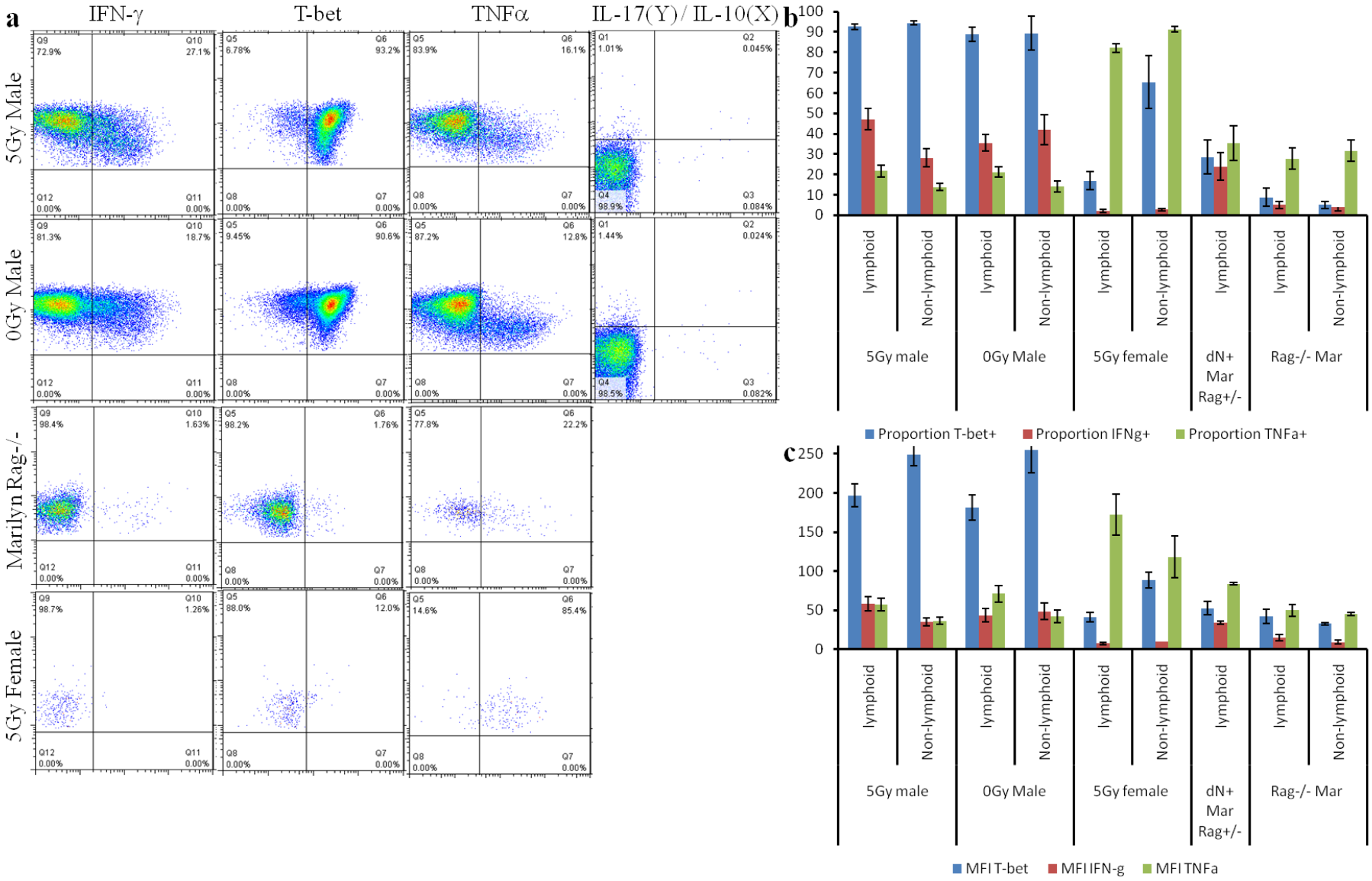

a
IFN-γ
T-bet
TNFα
IL-17(Y)/ IL-10(X)
5Gy Male
0Gy Male
Marilyn Rag-/-
5Gy Female
b
lymphoid
Non-lymphoid
5Gy male
0Gy Male
5Gy female
dN+ Mar Rag+/-
Rag-/- Mar
Proportion T-bet+
Proportion IFNg+
Proportion TNFa+
c
MFI T-bet
MFI IFN-g
MFI TNFa

Figure 7.7. Marilyn CD4 cytokine expression profile in GvHD mice
Donor Marilyn cells were harvested, purified by MACS and injected (i.v.) at 4 x 10^6 cells/mouse into irradiated (5Gy) male (n=4) and female (n=2) $Rag^{-/-}$ B6 mice and one group of non-irradiated male $Rag^{-/-}$ B6 mice (n=4), as described in Fig. 7.4. On day 8, 2 mice from each group were sacrificed along with a naive Marilyn $Rag2^{-/-}$ female mouse and a naïve double-transgenic mouse. Lymphoid (pLN, mLN, SP) and non-lymphoid organs (heart, kidney, liver, and lung) were harvested and half of the cells were surface stained using anti-$V\beta6^{FITC}$ and anti-$CD4^{PerCP}$, cells were fixed and intracellular stained with anti-T-bet^{PE}. The other half was stimulated for 4 hours with PMA (50ng/ml) + Ionomycin (250ng/ml) in the presence of Brefeldin A (1μg/ml), cells were harvested, and after removal of dead cells, the cells were surface stained for anti-$V\beta6^{FITC}$ and anti-$CD4^{PerCP}$. Cells were then fixed and stained for $TNF\alpha^{PE}$, $IFN\gamma^{APC}$ or $IL\text{-}17^{PE}$, $IL\text{-}10^{APC}$ and analysed by FACs gating on lymphocytes and then $V\beta6^+CD4^+$ cells.
a. Intracellular production of T-bet, INFγ and TNFα by donor T cells in pLN from 5Gy male mice, 0Gy male mice, 5Gy female mice and Marilyn $Rag^{-/-}$ mice is represented by plots of CD4 (Y axis) vs cytokine expression. Expression of IL-17 (Y axis) and IL-10 (X axis) is shown for the 5Gy male and 0Gy male groups only. One representative FACs plot for each group is shown.
b. Average proportions of cells in $CD4^+$ $V\beta6^+$ gate expressing T-bet (blue), IFNγ (red) and TNFα (Green) for lymphoid tissues (pLN, mLN, SP) and non-lymphoid organs (heart, kidney, liver, and lung). Error is expressed as ± standard error mean.
c. Average mean fluorescence intensity of cells in $CD4^+V\beta6^+$ gate expressing T-bet (blue), IFNγ (red) and TNFα (Green). Error is expressed as ± standard error mean.

7.2.8: Delayed Pre-conditioning (5Gy) of $Rag^{-/-}$ B6 male abrogates lethal GvHD

To determine whether timing of irradiation would be critical to the induction of lethal GvHD, one group of male $Rag^{-/-}$ B6 mice were given 5Gy irradiation ten days prior to T cell transfer (Fig. 7.8). Mice initially lost weight following 5Gy treatment at day -10 but recovered to within 5% of their original weight by day 0. On day 0, mice were injected (i.v.) with purified Marilyn CD4 T cells. From day 3 onwards mice showed a rapid loss of weight, as was seen in the control group of mice receiving 5Gy and T cells on the same day (day 0) (Fig .7.3-7 and Fig. 7.8a). By day 7, all mice showed clear signs of acute GvHD with hunched posture, reduced body temperature, skin problems and piloerection. All were expected to reach humane end point by day 8 as did control mice receiving T cells and 5Gy at day 0 (Fig. 7.8a). However mice in the group irradiated ten days before being given Marilyn cells began to recover and gain weight over the next 12 days and by day 20 none of them showed signs of acute GvHD (Fig. 7.8a and b), all recovering to their

original weight by day 30. This result indicates that T cell transfer following irradiation 10 days previously induces a weaker, transient form of acute GvHD which does not reach the humane end points. Therefore the initial *in vivo* environment (cytokine storm) created following radiation precondition is important in determining the level of acute GvHD induced.

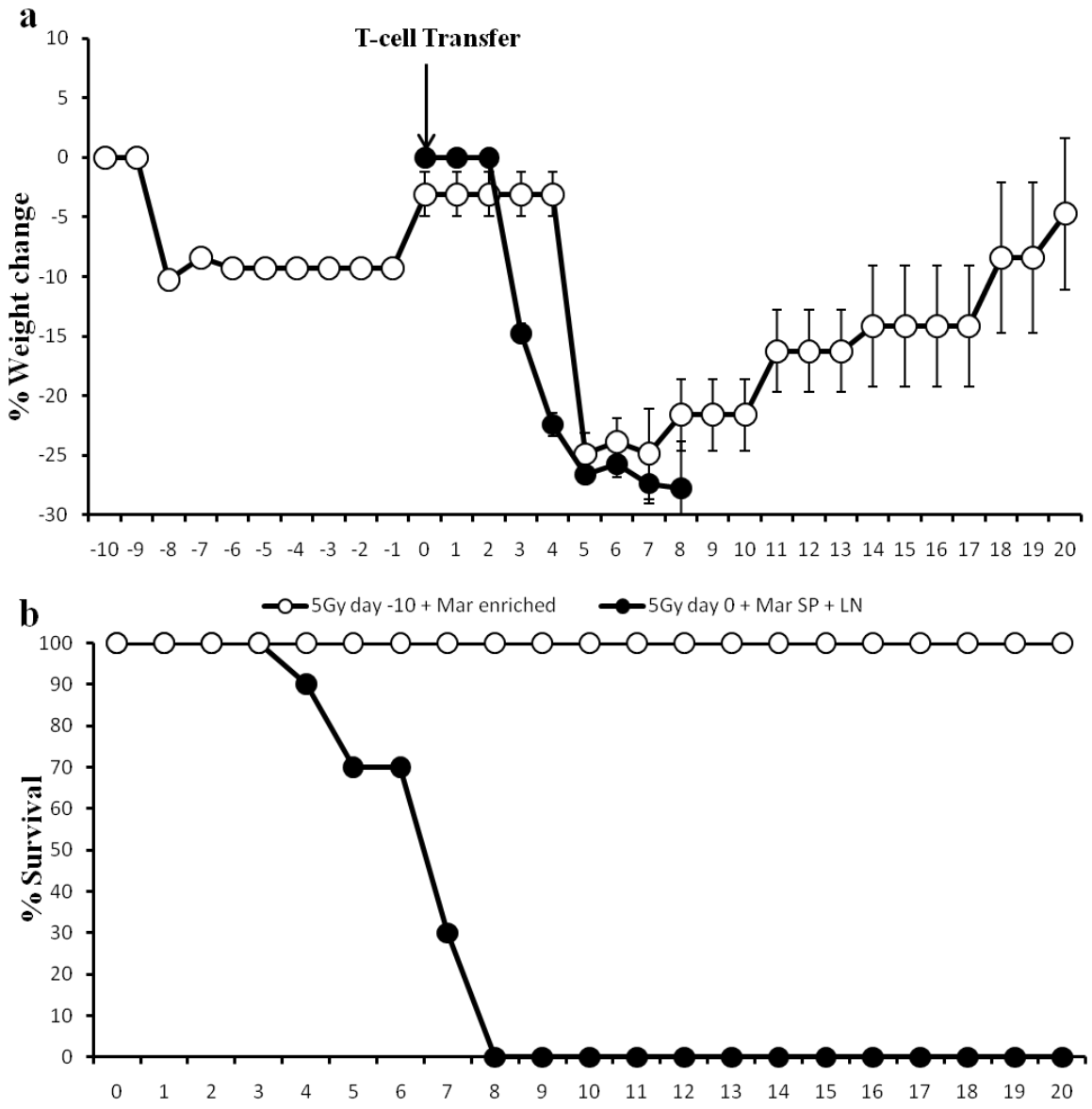


Figure 7.8. Timing of radiation pre-conditioning is critical to the development of lethal GvHD

SP and pLNs were harvested from a Marilyn Rag2$^{-/-}$ donor mouse and subjected to MACS purification of CD4 cells. T cells were injected (i.v.) at 4 x 10^6 cells/mouse at day -10 to sub-lethally irradiated (5Gy) Rag$^{-/-}$ mice (n=7). 5Gy and enriched T cells were also given to male mice on day 0 in a separate experiment (from Fig. 7.3-7) and is included as a reference (n=10).

a. Weight was measured at regular intervals and plotted as % weight change. Error is expressed as ± standard error mean.

b. Percentage survival for each group as described above.

7.2.9: T cells from male mice irradiated on day -10 exhibit adaptive tolerance

Male mice receiving 5Gy on day-10 prior to Marilyn CD4 T cell transfer became sick but did not die from acute GvHD (Fig. 7.8). Once they had recovered to their original starting weight by day 30, 2 mice were sacrificed and donor cells analysed for expression of surface markers CD44 and CD62L (as in Fig. 7.5.) and intracellular cytokines (as in Fig. 7.7.). Donor cells from male mice given 5Gy on day -10 become $CD44^+$ as seen with those isolated from mice given 5Gy on day 0 (Fig. 7.9a) however, these T cells display an intermediate CD44 expression, characteristic of chronically activated adapted T cells (Chiodetti, Choi et al. 2006). Donor cells isolated on day 8 from mice given Marilyn cells and 5Gy on day 0 were used as a reference. This is not a direct comparison, due to the different stage of T cell activation, as this group does not survive to day 30.

Donor cells isolated from lymphoid and non-lymphoid tissues were also stained for T-bet or cultured with PMA and Ionomycin in the presence of Brefeldin A for 4 hours. As shown in Fig. 7.7, the cells were then harvested, stained for IFN-γ and analysed by FACS to determine the proportions (Fig. 7.9c) and MFI (Fig. 7.9d) of T-bet and IFNγ expressing T cells. Donor cells from mice receiving 5Gy on day-10 show low production of the transcription factor T-bet and the Th1 cytokine IFN-γ (Fig. 7.9c and d) in donor cells isolated from lymphoid and non-lymphoid organs. This is reflected in both the MFI of T-bet and IFN-γ (Fig. 7.9b and c) and also the proportion of cells producing these molecules (Fig. 7.9b and c). 5Gy on day -10 before injection of Marilyn T cells prevents the severe acute GvHD seen in mice that receive 5Gy on day 0. Marilyn donor T cells from 5Gy day -10 male mice exhibit a CD44 intermediate phenotype with poor cytokine production.

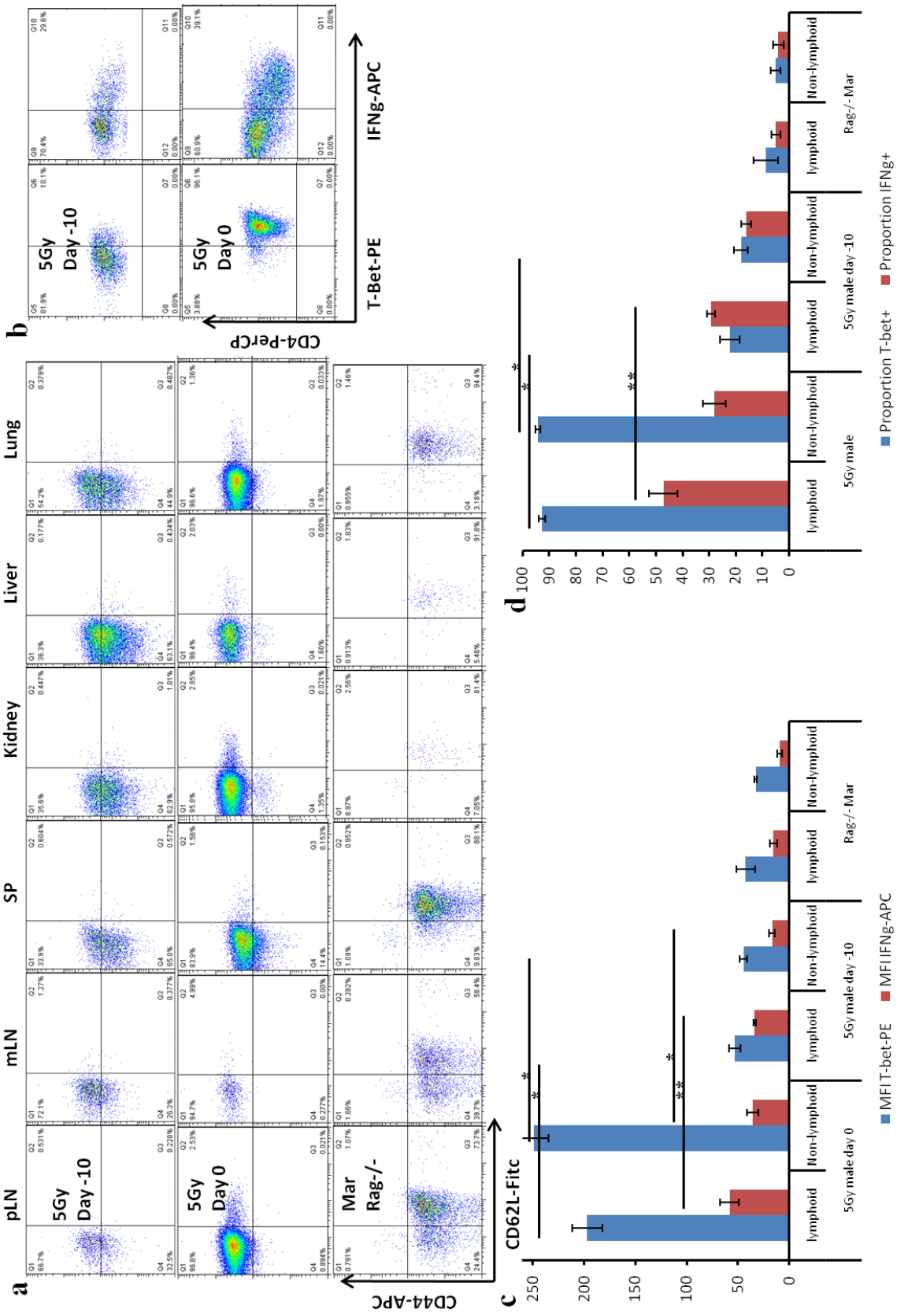


Figure 7.9. Donor CD4 T cells from 5Gy day-10 mice are not fully activated
SP and peripheral LNs were harvested from a Marilyn Rag2$^{-/-}$ donor mouse and subjected to MACS purification of CD4 cells. T cells were injected (i.v.) at 4 x 10^6 cells/mouse to sub-lethally irradiated (5Gy) Rag$^{-/-}$ mice (n=7). 5Gy and enriched T cells were also given to male mice on day 0 and included as a reference (n=10). On

day 30, two mice from the 5Gy day-10 group were sacrificed and analysed for T cell activation status.
a. Lymphoid (pLN, mLN, SP) and non-lymphoid organs (kidney, liver, lung) were harvested and cells were stained using anti-CD62L^{FITC}, anti-Vβ6^{PE}, anti-CD4^{PerCP} and anti-CD44^{APC}. Cells were gated on Vb6^{+} CD4^{+} lymphocytes; FACS plots represent CD44 and CD62L expression from this gate. One, representative set of plots is shown.
b. Donor T **c**ells were stained *ex vivo* for T-bet by intracellular staining (left) or cultured for 4 hours with PMA and Ionomycin in the presence of Brefeldin A (as in Fig. 7.7) and stained for intracellular INF-γ^{APC}, one representative FACS plot is shown.
c. Average mean fluorescence intensity of cells in the CD4^{+} Vβ6^{+} gate expressing T-bet (blue) and IFNγ (red). Error is expressed as ± standard error mean.
d. Average proportion of cells in CD4^{+} Vβ6^{+} gate expressing T-bet (blue) and IFNγ (red). Error is expressed as ± standard error mean. (* P value <0.01, ** <0.05).

7.2.10: T cells from mice that have recovered from acute GvHD fail to induce disease upon secondary adoptive transfer

Two mice that recovered from acute GvHD in the previous experiment (Fig. 7.8 and 7.9) were sacrificed on day 30 and donor Marilyn T cells were re-isolated using MACs purification. These T cells were then adoptively transferred into 3 sub-lethally irradiated (5Gy) B6 Rag$^{-/-}$ male recipients and weight loss measured at regular intervals (open circles) (Fig. 7.10). These mice did not develop any signs of acute GvHD or weight loss, compared with male recipients given 5Gy and T cells in the previous experiments (Fig. 7.3-7). These data indicate Marilyn CD4+ T cells have adapted so that on secondary adoptive cell transfer they are unable to induce acute GvHD following radiation pre-conditioning on day 0.

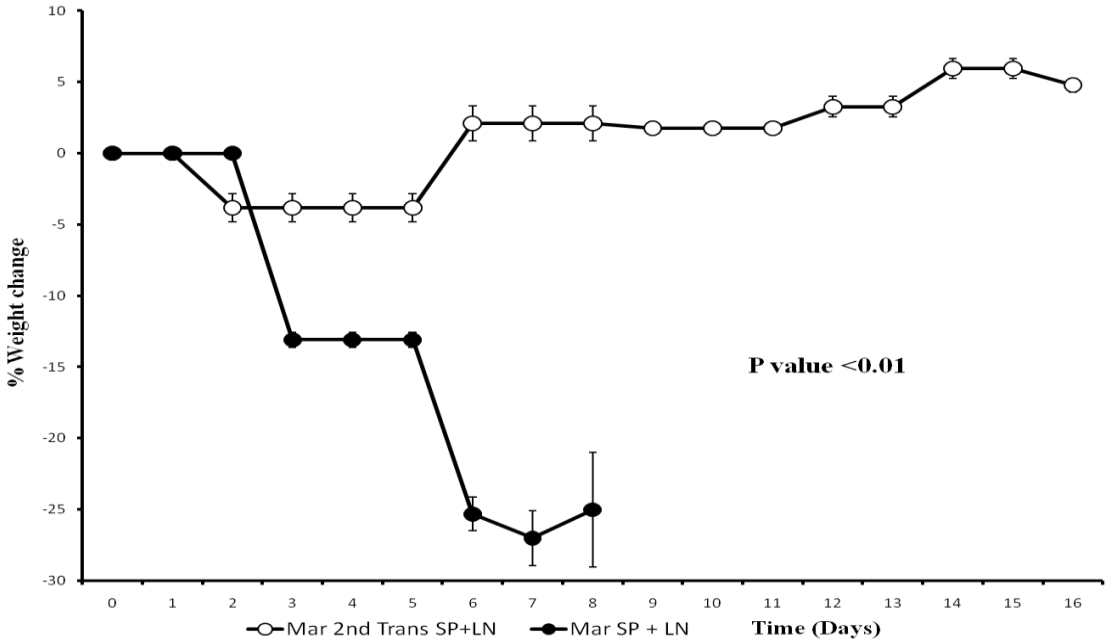


Figure 7.10. T cells from mice that have recovered from acute GvHD failed to induce disease upon secondary adoptive transfer
SP and pLNs were harvested from two male mice recovered from GVHD in the experiment depicted in Fig. 7.8. On day 30 and CD4 cells MACs purified. These re-isolated CD4 T cells were injected (i.v.) at 4 x 10^6 cells/mouse to 3 sub-lethally irradiated (5Gy) $Rag^{-/-}$ B6 male mice (open circles). Mice that receiving 5Gy (day 0) and SP+LN from a Marilyn $Rag2^{-/-}$ donor mouse in Fig. 7.3 were included for reference (closed circles). Weight was measured at regular intervals and plotted as % weight change. Error is expressed as ± standard error mean.

7.2.11: Marilyn CD4 T cells can induce acute GvHD in FoxP3-DTR male mice following Treg depletion

Mice with a novel diphtheria toxin receptor (DTR) knock-in strain were used as recipients for this set of experiments. These mice are on a WT B6 background with genes encoding GFP and DTR inserted into the endogenous FoxP3 locus, leading to co-expression at the cell surface of DTR on green Tregs cells (Kim, Rasmussen et al. 2007). This allows for efficient selective depletion of DTR-expressing Tregs by administration of a sub-lethal dose of diphtheria toxin (DT) (both DT and FoxP3-DTR mice kindly provided by the group of Prof Daniel Altman at the Hammersmith Hosp). Resting Marilyn T cells were purified as described previously and injected to sub-lethally irradiated (5Gy) male FoxP3-DTR mice. One group of mice received a single dose of DT (i.p.) (12.5μg) one day before T cell

transfer (closed squares) and the efficiency of DT-mediated Treg depletion confirmed by FACS.

Both DT treated and untreated mice lost weight rapidly following 5Gy radiation pre-conditioning and adoptive cell transfer (Fig. 7.11a) in a similar manner to lymphopenic recipients receiving Marilyn T cells alone (Fig. 7.3). Mice that received the additional DT treatment one day before CD4 T cell transfer however showed signs of acute GvHD as described previously and all reached the humane end points by day 7 (Fig. 7.11b). Mice that received no DT showed a similar degree of weight loss up to day 5 but did not display additional signs of acute GvHD, and these mice recovered to within 5% of their original weight by day 20 (2 mice were culled on day 7 for cytokine analysis, see next figure). These data indicate that the GvHD can be induced in WT male B6 mice by adoptive transfer of HY-specific CD4 T cells if the polyclonal Treg compartment is removed, highlighting a role for Treg cells in controlling the activation of Marilyn T cells *in vivo*.

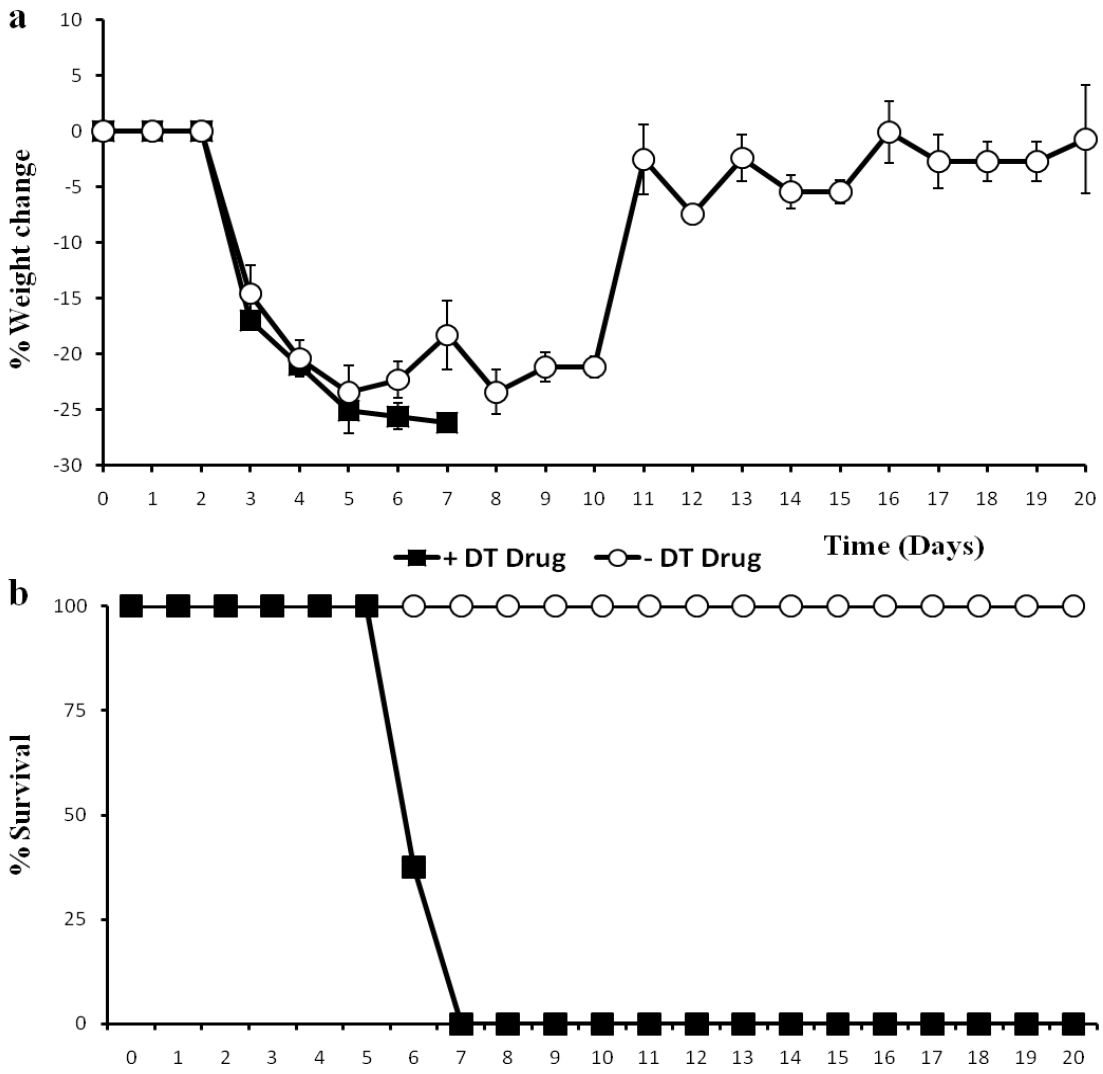


Figure 7.11. Tregs are crucial in preventing lethal GvHD induced by Marilyn CD4 T cells in male FoxP3-DTR mice
SP and pLNs were harvested from a $Rag2^{-/-}$ Marilyn female mouse and CD4 cells purified by MACS. Purified Marilyn CD4 T cells were injected (i.v.) at 4 x 10^6 cells/mouse to sub-lethally irradiated (5Gy) male FoxP3-DTR mice (n=12). A group of these mice (n=8) received a single dose of DT treatment (12.5μg) 1 day prior to CD4 transfer by (i.p.) injection.
a. Weight was measured at regular intervals and plotted as % weight change. Error is expressed as ±-standard error mean.
b. Percentage survival for each group as described above.

7.2.12: Donor T cell cytokine analysis from FoxP3-DTR male mice is consistent with male lymphopenic mice

On day 7, when all DT treated mice had reached the humane end point, 2 mice each from the DT treated and untreated group shown in Fig. 7.11 were culled. Lymphoid and non-lymphoid organs were harvested and the cells were plated out with PMA and Ionomycin in the presence of Brefeldin A, and analysed for the for Th1 cytokine profiles amongst adoptively transferred donor Marilyn CD4 T cells, as described previously (see Fig. 7.7). Again little discernable difference was seen in proportion (Fig. 7.12a) or MFI (Fig. 7.12b) of donor cells producing TNFα and IFNγ. These data in combination with donor cell analyses from lymphopenic acute GvHD mice (Fig. 7.7 and 7.9) suggests that Marilyn CD4 T cells are able to induce acute GvHD with radiation pre-conditioning in lymphopenic mice or in conjunction with Treg depletion in WT mice. However little functional or phenotypic differences were seen in the markers tested for donor Marilyn T cells from both groups, suggesting that the mechanisms for induction of lethal acute GvHD are complex and may directly or indirectly involve host cells.

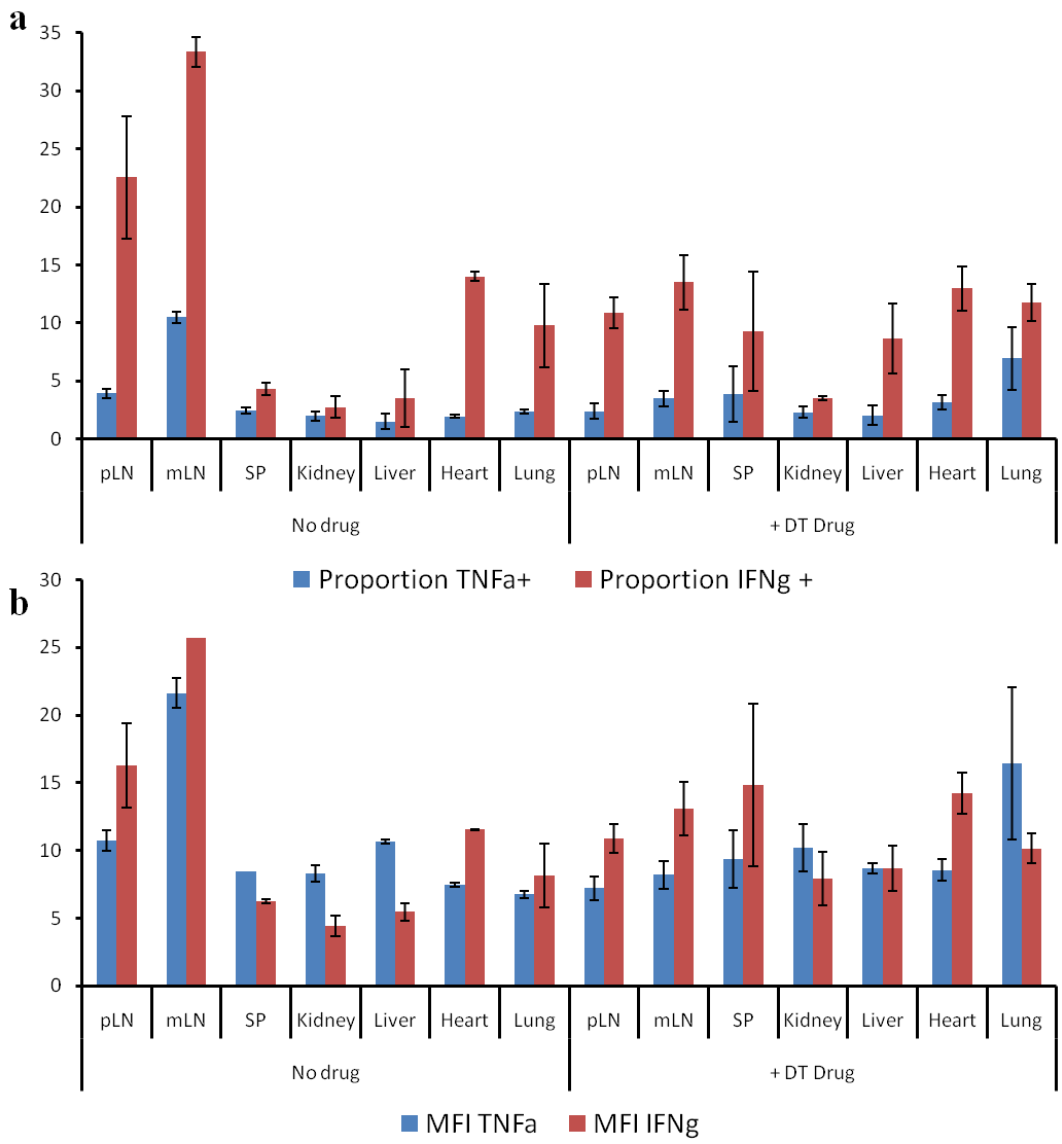


Figure 7.12. Comparison of IFNγ and TNFα production by donor CD4 T cells in DT treated and untreated mice

On day 7, 2 mice from the DT-treated and untreated group were culled and peripheral lymphoid (pLN, mLN, SP) and non-lymphoid organs (heart, kidney, liver, lung) were harvested and stimulated for 4 hours with PMA + Ionomycin in the presence of Brefeldin A. After removal of dead cells, cells were stained for anti-$V\beta6^{FITC}$ and anti-$CD4^{PerCP}$. Cells were then fixed and stained with anti-$TNF\alpha^{PE}$ and anti-$IFN\gamma^{APC}$. Samples were analysed by FACS and were gated on lymphocytes and then $V\beta6^{+}$ $CD4^{+}$ cells.

a. Average proportion of cells in $CD4^{+}$ $V\beta6^{+}$ gate expressing TNFα (blue) and IFNγ (red). Error is expressed as ± standard error mean.

b. Average mean fluorescence intensity of cells in $CD4^{+}V\beta6^{+}$ gate expressing TNFα (blue) and IFNγ (red). Error is expressed as ± standard error mean.

7.2.13: TNFα production is essential to induce GvHD

TNFα production has been shown to be strongly implicated in the severity of GvHD (Teshima, Ordemann et al. 2002), and higher serum levels of this cytokine increase Marilyn induced GvHD (Rivas, Weatherly et al. 2009; Kitamura, Sedlik et al. 2010). Anti-TNFα therapy has also been inhibit organ damage (Hill, Crawford et al. 1997) and reduce disease severity in humans (Uberti, Ayash et al. 2005). To explore this further, four groups of $Rag^{-/-}$ male mice were either untreated (G1) treated with T cells alone (G2), 5Gy alone (G3) or both (G4) and blood collected on days 1, 4 and 7, serum TNFα levels were determined using a mouse ELISA kit (Biolegend™) (Fig. 7.13a). Mice that received T cells (G2 and G4) produced significantly more TNFα than untreated mice and those given 5Gy alone (G1 and G3), while 5Gy alone did not induce more TNFα than untreated mice. Mice that received 5Gy and T cells (G4) however produced more TNFα at each time point than the T cell alone group (G2) with significantly higher levels on day 4. An increased TNFα level on day 4 correlates with peak GvHD severity and weight loss.

In another experiment $Rag^{-/-}$ male mice were given T cells alone (G1), 5Gy and Marilyn T cells (G2) or 5Gy, Marilyn T cells anti-TNFα drug Embrel® (i.p.) (G3), (Embrel® was developed by Immunex and kindly provided by Dr Richard Wilson, Kennedy Institute – Imperial college London). A clear protective effect was seen in the anti-TNFα treated group with significantly prolonged survival in this group (Fig. 7.13b and c) although this was not sufficient to prevent weight loss and eventual acute GvHD induced death in 6/7 mice. TNFα levels in serum were positively associated with onset of acute GvHD.

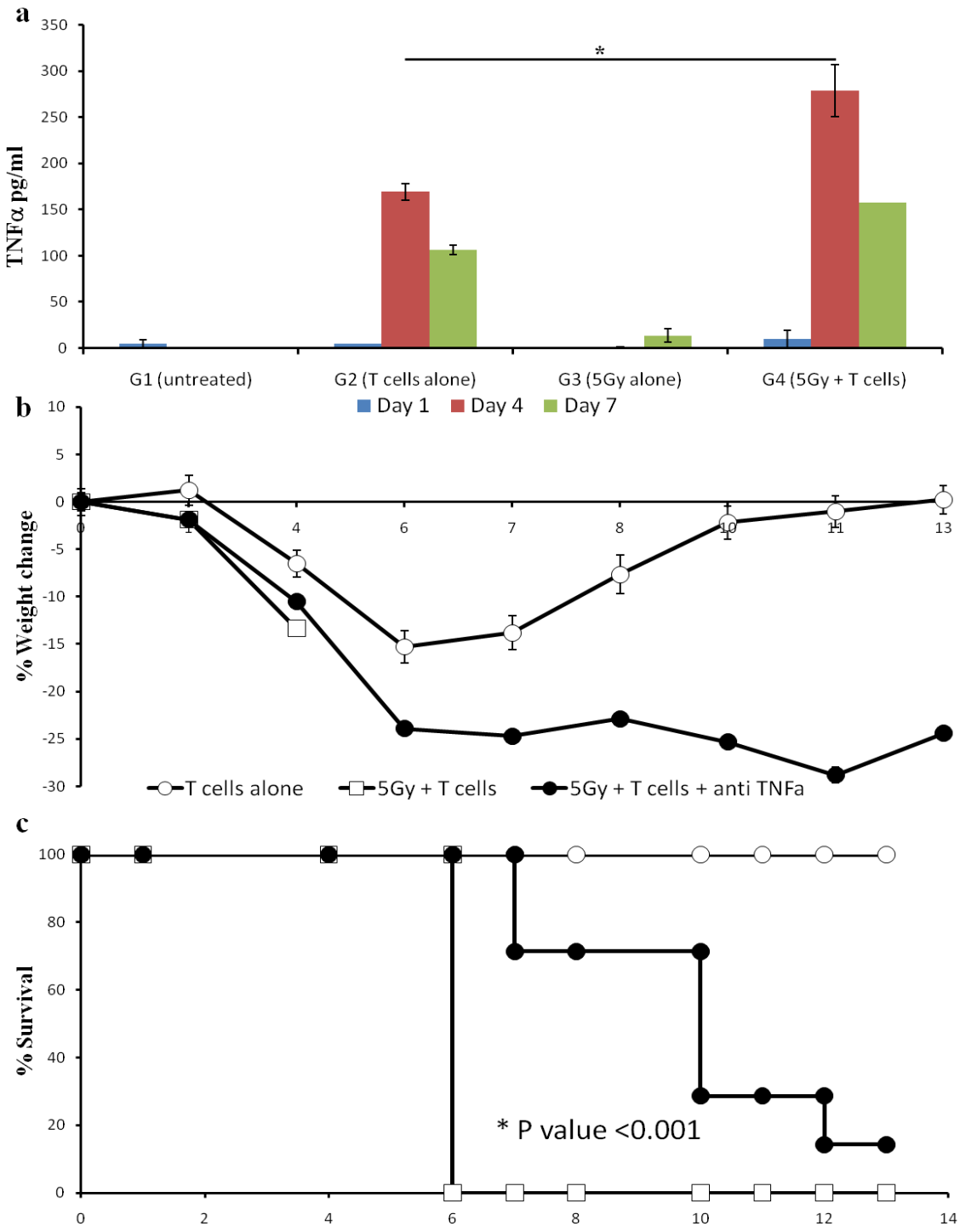


Figure 7.13. TNFα serum levels correlate to GvHD and anti-TNFα reduces GvHD disease severity

a. 4 groups of Rag$^{-/-}$ male mice were either untreated (G1, n=3), given 5Gy alone (G3, n=2) injected with 1 x 10^7 Marilyn SP+LN suspension alone (i.v.) (G2, n=2), injected with Marilyn cells with 5Gy (G4, n=2). Blood was collected on days 1, 4 and 7 and serum used for mouse TNFα ELISA (Biolegend™). Plates were read at 450nM absorbance (SpectramaxM2, Molecular Devices – SoftMax Pro) and protein concentrations calculated from the standard curve. Error is expressed as ±-standard error mean. (* P value 0.0329).

b. A separate group of $Rag^{-/-}$ male mice were given 1 x 10^6 Marilyn SP+LN suspension alone (i.v.) (n=3), Marilyn cells with 5Gy (n=5) or with additional anti-TNFα mAb (Embrel®) treatment, 400μg in 200μl (i.p.) in 4 doses (day 0, 3, 7 and 9) (n=7). Weight was measured at regular intervals and plotted as % weight change. Error is expressed as ±-standard error mean.
c. Percentage survival for each group as described above.

7.2.14: Neither HY-specific nor polyclonal CD8 T cells are able to induce GvHD in male lymphopenic mice

Matahari $CD8^+$ T cells express a transgenic, HY-specific TCR recognising the HY/UTY peptide in the context of $H2D^b$ (see chapters 3 and 4). In the next part of this study CD8 T cells were evaluated for their ability to induce GvHD in lymphopenic male mice given 5Gy irradiation (Fig. 7.14). Due to the numbers of CD8 T cells in Matahari $Rag^{-/-}$ mice, Matahari $Rag^{+/-}$ female mice were used as tissue donors to provide HY-specific CD8 T cells. As shown in Fig. **7**.14, adoptive transfer of resting Matahari CD8 T cells to $Rag^{-/-}$ B6 male mice receiving 5Gy did not induce significant weight loss (open circles) in comparison to those given 5Gy alone (closed circles). *In vitro* activated Matahari CD8 T cells were then used as donor cells to test for inducing GVHD. To prepare activated CD8 T cells, spleen and pooled lymph node cells from $Rag^{+/-}$ Matahari mice were cultured with HY-D^bUTY peptide and further expanded with rhIL-2. These *in vitro* activated CD8 cells were injected to $Rag^{-/-}$ B6 male mice that received 5Gy on day 0, but again no significant weight loss was seen compared to the 5Gy alone group (closed squares).

Matahari CD8 T cells represent a particular HY-specific CD8 T cell clone. These cells have also been identified as having a chronically activated self-reactive phenotype (chapter 4, Fig. 4.6). To evaluate whether polyclonal HY-specific CD8 T cells can induce GvHD in this GVHD model, we have taken advantage of the availability of the HY-D^bUTY tetramers. Polyclonal T cells were sourced from WT B6 females that had previously rejected an MB49 tumour and then boosted with male bone marrow derived dendritic cells (BMDCs) by i.p. injection. HY-D^bUTY tetramer and CD8-double positive cells were FACS purified from spleen and pooled lymph node cells of these mice (see FACS panel inset in Fig. 7.14). HY-specific CD8 T cells were injected (i.v.) into $Rag^{-/-}$ B6 male mice given 5Gy; none of these recipients lost a significant amount of weight, nor did they show any signs of acute

GvHD (closed squares). These data indicate that CD8 T cell responses to male antigen are insufficient to induce acute GvHD with radiation pre-conditioning.

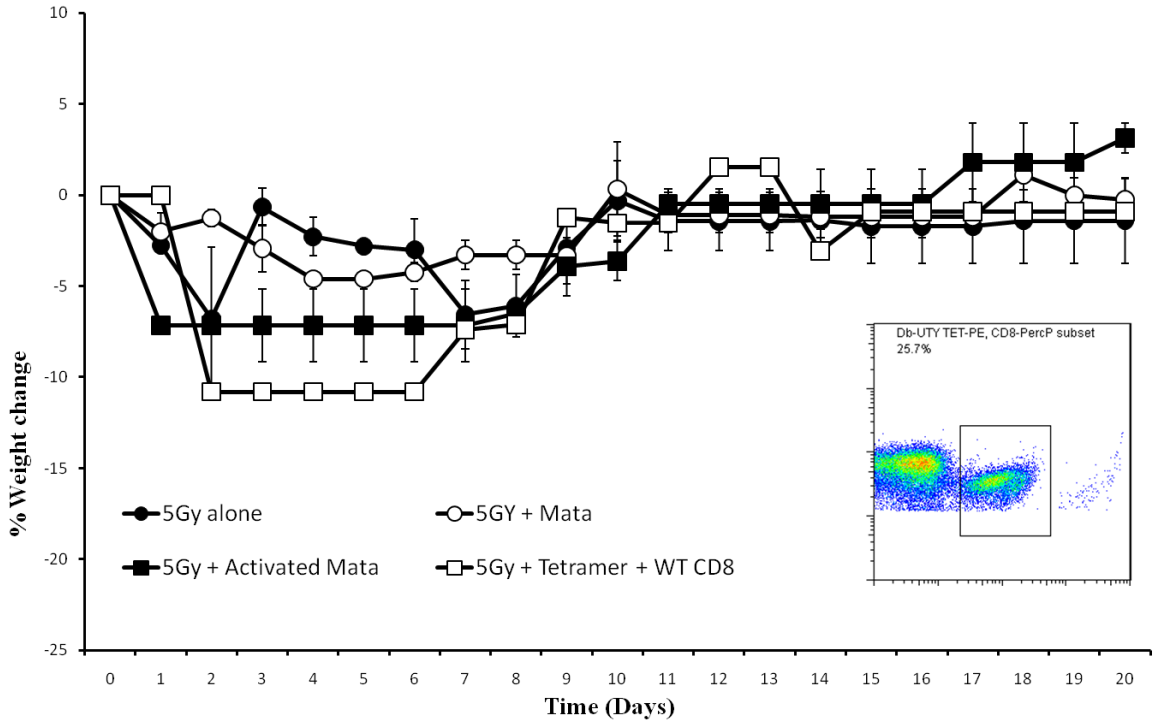


Figure 7.14. HY-specific CD8 T cells do not induce GvHD in lymphopenic male mice

SP and peripheral LNs were harvested from two Matahari $Rag^{+/-}$ donor mice. Cells were MACS purified for CD8 and FACS sorted for $V\beta8.3^{PE+}$ $CD8^{PerCP+}$ cells and injected (i.v.) at 4 x 10^6 cells/mouse to sub-lethally irradiated (5Gy) $Rag^{-/-}$ B6 male mice (n=10) (open circles). One group of $Rag^{-/-}$ B6 male mice (n=7) received sub-lethal irradiation (5Gy) alone (closed circles). SP and LN cells of Matahari $Rag^{+/-}$ donor mice were stimulated with 1nM D^bUTY peptide for 2 days, and activated CD8 T cells were further expanded for a further 2 days with 40IU/ml rhIL-2. On day 4, cells were harvested and FACS purified as above and injected (i.v.) at 1 x 10^7 cells/mouse to sub-lethally irradiated (5Gy) $Rag^{-/-}$ B6 male mice (n=4) (closed squares). Finally $tetramer^+$ $CD8^+$ cells were MACS purified for CD8 and FACS sorted from a B6 female mice that had cleared an MB49 tumour and received a male BMDC vaccine boost (inset FACS plot). 2 x 10^5 cells tetramer positive CD8 T cells were injected i.v. (open squares) (n=2). Weight was measured at regular intervals and plotted as % weight change. Error is expressed as ± standard error mean.

7.2.15: Matahari and WT tetramer positive CD8 T cells maintain activation markers but are poor Th1 cytokine producers

Two mice each from the two groups that received *in vitro* activated Matahari or freshly isolated HY-specific tetramer positive polyclonal CD8 T cells in the above experiment, shown in Fig. 7.14, were culled on day 20. Donor cells were analysed for the activation phenotype in comparison with T cells from a B6 female mouse as a control. Both types of donor cells mostly express CD44, indicative of a memory population, with few naive ($CD62L^{high}$ $CD44^{low}$) cells in each group (Fig. 7.15a). As CD44 is expressed at an intermediate level, these cells may have undergone adaptive tolerance resulting from chronic antigen exposure (Chiodetti, Choi et al. 2006) as seen with Marilyn cells in Fig. 7.9.

As shown in Fig. 7.7, cells were stained for intracellular T-bet or cultured for 4 hours with PMA and Ionomycin in the presence of Brefeldin A and stained for intracellular IFNγ and TNFα. Both Matahari and WT tetramer positive CD8 cells were poor producers of IFNγ and TNFα determined by MFI (Fig. 7.15b) and the proportion of expressing cells (Fig. 7.15c). Although Matahari cells expressed significantly more T-bet than B6 pLN and WT CD8 T cells, this was still low compared to Marilyn cells that induce GvHD (Fig. 7.7). In conclusion, Matahari and tetramer positive CD8 T cells maintain activation markers *in vivo* but may undergo adaptive tolerance and consequently are poor producers of Proinflammatory Th1 cytokines necessary for the induction of acute GvHD in male lymphopenic mice.

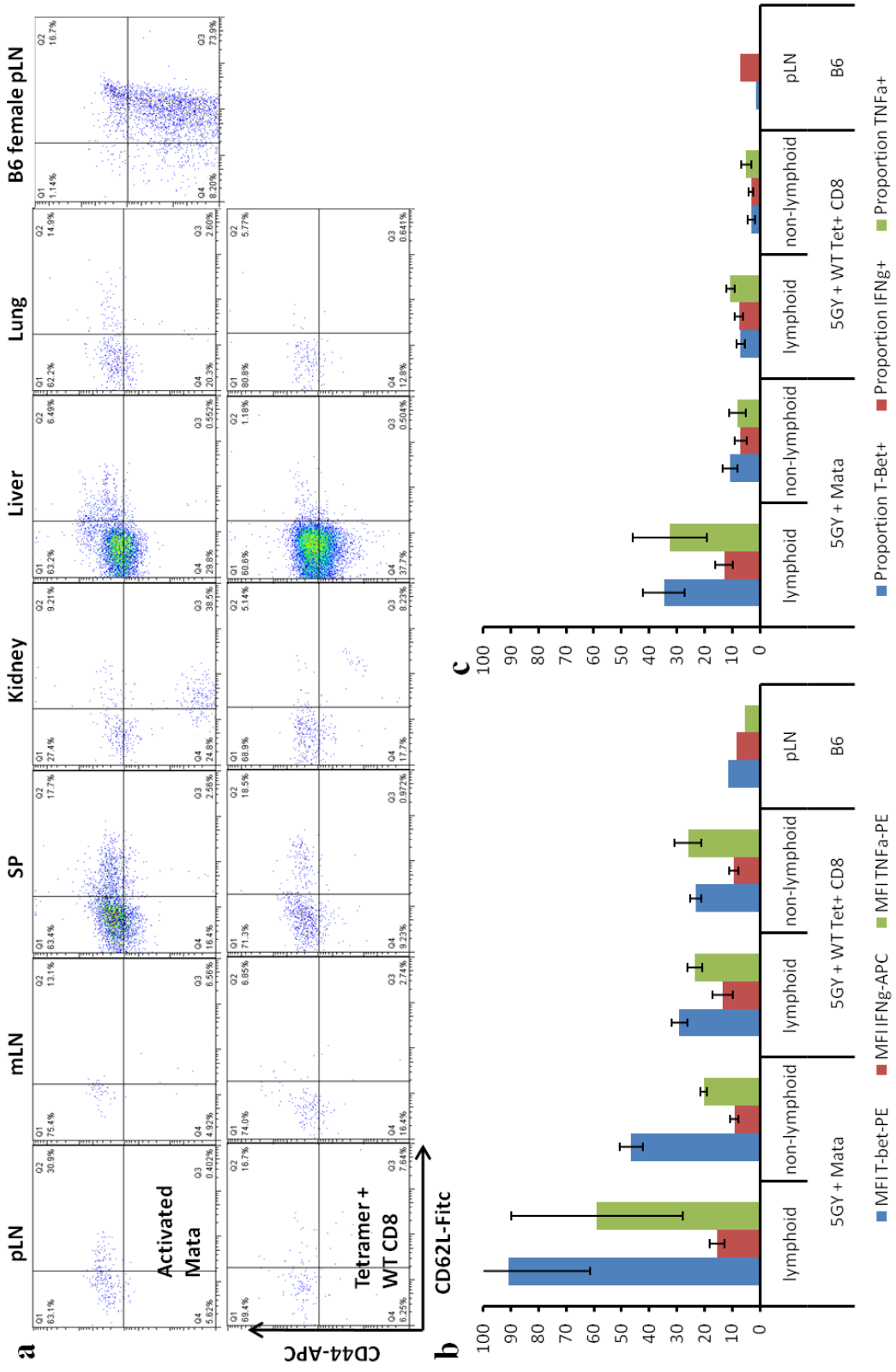


Figure 7.15. Matahari and WT CD8 are poor Th1 cytokine producers
pLN, mLN, SP, kidney, liver and lung were harvested from mice that received activated Matahari or tetramer positive CD8 T cells on day 20 (see Fig. 7.14) a WT B6 female pLN was used as a control.
a. Lymphoid (pLN, mLN, SP) and non-lymphoid organs (kidney, liver, lung) were harvested and cells were stained with anti-CD62L^{FITC}, anti-Vβ8.3^{PE}, anti-CD8^{PerCP}

and anti-CD44^{APC}. Cells were gated on lymphocytes and then Vβ8.3^{+} CD8^{+} cells, FACS plots represent CD44 and CD62L expression from this gate. One set of representative FACS plots shown.
b. Cells were stained for anti-T-bet^{PE} by intracellular staining (left) or cultured for 4 hours with PMA and ionomycin in the presence of Brefeldin A (as in Fig. 7.7), cells were stained for intracellular anti-TNFα^{PE} and anti-INF-γ^{APC}. A representative FACS plot is shown.
c. Average mean fluorescence intensity of cells in the CD8^{+} Vβ8.3^{+} gate expressing T-bet (blue), IFNγ (red) and TNFα (green). Error is expressed as ± standard error mean.
d. Average proportion of cells in CD8+ Vβ8.3+ gate expressing T-bet (blue), IFNγ (red) and TNFα (green). Error is expressed as ± standard error mean.

7.3: Discussion

It has generally not been possible to identify T cell epitopes exclusively expressed by human tumours. Tumour-associated antigens (TAA), expressed at higher levels in tumour cells but also expressed on non-malignant tissue have been more readily identified (see chapters 3 and 4). In addition, the increasing use of combination therapies such as lympho-depletion in conjunction with T cell adoptive transfer has the potential to cause adverse effects (Dudley and Rosenberg 2003). The HY system is an informative model for understanding anti-tumour T cell responses investigating such potential adverse effects. Evidence by several groups has demonstrated that when HY-specific T cell adoptive transfer is combined with additional therapies, this has the potential to induce potent anti-self responses (Rivas, Weatherly et al. 2009; Kitamura, Sedlik et al. 2010).

Two other groups have studied the role of Marilyn CD4 transgenic T cells in mouse models that have given GvHD or GvHD-like symptoms. Marilyn CD4 T cells infused into lymphopenic male mice expanded and displayed markers of activation but ultimately became 'adapted', a functionally unresponsive state (Rivas, Weatherly et al. 2009). Following systemic treatment with blockading antibody αPD-L1 with or without αCTLA-4 specific to co-inhibitory molecules, male lymphopenic mice infused with Marilyn CD4 T cells developed a multi organ wasting disease ultimately leading to pathology and mortality. A study by Kitamura

et al. has also reported a similar effect when WT and $Rag2^{-/-}$ B6 female mice are infused with Marilyn CD4 T cells and vaccinated with long chain peptides containing the cognate HY-Dby (Class II) and HY-Uty (Class I) sequence with adjuvant (IFA and CpG). Ten days later the mice boosted with long chain peptide developed shock like symptoms, rapid weight loss and acute GvHD, with death within 72 hours. However this did not happen if HY-Dby short chain peptide was used alone (Kitamura, Sedlik et al. 2010). Both of these studies showed that the acute GvHD effect was associated with a cytokine storm, demonstrating increased levels of Proinflammatory cytokines such as TNFα, IFNγ and IL-1 in mice with multiple organ wasting.

The novel approach used in this study is the induction of acute GvHD by adoptive transfer of Marilyn CD4 T cells into lymphopenic male mice following radiation conditioning, and into WT B6 male mice after *in vivo* depletion of Treg cells. This has implications for cancer therapy as most current treatment regimens use either radio- or chemo-therapy in conjunction with any novel therapy such as T cell adoptive transfer. The acute GvHD effect was initially noted during a tumour challenge experiment in which resting Marilyn T cells were transferred to tumour-bearing (for 7 days) $Rag^{-/-}$ B6 male and female hosts with or without sub-lethal radiation pre-conditioning with 5Gy (Fig. 7.1). The original aim of this experiment was to evaluate whether Marilyn CD4 T cells would control MB49 tumour in male recipients where cognate antigens were systemically expressed. However, the effect of acute GvHD in the male group was noted, with these mice reaching the humane end point by day 6. While the combined cell transfer/irradiation had little impact on tumour growth rate, it did induce a multiple organ wasting like disease analogous to GvHD. Mice receiving only radiation lost significantly more weight than those receiving T cells alone or female mice that received both. T cells expanded significantly in male mice but not female mice, indicating that this GvHD occurs in an antigen dependent manner (Fig. 7.4a). Surprisingly, T cells were no better at proliferating or infiltrating organs in the radiation pre-conditioned group compared to those receiving only T cells, suggesting that the distinct outcomes cannot be explained only by the presence of donor T cells (Fig. 7.4-6).

Clearly, the induction of acute GvHD is dependent on the direct effect of Marilyn CD4 cells. For triggering GvHD in lymphopenic male mice given 5Gy, highly purified Marilyn CD4 T cells were as efficient as un-fractioned spleen plus

lymph node cell suspensions from Marilyn mice (containing other cell types such as donor APC) (Fig. 7.2). This experiment did show however that transfer of large doses of purified Marilyn CD4 T cells alone were sufficient to induce an initial weight loss more that that shown by the group given 5Gy alone (Fig. 7.3), although this is insufficient to cause acute GvHD or death. Subsequent analysis of these mice showed that T cells are able to infiltrate non-lymphoid organs in both 5Gy treated and untreated mice (Fig. 7.4b and c) suggesting that induction of acute GvHD in these mice is not caused by quantitatively different T cell infiltration. Further analysis of the phenotype of these donor T cells (Fig. 7.5) showed that cells in both the 5Gy and non-irradiated group became activated ($CD44^{high}$) and maintained this status in the non-lymphoid organs (Fig. 7.5). The only noticeable difference in T cell activation was a significant skew towards effector memory T cells in 5Gy group (Fig. 7.6). This effect is likely to contribute towards the pathology of acute GvHD due to the ability of effector memory T cells to mediate killing through Granzyme B and Perforin (Okada, Kondo et al. 2008). Our results with the non-irradiated recipient group are consistent with those by Rivas *et al*. who showed Marilyn T cells can expand and activate in lymphopenic male mice but they become adapted and can only induce GvHD in their model upon additional negative co-stimulation blockade (αPD-L1) (Rivas, Weatherly et al. 2009). Kitamura *et al.* also demonstrate Marilyn T cells can induce GvHD in vaccinated B6 female mice following boosting with the long peptide, although they failed do so when short peptides were used for vaccination and boost (Kitamura, Sedlik et al. 2010). Together with our data this indicates that Marilyn T cells can induce GvHD only with additional stimulation (by PD-L1 blockade, vaccination or irradiation).

In order to elucidate the molecular mechanisms that prevent T cell adaptation in irradiated lymphopenic male mice, donor T cells were analysed for their ability to produce Th1 proinflammatory cytokines (Fig. 7.7) implicated in the induction of GvHD (Reddy and Ferrara 2003; Kitamura, Sedlik et al. 2010). T cells infused into male lymphopenic mice were able to produce significant amounts of T-bet, IFNγ and TNFα, however, no significant difference was seen between non-irradiated and 5Gy recipients. It is important to note that Marilyn CD4 T cells infused into lymphopenic male mice adopt a strong Th1 but not Th17 phenotype (Fig. 7.7 a). This again is in line with observation by Kitamura *et al.* who showed *ex vivo* Marilyn

T cells produce similar levels of IFNγ in both short peptide vaccinated and long peptide vaccinated B6 females and even higher levels of TNFα in the short peptide group. Rivas *et al*. however showed poor cytokine production from adapted Marilyn T cells from untreated lymphopenic male mice, although these T cells were evaluated at a much later stage (28 days). The cytokine production by Marilyn T cells is therefore not the major mechanism for the induction of GvHD.

Although the exact functional requirements for Marilyn T cells to escape adaptation remain elusive, it is clear radiation pre-conditioning is needed in this model. Male mice receiving 5Gy 10 days prior to T cell transfer showed an initial shock-like syndrome and rapid weight loss, a phenotype similar to mice receiving both 5Gy and T cells on day 0. The 5Gy -10 day mice however began to recover weight with 100% survival (Fig. 7.8) and donor T cells displayed a CD44 intermediate phenotype consistent with an adapted phenotype (Fig. 7.9a) (Choi and Schwartz 2007). Assessment of the cytokine profiles of the donor T cells of these mice from both lymphoid and non-lymphoid organs revealed low levels of T-bet and IFNγ expression (Fig. 7.9b,c and d). The phenotype of these T cells on day 30 displayed some similarity with the adapted Marilyn T cells described by Rivas *et al*. in untreated male mice, in that they were also poor cytokine producers and expressed markers of an adapted phenotype (Rivas, Weatherly et al. 2009). More importantly, the adapted T cells from male mice receiving 5Gy on day -10 were unable to induce GvHD or significant weight loss upon secondary transfer (Fig. 7.10). This is consistent with another report showing minor H antigen mismatched (B10.D2 (H-2^d) → BALB/c (H-2^d)) memory CD4+ T cells do not induce GvHD while naive CD4 T cells do (Anderson, McNiff et al. 2003). This may suggest that reconstitution of the immune system with memory or adapted T cells may abrogate these harmful effects.

Marilyn T cells can also induce acute GvHD in WT B6 male mice following Treg depletion (Fig. 7.11). This effect was also noticed by Kitamura *et al.* when B6 female mice boosted with HY-long peptide alone and Treg depleted (with no Marilyn T cell transfer) could develop GvHD like symptoms with some lethality (Kitamura, Sedlik et al. 2010). In the studies reported in this chapter, FoxP3-DTR male mice that received 5Gy, DT toxin and T cells lost weight at the same rate as mice that didn't receive DT toxin, but these mice also displayed signs of clinical acute GvHD reaching the humane end point by day 7 (Fig. 7.11b). No difference

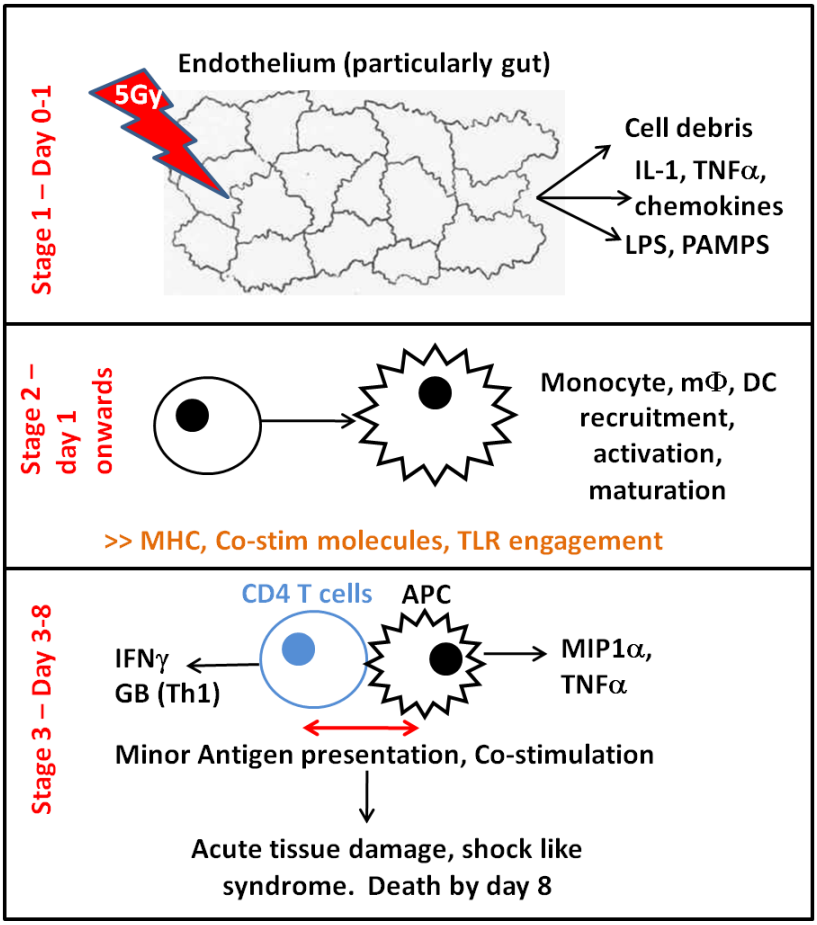
was seen in the Th1 cytokine profiles of donor T cells (Fig. 7.12), indicating that GvHD inducing signals may not be provided directly by T cells but more likely by professional APCs. These data indicate the requirement for Tregs in preventing GvHD in WT mice that receive radiation pre-conditioning and Marilyn adoptive transfer. Shevach *et al.* have shown polyclonal Treg cells are able to modulate antigen-specific CD4 T cells effectors by various mechanisms, including bystander suppression and limiting their migration into peripheral tissues (Davidson and Shevach 2011). This is an important finding to consider when designing adoptive transfer systems that require Treg abrogation or depletion.

Cytokine storms are a major factor in the induction of clinical acute GvHD (Hill and Ferrara 2000). Coupled with tissue damage and the release of factors such as LPS from the gut, this is believed to recuit, activate and mature APCs (Murai, Yoneyama et al. 2003) that are then able present antigen to and costimulate T cells in an immunogenic fashion (Beilhack, Schulz et al. 2005). IFNγ released from activated T cells activates macrophages which has been shown to lead to the production of TNFα by macrophages and DCs, the level of which has been shown to correlate to the severity of GvHD (Teshima, Ordemann et al. 2002). In our model, serum levels of TNFα were shown to increase in male lymphopenic mice receiving Marilyn T cells, with a significant increase of TNFα in mice that received both T cells and 5Gy (Fig. 7.13a). This suggests a synergistic effect of combining irradiation and T cells in the induction of GvHD. The highest levels of TNFα were also seen on day 4 in both T cell groups correlating with peak weight loss and onset of GvHD. Detectable TNFα was not produced in the 5Gy group indicating radiation treatment alone is not sufficient to induce TNFα production. This is consistent with an early study of the role TNFα in GvHD which showed high levels of TNFα mRNA in damaged tissues (Piguet, Grau et al. 1987).

Anti-TNFα therapy has been shown to inhibit organ damage (Hill, Crawford et al. 1997) and reduce GvHD severity in humans (Uberti, Ayash et al. 2005). Blocking TNFα *in vivo* using the commercially available drug Embrel® gave a significant protective effect and delayed death (Fig. 7.13b). This result confirms that of Kitamura *et al.* who showed TNFα production by APCs was essential for long peptide mediated death, with highest serum levels in this group, in addition, anti-TNFα mAb treatment could abrogate Marilyn CD4 T cell induced GvHD in long

peptide vaccinated mice. Indeed Rivas *et al.* have also shown LPS induced toxic shock occurs only with adapted Marilyn T cells not naive T cells in lymphopenic male mice, and this is correlated with a dramatic increase in TNFα blood serum levels. A model for GvHD induction following irradiation and T cell transfer is shown in the table below.

Table 9: Model of GvHD induction



In the second part of the study, Matahari CD8 T cells were evaluated for their ability to induce GvHD in radiation pre-conditioned lymphopenic male hosts. Resting and *in vitro* activated Matahari CD8 T cells infused into irradiated (5Gy) lymphopenic male recipients were unable to induce GvHD, nor were tetramer positive HY-specific polyclonal WT CD8s T cells able to do so (Fig. 7.14). Despite some expansion and infiltration of both types of T cell (Fig. 7.15), significant weight loss or GvHD symptoms were not seen following transfer of HY-specific CD8 T cells. Both WT CD8 and Matahari CD8 T cells displayed some Tc1 cytokine production (Fig. 7.15b) although this was low in comparison with Marilyn CD4 T cells (Fig. 7.7). HY-specific CD8 T cells are therefore unable to induce acute GvHD in lymphopenic male mice when combined with sub-lethal radiation pre-

conditioning. Consistent with these results Kitamua *et al.* also found Matahari T cells could not induce GvHD like symptoms or death in long peptide, short peptide and male splenocyte vaccinated mice

Acute GvHD in the model shown here with adoptive transfer of HY-specific Marilyn TCR CD4 T cells is likely caused by a runaway CD4 T cell response in the presence of a radiation-induced cytokine storm. Cytokines such as IL-1 released by the endothelium increases APC Th1 activation status (particularly DC) and therefore also increases positive co-stimulatory signalling to CD4 T cells while decreasing negative signals (Abhyankar, Gilliland et al. 1993) (Rivas, Weatherly et al. 2009). This results in a higher serum level production of the Th1 proinflammatory cytokine TNFα by APCs that likely acts to maintain activation signals and enhance antigen presentation preventing antigen-driven T cell adaptation as is seen in the non-irradiated groups (Rivas, Weatherly et al. 2009). In lymphopenic hosts this effect becomes 'runaway' due to the lack of regulation. This effect can be reproduced in WT DTR male mice by the depletion of Tregs prior to radiation pre-conditioning and donor T cell infusion. However, neither Matahari or polyclonal tetramer positive CD8 T cells are able to cause GvHD or weight loss in this model possibly due to their inability to provide help to mediators of killing such as NK cells and macrophages (van den Brink and Burakoff 2002).

In light of this and similar studies using negative co-stimulation blockade (Rivas, Weatherly et al. 2009) and long peptide vaccination (Kitamura, Sedlik et al. 2010), it is clear great care must be taken when using combinational T cell therapy. This highlights the need to understand the cross-talk between the innate and adaptive immune components when designing immuno-therapies. Attention must always be given to potential secondary effects of therapeutic immunotherapy. Conversely however, the study reported in this thesis does show that T cell adaptation can be reversed when combined with radiation pre-conditioning, which if targeted correctly, could be applied to boost therapeutic immunity against cancer.

Summary

This study has established MB49 as a relevant murine model for the study of tumour antigen-specific immune responses in cancer immunotherapy. MB49 is a HY^+ immunogenic tumour *in vivo* that is rejected at low doses by female C57BL/6 mice due to HY-specific cellular responses. MB49 utilises co-inhibitory ligands (PD-L1, FAS-L) and secretes local immunosuppressive factors (IDO, IL-10, TGF-β) to abrogate anti-tumour T cell responses and promote Treg development, both hallmarks of cancer (Zitvogel, Tesniere et al. 2006).

Using TCR transgenic T cells, HY-specific responses to malignant and non-malignant tissues were compared. These results confirm CTL responses are inhibited in tumour tissues, mediated by immunoevasive factors, but can be enhanced by the co-presence of a male skin graft. This mechanism of cross talk is assessed as a novel route to enhance antigen-specific T cell responses to tumour.

Conversely, tumours are shown to expand HY-specific Tregs, although this is associated with some cells losing FoxP3 expression. Using a FoxP3-GFP reporter system this work shows for the first time that antigen-specific Tregs (both thymic and peripheral) can become Th1-like effector T cells in tumour-bearing hosts. These cells have distinct gene expression profiles and are able to maintain this phenotype upon secondary adoptive cell transfer. This observation has profound implications for Treg therapy in autoimmune settings (Zhou, Bailey-Bucktrout et al. 2009) and potential protective effects if induced against tumours. I am indebted to Dr C. Addey, Dr D. Coe and Dr J-G. Chai for their assistance in preparing chapter 5 of this thesis for publication (submission and print) in the Journal of Immunology (Addey, White et al. 2011).

Utilising our ability to perform retroviral genetic modification, an MB49/rhIL-2 retrogenic tumour was produced. As with previous studies using tumours modified to produce IL-2, the MB/IL-2 tumour was shown to induce effective CTL responses, with long standing immunity (Fearon, Pardoll et al. 1990). Unlike previous studies however we also demonstrate that IL-2 in the local microenvironment can act to enhance tumour antigen-specific CD8 responses to a similar degree as polyclonal responses. It is also shown that rejection of IL-2 low producing tumours is mediated by CD8 T cells and these tumours are viable in

lymphopenic recipients. This work exposes the need for multiple approaches in inducing effective antigen-specific T cell mediated *in vivo* responses to tumour.

The principles of adoptive immunotherapy in the context of radiotherapy are considered using a model of T cell induced GvHD. Administration of Marilyn CD4 but not Matahari CD8 has the effect of inducing weight loss in lymphopenic male mice, and if combined with sublethal radiation preconditioning this is sufficient to induce death by GvHD. This mirrors systems using Marilyn T cells with negative co-stimulation blockade and long peptide vaccination (Rivas, Weatherly et al. 2009; Kitamura, Sedlik et al. 2010). GvHD could also be induced in lymphoreplete DTR males following the depletion of Tregs, consistent with studies by others (Tawara, Shlomchik et al. 2010). It is therefore important to assess the synergistic effect of combining multiple treatments in future adoptive immunotherapy strategies to minor H antigens.

Studies using adoptive cell transfer of antigen-specific T cell subsets in murine models are essential to the development of effective and safe treatments in human cancers. This work shows both polyclonal and antigen-specific T cell subsets can rarely be manipulated without consideration to other T cells, immune cells and treatments. Despite being over 20 years old, the field of adoptive immunotherapy in cancer has yet to yield widespread safe and effective treatments in a clinical setting. Using lessons from studies such as this it may be possible to refine current approaches to provide high efficacy treatments while minimising unwanted side effects.

References

(1999). "Complete sequence and gene map of a human major histocompatibility complex. The MHC sequencing consortium." Nature **401**(6756): 921-923.

Abad, J. D., C. Wrzensinski, et al. (2008). "T cell receptor gene therapy of established tumors in a murine melanoma model." J Immunother **31**(1): 1-6.

Abhyankar, S., D. G. Gilliland, et al. (1993). "Interleukin-1 Is a Critical Effector Molecule during Cytokine Dysregulation in Graft-Versus-Host Disease to Minor Histocompatibility Antigens." Transplantation **56**(6): 1518-1523.

Addey, C., M. White, et al. (2011). "Functional plasticity of antigen-specific regulatory T cells in context of tumor." Journal of immunology **186**(8): 4557-4564.

Aderem, A. and D. M. Underhill (1999). "Mechanisms of phagocytosis in macrophages." Annual review of immunology **17**: 593-623.

Almeida, A. R., B. Zaragoza, et al. (2006). "Competition controls the rate of transition between the peripheral pools of CD4+CD25- and CD4+CD25+ T cells." Int Immunol **18**(11): 1607-1613.

Alosco, T., B. A. Croy, et al. (1993). "Antitumor response independent of functional B or T lymphocytes induced by the local and sustained release of interleukin-2 by the tumor cells." Cancer immunology, immunotherapy : CII **36**(6): 364-372.

Anderson, B. E., J. McNiff, et al. (2003). "Memory CD4+ T cells do not induce graft-versus-host disease." The Journal of clinical investigation **112**(1): 101-108.

Ansari, M. J., A. D. Salama, et al. (2003). "The programmed death-1 (PD-1) pathway regulates autoimmune diabetes in nonobese diabetic (NOD) mice." The Journal of experimental medicine **198**(1): 63-69.

Antin, J. H., D. Weisdorf, et al. (2002). "Interleukin-1 blockade does not prevent acute graft-versus-host disease: results of a randomized, double-blind, placebo-controlled trial of interleukin-1 receptor antagonist in allogeneic bone marrow transplantation." Blood **100**(10): 3479-3483.

Antony, P. A., C. M. Paulos, et al. (2006). "Interleukin-2-dependent mechanisms of tolerance and immunity in vivo." J Immunol **176**(9): 5255-5266.

Antony, P. A., C. A. Piccirillo, et al. (2005). "CD8+ T cell immunity against a tumor/self-antigen is augmented by CD4+ T helper cells and hindered by naturally occurring T regulatory cells." J Immunol **174**(5): 2591-2601.

Arendt, C. W. and H. L. Ostergaard (1995). "CD45 protein-tyrosine phosphatase is specifically associated with a 116-kDa tyrosine-phosphorylated glycoprotein." The Journal of biological chemistry **270**(5): 2313-2319.

Arstila, T. P., A. Casrouge, et al. (1999). "A direct estimate of the human alphabeta T cell receptor diversity." Science **286**(5441): 958-961.

Assier, E., V. Jullien, et al. (2004). "NK cells and polymorphonuclear neutrophils are both critical for IL-2-induced pulmonary vascular leak syndrome." Journal of immunology **172**(12): 7661-7668.

Atkins, D., A. Breuckmann, et al. (2004). "MHC class I antigen processing pathway defects, ras mutations and disease stage in colorectal carcinoma." International journal of cancer. Journal international du cancer **109**(2): 265-273.

Auchincloss, H., Jr. and H. Sultan (1996). "Antigen processing and presentation in transplantation." Current opinion in immunology **8**(5): 681-687.

Auffermann-Gretzinger, S., I. S. Lossos, et al. (2002). "Rapid establishment of dendritic cell chimerism in allogeneic hematopoietic cell transplant recipients." Blood **99**(4): 1442-1448.

Azuma, M. (2006). "Fundamental mechanisms of host immune responses to infection." Journal of periodontal research **41**(5): 361-373.

Banchereau, J., F. Bazan, et al. (1994). "The Cd40 Antigen and Its Ligand." Annual Review of Immunology **12**: 881-922.

Banovic, T., K. P. A. MacDonald, et al. (2005). "TGF-beta in allogeneic stem cell transplantation: friend or foe?" Blood **106**(6): 2206-2214.

Barnes, D. W., J. F. Loutit, et al. (1962). ""Secondary disease" of radiation chimeras: a syndrome due to lymphoid aplasia." Annals of the New York Academy of Sciences **99**: 374-385.

Bassing, C. H., W. Swat, et al. (2002). "The mechanism and regulation of chromosomal V(D)J recombination." Cell **109 Suppl**: S45-55.

Beadling, C., K. W. Johnson, et al. (1993). "Isolation of interleukin 2-induced immediate-early genes." Proc Natl Acad Sci U S A **90**(7): 2719-2723.

Beadling, C. and K. A. Smith (2002). "DNA array analysis of interleukin-2-regulated immediate/early genes." Med Immunol **1**(1): 2.

Beilhack, A., S. Schulz, et al. (2005). "In vivo analyses of early events in acute graft-versus-host disease reveal sequential infiltration of T cell subsets." Blood **106**(3): 1113-1122.

Belladonna, M. L., P. Puccetti, et al. (2007). "Immunosuppression via tryptophan catabolism: the role of kynurenine pathway enzymes." Transplantation **84**(1 Suppl): S17-20.

Benghiat, F. S., L. Graca, et al. (2005). "Critical influence of natural regulatory CD25+ T cells on the fate of allografts in the absence of immunosuppression." Transplantation **79**(6): 648-654.

Benjamin, R. J., S. P. Cobbold, et al. (1986). "Tolerance to rat monoclonal antibodies. Implications for serotherapy." J Exp Med **163**(6): 1539-1552.

Bennett, M. W., J. O'Connell, et al. (2001). "Fas ligand upregulation is an early event in colonic carcinogenesis." Journal of clinical pathology **54**(8): 598-604.

Bettelli, E., T. Korn, et al. (2008). "Induction and effector functions of T(H)17 cells." Nature **453**(7198): 1051-1057.

Bhandoola, A., A. Sambandam, et al. (2003). "Early T lineage progenitors: new insights, but old questions remain." Journal of immunology **171**(11): 5653-5658.

Billingham, R. E. (1966). "The biology of graft-versus-host reactions." Harvey lectures **62**: 21-78.

Billingham, R. E., P. L. Krohn, et al. (1951). "Effect of cortisone on survival of skin homografts in rabbits." Br Med J **1**(4716): 1157-1163.

Blank, C., I. Brown, et al. (2004). "PD-L1/B7H-1 inhibits the effector phase of tumor rejection by T cell receptor (TCR) transgenic CD8+ T cells." Cancer Res **64**(3): 1140-1145.

Blank, C., T. F. Gajewski, et al. (2005). "Interaction of PD-L1 on tumor cells with PD-1 on tumor-specific T cells as a mechanism of immune evasion: implications for tumor immunotherapy." Cancer immunology, immunotherapy : CII **54**(4): 307-314.

Blazar, B. R., B. M. Carreno, et al. (2003). "Blockade of programmed death-1 engagement accelerates graft-versus-host disease lethality by an IFN-gamma-dependent mechanism." Journal of immunology **171**(3): 1272-1277.

Blazar, B. R., A. H. Sharpe, et al. (2003). "Ligation of OX40 (CD134) regulates graft-versus-host disease (GVHD) and graft rejection in allogeneic bone marrow transplant recipients." Blood **101**(9): 3741-3748.

Bluestone, J. A. and A. K. Abbas (2003). "Natural versus adaptive regulatory T cells." Nat Rev Immunol **3**(3): 253-257.

Bluestone, J. A., C. R. Mackay, et al. (2009). "The functional plasticity of T cell subsets." Nat Rev Immunol **9**(11): 811-816.

Bodmer, J. G., S. G. Marsh, et al. (1991). "Nomenclature for factors of the HLA system, 1990." Vox sanguinis **61**(2): 147-155.

Boissonnas, A., A. Scholer-Dahirel, et al. (2010). "Foxp3+ T cells induce perforin-dependent dendritic cell death in tumor-draining lymph nodes." Immunity **32**(2): 266-278.

Boniface, J. J., Z. Reich, et al. (1999). "Thermodynamics of T cell receptor binding to peptide-MHC: evidence for a general mechanism of molecular scanning." Proceedings of the National Academy of Sciences of the United States of America **96**(20): 11446-11451.

Borberg, H., H. F. Oettgen, et al. (1972). "Inhibition of established transplants of chemically induced sarcomas in syngeneic mice by lymphocytes from immunized donors." International journal of cancer. Journal international du cancer **10**(3): 539-547.

Bradfute, S. B., T. A. Graubert, et al. (2005). "Roles of Sca-1 in hematopoietic stem/progenitor cell function." Experimental hematology **33**(7): 836-843.

Bramson, J., M. Hitt, et al. (1996). "Construction of a double recombinant adenovirus vector expressing a heterodimeric cytokine: in vitro and in vivo production of biologically active interleukin-12." Human gene therapy **7**(3): 333-342.

Braun, M. Y., I. Grandjean, et al. (2001). "Acute rejection in the absence of cognate recognition of allograft by T cells." J Immunol **166**(8): 4879-4883.

Bubenik, J. (1993). "IL-2 gene therapy of cancer: at its beginning." Folia biologica **39**(6): 279-288.

Burnet, F. M. (1970). "The concept of immunological surveillance." Progress in experimental tumor research **13**: 1-27.

Bursuker, I. and R. J. North (1984). "Generation and decay of the immune response to a progressive fibrosarcoma. II. Failure to demonstrate postexcision immunity after the onset of T cell-mediated suppression of immunity." J Exp Med **159**(5): 1312-1321.

Butte, M. J., M. E. Keir, et al. (2007). "Programmed death-1 ligand 1 interacts specifically with the B7-1 costimulatory molecule to inhibit T cell responses." Immunity **27**(1): 111-122.

Cantrell, D. A. and K. A. Smith (1984). "The interleukin-2 T cell system: a new cell growth model." Science **224**(4655): 1312-1316.

Cao, X., S. F. Cai, et al. (2007). "Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance." Immunity **27**(4): 635-646.

Cao, Z. A., D. Daniel, et al. (2002). "Sub-lethal radiation enhances anti-tumor immunotherapy in a transgenic mouse model of pancreatic cancer." BMC Cancer **2**: 11.

Carroll, M. W., W. W. Overwijk, et al. (1998). "Construction and characterization of a triple-recombinant vaccinia virus encoding B7-1, interleukin 12, and a model tumor antigen." Journal of the National Cancer Institute **90**(24): 1881-1887.

Carswell, E. A., L. J. Old, et al. (1975). "An endotoxin-induced serum factor that causes necrosis of tumors." Proceedings of the National Academy of Sciences of the United States of America **72**(9): 3666-3670.

Casciari, J. J., H. Sato, et al. (1996). "Reference databases of cytokine structure and function." Cancer chemotherapy and biological response modifiers **16**: 315-346.

Cella, M., F. Sallusto, et al. (1997). "Origin, maturation and antigen-presenting function of dendritic cells." Current opinion in immunology **9**(1): 10-16.

Chai, J. G., D. Coe, et al. (2008). "In vitro expansion improves in vivo regulation by CD4+CD25+ regulatory T cells." J Immunol **180**(2): 858-869.

Chaput, N., G. Darrasse-Jeze, et al. (2007). "Regulatory T cells prevent CD8 T cell maturation by inhibiting CD4 Th cells at tumor sites." Journal of immunology **179**(8): 4969-4978.

Cheever, M. A. and C. S. Higano (2011). "PROVENGE (Sipuleucel-T) in prostate cancer: the first FDA-approved therapeutic cancer vaccine." Clinical cancer research : an official journal of the American Association for Cancer Research **17**(11): 3520-3526.

Chen, M. L., M. J. Pittet, et al. (2005). "Regulatory T cells suppress tumor-specific CD8 T cell cytotoxicity through TGF-beta signals in vivo." Proc Natl Acad Sci U S A **102**(2): 419-424.

Chen, W., W. Jin, et al. (2003). "Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3." J Exp Med **198**(12): 1875-1886.

Chen, Y., Y. Demir, et al. (2003). "The male minor transplantation antigen preferentially activates recipient CD4+ T cells through the indirect presentation pathway in vivo." J Immunol **171**(12): 6510-6518.

Chiodetti, L., S. Choi, et al. (2006). "Adaptive tolerance and clonal anergy are distinct biochemical states." Journal of immunology **176**(4): 2279-2291.

Choi, S. and R. H. Schwartz (2007). "Molecular mechanisms for adaptive tolerance and other T cell anergy models." Seminars in immunology **19**(3): 140-152.

Chorny, A., E. Gonzalez-Rey, et al. (2006). "Vasoactive intestinal peptide induces regulatory dendritic cells that prevent acute graft-versus-host disease while maintaining the graft-versus-tumor response." Blood **107**(9): 3787-3794.

Cignetti, A., A. Guarini, et al. (1994). "Transduction of the IL2 gene into human acute leukemia cells: induction of tumor rejection without modifying cell proliferation and IL2 receptor expression." Journal of the National Cancer Institute **86**(10): 785-791.

Clift, R. A., C. D. Buckner, et al. (1990). "Allogeneic marrow transplantation in patients with acute myeloid leukemia in first remission: a randomized trial of two irradiation regimens." Blood **76**(9): 1867-1871.

Cobbold, S. P., E. Adams, et al. (2009). "Infectious tolerance via the consumption of essential amino acids and mTOR signaling." Proceedings of the National Academy of Sciences of the United States of America **106**(29): 12055-12060.

Cobbold, S. P., A. Jayasuriya, et al. (1984). "Therapy with monoclonal antibodies by elimination of T cell subsets in vivo." Nature **312**(5994): 548-551.

Coe, D., C. Addey, et al. (2010). "The roles of antigen-specificity, responsiveness to transforming growth factor-beta and antigen-presenting cell subsets in tumour-induced expansion of regulatory T cells." Immunology **131**(4): 556-569.

Coe, D., S. Begom, et al. (2010). "Depletion of regulatory T cells by anti-GITR mAb as a novel mechanism for cancer immunotherapy." Cancer Immunol Immunother **59**(9): 1367-1377.

Cohen, P. A., L. Peng, et al. (2000). "CD4+ T cells in adoptive immunotherapy and the indirect mechanism of tumor rejection." Crit Rev Immunol **20**(1): 17-56.

Coleman, W. B. and G. J. Tsongalis (2009). Molecular pathology : the molecular basis of human disease. Burlington, Mass., Academic Press.

Connor, J., R. Bannerji, et al. (1993). "Regression of bladder tumors in mice treated with interleukin 2 gene-modified tumor cells." The Journal of experimental medicine **177**(4): 1127-1134.

Corthay, A., D. K. Skovseth, et al. (2005). "Primary antitumor immune response mediated by CD4(+) T cells." Immunity **22**(3): 371-383.

Coulie, P. G., T. Hanagiri, et al. (2001). "From tumor antigens to immunotherapy." International journal of clinical oncology / Japan Society of Clinical Oncology **6**(4): 163-170.

Cresswell, P. (2005). "Antigen processing and presentation." Immunol Rev **207**: 5-7.

Croce, C. M. (2008). "Oncogenes and cancer." The New England journal of medicine **358**(5): 502-511.

Cromme, F. V., P. F. van Bommel, et al. (1994). "Differences in MHC and TAP-1 expression in cervical cancer lymph node metastases as compared with the primary tumours." Br J Cancer **69**(6): 1176-1181.

Curiel, T. J. (2007). "Tregs and rethinking cancer immunotherapy." J Clin Invest **117**(5): 1167-1174.

Daley, S. R., J. Ma, et al. (2007). "A key role for TGF-beta signaling to T cells in the long-term acceptance of allografts." Journal of immunology **179**(6): 3648-3654.

Davey, G. M., S. L. Schober, et al. (1998). "Preselection thymocytes are more sensitive to T cell receptor stimulation than mature T cells." The Journal of experimental medicine **188**(10): 1867-1874.

Davidson, T. S. and E. M. Shevach (2011). "Polyclonal Tregs Modulate T Effector Cell Trafficking." European journal of immunology.

Davis, M. M. and P. J. Bjorkman (1988). "T cell antigen receptor genes and T cell recognition." Nature **334**(6181): 395-402.

de Waal Malefyt, R., J. Haanen, et al. (1991). "Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression." The Journal of experimental medicine **174**(4): 915-924.

de Waal Malefyt, R., H. Yssel, et al. (1993). "Direct effects of IL-10 on subsets of human CD4+ T cell clones and resting T cells. Specific inhibition of IL-2 production and proliferation." Journal of immunology **150**(11): 4754-4765.

de Witte, M. A., G. M. Bendle, et al. (2008). "TCR gene therapy of spontaneous prostate carcinoma requires in vivo T cell activation." Journal of immunology **181**(4): 2563-2571.

de Witte, M. A., A. Jorritsma, et al. (2008). "Requirements for effective antitumor responses of TCR transduced T cells." Journal of immunology **181**(7): 5128-5136.

Deaglio, S., K. M. Dwyer, et al. (2007). "Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression." J Exp Med **204**(6): 1257-1265.

den Haan, J. M., L. M. Meadows, et al. (1998). "The minor histocompatibility antigen HA-1: a diallelic gene with a single amino acid polymorphism." Science **279**(5353): 1054-1057.

Ding, L. and E. M. Shevach (1992). "IL-10 inhibits mitogen-induced T cell proliferation by selectively inhibiting macrophage costimulatory function." Journal of immunology **148**(10): 3133-3139.

Dong, H., S. E. Strome, et al. (2002). "Tumor-associated B7-H1 promotes T cell apoptosis: a potential mechanism of immune evasion." Nature medicine **8**(8): 793-800.

Doren, M. (2010). "Assessing the effectiveness of human papillomavirus (HPV) vaccination to prevent cervical cancer: perspectives from Germany." Journal of epidemiology and community health **64**(2): 103-104.

Dranoff, G. (2009). "Targets of protective tumor immunity." Ann N Y Acad Sci **1174**: 74-80.

Du Pasquier, L. (1992). "Origin and evolution of the vertebrate immune system." APMIS : acta pathologica, microbiologica, et immunologica Scandinavica **100**(5): 383-392.

Duarte, J. H., S. Zelenay, et al. (2009). "Natural Treg cells spontaneously differentiate into pathogenic helper cells in lymphopenic conditions." Eur J Immunol **39**(4): 948-955.

Dudley, M. E. and S. A. Rosenberg (2003). "Adoptive-cell-transfer therapy for the treatment of patients with cancer." Nat Rev Cancer **3**(9): 666-675.

Dudley, M. E. and S. A. Rosenberg (2007). "Adoptive cell transfer therapy." Semin Oncol **34**(6): 524-531.

Dudley, M. E., J. R. Wunderlich, et al. (2002). "Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes." Science **298**(5594): 850-854.

Dudley, M. E., J. R. Wunderlich, et al. (2005). "Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma." J Clin Oncol **23**(10): 2346-2357.

Duffner, U. A., Y. Maeda, et al. (2004). "Host dendritic cells alone are sufficient to initiate acute graft-versus-host disease." Journal of Immunology **172**(12): 7393-7398.

Dummer, R., J. C. Hassel, et al. (2004). "Adenovirus-mediated intralesional interferon-gamma gene transfer induces tumor regressions in cutaneous lymphomas." Blood **104**(6): 1631-1638.

Dummer, R., C. Rochlitz, et al. (2008). "Intralesional adenovirus-mediated interleukin-2 gene transfer for advanced solid cancers and melanoma." Molecular therapy : the journal of the American Society of Gene Therapy **16**(5): 985-994.

Dunn, G. P., L. J. Old, et al. (2004). "The immunobiology of cancer immunosurveillance and immunoediting." Immunity **21**(2): 137-148.

Eichwald, E. J. and C. R. Silmser (1955). "Skin." Transplantation bulletin **2**: 148-149.

Elias, E. G., J. L. Zapas, et al. (2005). "GM-CSF and IL-2 combination as adjuvant therapy in cutaneous melanoma: early results of a phase II clinical trial." Oncology **19**(4 Suppl 2): 15-18.

Ettinghausen, S. E. and S. A. Rosenberg (1995). "Immunotherapy and gene therapy of cancer." Adv Surg **28**: 223-254.

Fearon, E. R., D. M. Pardoll, et al. (1990). "Interleukin-2 production by tumor cells bypasses T helper function in the generation of an antitumor response." Cell **60**(3): 397-403.

Feng, X., K. M. Hui, et al. (2008). "Targeting minor histocompatibility antigens in graft versus tumor or graft versus leukemia responses." Trends in immunology **29**(12): 624-632.

Fernandez, E. J. and E. Lolis (2002). "Structure, function, and inhibition of chemokines." Annual review of pharmacology and toxicology **42**: 469-499.

Fidler, I. J. (1973). "Selection of successive tumour lines for metastasis." Nat New Biol **242**(118): 148-149.

Fife, B. T. and J. A. Bluestone (2008). "Control of peripheral T cell tolerance and autoimmunity via the CTLA-4 and PD-1 pathways." Immunological reviews **224**: 166-182.

Fisher, R. I., S. A. Rosenberg, et al. (2000). "Long-term survival update for high-dose recombinant interleukin-2 in patients with renal cell carcinoma." Cancer J Sci Am **6 Suppl 1**: S55-57.

Floess, S., J. Freyer, et al. (2007). "Epigenetic control of the foxp3 locus in regulatory T cells." PLoS Biol **5**(2): e38.

Fontenot, J. D., M. A. Gavin, et al. (2003). "Foxp3 programs the development and function of CD4+CD25+ regulatory T cells." Nat Immunol **4**(4): 330-336.

Fontenot, J. D., J. P. Rasmussen, et al. (2005). "A function for interleukin 2 in Foxp3-expressing regulatory T cells." Nat Immunol **6**(11): 1142-1151.

Fontenot, J. D., J. P. Rasmussen, et al. (2005). "Regulatory T cell lineage specification by the forkhead transcription factor foxp3." Immunity **22**(3): 329-341.

Fraser, I. P., H. Koziel, et al. (1998). "The serum mannose-binding protein and the macrophage mannose receptor are pattern recognition molecules that link innate and adaptive immunity." Seminars in immunology **10**(5): 363-372.

Fugmann, S. D., A. I. Lee, et al. (2000). "The RAG proteins and V(D)J recombination: complexes, ends, and transposition." Annual review of immunology **18**: 495-527.

Fukuyama, T., Y. Ichiki, et al. (2007). "Cytokine production of lung cancer cell lines: Correlation between their production and the inflammatory/immunological responses both in vivo and in vitro." Cancer Sci **98**(7): 1048-1054.

Fyfe, G., R. I. Fisher, et al. (1995). "Results of treatment of 255 patients with metastatic renal cell carcinoma who received high-dose recombinant interleukin-2 therapy." J Clin Oncol **13**(3): 688-696.

Gansbacher, B., K. Zier, et al. (1992). "Retroviral gene transfer induced constitutive expression of interleukin-2 or interferon-gamma in irradiated human melanoma cells." Blood **80**(11): 2817-2825.

Gansbacher, B., K. Zier, et al. (1990). "Interleukin 2 gene transfer into tumor cells abrogates tumorigenicity and induces protective immunity." The Journal of experimental medicine **172**(4): 1217-1224.

Garcia, K. C. and E. J. Adams (2005). "How the T cell receptor sees antigen--a structural view." Cell **122**(3): 333-336.

Garin, M. I., C. C. Chu, et al. (2007). "Galectin-1: a key effector of regulation mediated by CD4+CD25+ T cells." Blood **109**(5): 2058-2065.

Gastl, G., C. L. Finstad, et al. (1992). "Retroviral vector-mediated lymphokine gene transfer into human renal cancer cells." Cancer research **52**(22): 6229-6236.

Gattinoni, L., C. A. Klebanoff, et al. (2005). "Acquisition of full effector function in vitro paradoxically impairs the in vivo antitumor efficacy of adoptively transferred CD8+ T cells." J Clin Invest **115**(6): 1616-1626.

Gattinoni, L., D. J. Powell, Jr., et al. (2006). "Adoptive immunotherapy for cancer: building on success." Nature reviews. Immunology **6**(5): 383-393.

Gaur, U. and B. B. Aggarwal (2003). "Regulation of proliferation, survival and apoptosis by members of the TNF superfamily." Biochemical pharmacology **66**(8): 1403-1408.

Gavin, M. A., S. R. Clarke, et al. (2002). "Homeostasis and anergy of CD4(+)CD25(+) suppressor T cells in vivo." Nat Immunol **3**(1): 33-41.

Gavin, M. A., J. P. Rasmussen, et al. (2007). "Foxp3-dependent programme of regulatory T cell differentiation." Nature **445**(7129): 771-775.

Ghiringhelli, F., P. E. Puig, et al. (2005). "Tumor cells convert immature myeloid dendritic cells into TGF-beta-secreting cells inducing CD4+CD25+ regulatory T cell proliferation." J Exp Med **202**(7): 919-929.

Gillis, S., M. M. Ferm, et al. (1978). "T cell growth factor: parameters of production and a quantitative microassay for activity." J Immunol **120**(6): 2027-2032.

Gordon, J. and L. D. MacLean (1965). "A lymphocyte-stimulating factor produced in vitro." Nature **208**(5012): 795-796.

Gorelik, L. and R. A. Flavell (2001). "Immune-mediated eradication of tumors through the blockade of transforming growth factor-beta signaling in T cells." Nature medicine **7**(10): 1118-1122.

Gorer, P. A. (1948). "The significance of studies with transplanted tumours." British journal of cancer **2**(2): 103-107.

Graff, R. J. and D. W. Bailey (1973). "The non-H-2 histocompatibility loci and their antigens." Transplantation reviews **15**: 26-49.

Granucci, F., C. Vizzardelli, et al. (2001). "Inducible IL-2 production by dendritic cells revealed by global gene expression analysis." Nat Immunol **2**(9): 882-888.

Gratama, J. W., R. J. Bruin, et al. (1993). "Activation of the immune system of cancer patients by continuous i.v. recombinant IL-2 (rIL-2) therapy is dependent on dose and schedule of rIL-2." Clin Exp Immunol **92**(2): 185-193.

Gray, P. W. and D. V. Goeddel (1982). "Structure of the human immune interferon gene." Nature **298**(5877): 859-863.

Greenfield, A., D. Scott, et al. (1996). "An H-YDb epitope is encoded by a novel mouse Y chromosome gene." Nat Genet **14**(4): 474-478.

Greenwald, R. J., V. A. Boussiotis, et al. (2001). "CTLA-4 regulates induction of anergy in vivo." Immunity **14**(2): 145-155.

Greenwald, R. J., G. J. Freeman, et al. (2005). "The B7 family revisited." Annual Review of Immunology **23**: 515-548.

Griffith, T. S., M. Kawakita, et al. (2001). "Inhibition of murine prostate tumor growth and activation of immunoregulatory cells with recombinant canarypox viruses." Journal of the National Cancer Institute **93**(13): 998-1007.

Grinyo, J. M., O. Bestard, et al. (2010). "Optimal immunosuppression to prevent chronic allograft dysfunction." Kidney international. Supplement(119): S66-70.

Grohmann, U., F. Fallarino, et al. (2003). "Tolerance, DCs and tryptophan: much ado about IDO." Trends in Immunology **24**(5): 242-248.

Guarini, A., L. Riera, et al. (1997). "Transfer of the interleukin-2 gene into human cancer cells induces specific antitumor recognition and restores the expression of CD3/T cell receptor associated signal transduction molecules." Blood **89**(1): 212-218.

Guarini, A., L. Riera, et al. (1996). "Human lung carcinoma cells engineered to release IL2, IL7, GM-CSF and TNF alpha." International journal of oncology **8**(4): 765-772.

Guirguis, L. M., J. C. Yang, et al. (2002). "Safety and efficacy of high-dose interleukin-2 therapy in patients with brain metastases." Journal of immunotherapy **25**(1): 82-87.

Guminski, A. D. and J. F. Thompson (2009). "Predicting response to IL-2 therapy for metastatic melanoma." Expert review of anticancer therapy **9**(11): 1571-1575.

Halak, B. K., H. C. Maguire, Jr., et al. (1999). "Tumor-induced interleukin-10 inhibits type 1 immune responses directed at a tumor antigen as well as a non-tumor antigen present at the tumor site." Cancer research **59**(4): 911-917.

Hale, G., T. Hoang, et al. (1983). "Removal of T cells from bone marrow for transplantation. Comparison of rat monoclonal anti-lymphocyte antibodies of different isotypes." Molecular biology & medicine **1**(3): 305-319.

Hambach, L. and E. Goulmy (2005). "Immunotherapy of cancer through targeting of minor histocompatibility antigens." Current opinion in immunology **17**(2): 202-210.

Hanahan, D. and R. A. Weinberg (2011). "Hallmarks of cancer: the next generation." Cell **144**(5): 646-674.

Hanson, H. L., D. L. Donermeyer, et al. (2000). "Eradication of established tumors by CD8+ T cell adoptive immunotherapy." Immunity **13**(2): 265-276.

Hara, I., H. Hotta, et al. (1996). "Rejection of mouse renal cell carcinoma elicited by local secretion of interleukin-2." Japanese journal of cancer research : Gann **87**(7): 724-729.

Harding, F. A., J. G. McArthur, et al. (1992). "CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T cell clones." Nature **356**(6370): 607-609.

Harman, B. C., E. J. Jenkinson, et al. (2003). "Microenvironmental regulation of Notch signalling in T cell development." Seminars in immunology **15**(2): 91-97.

Harty, J. T. and V. P. Badovinac (2008). "Shaping and reshaping CD8+ T cell memory." Nature reviews. Immunology **8**(2): 107-119.

Hattori, K., T. Hirano, et al. (1998). "Differential effects of anti-Fas ligand and anti-tumor necrosis factor alpha antibodies on acute graft-versus-host disease pathologies." Blood **91**(11): 4051-4055.

Hill, G. R., J. M. Crawford, et al. (1997). "Total body irradiation and acute graft-versus-host disease: The role of gastrointestinal damage and inflammatory cytokines." Blood **90**(8): 3204-3213.

Hill, G. R. and J. L. Ferrara (2000). "The primacy of the gastrointestinal tract as a target organ of acute graft-versus-host disease: rationale for the use of cytokine shields in allogeneic bone marrow transplantation." Blood **95**(9): 2754-2759.

Hinrichs, C. S., Z. A. Borman, et al. (2009). "Adoptively transferred effector cells derived from naive rather than central memory CD8+ T cells mediate superior antitumor immunity." Proc Natl Acad Sci U S A **106**(41): 17469-17474.

Hinrichs, C. S., L. Gattinoni, et al. (2006). "Programming CD8+ T cells for effective immunotherapy." Curr Opin Immunol **18**(3): 363-370.

Hinrichs, C. S., A. Kaiser, et al. (2009). "Type 17 CD8+ T cells display enhanced antitumor immunity." Blood **114**(3): 596-599.

Hinrichs, C. S., R. Spolski, et al. (2008). "IL-2 and IL-21 confer opposing differentiation programs to CD8+ T cells for adoptive immunotherapy." Blood **111**(11): 5326-5333.

Hoag, W. G. (1963). "Spontaneous Cancer in Mice." Ann N Y Acad Sci **108**: 805-831.

Hoption Cann, S. A., J. P. van Netten, et al. (2003). "Dr William Coley and tumour regression: a place in history or in the future." Postgraduate medical journal **79**(938): 672-680.

Hori, S., T. Nomura, et al. (2003). "Control of regulatory T cell development by the transcription factor Foxp3." Science **299**(5609): 1057-1061.

Horwitz, D. A., S. G. Zheng, et al. (2008). "Natural and TGF-beta-induced Foxp3(+)CD4(+) CD25(+) regulatory T cells are not mirror images of each other." Trends in immunology **29**(9): 429-435.

Hsieh, C. S., S. E. Macatonia, et al. (1995). "T cell genetic background determines default T helper phenotype development in vitro." J Exp Med **181**(2): 713-721.

Hunder, N. N., H. Wallen, et al. (2008). "Treatment of metastatic melanoma with autologous CD4+ T cells against NY-ESO-1." The New England journal of medicine **358**(25): 2698-2703.

Hung, K., R. Hayashi, et al. (1998). "The central role of CD4(+) T cells in the antitumor immune response." Journal of Experimental Medicine **188**(12): 2357-2368.

Hutloff, A., A. M. Dittrich, et al. (1999). "ICOS is an inducible T cell co-stimulator structurally and functionally related to CD28." Nature **397**(6716): 263-266.

Hwang, E. S., J. H. Hong, et al. (2005). "IL-2 production in developing Th1 cells is regulated by heterodimerization of RelA and T-bet and requires T-bet serine residue 508." J Exp Med **202**(9): 1289-1300.

Inaba, K., M. Inaba, et al. (1992). "Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor." The Journal of experimental medicine **176**(6): 1693-1702.

Iwadate, Y., A. Yamaura, et al. (2001). "Induction of immunity in peripheral tissues combined with intracerebral transplantation of interleukin-2-producing cells eliminates established brain tumors." Cancer research **61**(24): 8769-8774.

Iwai, Y., M. Ishida, et al. (2002). "Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade." Proc Natl Acad Sci U S A **99**(19): 12293-12297.

Iwai, Y., S. Terawaki, et al. (2005). "PD-1 blockade inhibits hematogenous spread of poorly immunogenic tumor cells by enhanced recruitment of effector T cells." Int Immunol **17**(2): 133-144.

Iwasaki, A. and R. Medzhitov (2004). "Toll-like receptor control of the adaptive immune responses." Nature Immunology **5**(10): 987-995.

Jain, J., C. Loh, et al. (1995). "Transcriptional regulation of the IL-2 gene." Curr Opin Immunol **7**(3): 333-342.

Jaksch, M. and J. Mattsson (2005). "The pathophysiology of acute graft-versus-host disease." Scandinavian Journal of Immunology **61**(5): 398-409.

James, E., D. Scott, et al. (2002). "HY peptides modulate transplantation responses to skin allografts." International immunology **14**(11): 1333-1342.

Janeway, C. (2005). Immunobiology : the immune system in health and disease. New York, Garland Science.

Janeway, C. A., Jr., J. Ron, et al. (1987). "The B cell is the initiating antigen-presenting cell in peripheral lymph nodes." Journal of immunology **138**(4): 1051-1055.

Janssen, E. M., E. E. Lemmens, et al. (2003). "CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes." Nature **421**(6925): 852-856.

Jemal, A., F. Bray, et al. (2011). "Global cancer statistics." CA: a cancer journal for clinicians **61**(2): 69-90.

Jiang, Z., E. Podack, et al. (2001). "Major histocompatibility complex-mismatched allogeneic bone marrow transplantation using perforin and/or Fas ligand double-defective CD4(+) donor T cells: involvement of cytotoxic function by donor lymphocytes prior to graft-versus-host disease pathogenesis." Blood **98**(2): 390-397.

Johnson, L. A., R. A. Morgan, et al. (2009). "Gene therapy with human and mouse T cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen." Blood **114**(3): 535-546.

Kaplan, D. H., B. E. Anderson, et al. (2004). "Target antigens determine graft-versus-host disease phenotype." Journal of immunology **173**(9): 5467-5475.

Karim, M., G. Feng, et al. (2005). "CD25+CD4+ regulatory T cells generated by exposure to a model protein antigen prevent allograft rejection: antigen-specific reactivation in vivo is critical for bystander regulation." Blood **105**(12): 4871-4877.

Kasakura, S. and L. Lowenstein (1965). "A factor stimulating DNA synthesis derived from the medium of leukocyte cultures." Nature **208**(5012): 794-795.

Kernan, N. A., N. H. Collins, et al. (1986). "Clonable T lymphocytes in T cell-depleted bone marrow transplants correlate with development of graft-v-host disease." Blood **68**(3): 770-773.

Kershaw, M. H., C. Hsu, et al. (2001). "Immunization against endogenous retroviral tumor-associated antigens." Cancer Res **61**(21): 7920-7924.

Khattar, M., W. Chen, et al. (2009). "Expanding and converting regulatory T cells: a horizon for immunotherapy." Arch Immunol Ther Exp (Warsz) **57**(3): 199-204.

Khattri, R., T. Cox, et al. (2003). "An essential role for Scurfin in CD4+CD25+ T regulatory cells." Nat Immunol **4**(4): 337-342.

Kianmanesh, A., N. R. Hackett, et al. (2001). "Intratumoral administration of low doses of an adenovirus vector encoding tumor necrosis factor alpha together with naive dendritic cells elicits significant suppression of tumor growth without toxicity." Human gene therapy **12**(17): 2035-2049.

Kim, C. J., J. Cormier, et al. (1998). "Use of recombinant poxviruses to stimulate anti-melanoma T cell reactivity." Annals of surgical oncology **5**(1): 64-76.

Kim, J. M., J. P. Rasmussen, et al. (2007). "Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice." Nat Immunol **8**(2): 191-197.

Kim, R., M. Emi, et al. (2006). "Tumor-driven evolution of immunosuppressive networks during malignant progression." Cancer Res **66**(11): 5527-5536.

Kinsella, T. M. and G. P. Nolan (1996). "Episomal vectors rapidly and stably produce high-titer recombinant retrovirus." Human gene therapy **7**(12): 1405-1413.

Kioi, M., T. Shimamura, et al. (2009). "IL-13 cytotoxin has potent antitumor activity and synergizes with paclitaxel in a mouse model of oral squamous cell carcinoma." International journal of cancer. Journal international du cancer **124**(6): 1440-1448.

Kitamura, H., C. Sedlik, et al. (2010). "Long Peptide Vaccination Can Lead to Lethality through CD4(+) T Cell-Mediated Cytokine Storm." Journal of Immunology **185**(2): 892-901.

Kitoh, A., M. Ono, et al. (2009). "Indispensable role of the Runx1-Cbfbeta transcription complex for in vivo-suppressive function of FoxP3+ regulatory T cells." Immunity **31**(4): 609-620.

Klebanoff, C. A., L. Gattinoni, et al. (2006). "CD8+ T cell memory in tumor immunology and immunotherapy." Immunol Rev **211**: 214-224.

Klebanoff, C. A., L. Gattinoni, et al. (2005). "Central memory self/tumor-reactive CD8+ T cells confer superior antitumor immunity compared with effector memory T cells." Proc Natl Acad Sci U S A **102**(27): 9571-9576.

Klebb, G., I. B. Autenrieth, et al. (1996). "Interleukin-2 is indispensable for development of immunological self-tolerance." Clin Immunol Immunopathol **81**(3): 282-286.

Klunker, S., M. M. Chong, et al. (2009). "Transcription factors RUNX1 and RUNX3 in the induction and suppressive function of Foxp3+ inducible regulatory T cells." J Exp Med **206**(12): 2701-2715.

Ko, K., S. Yamazaki, et al. (2005). "Treatment of advanced tumors with agonistic anti-GITR mAb and its effects on tumor-infiltrating Foxp3+CD25+CD4+ regulatory T cells." The Journal of experimental medicine **202**(7): 885-891.

Kochenderfer, J. N., W. H. Wilson, et al. (2010). "Eradication of B-lineage cells and regression of lymphoma in a patient treated with autologous T cells genetically engineered to recognize CD19." Blood **116**(20): 4099-4102.

Kohm, A. P., J. S. McMahon, et al. (2006). "Cutting Edge: Anti-CD25 monoclonal antibody injection results in the functional inactivation, not depletion, of CD4+CD25+ T regulatory cells." J Immunol **176**(6): 3301-3305.

Koizumi, K., S. Hojo, et al. (2007). "Chemokine receptors in cancer metastasis and cancer cell-derived chemokines in host immune response." Cancer science **98**(11): 1652-1658.

Komatsu, N., M. E. Mariotti-Ferrandiz, et al. (2009). "Heterogeneity of natural Foxp3+ T cells: a committed regulatory T cell lineage and an uncommitted minor population retaining plasticity." Proc Natl Acad Sci U S A **106**(6): 1903-1908.

Korn, T., E. Bettelli, et al. (2009). "IL-17 and Th17 Cells." Annual review of immunology **27**: 485-517.

Korngold, R. and J. Sprent (1987). "T cell subsets and graft-versus-host disease." Transplantation **44**(3): 335-339.

Krensky, A. M., A. Weiss, et al. (1990). "T-lymphocyte-antigen interactions in transplant rejection." The New England journal of medicine **322**(8): 510-517.

Kretschmer, K., I. Apostolou, et al. (2005). "Inducing and expanding regulatory T cell populations by foreign antigen." Nature immunology **6**(12): 1219-1227.

Krueger, G. G., C. M. Jorgensen, et al. (1999). "Persistent transgene expression and normal differentiation of immortalized human keratinocytes in vivo." The Journal of investigative dermatology **112**(2): 233-239.

Kryczek, I., S. Wei, et al. (2007). "Relationship between B7-H4, regulatory T cells, and patient outcome in human ovarian carcinoma." Cancer research **67**(18): 8900-8905.

Kryczek, I., S. Wei, et al. (2007). "Cutting edge: Th17 and regulatory T cell dynamics and the regulation by IL-2 in the tumor microenvironment." J Immunol **178**(11): 6730-6733.

Kuhn, R., J. Lohler, et al. (1993). "Interleukin-10-Deficient Mice Develop Chronic Enterocolitis." Cell **75**(2): 263-274.

Kwon, B., K. Y. Yu, et al. (1999). "Identification of a novel activation-inducible protein of the tumor necrosis factor receptor superfamily and its ligand." Faseb Journal **13**(5): A948-A948.

Kyewski, B. and L. Klein (2006). "A central role for central tolerance." Annual review of immunology **24**: 571-606.

Lal, G., N. Zhang, et al. (2009). "Epigenetic regulation of Foxp3 expression in regulatory T cells by DNA methylation." J Immunol **182**(1): 259-273.

Lanier, L. L., S. O'Fallon, et al. (1995). "CD80 (B7) and CD86 (B70) provide similar costimulatory signals for T cell proliferation, cytokine production, and generation of CTL." J Immunol **154**(1): 97-105.

Lantz, O., I. Grandjean, et al. (2000). "Gamma chain required for naive CD4+ T cell survival but not for antigen proliferation." Nat Immunol **1**(1): 54-58.

Lawlor, D. A., J. Zemmour, et al. (1990). "Evolution of class-I MHC genes and proteins: from natural selection to thymic selection." Annual review of immunology **8**: 23-63.

Lechler, R., W. F. Ng, et al. (2001). "Dendritic cells in transplantation - Friend or foe?" Immunity **14**(4): 357-368.

Lecoeur, H. (2002). "Nuclear apoptosis detection by flow cytometry: influence of endogenous endonucleases." Experimental cell research **277**(1): 1-14.

Lee, K. H., E. Wang, et al. (1999). "Increased vaccine-specific T cell frequency after peptide-based vaccination correlates with increased susceptibility to in vitro stimulation but does not lead to tumor regression." Journal of immunology **163**(11): 6292-6300.

Leung, D. T., S. Morefield, et al. (2000). "Regulation of lymphoid homeostasis by IL-2 receptor signals in vivo." J Immunol **164**(7): 3527-3534.

Levings, M. K., R. Sangregorio, et al. (2002). "Human CD25+CD4+ T suppressor cell clones produce transforming growth factor beta, but not interleukin 10, and are distinct from type 1 T regulatory cells." J Exp Med **196**(10): 1335-1346.

Li, M. O., S. Sanjabi, et al. (2006). "Transforming growth factor-beta controls development, homeostasis, and tolerance of T cells by regulatory T cell-dependent and -independent mechanisms." Immunity **25**(3): 455-471.

Li, Y., M. Bleakley, et al. (2005). "IL-21 influences the frequency, phenotype, and affinity of the antigen-specific CD8 T cell response." Journal of immunology **175**(4): 2261-2269.

Lichtor, T., R. P. Glick, et al. (2002). "Application of interleukin-2-secreting syngeneic/allogeneic fibroblasts in the treatment of primary and metastatic brain tumors." Cancer gene therapy **9**(5): 464-469.

Lin, M., B. Storer, et al. (2003). "Relation of an interleukin-10 promoter polymorphism to graft-versus-host disease and survival after hematopoietic cell transplantation." New England Journal of Medicine **349**(23): 2201-2210.

Liu, E. H., R. M. Siegel, et al. (2007). "T cell-directed therapies: lessons learned and future prospects." Nature Immunology **8**(1): 25-30.

Liyanage, U. K., T. T. Moore, et al. (2002). "Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma." Journal of immunology **169**(5): 2756-2761.

Lohr, J., B. Knoechel, et al. (2005). "T cell tolerance and autoimmunity to systemic and tissue-restricted self-antigens." Immunological reviews **204**: 116-127.

Loskog, A., C. Ninalga, et al. (2005). "Optimization of the MB49 mouse bladder cancer model for adenoviral gene therapy." Laboratory animals **39**(4): 384-393.

Lowenthal, J. W., P. Corthesy, et al. (1985). "High and low affinity IL 2 receptors: analysis by IL 2 dissociation rate and reactivity with monoclonal anti-receptor antibody PC61." J Immunol **135**(6): 3988-3994.

Lu, L. F., M. A. Gavin, et al. (2007). "G protein-coupled receptor 83 is dispensable for the development and function of regulatory T cells." Mol Cell Biol **27**(23): 8065-8072.

Luboshits, G., S. Shina, et al. (1999). "Elevated expression of the CC chemokine regulated on activation, normal T cell expressed and secreted (RANTES) in advanced breast carcinoma." Cancer research **59**(18): 4681-4687.

Macatonia, S. E., N. A. Hosken, et al. (1995). "Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells." Journal of immunology **154**(10): 5071-5079.

Medzhitov, R. and C. A. Janeway, Jr. (1997). "Innate immunity: the virtues of a nonclonal system of recognition." Cell **91**(3): 295-298.

Melchionda, F., M. K. McKirdy, et al. (2004). "Escape from immune surveillance does not result in tolerance to tumor-associated antigens." Journal of Immunotherapy **27**(5): 329-338.

Mellor, A. L. and D. H. Munn (2004). "IDO expression by dendritic cells: tolerance and tryptophan catabolism." Nature reviews. Immunology **4**(10): 762-774.

Menning, A., U. E. Hopken, et al. (2007). "Distinctive role of CCR7 in migration and functional activity of naive- and effector/memory-like Treg subsets." Eur J Immunol **37**(6): 1575-1583.

Meunier, M. C., J. S. Delisle, et al. (2005). "T cells targeted against a single minor histocompatibility antigen can cure solid tumors." Nature medicine **11**(11): 1222-1229.

Milella, M., J. Jacobelli, et al. (1999). "Interleukin-2 gene transfer into human transitional cell carcinoma of the urinary bladder." British journal of cancer **79**(5-6): 770-779.

Miller, A. D., D. G. Miller, et al. (1993). "Use of retroviral vectors for gene transfer and expression." Methods in enzymology **217**: 581-599.

Millrain, M., P. Chandler, et al. (2001). "Examination of HY response: T cell expansion, immunodominance, and cross-priming revealed by HY tetramer analysis." Journal of immunology **167**(7): 3756-3764.

Mitchison, N. A. (1955). "Studies on the immunological response to foreign tumor transplants in the mouse. I. The role of lymph node cells in conferring immunity by adoptive transfer." The Journal of experimental medicine **102**(2): 157-177.

Mizoguchi, H., J. J. O'Shea, et al. (1992). "Alterations in signal transduction molecules in T lymphocytes from tumor-bearing mice." Science **258**(5089): 1795-1798.

Morgan, D. A., F. W. Ruscetti, et al. (1976). "Selective in vitro growth of T lymphocytes from normal human bone marrows." Science **193**(4257): 1007-1008.

Morgan, R. A., M. E. Dudley, et al. (2010). "Adoptive cell therapy: genetic modification to redirect effector cell specificity." Cancer J **16**(4): 336-341.

Moroz, A., C. Eppolito, et al. (2004). "IL-21 enhances and sustains CD8+ T cell responses to achieve durable tumor immunity: comparative evaluation of IL-2, IL-15, and IL-21." Journal of immunology **173**(2): 900-909.

Morris, A. G. and P. T. Tomkins (1989). "Interactions of interferons in the induction of histocompatibility antigens in mouse fibroblasts and glial cells." Immunology **67**(4): 537-539.

Mortarini, R., A. Piris, et al. (2003). "Lack of terminally differentiated tumor-specific CD8+ T cells at tumor site in spite of antitumor immunity to self-antigens in human metastatic melanoma." Cancer research **63**(10): 2535-2545.

Mosmann, T. R. and S. Sad (1996). "The expanding universe of T cell subsets: Th1, Th2 and more." Immunology today **17**(3): 138-146.

Mumberg, D., P. A. Monach, et al. (1999). "CD4(+) T cells eliminate MHC class II-negative cancer cells in vivo by indirect effects of IFN-gamma." Proceedings of the National Academy of Sciences of the United States of America **96**(15): 8633-8638.

Munn, D. H. and A. L. Mellor (2007). "Indoleamine 2,3-dioxygenase and tumor-induced tolerance." The Journal of clinical investigation **117**(5): 1147-1154.

Munn, D. H., M. D. Sharma, et al. (2004). "Expression of indoleamine 2,3-dioxygenase by plasmacytoid dendritic cells in tumor-draining lymph nodes." The Journal of clinical investigation **114**(2): 280-290.

Murai, M., H. Yoneyama, et al. (2003). "Peyer's patch is the essential site in initiating murine acute and lethal graft-versus-host reaction." Nature Immunology **4**(2): 154-160.

Muranski, P., A. Boni, et al. (2008). "Tumor-specific Th17-polarized cells eradicate large established melanoma." Blood **112**(2): 362-373.

Murphy, A., J. A. Westwood, et al. (2005). "Gene modification strategies to induce tumor immunity." Immunity **22**(4): 403-414.

Nakae, S., Y. Komiyama, et al. (2002). "Antigen-specific T cell sensitization is impaired in IL-17-deficient mice, causing suppression of allergic cellular and humoral responses." Immunity **17**(3): 375-387.

Nakagawa, T. Y. and A. Y. Rudensky (1999). "The role of lysosomal proteinases in MHC class II-mediated antigen processing and presentation." Immunological reviews **172**: 121-129.

Nishikawa, H., E. Jager, et al. (2005). "CD4+ CD25+ regulatory T cells control the induction of antigen-specific CD4+ helper T cell responses in cancer patients." Blood **106**(3): 1008-1011.

Nishikawa, H., T. Kato, et al. (2005). "IFN-gamma controls the generation/activation of CD4+ CD25+ regulatory T cells in antitumor immune response." J Immunol **175**(7): 4433-4440.

O'Farrell, A. M., Y. Liu, et al. (1998). "IL-10 inhibits macrophage activation and proliferation by distinct signaling mechanisms: evidence for Stat3-dependent and -independent pathways." EMBO J **17**(4): 1006-1018.

Okada, R., T. Kondo, et al. (2008). "Phenotypic classification of human CD4+ T cell subsets and their differentiation." International immunology **20**(9): 1189-1199.

Okazaki, T. and T. Honjo (2007). "PD-1 and PD-1 ligands: from discovery to clinical application." International immunology **19**(7): 813-824.

Onizuka, S., I. Tawara, et al. (1999). "Tumor rejection by in vivo administration of anti-CD25 (interleukin-2 receptor alpha) monoclonal antibody." Cancer research **59**(13): 3128-3133.

Overwijk, W. W. (2005). "Breaking tolerance in cancer immunotherapy: time to ACT." Curr Opin Immunol **17**(2): 187-194.

Overwijk, W. W., M. R. Theoret, et al. (2003). "Tumor regression and autoimmunity after reversal of a functionally tolerant state of self-reactive CD8+ T cells." J Exp Med **198**(4): 569-580.

Pantuck, A. J. and A. S. Belldegrun (2001). "Phase I clinical trial of interleukin 2 (IL-2) gene therapy for prostate cancer." Current urology reports **2**(1): 33.

Paris, F., Z. Fuks, et al. (2001). "Endothelial apoptosis as the primary lesion initiating intestinal radiation damage in mice." Science **293**(5528): 293-297.

Parker, D. C. (1993). "T cell-dependent B cell activation." Annual review of immunology **11**: 331-360.

Parkinson, D. R., J. S. Abrams, et al. (1990). "Interleukin-2 therapy in patients with metastatic malignant melanoma: a phase II study." Journal of clinical oncology : official journal of the American Society of Clinical Oncology **8**(10): 1650-1656.

Perez-Diez, A., N. T. Joncker, et al. (2007). "CD4 cells can be more efficient at tumor rejection than CD8 cells." Blood **109**(12): 5346-5354.

Perez-Diez, A. and F. M. Marincola (2002). "Immunotherapy against antigenic tumors: a game with a lot of players." Cell Mol Life Sci **59**(2): 230-240.

Perez-Diez, A., A. Martinez-Crespo, et al. (1996). "Immunomodulation induced by synthetic peptides derived from tumor-rejection antigens." Int J Dev Biol **Suppl 1**: 311S.

Perez, V. L., L. Van Parijs, et al. (1997). "Induction of peripheral T cell tolerance in vivo requires CTLA-4 engagement." Immunity **6**(4): 411-417.

Petersdorf, E. W. and M. Malkki (2006). "Genetics of risk factors for graft-versus-host disease." Seminars in hematology **43**(1): 11-23.

Piguet, P. F., G. E. Grau, et al. (1987). "Tumor-Necrosis-Factor Cachectin Is an Effector of Skin and Gut Lesions of the Acute Phase of Graft-Vs-Host Disease." Journal of Experimental Medicine **166**(5): 1280-1289.

Poeschla, E. M., F. Wong-Staal, et al. (1998). "Efficient transduction of nondividing human cells by feline immunodeficiency virus lentiviral vectors." Nature medicine **4**(3): 354-357.

Potts, W. K. and E. K. Wakeland (1990). "Evolution of diversity at the major histocompatibility complex." Trends in ecology & evolution **5**(6): 181-187.

Powell, S. and A. Z. Dudek (2009). "Single-institution outcome of high-dose interleukin-2 (HD IL-2) therapy for metastatic melanoma and analysis of favorable response in brain metastases." Anticancer research **29**(10): 4189-4193.

Probst, H. C., K. McCoy, et al. (2005). "Resting dendritic cells induce peripheral CD8+ T cell tolerance through PD-1 and CTLA-4." Nat Immunol **6**(3): 280-286.

Reddy, P. and J. L. Ferrara (2003). "Immunobiology of acute graft-versus-host disease." Blood reviews **17**(4): 187-194.

Reddy, P., R. Negrin, et al. (2008). "Mouse models of bone marrow transplantation." Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation **14**(1 Suppl 1): 129-135.

Reed, S. G. (1999). "TGF-beta in infections and infectious diseases." Microbes and infection / Institut Pasteur **1**(15): 1313-1325.

Rees, R. C., S. McArdle, et al. (2002). "Disabled infectious single cycle-herpes simplex virus (DISC-HSV) as a vector for immunogene therapy of cancer." Current opinion in molecular therapeutics **4**(1): 49-53.

Reichert, J. M. and V. E. Valge-Archer (2007). "Development trends for monoclonal antibody cancer therapeutics." Nature reviews. Drug discovery **6**(5): 349-356.

Rivas, M. N., K. Weatherly, et al. (2009). "Reviving Function in CD4(+) T Cells Adapted to Persistent Systemic Antigen." Journal of Immunology **183**(7): 4284-4291.

Robb, R. J., A. Munck, et al. (1981). "T cell growth factor receptors. Quantitation, specificity, and biological relevance." J Exp Med **154**(5): 1455-1474.

Robb, R. J. and K. A. Smith (1981). "Heterogeneity of human T cell growth factor(s) due to variable glycosylation." Mol Immunol **18**(12): 1087-1094.

Robertson, N. J., J. G. Chai, et al. (2007). "Natural regulation of immunity to minor histocompatibility antigens." Journal of immunology **178**(6): 3558-3565.

Roopenian, D. C. (1992). "What are minor histocompatibility loci? A new look at an old question." Immunology today **13**(1): 7-10.

Rosenberg, S. A. (1988). "Development of new immunologic approaches to cancer therapy." Blood Purif **6**(2): 69-76.

Rosenberg, S. A. (1999). "A new era for cancer immunotherapy based on the genes that encode cancer antigens." Immunity **10**(3): 281-287.

Rosenberg, S. A., W. F. Anderson, et al. (1993). "The development of gene therapy for the treatment of cancer." Ann Surg **218**(4): 455-463; discussion 463-454.

Rosenberg, S. A. and M. T. Lotze (1986). "Cancer immunotherapy using interleukin-2 and interleukin-2-activated lymphocytes." Annu Rev Immunol **4**: 681-709.

Rosenberg, S. A., J. J. Mule, et al. (1985). "Regression of established pulmonary metastases and subcutaneous tumor mediated by the systemic administration of high-dose recombinant interleukin 2." The Journal of experimental medicine **161**(5): 1169-1188.

Rosenberg, S. A., B. S. Packard, et al. (1988). "Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. A preliminary report." The New England journal of medicine **319**(25): 1676-1680.

Rosenberg, S. A., J. C. Yang, et al. (1994). "Treatment of 283 consecutive patients with metastatic melanoma or renal cell cancer using high-dose bolus interleukin 2." JAMA **271**(12): 907-913.

Rotzschke, O., K. Falk, et al. (1990). "Characterization of naturally occurring minor histocompatibility peptides including H-4 and H-Y." Science **249**(4966): 283-287.

Rouvier, E., M. F. Luciani, et al. (1993). "CTLA-8, cloned from an activated T cell, bearing AU-rich messenger RNA instability sequences, and homologous to a herpesvirus saimiri gene." Journal of immunology **150**(12): 5445-5456.

Rubtsov, Y. P., R. E. Niec, et al. (2010). "Stability of the regulatory T cell lineage in vivo." Science **329**(5999): 1667-1671.

Rudd, C. E., A. Taylor, et al. (2009). "CD28 and CTLA-4 coreceptor expression and signal transduction." Immunological reviews **229**(1): 12-26.

Russell, E. S., E. F. Neufeld, et al. (1951). "Comparison of normal blood picture of young adults from 18 inbred strains of mice." Proc Soc Exp Biol Med **78**(3): 761-766.

Sad, S., R. Marcotte, et al. (1995). "Cytokine-induced differentiation of precursor mouse CD8+ T cells into cytotoxic CD8+ T cells secreting Th1 or Th2 cytokines." Immunity **2**(3): 271-279.

Sadlack, B., J. Lohler, et al. (1995). "Generalized autoimmune disease in interleukin-2-deficient mice is triggered by an uncontrolled activation and proliferation of CD4+ T cells." Eur J Immunol **25**(11): 3053-3059.

Sakaguchi, S., M. Ono, et al. (2006). "Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease." Immunological reviews **212**: 8-27.

Sakaguchi, S., N. Sakaguchi, et al. (1995). "Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases." J Immunol **155**(3): 1151-1164.

Sallusto, F., C. R. Mackay, et al. (2000). "The role of chemokine receptors in primary, effector, and memory immune responses." Annual Review of Immunology **18**: 593-+.

Savelkoul, H. F., B. W. Seymour, et al. (1991). "IL-4 can correct defective IgE production in SJA/9 mice." J Immunol **146**(6): 1801-1805.

Schluns, K. S., W. C. Kieper, et al. (2000). "Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo." Nature immunology **1**(5): 426-432.

Schluns, K. S., K. Williams, et al. (2002). "Cutting edge: requirement for IL-15 in the generation of primary and memory antigen-specific CD8 T cells." Journal of immunology **168**(10): 4827-4831.

Schroder, K., P. J. Hertzog, et al. (2004). "Interferon-gamma: an overview of signals, mechanisms and functions." Journal of leukocyte biology **75**(2): 163-189.

Scott, D., C. Addey, et al. (2000). "Dendritic cells permit identification of genes encoding MHC class II-restricted epitopes of transplantation antigens." Immunity **12**(6): 711-720.

Scott, D. M., I. E. Ehrmann, et al. (1995). "Identification of a mouse male-specific transplantation antigen, H-Y." Nature **376**(6542): 695-698.

Serody, J. S., S. E. Burkett, et al. (2000). "T-lymphocyte production of macrophage inflammatory protein-1 alpha is critical to the recruitment of CD8(+) T cells to the liver, lung, and spleen during graft-versus-host disease." Blood **96**(9): 2973-2980.

Sharma, M. D., D. Y. Hou, et al. (2010). "Reprogrammed foxp3(+) regulatory T cells provide essential help to support cross-presentation and CD8(+) T cell priming in naive mice." Immunity **33**(6): 942-954.

Sharma, M. D., D. Y. Hou, et al. (2009). "Indoleamine 2,3-dioxygenase controls conversion of Foxp3+ Tregs to TH17-like cells in tumor-draining lymph nodes." Blood **113**(24): 6102-6111.

Sharma, S., M. Stolina, et al. (1999). "T cell-derived IL-10 promotes lung cancer growth by suppressing both T cell and APC function." Journal of immunology **163**(9): 5020-5028.

Shimizu, J., S. Yamazaki, et al. (1999). "Induction of tumor immunity by removing CD25+CD4+ T cells: a common basis between tumor immunity and autoimmunity." J Immunol **163**(10): 5211-5218.

Shimizu, J., S. Yamazaki, et al. (2002). "Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance." Nat Immunol **3**(2): 135-142.

Shinkai, Y., G. Rathbun, et al. (1992). "RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement." Cell **68**(5): 855-867.

Shlomchik, W. D. (2003). "Antigen presentation in graft-vs-host disease." Experimental Hematology **31**(12): 1187-1197.

Shortman, K. and Y. J. Liu (2002). "Mouse and human dendritic cell subtypes." Nature reviews. Immunology **2**(3): 151-161.

Shortman, K., D. Vremec, et al. (1991). "The kinetics of T cell antigen receptor expression by subgroups of CD4+8+ thymocytes: delineation of CD4+8+3(2+)

thymocytes as post-selection intermediates leading to mature T cells." The Journal of experimental medicine **173**(2): 323-332.

Shurin, M. R. (1996). "Dendritic cells presenting tumor antigen." Cancer immunology, immunotherapy : CII **43**(3): 158-164.

Sica, G. L., I. H. Choi, et al. (2003). "B7-H4, a molecule of the B7 family, negatively regulates T cell immunity." Immunity **18**(6): 849-861.

Siegel, J. P. and R. K. Puri (1991). "Interleukin-2 toxicity." Journal of clinical oncology : official journal of the American Society of Clinical Oncology **9**(4): 694-704.

Sillanpaa, S., M. A. Anttila, et al. (2003). "CD44 expression indicates favorable prognosis in epithelial ovarian cancer." Clinical cancer research : an official journal of the American Association for Cancer Research **9**(14): 5318-5324.

Simonsen, M. (1965). "Graft versus host reactions and their possible implications in man." Bibliotheca haematologica **23**: 115-121.

Simpson, E., D. Scott, et al. (1997). "The male-specific histocompatibility antigen, H-Y: a history of transplantation, immune response genes, sex determination and expression cloning." Annu Rev Immunol **15**: 39-61.

Simpson, E., D. Scott, et al. (2001). "Minor H antigens: genes and peptides." European journal of immunogenetics : official journal of the British Society for Histocompatibility and Immunogenetics **28**(5): 505-513.

Simpson, W. G., S. D. Heys, et al. (1995). "Acute phase proteins and recombinant IL-2 therapy: prediction of response and survival in patients with colorectal cancer." Clin Exp Immunol **99**(2): 143-147.

Singh, N. J. and R. H. Schwartz (2003). "The strength of persistent antigenic stimulation modulates adaptive tolerance in peripheral CD4+ T cells." The Journal of experimental medicine **198**(7): 1107-1117.

Small, E. J., N. S. Tchekmedyian, et al. (2007). "A pilot trial of CTLA-4 blockade with human anti-CTLA-4 in patients with hormone-refractory prostate cancer." Clinical cancer research : an official journal of the American Association for Cancer Research **13**(6): 1810-1815.

Smit, M. J., P. Verdijk, et al. (2003). "CXCR3-mediated chemotaxis of human T cells is regulated by a Gi- and phospholipase C-dependent pathway and not via activation of MEK/p44/p42 MAPK nor Akt/PI-3 kinase." Blood **102**(6): 1959-1965.

Smith, K. A. (1988). "Interleukin-2: inception, impact, and implications." Science **240**(4856): 1169-1176.

Smith, K. A., M. F. Favata, et al. (1983). "Production and characterization of monoclonal antibodies to human interleukin 2: strategy and tactics." J Immunol **131**(4): 1808-1815.

Smith, K. A., K. J. Gilbride, et al. (1980). "Lymphocyte activating factor promotes T cell growth factor production by cloned murine lymphoma cells." Nature **287**(5785): 853-855.

Smith, K. A., L. B. Lachman, et al. (1980). "The functional relationship of the interleukins." J Exp Med **151**(6): 1551-1556.

Spierings, E. (2008). "Minor histocompatibility antigens: targets for tumour therapy and transplant tolerance." Int J Immunogenet **35**(4-5): 363-366.

Spierings, E., B. Wieles, et al. (2004). "Minor histocompatibility antigens--big in tumour therapy." Trends Immunol **25**(2): 56-60.

Spitalny, G. L. and E. A. Havell (1984). "Monoclonal antibody to murine gamma interferon inhibits lymphokine-induced antiviral and macrophage tumoricidal activities." The Journal of experimental medicine **159**(5): 1560-1565.

Stein, U., W. Walther, et al. (1996). "Reversal of multidrug resistance by transduction of cytokine genes into human colon carcinoma cells." Journal of the National Cancer Institute **88**(19): 1383-1392.

Steinman, R. M. and Z. A. Cohn (1973). "Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution." The Journal of experimental medicine **137**(5): 1142-1162.

Stern, J. B. and K. A. Smith (1986). "Interleukin-2 induction of T cell G1 progression and c-myb expression." Science **233**(4760): 203-206.

Su, H. C., L. P. Cousens, et al. (1998). "CD4+ and CD8+ T cell interactions in IFN-gamma and IL-4 responses to viral infections: requirements for IL-2." J Immunol **160**(10): 5007-5017.

Sugimoto, N., T. Oida, et al. (2006). "Foxp3-dependent and -independent molecules specific for CD25+CD4+ natural regulatory T cells revealed by DNA microarray analysis." Int Immunol **18**(8): 1197-1209.

Summerhayes, I. C. and L. M. Franks (1979). "Effects of donor age on neoplastic transformation of adult mouse bladder epithelium in vitro." J Natl Cancer Inst **62**(4): 1017-1023.

Sun, J., Y. Zhang, et al. (2010). "Hypoxia induces T cell apoptosis by inhibiting chemokine C receptor 7 expression: the role of adenosine receptor A(2)." Cellular & molecular immunology **7**(1): 77-82.

Suri-Payer, E. and B. Fritzsching (2006). "Regulatory T cells in experimental autoimmune disease." Springer Semin Immunopathol **28**(1): 3-16.

Surman, D. R., M. E. Dudley, et al. (2000). "Cutting edge: CD4+ T cell control of CD8+ T cell reactivity to a model tumor antigen." J Immunol **164**(2): 562-565.

Szabo, S. J., S. T. Kim, et al. (2000). "A novel transcription factor, T-bet, directs Th1 lineage commitment." Cell **100**(6): 655-669.

Takahashi, A., K. Kono, et al. (2001). "Elevated caspase-3 activity in peripheral blood T cells coexists with increased degree of T cell apoptosis and down-regulation of TCR zeta molecules in patients with gastric cancer." Clin Cancer Res **7**(1): 74-80.

Tan, Y., M. Xu, et al. (1996). "IL-2 gene therapy of advanced lung cancer patients." Anticancer research **16**(4A): 1993-1998.

Tanaka, H., J. Tanaka, et al. (2002). "Depletion of CD4+ CD25+ regulatory cells augments the generation of specific immune T cells in tumor-draining lymph nodes." J Immunother **25**(3): 207-217.

Tanchot, C., D. L. Barber, et al. (2001). "Adaptive tolerance of CD4+ T cells in vivo: multiple thresholds in response to a constant level of antigen presentation." Journal of immunology **167**(4): 2030-2039.

Tang, Q., J. Y. Adams, et al. (2008). "Central role of defective interleukin-2 production in the triggering of islet autoimmune destruction." Immunity **28**(5): 687-697.

Taniguchi, T., H. Matsui, et al. (1983). "Structure and expression of a cloned cDNA for human interleukin-2." Nature **302**(5906): 305-310.

Tawara, I., W. D. Shlomchik, et al. (2010). "A crucial role for host APCs in the induction of donor CD4+CD25+ regulatory T cell-mediated suppression of experimental graft-versus-host disease." J Immunol **185**(7): 3866-3872.

Teshima, T., R. Ordemann, et al. (2002). "Acute graft-versus-host disease does not require alloantigen expression on host epithelium." Nature medicine **8**(6): 575-581.

Thornton, A. M., E. E. Donovan, et al. (2004). "Cutting edge: IL-2 is critically required for the in vitro activation of CD4+CD25+ T cell suppressor function." J Immunol **172**(11): 6519-6523.

Thornton, A. M., P. E. Korty, et al. (2010). "Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells." J Immunol **184**(7): 3433-3441.

Till, B. G., M. C. Jensen, et al. (2008). "Adoptive immunotherapy for indolent non-Hodgkin lymphoma and mantle cell lymphoma using genetically modified autologous CD20-specific T cells." Blood **112**(6): 2261-2271.

Tivol, E. A., F. Borriello, et al. (1995). "Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4." Immunity **3**(5): 541-547.

Townsend, S. E., F. W. Su, et al. (1994). "Specificity and longevity of antitumor immune responses induced by B7-transfected tumors." Cancer Res **54**(24): 6477-6483.

Tsuji, M., N. Komatsu, et al. (2009). "Preferential generation of follicular B helper T cells from Foxp3+ T cells in gut Peyer's patches." Science **323**(5920): 1488-1492.

Turk, M. J., J. A. Guevara-Patino, et al. (2004). "Concomitant tumor immunity to a poorly immunogenic melanoma is prevented by regulatory T cells." J Exp Med **200**(6): 771-782.

Uberti, J. P., L. Ayash, et al. (2005). "Pilot trial on the use of etanercept and methylprednisolone as primary treatment for acute graft-versus-host disease." Biology of Blood and Marrow Transplantation **11**(9): 680-687.

Valujskikh, A., W. M. Baldwin, 3rd, et al. (2010). "Recent progress and new perspectives in studying T cell responses to allografts." Am J Transplant **10**(5): 1117-1125.

Valujskikh, A., O. Lantz, et al. (2002). "Cross-primed CD8(+) T cells mediate graft rejection via a distinct effector pathway." Nat Immunol **3**(9): 844-851.

Valujskikh, A., D. Matesic, et al. (1999). "Characterization and manipulation of T cell immunity to skin grafts expressing a transgenic minor antigen." Transplantation **68**(7): 1029-1036.

van den Brink, M. R. M. and S. J. Burakoff (2002). "Cytolytic pathways in haematopoietic stem-cell transplantation." Nature Reviews Immunology **2**(4): 273-281.

VanderVegt, F. P. and L. L. Johnson (1993). "Induction of long-term H-Y-specific tolerance in female mice given male lymphoid cells while transiently depleted of CD4+ or CD8+ T cells." The Journal of experimental medicine **177**(6): 1587-1592.

Vendetti, S., T. S. Davidson, et al. (2010). "Polyclonal Treg cells enhance the activity of a mucosal adjuvant." Immunol Cell Biol **88**(7): 698-706.

Verginis, P., K. A. McLaughlin, et al. (2008). "Induction of antigen-specific regulatory T cells in wild-type mice: visualization and targets of suppression." Proc Natl Acad Sci U S A **105**(9): 3479-3484.

Vieira, P. L., J. R. Christensen, et al. (2004). "IL-10-secreting regulatory T cells do not express Foxp3 but have comparable regulatory function to naturally occurring CD4+CD25+ regulatory T cells." J Immunol **172**(10): 5986-5993.

Villarino, A. V., C. M. Tato, et al. (2007). "Helper T cell IL-2 production is limited by negative feedback and STAT-dependent cytokine signals." J Exp Med **204**(1): 65-71.

Vitale, M., G. Pelusi, et al. (2005). "HLA class I antigen down-regulation in primary ovary carcinoma lesions: association with disease stage." Clinical cancer research : an official journal of the American Association for Cancer Research **11**(1): 67-72.

von Boehmer, H., I. Aifantis, et al. (2003). "Thymic selection revisited: how essential is it?" Immunological reviews **191**: 62-78.

Wagner, P., M. Koch, et al. (2008). "Detection and functional analysis of tumor infiltrating T-lymphocytes (TIL) in liver metastases from colorectal cancer." Annals of surgical oncology **15**(8): 2310-2317.

Wahl, S. M. and W. Chen (2005). "Transforming growth factor-beta-induced regulatory T cells referee inflammatory and autoimmune diseases." Arthritis Res Ther **7**(2): 62-68.

Wajant, H., K. Pfizenmaier, et al. (2003). "Tumor necrosis factor signaling." Cell death and differentiation **10**(1): 45-65.

Walunas, T. L., C. Y. Bakker, et al. (1996). "CTLA-4 ligation blocks CD28-dependent T cell activation." The Journal of experimental medicine **183**(6): 2541-2550.

Wan, Y. Y. and R. A. Flavell (2007). "Regulatory T cell functions are subverted and converted owing to attenuated Foxp3 expression." Nature **445**(7129): 766-770.

Wang, K. S., D. A. Frank, et al. (2000). "Interleukin-2 enhances the response of natural killer cells to interleukin-12 through up-regulation of the interleukin-12 receptor and STAT4." Blood **95**(10): 3183-3190.

Wang, L., T. S. Yi, et al. (2009). "IL-17 can promote tumor growth through an IL-6-Stat3 signaling pathway." Journal of Experimental Medicine **206**(7): 1457-1464.

Wang, L. X., W. X. Huang, et al. (2004). "Adoptive immunotherapy of cancer with polyclonal, 108-fold hyperexpanded, CD4+ and CD8+ T cells." Journal of translational medicine **2**(1): 41.

Wang, L. X., W. X. Huang, et al. (2004). "Adoptive immunotherapy of cancer with polyclonal, 108-fold hyperexpanded, CD4+ and CD8+ T cells." J Transl Med **2**(1): 41.

Wang, R. F. (2006). "Immune suppression by tumor-specific CD4+ regulatory T cells in cancer." Semin Cancer Biol **16**(1): 73-79.

Wang, S. and L. Chen (2004). "T lymphocyte co-signaling pathways of the B7-CD28 family." Cellular & molecular immunology **1**(1): 37-42.

Wang, Y., A. Kissenpfennig, et al. (2008). "Th2 lymphoproliferative disorder of LatY136F mutant mice unfolds independently of TCR-MHC engagement and is insensitive to the action of Foxp3+ regulatory T cells." J Immunol **180**(3): 1565-1575.

Watts, T. H. (2005). "Tnf/tnfr family members in costimulation of T cell responses." Annual Review of Immunology **23**: 23-68.

Watts, T. H. and M. A. DeBenedette (1999). "T cell co-stimulatory molecules other than CD28." Current Opinion in Immunology **11**(3): 286-293.

Weiss, M. J., J. R. Wong, et al. (1987). "Dequalinium, a Topical Antimicrobial Agent, Displays Anticarcinoma Activity Based on Selective Mitochondrial Accumulation." Proceedings of the National Academy of Sciences of the United States of America **84**(15): 5444-5448.

Welniak, L. A., B. R. Blazar, et al. (2007). "Immunobiology of allogeneic hematopoietic stem cell transplantation." Annual review of immunology **25**: 139-170.

Weston, L., A. Geczy, et al. (1998). "A convenient and reliable IL-2 bioassay using frozen CTLL-2 to improve the detection of helper T lymphocyte precursors." Immunology and cell biology **76**(2): 190-192.

Whedon, M. B. and D. Wujcik (1997). Blood and marrow stem cell transplantation : principles, practice, and nursing insights. Sudbury, Mass. London, Jones and Bartlett.

White, R. L., Jr., D. J. Schwartzentruber, et al. (1994). "Cardiopulmonary toxicity of treatment with high dose interleukin-2 in 199 consecutive patients with metastatic melanoma or renal cell carcinoma." Cancer **74**(12): 3212-3222.

Williams, L. M. and A. Y. Rudensky (2007). "Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3." Nat Immunol **8**(3): 277-284.

Williams, N. S. and V. H. Engelhard (1996). "Identification of a population of CD4+ CTL that utilizes a perforin- rather than a Fas ligand-dependent cytotoxic mechanism." Journal of immunology **156**(1): 153-159.

Willimsky, G. and T. Blankenstein (2005). "Sporadic immunogenic tumours avoid destruction by inducing T cell tolerance." Nature **437**(7055): 141-146.

Wing, K., Y. Onishi, et al. (2008). "CTLA-4 control over Foxp3+ regulatory T cell function." Science **322**(5899): 271-275.

Wortzel, R. D., C. Philipps, et al. (1983). "Multiple tumour-specific antigens expressed on a single tumour cell." Nature **304**(5922): 165-167.

Wysocki, C. A., A. Panoskaltsis-Mortari, et al. (2005). "Leukocyte migration and graft-versus-host disease." Blood **105**(11): 4191-4199.

Xu, L., A. Kitani, et al. (2007). "Cutting edge: regulatory T cells induce CD4+CD25-Foxp3- T cells or are self-induced to become Th17 cells in the absence of exogenous TGF-beta." J Immunol **178**(11): 6725-6729.

Yamaguchi, T., K. Hirota, et al. (2007). "Control of immune responses by antigen-specific regulatory T cells expressing the folate receptor." Immunity **27**(1): 145-159.

Yang, J. C., S. L. Topalian, et al. (1994). "Randomized comparison of high-dose and low-dose intravenous interleukin-2 for the therapy of metastatic renal cell

carcinoma: an interim report." Journal of clinical oncology : official journal of the American Society of Clinical Oncology **12**(8): 1572-1576.

Yang, L. and D. Baltimore (2005). "Long-term in vivo provision of antigen-specific T cell immunity by programming hematopoietic stem cells." Proceedings of the National Academy of Sciences of the United States of America **102**(12): 4518-4523.

Yang, X. O., R. Nurieva, et al. (2008). "Molecular antagonism and plasticity of regulatory and inflammatory T cell programs." Immunity **29**(1): 44-56.

Yao, H., S. S. Ng, et al. (2011). "Effective melanoma immunotherapy with interleukin-2 delivered by a novel polymeric nanoparticle." Molecular cancer therapeutics **10**(6): 1082-1092.

Yao, Z., W. C. Fanslow, et al. (1995). "Herpesvirus Saimiri encodes a new cytokine, IL-17, which binds to a novel cytokine receptor." Immunity **3**(6): 811-821.

Yee, C. (2010). "Adoptive therapy using antigen-specific T cell clones." Cancer journal **16**(4): 367-373.

Yee, C., J. A. Thompson, et al. (2002). "Adoptive T cell therapy using antigen-specific CD8+ T cell clones for the treatment of patients with metastatic melanoma: in vivo persistence, migration, and antitumor effect of transferred T cells." Proceedings of the National Academy of Sciences of the United States of America **99**(25): 16168-16173.

Youssef, A. R., C. Otley, et al. (2004). "Role of CD4+ and CD8+ T cells in murine skin and heart allograft rejection across different antigenic desparities." Transplant immunology **13**(4): 297-304.

Yron, I., T. A. Wood, Jr., et al. (1980). "In vitro growth of murine T cells. V. The isolation and growth of lymphoid cells infiltrating syngeneic solid tumors." Journal of immunology **125**(1): 238-245.

Yu, J. S., P. K. Lee, et al. (2003). "Intratumoral T cell subset ratios and Fas ligand expression on brain tumor endothelium." Journal of neuro-oncology **64**(1-2): 55-61.

Yui, M. A., L. L. Sharp, et al. (2004). "Preferential activation of an IL-2 regulatory sequence transgene in TCR gamma delta and NKT cells: subset-specific differences in IL-2 regulation." J Immunol **172**(8): 4691-4699.

Zelenay, S., T. Lopes-Carvalho, et al. (2005). "Foxp3+ CD25- CD4 T cells constitute a reservoir of committed regulatory cells that regain CD25 expression upon homeostatic expansion." Proc Natl Acad Sci U S A **102**(11): 4091-4096.

Zeng, R., R. Spolski, et al. (2005). "Synergy of IL-21 and IL-15 in regulating CD8+ T cell expansion and function." J Exp Med **201**(1): 139-148.

Zheng, H., C. Matte-Martone, et al. (2009). "Central memory CD8+ T cells induce graft-versus-host disease and mediate graft-versus-leukemia." Journal of immunology **182**(10): 5938-5948.

Zhou, G., C. G. Drake, et al. (2006). "Amplification of tumor-specific regulatory T cells following therapeutic cancer vaccines." Blood **107**(2): 628-636.

Zhou, G. and H. I. Levitsky (2007). "Natural regulatory T cells and de novo-induced regulatory T cells contribute independently to tumor-specific tolerance." J Immunol **178**(4): 2155-2162.

Zhou, S., R. Ou, et al. (2004). "Differential tissue-specific regulation of antiviral CD8+ T cell immune responses during chronic viral infection." J Virol **78**(7): 3578-3600.

Zhou, X., S. Bailey-Bucktrout, et al. (2009). "Plasticity of CD4(+) FoxP3(+) T cells." Curr Opin Immunol **21**(3): 281-285.

Zhou, X., S. L. Bailey-Bucktrout, et al. (2009). "Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo." Nat Immunol **10**(9): 1000-1007.

Zitvogel, L., A. Tesniere, et al. (2006). "Cancer despite immunosurveillance: immunoselection and immunosubversion." Nat Rev Immunol **6**(10): 715-727.