

Functional analysis of tumour specific effector and regulatory T cells in *vivo*: Implications for cancer immunotherapy

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Abstract

MB49, an H2^{b+} murine bladder carcinoma cell line, naturally expresses the male-specific minor histocompatibility antigen, HY, which can be used as a proxy tumour associated antigen. The MB49 cell line, together with two HY-specific TCR-transgenic mouse strains (as sources of tumour-specific CD4 and CD8 T cells), form a unique system in which anti-tumour T cell responses can be manipulated and analysed. I have studied some basic but critical immunological aspects of MB49 cells, adoptively transferred T cells and an immunotherapeutic antibody.

I confirmed that MB49 cells express all three defined HY genes (Dby, Uty and Smcy), and demonstrated that cultured and freshly isolated tumour cells display a distinct phenotype. I found that MB49 tumours are very aggressive even though they are clearly immunogenic. Female B6 mice are more efficient than males at controlling MB49 tumours, especially when inoculated with a low tumour dose, suggesting that HY-specific T cell responses make a significant contribution to immune control of MB49 tumours.

I evaluated three different immunotherapies for treating MB49 tumours: Whole body irradiation followed by the adoptive transfer of Tumour Associated Antigen specific CD4 T cells, or IL-15 expressing CD8 T cells, were found to inhibit the growth of MB49 tumours. Administration of anti-GITR mAbs was found to be very effective at eliminating established MB49 tumours.

I observed a novel role for IL-2 in immunoregulation; by the suppression of the differentiation of IL-17-producing Th17 cells.

Finally, I demonstrated that Transforming Growth Factor β is essential for the tumour-induced expansion of natural regulatory T cells.

This study not only extends the current knowledge of the immunobiology of MB49 tumours but also lays a foundation for further investigation with respect to designing rational T cell cancer immunotherapy using genetically manipulated, tumour-specific T cells.

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List of Abbreviations

BCR : B cell receptor

CD : Cluster of differentiation x

CFSE : Carboxyfluorescein Succinimidyl Ester

CTL : Cytotoxic T Lymphocyte

CTLA-4 : Cytotoxic T Lymphocyte Antigen

DMSO : Dimethyl sulphoxide

DMBA : 7,12-Dimethylbenz(a)anthracene

EDTA : Ethylenediaminetetraacetic acid

FACS : Fluorescent activated cell sorting

FCS : Foetal Calf Serum

FoxP3 : Forkhead box P3

GFP : Green fluorescent protein

GITR : Glucocorticoid Induced Tumour Necrosis Factor Receptor related protein

ICOS : Inducible T cell COstimulator

IDO: Indoleamine 2,3-DiOxygenase

IFN γ : Interferon gamma

IL-x : Interleukin-x

i.p. : intraperitoneal

iTreg : induced regulatory T cell

i.v. : Intravenous

mAb : Monoclonal antibody

MACS : Magnetic activated cell sorting

MB49 : Murine Bladder Carinoma-49

MHC: Major Histocompatibility Complex

nTreg : Natural regulatory T cells

PBS : Phosphate Buffered Saline

PBL : Peripheral Blood Lymphocytes

PBMC : Peripheral blood mononuclear cells

PD-1: Programmed Death 1

PDL-1/2: Programmes Death Ligand 1/2

PMA : Phorbol 12-myristate 13-acetate

pMHC : peptide-MHC complex

RBC : Red Blood Cells

s.c. : subcutaneous

TAA : Tumour Associated Antigen

Tc : T cell

Tcm : Central memory T cell

TCR : T cell receptor

Tem : Effector memory T cell

Tg : Transgenic

TIL : Tumour Infiltrating Lymphocyte

TNF : Tumour necrosis factor

TNFR : TNF Receptor

Treg : regulatory T cell

WT : Wild Type

YFP : Yellow fluorescent protein

Chapter 1 : Introduction

1.1 : Immunotherapy for Cancer

Aetiology of human cancers is very varied with tumours arising in numerous tissues. The approximate number of deaths from cancer, worldwide, in 2008 was 7.6 million (M), the most common forms being lung cancer (1.3M), stomach cancer (0.8M) and colorectal cancer (0.639M)¹. Cancer represents an enormous challenge to health care systems and scientists across the globe; being a major cost to health care systems and a complex disease that scientists strive to understand and be able to modulate.

Over the last 20 years, a number of Tumour Associated Antigens (TAA) 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 proteins²⁻⁴. Whilst not all TAA are tumour-specific antigens they do represent a valid target for immunotherapy as they are more limited in their distribution, thus making any targeting therapies more specific than the systemic chemotherapy treatments most widely used in clinics. The identification of TAAs and TAA-specific T cells in a range of different tumours, especially from melanoma patients, has meant that augmentation of adaptive immune responses to overcome tumours has become a clinical reality.

There are three general strategies for cancer immunotherapy. The first uses monoclonal antibodies (mAb) to directly target tumour cells or modulate the immune system. The second uses various cancer vaccines including tumour-specific peptides, peptide-loaded DCs and/or novel adjuvants designed to activate endogenous DCs and T cells. The third utilises adoptive transfer of TAA-specific lymphocytes. This latter approach, here-in called adoptive immunotherapy, has yielded encouraging clinical responses in melanoma patients⁵.

Adoptive immunotherapy for the treatment of cancer was first realised in mice in the 1950s and 60s when it was demonstrated that the transfer of leukocytes, before tumour inoculation, could lead to tumour rejection^{6, 7}. As the area developed, it was found that leukocytes from immunised mice, adoptively transferred to mice with established tumours, could cause regression⁸.

Soon after these fundamental observations were made, the discovery of Interleukin-2, an important cytokine required for the growth and survival of T cells, led to the ability to

generate large numbers of leukocytes *in vitro*^{9, 10}. This capability has allowed the field of cancer immunology to develop rapidly. One of the most significant findings being that the adoptive transfer of tumour infiltrating lymphocytes, isolated from tumour biopsies and expanded *in vitro* with IL-2 is effective at reducing tumour burden in mice^{11, 12}.

Rosenberg *et al.* have been pioneers in applying adoptive cell transfer of *ex vivo* expanded melanoma specific T cells to melanoma patients. In original trials, lymphocytes were harvested from freshly resected melanomas, subsequently expanded *in vitro* then adoptively transferred back to patients combined with high doses of IL-2. Results were promising with many patients showing an objective response^{13, 14}. More recently, Dudley *et al.* have demonstrated that lympho-depletion, with cyclophosphamide and fludarabine, before adoptive cell transfer greatly improves objective responses¹⁵. Originally, adoptive immunotherapy was solely used in the treatment of metastatic melanoma, where the Melan-A/Mart-1 tumour antigen is well defined. However, recent trials have been extended to other forms of cancer including B cell lymphomas. Table 1, lists some of the most recent adoptive immunotherapy clinical trials.

Table 1. Clinical trials using adoptive transfer of T cells.

Date	Cell Type	Treatment regimen	Tumour	Responses	Reference
1988	<i>In vitro</i> expanded TIL	High IL-2	MM	11/15 OR	¹³
1994	<i>In vitro</i> expanded TIL	Cyclo. High IL-2 TIL and IL-2 in two week cycles	MM	24/86 PR 5/86 CR	¹⁶
1990	<i>In vitro</i> expanded TIL	High IL-2. TIL and IL-2 in two week cycles	MM		¹⁷
2002	<i>In vitro</i> expanded Mart-1 or gp-100 T cells from PBMC	Increasing doses of IL-2 s.c.	MM	5/10 SD 1/10 MR 2/10 MiR	¹⁸
2002	<i>In vitro</i> expanded TIL (7.8 x 10 ¹⁰ cells i.v.)	Cyclo and Flur for 7 days, cell transfer. High IL-2.	MM	6/13 OR 4/13 MR	¹⁹
2005	<i>In vitro</i> expanded TIL	Cyclo (2d) then Flur (5d) transfer then high IL-2	MM	15/35 PR 3/35 CR	²⁰
2006	PBMC transduced with Mart-1 specific TCR.	Cyclo (2d) then Flur (5d) transfer then high IL-2 and Mart-1 vaccine	MM	2/17 PR 1/17 MR	²¹
2008	<i>In vitro</i> expanded NY-ESO-1 specific CD4 from PBL	N/A	MM	1/1 CR	²²
2008	Genetically modified T cells expressing CD20 specific chimeric receptor	Varied cytoreductive therapy before cell transfer then 14 days low IL-2	Non-HL MCL	2/7 CR 4/7 SD 1/7 PR	²³
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	FL	1/1 PR	²⁴

Abbreviations: CR; Complete response, Cyclo; Cyclophosphamide, FL; Follicular Lymphoma, Flur; Fludarabine, hrs; hours, i.v.; intra venous, MCL; Mantel cell lymphoma, MiR; Minor response, MM; Metastatic Melanoma, MR; Mixed response, NHL; Non-Hodgkin Lymphoma, OR; Objective response, PR; Partial response, s.c.; sub cutaneous, SD; TIL; Tumour Infiltrating Lymphocyte. Definitions: gp-100 T cells; High IL-2; High dose IL-2 (720,000 IU/kg), Low IL-2; 500,000 IU/m².

Despite numerous trials, new protocols and encouraging reports of donor T cell persistence^{17, 18, 24} and infiltration into the tumour mass¹⁸, the full potential of adoptive immunotherapy has yet to be realised. However, since Rosenberg *et al.* started their adoptive immunotherapy trials, many studies of murine models of cancer have taken place and a pantheon of new knowledge has been discovered about the mammalian immune

system, how it interacts with tumours and how it can be manipulated to enhance adoptive immunotherapy.

1.2 : The Adaptive immune system

Mammals have developed a complex immune system because of evolutionary pressure to protect the body from foreign substances including toxins and pathogenic micro-organisms. Evolution has required the immune system to develop a huge diversity of responses; this reflects the vast number of harmful chemicals, viruses and bacteria that are encountered in the environment.

The immune system can be divided into two branches; the innate immune system and the adaptive immune system.

The innate immune system utilises the following four mechanisms to prevent toxins and pathogenic microbes from entering the body:

1. Anatomical. Including the skin (epidermis and dermis) and mucous membranes
2. Physiologic. Includes thermoregulation, PH (i.e. acidity in the stomach) and chemical mediators (i.e. lysozymes, interferons)
3. Phagocytic/Endocytic. Neutrophils and macrophages can phagocytose micro-organisms
4. Inflammatory. Damage and infection cause leakage of vascular fluid into tissues which contains serum proteins, antibodies and phagocytic cells.

The innate immune system exhibits limited specificity to toxins and pathogens whereas the adaptive immune system is characterised by its ability to discriminate between a huge variety of foreign and self-antigens. In addition, activation of the innate immune system does not lead to the development of memory responses whereas activation of the adaptive immune system does.

The adaptive immune system comprises B cells that express B cell receptors (BCR) and generate antibodies and T cells that express T cell receptors (TCR) and produce cytokines and can in some cases, directly kill specific cells. Each B cell or T cell clone has a unique specificity for antigen; this specificity is generated by the rearrangement of a set of gene segments to generate antibodies (BCR shed from the surface of B cells), and TCR.

T cells are only able to recognise specific peptide epitopes, derived from self or foreign proteins, when they are presented on the surface of a cell in association with a major histocompatibility complex (MHC) molecule. T cells only generate an effective

response when their TCR is engaged by MHC-peptide at the same time as they are co-stimulated by ligands on an antigen presenting cell (APC) (Described in more detail in Section 1.2.13 :). Engagement of the TCR alone leads to anergy, deletion or tolerance. Co-stimulatory molecules can have positive or negative effect on the activation of T cells²⁵.

1.2.1 : The Major Histocompatibility Complex

The major histocompatibility complex (MHC) was discovered in the 50s and 60s by Peter Gorer and George Snell when they identified a group of genes that were responsible for the rejection of transplantable tumours and other tissues in mice²⁶. It was subsequently established, in both mice and humans, that MHC Class I genes encode glycoproteins that are expressed on all nucleated cells and present antigen to CD8 T cells and that the MHC class II genes encode glycoproteins that are expressed on APCs and present antigen to CD4 T cells.

The MHC Class I and Class II genes are highly polymorphic with a huge number of different alleles at each locus which can differ in their DNA sequence from one human to another by 5 – 10%.

MHC Class I proteins (MHC-I here-in) present peptides produced in the “MHC-I pathway”. The MHC-I pathway processes intracellular proteins for presentation on the surface of most cells: The assembly of MHC-I molecules occurs in the endoplasmic reticulum (ER) where the MHC-I heavy chain and β 2- microglobulin (β 2m; a non-polymorphic, non-MHC encoded protein) proteins form dimers. Peptides generated, by proteasomes, from proteins in the cytosol are translocated into the ER by transporting proteins (TAP1 and TAP2). TAP proteins form a complex with MHC-I molecules and are essential for the loading of peptide and assembly of the MHC-I-peptide complex before it is released into the secretory pathway in the ER and subsequently transported to the cell surface for presentation of the loaded peptide²⁷.

MHC Class II proteins (MHC-II here-in) present peptides produced in the “MHC-II pathway”. The MHC-II pathway primarily processes proteins, derived from extracellular sources, via the endosome/lysosome/phagosome compartments for presentation on the surface of professional APC. MHC-II molecules are heterodimers that assemble in the ER before being transported to the endocytic compartments. In these compartments, self and non-self peptides, that are derived from degraded larger proteins, are loaded onto the

MHC-II molecule before it is transported to the cell surface²⁸ for presentation of the loaded peptide.

1.2.2 : Antigen presenting cells

All normal nucleated cells express MHC-I molecules. Once loaded with peptide, these complexes (pMHC-I) can potentially be recognised by CD8 T cells with the relevant TCR.

MHC-II molecules loaded with peptide (pMHC-II) are only normally expressed by professional antigen presenting cells (pAPC), a group of cells which includes dendritic cells (DC), B cells and macrophages. pAPC can internalise antigen from their microenvironment by endocytosis or phagocytosis and process it, as described above, to be presented at the cell surface in combination with MHC-II.

1.2.3 : B cells

B cells are generated from lymphoid stem cells within the bone marrow. Progenitor B cells proliferate within the bone marrow and differentiate into precursor B cells. Development of immature B cells from precursor B cells, is dependent on the rearrangement of immunoglobulin DNA and is driven by IL-7. After a productive rearrangement and expression of a non-self reactive BCR with a particular antigen specificity, immature B cells are released into the blood stream and migrate thence to the periphery. An immature B cell is activated when the BCR recognises its cognate antigen, resulting in up-regulation of pMHCII which allows interaction with CD4 T cells. The B cell receives a co-stimulatory signal from CD4 T cells through CD40-CD40L ligation (CD40 expressed on the CD4 cell and CD40L on the B cell) which leads to proliferation and generation of plasma cells and memory B cells²⁹. Plasma cells secrete high levels of monoclonal antibody, the isotypes of which are dependent on the cytokine milieu.

1.2.4 : Dendritic cells

Dendritic cells (DC) are derived from bone marrow progenitor cells, found in different areas throughout the body and are classified according to their location. Langerhans cells are found in the epidermis and mucous membranes. Interstitial DC are found in most

organs. Interdigitating DC populate the secondary lymphoid organs and thymus. Circulating DC are found in the blood, lymph node and spleen. DC can be divided into two subsets, lymphoid DC are characterised by $CD11c^+CD8\alpha^+DEC205^+CD11b^-$ and myeloid DC by $CD11c^+CD8\alpha^-DEC205^+CD11b^+$ ^{30, 31}. Immature DC efficiently endocytose and phagocytose antigens in peripheral tissues, they subsequently degrade antigens and present the resultant peptides on their surface in combination with MHC-I and MHC-II. Inflammatory signals such as bacterial products (e.g. LPS and CpG etc), cytokines and chemokines (e.g. GM-CSF and IL-4) cause the activation and maturation of DCs. Upon activation, DCs migrate to the lymphoid organs, up-regulate co-stimulatory molecules and MHC-I and MHC-II, secrete stimulatory cytokines (e.g. IL-12 and IL-6 etc) and present antigen to CD4 and CD8 T cells^{32, 33}.

1.2.5 : Monocytes/Macrophages

Mononuclear cells which comprise monocytes circulating in the blood and macrophages in the periphery, are derived from granulocyte-monocyte progenitor cells in the bone marrow. Progenitor cells develop into pro-monocytes and enter the blood stream. Pro-monocytes migrate into peripheral tissues and mature into tissue specific macrophages. Macrophages express a restricted repertoire of phagocytic receptors that allows them to phagocytose dying cells, whole micro-organisms, activated clotting factors and cellular debris³⁴. Macrophages can process antigen and present pMHC-I, pMHC-II and co-stimulatory molecules for T cell activation.

1.2.6 : T cell development

Negative and positive selection of the T cell repertoire during T cell development in the thymus determines the ability of the immune system to distinguish self from non-self.

T cells originate in the bone marrow (BM) as haematopoietic stem cells (HSCs). HSC's differentiate into T lineage progenitors (TLP) which migrate into the blood stream and then into the thymus³⁵. In the thymus T cell progenitors expand and differentiate through several distinct phases in the cortex and medulla of the thymus³⁶.

TLP enter the thymus and progress into the inner cortex where they interact with cortical epithelial cells that are resident in the thymus. Early thymocytes are negative

for CD4 and CD8 and described as double negative (DN) thymocytes. Signalling through the Notch pathway commits TLP to the T cell lineage and they become DN cells³⁷ expressing the pre-TCR³⁸. A positive signal through the pre-TCR ensures the thymocytes survival and they become CD4⁺CD8⁺ double positive (DP) thymocytes which express a mature TCR³⁹. Mature TCR molecules are dimers formed by the varying combination and interaction of the TCR proteins; TCR α , β , δ and γ . The huge diversity of the TCR repertoire is generated by the rearrangement of the four genes (*Tcra*, *Tcrb*, *Tcrd* and *Tcrg*), that encode these proteins, during T cell development in the thymus⁴⁰. Rearrangement is driven by the Recombinase Activating Gene 1 and 2 (RAG1/2) proteins that create double stranded breaks at specific Recombination Signal Sequences (RSSs) that flank the TCR variable (V), diversity (D) and joining (J) gene segments. When the segments are rejoined they generate a TCR sequence⁴¹ that is translated into the TCR. Each T cell clone expresses a unique TCR on its cell surface.

DP thymocytes with a mature TCR interact with APCs, including thymic B cells, DCs, cortical thymic epithelial cells (cTECs) and medullary thymic epithelial cells (mTECs). DP thymocytes are positively selected if they interact, with sufficient affinity, with self-peptide-MHC complexes on cTECs. At this point, DP thymocytes that interact with MHC-I restricted peptides are destined to become CD8 T cells and those that interact with MHC-II are destined to become CD4 T cells. Positively selected thymocytes migrate to the medulla and interact with mTECs. mTECs express MHC-I and -II and because of their promiscuous gene expression, under the influence of the autoimmune regulator (AIRE), can present virtually every possible self epitope to the TCR of DP thymocytes⁴². In the medulla, DP thymocytes that interact with self-peptide-MHC with very high affinity are negatively selected⁴³.

The results of the thymic process of positive and negative selection is a repertoire of naïve CD4 and CD8 T cells that have a controlled affinity for self-peptide-MHC complexes. Crucially thymocytes that have a strong affinity for self-peptide MHC complexes are deleted and this prevents auto-immunity⁴⁴. It is this tolerance to self peptides that contributes to the persistence of tumours; most TAAs, which are derived from self proteins, are expressed by mTECs in the thymus during T cell selection and as a consequence, high avidity auto-reactive T cells, that could later recognise tumours expressing these TAAs, are deleted and a repertoire is created in which the remaining T cells have only a low to intermediate avidity for TAA, and thus minimal effector function upon TAA recognition⁴⁵. This is discussed in more detail in Section 1.4 : on page 31.

After release from the thymus, mature, naïve CD4 and CD8 T cells are found continually circling through the blood, peripheral tissues and lymph nodes. The effectors of the adaptive immune system are largely comprised of T cells that express TCR α and TCR β , transcribed from the *Tcra* and *Tcrb* genes, and it is these $\alpha\beta$ -T cells that will be discussed.

Naïve CD4 and CD8 T cells are characterised by the low expression of CD44 and high expression of CD62L. CD62L (L-selectin) is a cell adhesion molecule that facilitates the entry of T cells into the secondary lymphoid organs.

To become activated, T cells require at least two signals, one through the TCR and one through a co-stimulatory molecule, most commonly CD28⁴⁶, to become activated (A more detailed description is given in Section 1.2.13 : T cell co-stimulation, below). Stimulation through the TCR alone, as happens in a tolerant situation when there are no additional signals from invading pathogens or damaged cells, leads to T cell anergy⁴⁷ and tolerance⁴⁸.

Activated T cells have increased expression of CD44 and chemokine and homing receptors (e.g. CCR5/7) and reduced expression of CD62L; this expression profile facilitates their migration to the periphery and sites of inflammation and infection. The type of cytokines and cell surface markers that are expressed, by the T cells, after activation are influenced by the microenvironment, the cytokines secreted by the APC and other cells in close proximity and the co-stimulatory signals received by the T cell from the APC.

In addition to the expression of molecules that contribute to activation, activated T cells also begin to express inhibitory molecules such as Programmed Death-1 (PD-1) and Cytotoxic T Lymphocyte Antigen 4 (CTLA-4) which, when bound to their cognate antigen on APC, causes the death and inactivation of activated T cells. This feedback system regulates the strength and extent of the immune response ensuring that cytotoxic effects are limited to a small area and short time frame to minimise damage to healthy tissue.

1.2.7 : CD4 T cells

As mentioned above, DP thymocytes that recognise MHC-II restricted peptides, presented by cTECs during thymocyte development, are destined to become CD4 T cells; upon interaction with MHC-II in the thymus, they gradually down regulate CD8 and up regulate CD4 and migrate out of the thymus as mature naïve CD4 T cells.

Activated CD4 T cells can develop cytotoxic functions⁴⁹ but their primary function is to maintain and regulate B cell and CD8 T cell responses⁵⁰. Following antigen clearance, a small proportion of activated CD4 T cells survive and persist in the blood stream and lymph nodes as memory CD4 T cells which are rapidly reactivated if the same antigen is presented again.

CD4 T cells can be separated into four distinct subsets, Th1, Th2, Th17 and regulatory T cells (Treg) based on their function and cytokine profiles.

1.2.8 : Th1

A type 1 T helper response (Th1) is generated in response to intracellular pathogens such as bacteria and viruses. It is characterised by CD4 T cell secretion of Interferon gamma (IFN γ), IL-2 and Tumour Necrosis Factor alpha (TNF α), activation of CD8 T cells and macrophages and the production of opsonising antibodies from B cells. IFN γ increases the production of IL-12 by DC and macrophages which in turn stimulates the secretion of IFN γ by Th1 cells thus providing a feedback loop that drives the Th1 response⁵¹.

1.2.9 : Th2

A type 2 T helper (Th2) response is generated against multi-cellular organisms and is typified by the activation of B cells and secretion of IL-4, 5, 6, 9 10 and 13 by CD4 T cells⁵¹.

1.2.10 : Th17

A distinct subset of CD4 T cells was recently identified as secreting IL-17⁵². These cells have been termed Th17 cells. Th17 cells are found in inflammatory environments and are induced by TCR stimulation in the presence of Transforming Growth Factor- β (TGF β) and IL-6^{53, 54}. Th17 cells secrete IL-17A, IL-17F and IL-22 which induce a wide range of responses from surrounding tissue because of the ubiquitous expression of the IL-17 and IL-22 receptors⁵⁵.

1.2.11 : Regulatory T cells

CD4 regulatory T cells (Tregs) are characterised by the expression of the forkhead/winged helix transcription factor (FoxP3)⁵⁶ and the α chain of IL-2 receptor (CD25)⁵⁷. They suppress T cell responses by the secretion of suppressive cytokines IL-10 and TGF β ⁵⁸ and by cell contact dependent mechanisms including perforin mediated cytotoxicity and membrane bound TGF β and CTLA-4. Foxp3⁺ Tregs can be grouped into natural (nTregs) and induced Tregs (iTregs)⁵⁹.

nTreg differentiate and mature in the thymus; cells are committed to the Treg lineage when they recognise self-peptide-MHC with an avidity ranging between that required for positive and negative selection. Development in the thymus may not be consistent for all thymocytes and therefore it is possible that both conventional and regulatory T cells with the same or similar specificities can develop⁵⁶.

The primary function of nTregs is to control self-reactive peripheral T cells that escape thymic deletion, as suggested by early studies using neonatal thymectomy or depleting anti-CD25 antibodies^{57, 60}, and verified by recent investigations using Foxp3^{DTR} mice⁶¹.

iTregs are induced in the periphery and their primary function is similar to that of nTreg. However, iTreg differ from nTreg in that they can be induced in response to foreign antigen and can block other cells from producing IL-17⁶², suggesting that they have a distinct function from nTreg, possibly in mediating non-responsiveness to environmental antigens. The development of iTregs needs a low dose of antigen presented by resting APC and engagement of CTLA-4⁶²⁻⁶⁴.

1.2.12 : CD8 T cells

DP thymocytes that recognise MHC-I restricted peptides, presented by cTECs during thymocyte development, gradually down regulate CD4 and up regulate CD8 and migrate out of the thymus as mature CD8 T cells. CD8 T cells are then found in the blood, periphery and lymph nodes. Upon ligation of TCR with pMHC-I on APC and sufficient co-stimulation, CD8 T cells become activated and begin to proliferate. Upon activation CD8 T cells, also known as cytotoxic or killer T cells, secrete perforins, granzymes and up-regulate Fas-Ligand (Fas-L) effecting the death of the target cell. CD8 T cell proliferation and activation is enhanced by IL-2⁹, IL-7⁶⁵ and IL-15⁶⁶. CD8 T cells spontaneously secrete

Th1 cytokines IFN γ and IL-2 but can be induced to secrete Th2 cytokines in the presence of IL-4⁶⁷.

Whether or not the source of antigen is cleared, CD8 T cells numbers fall dramatically during a phase of contraction. However, not all antigen specific CD8 T cells are lost and a small proportion survive and persist in the blood stream and lymph nodes as memory CD8 T cells which are rapidly reactivated if the same antigen is presented again⁶⁸.

1.2.13 : T cell co-stimulation

Co-stimulation is required for optimal T cell activation, differentiation and survival. Co-stimulatory molecules fall into two families, the immunoglobulin family, including CD28-CD80/86 and the tumour necrosis factor receptor super-family, including OX40, GITR and 4-1BB. A brief description of T cell costimulatory receptors is laid out in Table 2.

Table 2. Co-stimulatory molecules, the cells that express them, their ligands and the T cell response following ligation of the molecule.

Receptor/Ligand (T cell)	Cognate Receptor/Ligand (Expressed by)	Effect on T cell	Ref.
CD28 (CD4; CD8)	CD80/86 (Bc;DC;Tc)	Activation; Proliferation; Cytokine secretion	⁶⁹
CTLA-4 (CD4; CD8; Treg)	CD80/86 (Bc;DC;Tc)	Inhibition; Tolerance Th1	⁷⁰
ICOS (act CD4; act CD8; Treg)	ICOS-L (Bc;DC)	Increased IL-10. Th2	⁷¹
GITR (Treg; act CD4; act CD8)	GITR-L (Bc; DC; Mθ)	Th1 or Th2	⁷²
CD40L (CD4)	CD40 (Bc; Mθ)	Th2	⁷³
4-1-BB (CD8 more than CD4)	4-1-BBL (Bc; DC; Mθ)	Increased CD4/8 proliferation. Th1 or Th2.	⁷⁴
OX-40 (mainly CD4; some CD8)	OX-40L (Bc; DC; act Tc)	Th2, but sustains Th1 proliferation and survival	^{74, 75}
PD-1 (act CD4; act CD8)	PD-L1/PD-L2 (Bc; DC; Mθ; Tc)	Negative regulation of PD-1 expressing cell	^{76, 77}

Abbreviations: 4-1-1BBL; 4-1-BB Ligand, act; activated, Bc; B cell, DC; Dendritic cell, GITR; Glucocorticoid Induced Tumor necrosis factor Receptor family related gene, ICOS; Inducible Costimulator, GITR-L; GITR-Ligand, ICOS-L; ICOS-Ligand, Mθ; Macrophage, Tc; T cell. Definitions: Th1; induction of Th1 cytokines, Th2; induction of Th2 cytokines.

1.3 : Cytokines

Cytokines are pluripotent mediators of the immune system, they are produced from many sources, including non-hematopoietic cells, under differential conditions. Cytokines are usually soluble although there are some that are membrane bound. They bind receptors on their target cell and induce various responses including migration, activation, proliferation, differentiation and inhibition.

Here follows a brief description of the cytokines that are important to this study.

1.3.1 : IL-2

IL-2 is secreted by CD4 and CD8 T cells after TCR stimulation and acts in an autocrine manner to enhance expansion, differentiation and cytolytic effector functions⁷⁸. IL-2 is also required for the maintenance of Treg⁷⁹. Indeed, CD25, the alpha chain of IL-2 receptor is expressed transiently on CD4 and CD8 T cells after activation but ubiquitously on nTreg cells. The ubiquitous expression of CD25 on nTreg may prevent autoimmunity by sequestering excess IL-2 in the local microenvironment⁸⁰.

1.3.2 : IL-10

IL-10 is an anti-inflammatory cytokine that is secreted by monocytes and lymphocytes. IL-10 can modulate T cell responses directly or indirectly via APC⁸¹. Importantly, IL-10 is a key cytokine controlling and driving the development and function of T regulatory type 1 (Tr1) cells which are an independent regulatory T cell population (unlike Treg, Tr1 cells do not express Foxp3⁸²) that can inhibit CD4 responses⁸³.

IL-10 inhibits cellular immune responses and antigen induced proliferation by down regulating MHC-II⁸⁴ and co-stimulatory CD80/86 molecules on DCs, monocytes and macrophages⁸⁴⁻⁸⁷ thus inhibiting the ability of APC to prime T cells⁸⁸. Furthermore, IL-10 inhibits cytokine production by macrophages⁸⁹ and can directly inhibit IL-2 production by CD4 T cells⁹⁰.

1.3.3 : IL-15

The role of IL-15 in the differentiation and survival of CD8 T cells is well established^{91, 92}. The IL-15 receptor is one of the common γ chain cytokine receptors which includes IL-2R and IL-7R which are critical regulators of activated T cell proliferation, survival and memory generation. IL-15 is a pleiotropic cytokine that influences both the innate and adaptive immune responses; it promotes the survival and expansion of CD8 T cells^{66, 93} and stimulates CD8 T cell cytotoxicity as well as playing an important role in the differentiation and survival of memory CD8 T cells and stimulating DC maturation^{94, 95}.

IL-15 has also been implicated in the *de novo* generation of iTreg whilst simultaneously abrogating the effect of Tregs on CD4 and CD8 T cell effector function. Together, these effects of IL-15 may allow rapid but transient responses against pathogens⁹⁶.

1.3.4 : IL-17A

IL-17A is a pro-inflammatory cytokine that promotes cytotoxic T cells and angiogenesis and has been implicated in autoimmune diseases such as rheumatoid arthritis⁵⁵. The majority of IL-17A is produced by CD4 T cells but CD8 T cells can also produce some IL-17⁹⁷. The IL-17 receptor (IL-17R) is expressed ubiquitously⁵². Signalling through the IL-17R causes the secretion of IL-6 and IL-8 from fibroblasts, keratinocytes and epithelial cells⁹⁷.

1.3.5 : IFN γ

IFN γ , a signature cytokine released by CD4 and CD8 T cells after activation, causes the up regulation of MHC-I and -II molecules in numerous cell types^{98, 99}. IFN γ promotes anti-microbial and anti-tumour responses from macrophages; consistent with this, the neutralisation of IFN γ with the monoclonal antibody R46/A2 inhibits anti-viral and anti-tumour responses¹⁰⁰. IFN γ also regulates B cell functions including immunoglobulin production and class switching¹⁰¹.

1.3.6 : TGF β

TGF β is an important mediator of suppression; it can cause apoptosis, inhibit proliferation, control inflammatory responses¹⁰² and can prevent the induction of CD8 mediated responses¹⁰³. There are three TGF β isotypes, TGF β -1, TGF β -2 and TGF β -3. TGF β -1 binds to TGF β receptor II (TGF β RII) and the signal is propagated through the Smad pathway¹⁰⁴.

In addition, TGF β can convert naïve CD4 T cells to Foxp3-expressing Treg cells¹⁰⁵. TGF- β is secreted by numerous lung cancer cell lines *in vitro* and is produced by tumour cells and natural or induced Treg cells⁵⁸.

1.4 : Tumour immunology

It is unclear what pushes a healthy cell towards becoming cancerous but it is known that genetic instability in cancer cells results in the rapid acquisition of mutations that enhance their survival and the development of tumours. Cancer cells provide their own growth signals, ignore inhibitory signals, avoid cell death, replicate uncontrollably, activate angiogenesis and in some cases, metastasise¹⁰⁶. Cancer cells may also evade the immune system in a process termed immunoevasion. Those that do evade the immune system, thrive and eventually represent the majority of cells within a tumour in a process termed immunoselection^{107,108}. It is not clear whether immunoevasion is necessary for tumour development but it definitely enhances the process of tumour development.

There are several mechanisms associated with a tumours ability to evade immune responses. In brief, tumour cells can reduce the expression of MHC class I and co-stimulatory molecules, thereby protecting them from immune recognition. Furthermore, tumour cells can actively suppress APC function by reducing expression of co-stimulatory molecules but increasing expression of inhibitory molecules. Tumour cells not only make suppressive factors themselves but also induce host immune cells to produce inhibitory factors. In addition, tumour cells express Fas-L which can induce apoptosis in Fas-expressing T cells. Finally, tumour cells and/or tumour-derived factors can directly or indirectly down-regulate TCR expression, thus avoiding immune recognition by T cells.

Tumour growth and progression is also influenced by the normal function of the immune system; Tregs, whose function it is to prevent autoimmunity by suppressing self-

specific immune responses, suppress immune responses to tumours whose TAAs are, for example, over-expressed or aberrantly expressed self antigens.

I shall now describe the above factors, in more detail.

1.4.1 : Tumour immune evasion

Defects in antigen processing and presentation in tumour cells confers an advantage for tumour growth due to reduced recognition by T cells. The classical MHC-I pathway processes intracellular proteins for presentation on the cell surface. Deletion of MHC-I and/or accessory proteins required for antigen loading is common in tumours. For example, human ovarian carcinoma and colorectal carcinoma can down-regulate TAP1, TAP2 and $\beta 2m$ ^{109, 110} which in turn reduces the cell surface expression of MHC-I molecules.

As well as possibly having defects in antigen presentation, tumour cells are generally also poor stimulators of T cells, as they express limited T cell co-stimulatory molecules. Evidence suggests that this is an important factor in immune evasion: Melanoma transfected with CD80 causes tumour rejection and concomitant immunity to challenge by the untransfected tumour¹¹¹, suggesting that non-immunogenic tumours do not express CD80 and that enhancing T cell co-stimulation causes tumour rejection.

Compounding the limited expression of positive costimulatory molecules, tumour cells can express abundant T cell inhibitory molecules, some of which are detailed below.

PD-L1 expressed on DC and macrophages is able to bind PD-1 on T cells¹¹²⁻¹¹⁴. Ligation of PD-L1 by PD-1 on activated T cells inhibits T cell proliferation and cytokine production. Moreover, interaction of PD-L1 and PD-1 is essential for the induction of peripheral CD8 T cell tolerance⁷⁶. Interestingly, tumour cells also express PD-L1 which can induce apoptosis in activated T cells expressing PD-1¹¹⁵. PD-L1 has been found to be expressed on human lung, ovarian and colon carcinomas and melanoma¹¹⁵.

B7-H4 can be induced on human T cells, B cells, macrophages and DC. B7-H4 is also expressed on tumour cells, tumour infiltrating macrophages and tumour associated endothelial cells. IL6 and IL-10 increase B7-H4 expression whereas GM-CSF and IL-4 reduce B7-H4 expression. B7-H4 inhibits T cell responses¹¹⁶. B7-H4 was found to be expressed in ovarian carcinoma: in a study by Kryczek *et al.* ovarian carcinomas from 103 patients were studied: B7-H4 expression on tumour-associated macrophages was found to be associated with increased numbers of Treg and a poorer prognosis when compared to expression of B7-H4 on tumour cells themselves¹¹⁷.

The tumour microenvironment contains immunosuppressive factors that can be secreted directly from the tumour or from APC and T cells. Indoleamine-2,3-dioxygenase (IDO) is an enzyme expressed by a subset of APC and some tumour cells. IDO catabolises tryptophan, decreased tryptophan and the presence of tryptophan metabolites inhibits T cells proliferation and induces apoptosis^{118, 119}. Tumour cells can induce IDO expression in DC in tumour draining LN, thus rendering them tolerogenic¹²⁰. Immunosuppressive factors directly secreted from tumours include gangliosides, IL-10, prostaglandins, TGF β and VEGF. IL-10 and TGF β are the two best characterised suppressive cytokines in the tumour microenvironment (See also Sections 1.3.3 :, page 29 and 1.3.6 :, page 31).

IL-10 can modulate T cell responses directly or indirectly via APCs⁸¹. Importantly, IL-10 is a key cytokine controlling the development and function of Tr1 cells which is an independent regulatory T cell population, as Tr1 cells do not express Foxp3⁸².

TGF β can convert naïve CD4 T cells to Foxp3-expressing Treg cells¹⁰⁵, it is secreted by numerous lung cancer cell lines *in vitro* and when secreted in high quantities (>109pg/ml), by tumours, it prevents the induction of CD8 mediated responses¹⁰³.

In summary, tumours can hi-jack many naturally occurring immuno-suppressive mechanisms to enhance their survival. Understanding these mechanisms at the cellular and molecular level is essential for us to rationally design new cancer immunotherapies.

1.4.2 : Regulatory T cells and cancer

CD4 and CD8 T cells, expressing TCR specific for TAA, are found in human melanoma¹²¹, ovarian carcinomas¹²², and colorectal cancer¹²³ as well as in numerous murine tumours. However, tumours are rarely rejected by their hosts. One reason for the failure of anti-tumour immunity is the presence of tumour-specific regulatory T cells which play a key role in suppressing anti-tumour responses: As many TAAs are aberrantly expressed normal self-proteins¹²⁴, the activity of self-reactive nTregs is proposed to mediate an inhibitory influence over the development of effective tumour specific T cell responses. Indeed, *in vivo* depletion of nTregs by anti-CD25 treatment leads to improved tumour immunosurveillance and inhibition of tumour growth^{125, 126}. Moreover, Treg cells, specific for TAA, can suppress anti-tumour immune responses¹²⁷. Treg cells are found at elevated numbers in the PBL and tumours of patients with invasive breast or pancreatic cancer¹²⁸. It

has been shown that tumour infiltrating Treg cells inhibit the effector functions of Th cells¹²⁹ and CD8 CTL¹³⁰.

1.5 : Immunotherapy for tumour mediated immune suppression

As has been described above, tumours have many and varied ways of suppressing active immune responses that inhibit early tumour development¹⁰⁸. However, the presence of T cell responses to TAA is heartening because it means that the immune system is trying to, and with a little help might eventually be able to, destroy tumours.

There are two key methods of targeting the pathways that tumours hijack to evade the immune system; antibody immunotherapy and adoptive immunotherapy.

1.5.1 : Antibody immunotherapy

Antibody immunotherapy can be broken down into two categories, immuno-targeting therapy and immuno-modulation therapy. Both therapies make use of monoclonal antibodies (mAb) specific for unique markers on the cell surface.

Immuno-targeting therapy uses mAb directed at TAAs on the surface of cancerous cells or, factors associated with the tumours growth. The binding of a mAb to tumour or tumour associated cells, leads to their death by antibody mediated immunity (AMI). An example of this group of antibodies is Alemtuzumab (aka, Campeth) which is a humanised rat anti-human CD52 mAb which destroys cells of chronic lymphocytic leukaemia by AMI^{131, 132}. Monoclonal antibodies can also be engineered to carry a toxin which kills the cell that the mAb binds for example MylotargTM which is an anti-CD33 mAb conjugated to an immunotoxin¹³³.

Immuno-modulation therapy uses mAbs that bind receptors and chemokines and influence the natural immune response to the tumour. For example, immuno-modulating mAbs can be used to block inhibitory pathways such as the PD-1/PDL-1/2 or activate stimulatory pathways for APC and/or T cells.

There are several promising immuno-modulating mAb in early clinical trials or murine trials that are of note. These are described below.

1.5.2 : Anti-CTLA-4 (4F10)

CTLA-4 is a potent immuno-modulatory receptor that is constitutively expressed on the surface of Treg¹³⁴ and up-regulated after activation on conventional CD4 and CD8 T cells¹³⁵. It competes with CD28 for binding to the co-stimulatory molecules CD80 and CD86. CD80/86-CTLA-4 signalling inhibits T cell proliferation and increases the activation threshold of the TCR⁷⁰.

Blocking CTLA-4¹³⁶ with the anti-CTLA4 mAb, 4F10, increases production of IL-2 and IFN γ and enhances tumour rejection^{137, 138}. Therapy with anti-CTLA4 has shown promising results in a Phase I trial for the treatment of prostate cancer¹³⁹.

1.5.3 : Anti-PD-L1

As described earlier (Section 1.4.1 :, page 32), the PD-1/PD-L1 pathway is inhibitory, with ligation of PD-1 on T cells, by PD-L1 on APCs and tumour cells, resulting in down regulation of T cell responses. Furthermore, it was noted that PD-L1 has been found to be expressed by various cancers¹¹⁵. Therefore, blocking PD-1/PDL1 interactions has the potential to abrogate tumour mediated T cell tolerance and anergy.

The anti-PD-L1 mAb¹⁴⁰, increases CD8 T cell cytotoxicity and IFN γ secretion *in vitro* and inhibits the growth of the murine P815 tumour *in vivo*.. This response is specific as the anti-PD-L1 mAb did not have any significant effect on the growth of B16 melanomas which do not express PD-L1¹⁴¹. A separate study demonstrated that PD-1^{-/-} T cells increased cytokine production, proliferation and cytolytic activity in response to PD-L1 expressing tumour cell lines¹⁴².

A recent phase I clinical trial of a humanised anti-PD-L1 mAb in patients with mixed advanced metastatic cancers produced a durable complete response in 1/39 patients and partial responses in 2/39 patients¹⁴³.

Together these reports suggest that blockade of PD-L1 is therapeutically advantageous.

1.5.4 : Anti-GITR (DTA-1)

GITR is expressed at high levels on activated CD4 and CD8 T cells and Treg cells as well as at low levels on resting CD4 and CD8 T cells^{144, 145}. GITR-L is expressed on murine B cells, DC and macrophages¹⁴⁶. Ligation of GITR on Treg cells with DTA-1, an agonistic anti-GITR mAb, abrogates suppressive function of Treg cells¹⁴⁷. Whereas, interestingly, engagement of GITR with DTA-1 mAbs or its natural ligand (GITR-L) activates resting T cells and causes up-regulation of CD25, CD69 and expression of IFN γ , IL-2, IL-4 and high amounts of IL-10¹⁴⁸.

Administration of DTA-1 mAbs to mice eliminates established tumours, although it is unclear if DTA-1 abrogates Treg suppression or activates CD4 and CD8 effector cells¹²⁶. Further experiments with the B16 model showed that DTA-1 enhances vaccine-induced CD8 T cell responses¹⁴⁹.

The suppressive effects of Treg cells are contact-dependent and activated at the site of antigen presentation, which in cancer patients is predominantly the tumour site. The local reversal of suppression, therefore, is a promising target for the treatment of established tumours. The potential ability of DTA-1 to reverse suppression and concomitantly activate CD4 and CD8 T cells¹⁴⁷ make DTA-1 a promising mAb to develop for the treatment of cancer^{126, 147, 149, 150}.

1.5.5 : Adoptive Immunotherapy

As described at the beginning of this Introduction, the clinical application of adoptive immunotherapy has met with limited success, probably because of immuno-suppressive mechanisms in the tumour microenvironment. To try and improve the clinical application of adoptive immunotherapy, numerous murine models have been developed that have offered some useful insights.

1.5.6 : T cells engineered to express an antigen specific TCR

In tumour-bearing hosts, the induction of CD8 T cell tolerance and anergy is very common, representing one of the major challenges to successful adoptive immunotherapy¹⁵¹. A

promising strategy to optimise adoptive immunotherapy is to use antigen-specific CD8 T cells genetically modified to counteract tumour mediated suppression¹⁵².

Three independent pre-clinical animal studies using T cells with genetically modified TCRs have demonstrated that this methodology can cause tumour rejection.

Abad *et al.* used T cells expressing a genetically engineered TCR, designated pmel1, that recognises the gp100₂₅₋₃₃ peptide (expressed by the B16 melanoma) presented in the context of H2Db¹⁵³. The adoptive transfer of these modified T cells, into mice with B16 melanomas, successfully reduced the growth of tumours in a dose dependent manner. When unmodified lymphocytes were added concurrently with the pmel1 cells they abrogated tumour inhibition¹⁵⁴.

These results were repeated and confirmed by DeWitte *et al.*, who demonstrated that an increasing number of unmodified lymphocytes inhibits the anti-tumour function of co-transferred genetically engineered OT-1 T cells that recognise the RIP-OVA antigen expressed on B16-OVA cell line¹⁵⁵. In the same set of experiments, irradiation was found to boost the anti-tumour response more efficiently than vaccination. DeWitte *et al.* also demonstrated that a small increase in the efficiency of transduction, leading to better expression of the TCR, resulted in a significantly better anti-tumour response¹⁵⁶. A complementary study by Wrzesinski *et al.* confirmed the importance of myeloablation by irradiation before adoptive T cells transfer¹⁵⁷.

Both of these experiments used immunogenic transplantable tumours which may not correlate with the clinical situation where tumours are naturally arising and express normal self antigens. However similar results have been obtained in a system with a 'spontaneously' arising tumour: De Witte *et al.* generated Transgenic Adenocarcinoma of Mouse Prostate (TRAMP) mice which express the transforming SV40 large T antigen under the control of the prostate specific probasin promoter. In these mice the SV40 antigen is expressed as self so the TRAMP mice are tolerant to it. Subsequently, male TRAMP mice develop 'naturally' arising prostatic neoplasia between 8 and 12 weeks of age. Autologous T cells isolated and transduced with a TCR specific for an epitope from the SV40 antigen were able to cause tumour remission upon adoptive transfer to the TRAMP mice. Genetically modified T cells alone reduced tumour progression however, for long term tumour remission vaccination was also required¹⁵⁸.

Together these experiments demonstrate that it is highly feasible to expect tumour regression after therapy using genetically modified T cells expressing a tumour-specific TCR, even in animals tolerant to the antigen. They also suggest that preconditioning with radiotherapy and boosting with vaccination are essential for effective tumour remission.

These observations are repeated in numerous studies of adoptive T cell transfer that have used sub-lethal irradiation of between 4 – 8Gy to boost anti-tumour responses¹⁵⁹⁻¹⁶¹. Irradiation enhances T cell adoptive transfer because it reduces the number of suppressive Treg, increases antigen shedding from tumours and increases the amount of ‘space’ and cytokine availability by depleting competing T cells, all of which combined mean that adoptively transferred T cells have better anti-tumour potential¹⁶².

1.5.7 : Gene transfer to T cells aimed at enhancing co-stimulation and migration to tumour tissues

Tumours secrete suppressive cytokines and express low levels of pMHC-I and costimulatory molecules. One approach to overcome this form of suppression is to create genetically modified T cells that express high levels of costimulatory molecules such as CD28. For example, Haynes *et al.* generated a chimeric receptor by fusing the antigen binding fragment of the anti-erbB mAb to the hinge region of CD8 and the transgenic and intracellular signalling domains of the human TCR- ζ chain (scFv-antierbB2- ζ). They compared this chimeric receptor with a similar receptor that has the addition of the transmembrane region and cytoplasmic region of CD28 (scFv-antierbB2- ζ -CD28). The adoptive transfer of the engineered T cells into SCID mice with Colo 205 tumours led to complete eradication of the tumours from 7 out of 10 mice. The anti-tumour response was even evident when tumours that had been established for 3 days were treated¹⁶³.

Weakly immunogenic tumours may not cause sufficient inflammation and cytokine release to attract tumour antigen-specific T cells, thus modifying T cells to increase their sensitivity or enhance their responses to mitogens could increase anti-tumour responses after adoptive therapy. For example, IL-8 (CXCL8) is a chemokine that acts as an autocrine factor for melanoma, liver and pancreatic tumours and it is expressed by melanoma cells¹⁶⁴. Genetically modified T cells that can migrate in response to chemokines may be able to infiltrate the tumour and exhibit beneficial anti-tumour effects. Indeed, Kershaw *et al.* identified a cytokine designated Gro- α (the receptor for which is CXCR2) that is secreted from melanoma cells. When they transduced T cells with CXCR2 the T cells were able to preferentially migrate towards the chemokine and it also stimulated them to produce IFN γ . Although the group were not able to directly show that the modified T cells had enhanced anti-tumour properties they demonstrated a unique way of modifying T cells that may have potential applications in adoptive immunotherapy¹⁶⁵.

1.5.8 : Genetic T cell modification by over-expressing exogenous cytokines

Following activation, T cells respond to cytokines that can reduce their activation threshold, enhance their cytotoxicity and prevent activation induced cell death. In the clinical trials previously described, IL-2 is the cytokine of choice to enhance adoptive T cell therapy. However, because of the requirement of IL-2 for Treg suppression as well as conventional CD4 and CD8 proliferation, it may also have deleterious effects on anti-tumour responses. IL-15 represents a rational alternative to IL-2 because it elicits similar responses from conventional T cells, i.e. prolonged survival, without enhancing suppressive Treg capacities⁹².

Overwijk *et al.* found that IL-2 administration, combined with peptide vaccination and adoptive transfer of pmel1 T cells (gp100₂₅₋₃₃ peptide specific), led to long term remission in mice with established B16 melanomas. However, they did note that even this triple combination did not always completely cure mice¹⁵³.

A study by Oelert *et al.* demonstrated that both preconditioning with irradiation and the presence of IL-15 are required to cause tumour rejection in mice adoptively transferred with antigen specific TCR transgenic T cells¹⁶⁶. A result that is corroborated by a study from Klebanoff *et al.* in which pmel TCR transgenic mice were crossed with human IL-15 transgenic mice to create tumour specific T cells that express hIL-15. These double transgenic T cells were efficient at inhibiting growth of B16 tumours. Moreover, pmel cells that were cultured in IL-15, before adoptive transfer, were significantly better at reducing tumour growth than those cultured with IL-2¹⁶⁷.

1.5.9 : Adoptive T cell therapy using in vitro activated CD8 T cells

The studies using the Pmel1/B16 melanoma model also revealed that the anti-tumour efficiency of adoptively transferred CD8 T cells is critically dependent on their differentiation status, with progressive differentiation inversely correlated with *in vivo* anti-tumour effectiveness¹⁶². CD8 T cells, that have been fully differentiated *in vitro*, show profoundly impaired anti-tumour activity *in vivo*¹⁶⁸. A similar pattern is seen in the CD8 memory compartment whereby tumour-specific central memory CD8 T cells (T_{CM}) are

superior to effector memory cells (T_{EM}) for causing the regression of established B16 melanomas¹⁶⁹. This is because the fully differentiated T cells have reduced expression of lymphoid-homing and costimulatory molecules, lose the ability to produce IL-2 and to respond to homeostatic cytokines, and have decreased survival and proliferative capacity *in vivo*¹⁶². It is therefore very important to consider the activation conditions and phenotype of T cells before their adoptive transfer.

1.5.10 : Adoptive T cell therapy using CD4 T cells

CD4 T cells are critical for anti-tumour responses against a variety of murine tumours¹⁷⁰⁻¹⁷³. The ability of CD4 T cells to cause tumour rejection is largely based on their ability to secrete IFN γ ^{171, 174, 175}. Despite the ability of CD4 T cells to cause tumour rejection alone or in combination with other treatments, the field of CD4 adoptive immunotherapy has not advanced very far. Basic^{168, 170, 176} and clinical studies^{13, 16, 19} have concentrated on the adoptive transfer of CD8 T cells alone or in some cases with CD4 T cells^{170, 173} or CD4 peptide vaccines¹⁷⁷.

Mumberg *et al.* 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 IFN γ ¹⁷⁴. This result was confirmed by Perez-Diez *et al.* who compared the anti-tumour efficiency of TCR transgenic CD4 and CD8 T cells directed at the male HY antigen expressed by MB49 tumours. This study suggested that CD4 T cells were better than CD8 T cells at causing tumour rejection¹⁷⁸.

CD4 T cells can be differentiated *in vitro* into Th17 cells. Th17 cells produce pro-inflammatory cytokines including IL-17A, IL-6, and Granulocyte-Colony Stimulating Factor (G-CSF) which activate other immune cells including neutrophils leading to local inflammation¹⁷⁹. Muranski *et al.* demonstrated that tumour specific Th17 cells are more effective than Th1 cells at destroying established B16 melanomas¹⁸⁰.

It has become clear that a wide variety of factors influence the anti-tumour effects of adoptively transferred T cells and these factors need to be carefully considered before clinical adoptive immunotherapy can be successful.

1.6 : Hypothesis

Fundamental to this thesis is the use of the MB49-HY murine tumour system; the same as that used by Perez-Diez *et al.*¹⁷⁸. This system was chosen because of the availability of CD4 (Marilyn) and CD8 (MataHari) T cells that respond to male HY antigen expressed on the MB49 tumour and male tissues (detailed in Chapter 3). Taking advantage of extensive experience of anti-HY T cell studies in this laboratory¹⁸¹⁻¹⁸⁸, and the availability of CD4 and CD8 tumour specific T cells, it has been possible to address some of the key issues relating to the development of successful adoptive T cell cancer immunotherapy.

Previous studies have suggested that MB49 tumours are rejected in female mice when mice are inoculated s.c. with 1×10^5 cells but that tumours develop when the number of cells is increased¹⁸⁹ to 7×10^5 . This suggests that although female C57BL/6 mice are capable of eliciting an immune response to HY antigen derived from the MB49 tumour the response is suppressed when more MB49 cells are used. We hypothesised that MB49 tumour cells were capable of immunoevasion. And so, to establish a mechanism by which immunoevasion may be happening we will test a panel of cell surface markers that have been implicated in tumour escape from the immune system including PDL-1 and PDL-2 as well as common co-stimulatory molecules CD80, CD86 and CD40.

Low numbers of HY⁺ MB49 cells are rejected by female mice suggesting that a T cell mediated antigen specific immune response is active in these mice and therefore we expect to be able to detect CD4 and CD8 tumour infiltrating lymphocytes (TIL). We will deplete CD4, CD8 and Treg cells from tumour bearing mice to evaluate the relative contributions of the various T cell subsets to tumour growth. We expect that depletion of CD4 and CD8 effector T cells will enhance, and depletion of regulatory T cells will inhibit, tumour growth.

Cytokines that enhance or inhibit the immune response to tumour can have a significant impact on tumour growth. We have the reagents to test the effect of IL-10, IL-15, IL-17, IFN γ and TGF β on the growth of the MB49 tumours in C57BL/6 mice.

MB49 tumours, growing in female C57BL/6 mice, induce tumour infiltrating cells to produce IL-10 which in turn inhibits Th1 responses and decreases the survival time of the mice when compared to IL-10KO C57BL/6 female mice¹⁹⁰. We predict that using the same system, i.e. IL-10 knockout¹⁹¹ C57BL/6 mice with MB49 tumours we will reproduce the results of Halak *et al*¹⁹⁰. To confirm these results we will also use an IL-10R blocking antibody (1B1.3a)¹⁹² to inhibit IL-10 signalling in MB49 tumour bearing mice.

IL-15 causes the activation and differentiation of CD8 T cells which enhances anti-viral immunity^{66, 93}. Additionally, antigen specific T cells expressing transgenic IL-15 have been shown to inhibit the growth of B16 melanomas¹⁶⁷. Taking advantage of the availability of hIL-15 transgenic (Tg) mice¹⁹³, the kinetics of MB49 growth in WT versus Tg mice will be compared. We predict that the growth of MB49 tumours will be inhibited in IL-15Tg mice when compared to WT mice.

As described later, in section 3.1.3.5, there is a debate over whether IL-17A is pro-tumour or anti-tumour¹⁹⁴ because IL-17A enhances the function of cytotoxic T cells but also promotes angiogenesis and metastasis. IL-17A KO¹⁹⁵ mice have previously been shown to be more resistant than wild type control mice to the MC38 tumour¹⁹⁶ suggesting that IL17A is pro-tumour. We predict that IL-17A KO mice bearing MB49 tumours will react in a similar way.

IFN γ , is a signature cytokine for Th1 cells and it plays a critical role in anti-tumour immunity¹⁹⁷⁻¹⁹⁹. Neutralisation of IFN γ with the mAb R46/A2 inhibits anti-viral and anti-tumour responses¹⁰⁰. We expect that IFN γ will be critical in the immune response to MB49 tumours.

Glucocorticoid Induced TNFR related protein (GITR) is a member of the Tumour Necrosis Factor Receptor (TNFR) family that is expressed on T cells with the primary purpose of protecting them from activation-induced cell death. DTA-1, an antagonistic anti-GITR mAb, has been shown to cause tumour rejection in numerous murine models^{147, 149, 200, 201} and has the potential to be an extremely effective treatment for a variety of different cancers. Although administration of DTA-1 can eliminate established tumours it is unclear whether DTA-1 abrogates Treg suppression or activates CD4 and CD8 effector cells¹²⁶. We will, for the first time, evaluate the effect of DTA-1 in the MB49 tumour system. Additionally, we will investigate which T cell population is affected by the DTA-1 mAb.

We surmised that the adoptive transfer of Marilyn CD4 T cells would be more effective than the adoptive transfer of Matahari CD8 T cells at causing the rejection of MB49 tumours in immunodeficient (Rag^{-/-}) and immunocompetant (Rag⁺) mice based on the study by Perez-Diez *et al.*¹⁷⁸. CD8 T cells that are effective at killing tumour cells *in vitro* are not necessarily effective at killing the same tumour cells *in vivo*. However CD4 T cells are multi-potent cells that can directly kill tumour cells, *in vitro* and *in vivo*, activate antigen presenting cells and NK cells. To test this hypothesis HY-antigen specific, Marilyn CD4⁺ or Matahari CD8⁺ T cells will be adoptively transferred into MB49 tumour bearing mice and the growth of the tumour will be analysed. We will also study the effect of

combined therapies involving the use of T cell preconditioning and host irradiation to optimise the adoptive transfer of T cells.

Regulatory T cells represent a significant barrier to effective adoptive T cell immunotherapy for cancer as evidenced by reports that *in vivo* depletion of nTreg cells improves anti-tumour responses and enhances the efficiency of therapeutic cancer vaccines^{125, 126}. We will design experiments using Marilyn Treg to study the expansion and distribution of antigen specific Treg cells in the MB49 system. We predict that antigen specific Treg will potentially inhibit T cell responses to the MB49 tumour, potentially by direct cell-cell contact within the tumour.

1.7 : Aims of this PhD project

The aim of this study was to address some of the key questions that remain unanswered in the quest for an effective tumour immunotherapy. These questions were:

1. What is the most effective cell type to transfer; CD4 or CD8?
2. Within the CD4 and CD8 compartments which is the more potent subset; naïve, activated, EM or CM, Th1 or Th17?
3. Does pre-conditioning with irradiation increase anti-tumour reactivity of adoptively transferred T cells?
4. Are tumour-specific CD8 T cells, that over-express IL-2 or hIL-15, better at clearing tumours than tumour-specific CD8 T cells not over-expressing these cytokines?
5. What are the roles played by Treg cells in anti-tumour immunity?
6. Can anti-GITR mAb (DTA-1) be used for cancer immunotherapy?

Chapter 2 : Materials and Methods

The materials and methods used in this project are detailed in this chapter. Firstly, buffers and chemical reagents are described, followed by the protocols used. The precise details (i.e. conjugated antibodies used for Fluorescence Activated Cell Sorting (FACS) and flow cytometry) of a protocol used for each section is described in the results.

2.1 : Media

Table 3. Media, use and composition

Medium	Use	Composition
Ammonium Sulphate Solution	Purification of mAb from hybridomas	4.1M (NH ₄) ₂ SO ₄ in dH ₂ O
Blood Buffer	Collection of blood	PBS, 100U/ml Heparin, 2mM EDTA
Complete culture medium	Maintenance of hybridomas / tumour cell lines / <i>in vitro</i> cultures	RPMI 1640 Medium, supplemented with 10% FCS, 10mM HEPES, 100 IU/ml Penicillin, 100µg/ml Streptomycin, 2mM L-glutamine and 2x10 ⁻⁵ M β2-Mercaptoethanol*.
Freezing Medium	Storing cells in liquid nitrogen	8ml complete medium, 1ml DMSO, 1 ml 20% FCS
MACS® buffer	Washing of single cell suspensions before MACS/FACS	PBS, 2mM EDTA, 1% FCS
PBS	Resuspending of cells/reagents for <i>in vivo</i> use	NaCl, 137mmol/L; KCl, 2.7mmol/L; Na ₂ HPO ₄ , 8mmol/L; KH ₂ HPO ₄ , 2mmol/L. pH 7.3. Oxoid, Hampshire, UK.
RBC lysis buffer (Qiagen, Hilden, Germany).	Depletion of red blood cells before counting/FACS	Unspecified concentrations of NaCl, EDTA and Sodium Bicarbonate

Abbreviations: FACS; Fluorescence Activated Cell Sorting, PBS; Phosphate Buffered Saline, RBC; Red Blood Cell. *Ingredients of complete culture medium (Invitrogen, Paisley, UK) except for FCS (Autogen Bioclear, Calne, UK).

2.2 : Mice

The mice used in this project were housed under Special Pathogen-Free conditions in the Biological Services Unit, Clinical Sciences Centre, Hammersmith Hospital and used in accordance with Home Office guidelines as defined in the project licence. All the mice used in this project were on a C57BL/6 background.

Table 4. Mice, strain, phenotype and primary reference

Strain	Phenotype	Reference
C57BL/6	Wild type, Thy1.2 ⁺	202, 203
C57BL/6 Thy1.1 ⁺	Wild type, Thy1.1 ⁺	202
Rag2 ^{-/-}	No TCR rearrangement and subsequently no B cells or T cells	204
Marilyn	TCR transgenic for Dby-A ^b	205
MataHari	TCR transgenic for Uty-D ^b	206
hIL-15Tg	Transgenic over-expressing human IL-15	193
dnTGFβIIIR	Transgenic for dominant negative TGFβ receptor type II	207
FoxP3-GFP	Foxp3-reporter mice	208
IL-10 KO	IL-10KO	191
IL-17 KO	IL-17KO	195

Thy-1.2⁺ Wild Type (WT) C57BL/6 (B6) mice are an inbred strain produced in 1921 by Clarence Little. The MHC haplotype of B6 is H-1^c, H-2^b, H-3^a. B6 have a high total leukocyte count²⁰² and a low incidence of spontaneous tumour development²⁰⁹ and are therefore an ideal strain for the study of immunology and tumour development. B6 were purchased from Harlan Laboratories (Bicester, UK).

Thy-1.1⁺ C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME).

Marilyn mice are HY-specific Rag2-deficient TCR-transgenic on a C57BL/6, H2^b background kindly provided by Dr O. Lantz (Institut Curie, Paris, France). Marilyn mice express a transgenic V β 6 T-cell receptor (TCR) isolated from a CD4 T cell that recognised the HY/Ab/Dby peptide (NAGFNSNRANSSRSS) presented by the class II molecule H2-A^{b205}. Marilyn mice were originally on a Rag2^{-/-}Thy-1.2⁺ background. Thy-1.2⁺, Thy-1.1⁺ and Thy1.1⁺1.2⁺ Marilyn mice on either a Rag2^{+/-} or a Rag2^{-/-} background were produced after crossing with Thy-1.1⁺ B6 mice.

MataHari mice are HY-specific Rag-deficient TCR-transgenic mouse lines on a C57BL/6, H2^b background also provided by Dr O. Lantz (Institut Curie, Paris, France). MataHari mice express a transgenic V β 8.3 TCR isolated from a CD8 T cell specific for the HY/Db/Uty peptide (WMHHNMDLI) restricted by the class I molecule H2-D^{b206}. MataHari mice were originally on a Rag1^{-/-}Thy-1.2⁺ background. Thy-1.2⁺, Thy-1.1⁺ and Thy1.1⁺1.2⁺ MataHari mice on either a Rag1^{+/-} or a Rag1^{-/-} background were produced after crossing with Thy-1.1⁺ B6 mice.

Dominant-negative TGF β IIR (dnTGF β IIR) mice express a dominant negative form of TGF β IIR that has a truncated intracellular kinase domain. Despite being able to bind all three isoforms of TGF β the receptor cannot mediate signal transduction²⁰⁷. The dnTGF β IIR mice on a C57BL/6 background were kindly provided by Richard Flavell (Yale University School of Medicine, New Haven, CT). dnTGF β IIR mice were mated with Rag2^{-/-} Marilyn mice to produce F1 mice, from which double-transgenic naïve (CD4⁺ V β 6⁺ CD25⁻ dnTGF β IIR⁺) or nTreg (CD4⁺ V β 6⁺ CD25⁺ dnTGF β IIR⁺) T cells were purified.

IL-15 transgenic (IL-15Tg) mice over-express human IL-15 (hIL-15). The IL-15Tg mice were generated on a C57BL/6 background and kindly provided by Thomas A. Waldmann *et al.* (National Cancer Institute, Bethesda, MD)¹⁹³. IL-15Tg mice were mated with Rag2^{-/-} MataHari mice to produce F1 mice, from which double-transgenic (CD8⁺ V β 8.3⁺ IL-15Tg) T cells were purified.

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)¹⁹¹. 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)¹⁹⁵.

Rag2^{-/-} mice do not express recombination activating gene 2 (Rag2) which is essential for T and B cell development. Rag2^{-/-} mice do not have T or B cells. Rag2^{-/-} mice on a C57BL/6 background were originally produced by Fredrick Alt *et al.* (Columbia University, NY)²⁰⁴. A Colony of Rag2^{-/-} mice were produced by us during the mating of Rag2^{-/-} Marilyn females with WT B6 males.

Foxp3/green fluorescent protein (GFP) knock-in mice on a B6 background were generated and kindly provided by Dr B Malissen *et al.* (Centre d'Immunologie de Marseille-Luminy, Marseille, France)²⁰⁸. FoxP3/GFP mice were mated with Rag2^{-/-} Marilyn mice to produce F1 mice, from which double-transgenic nTreg cells (Mar-nTregGFP+) were purified.

2.3 : Peptides

The HY-A^b-Dby peptide, NAGFNSNRANSSRSS¹⁸⁶, and the HY-D^b-Uty peptide, WMHHNMDLI¹⁸⁵, 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 : Marilyn and MataHari T cells

CD4⁺ Marilyn and CD8⁺ MataHari T cells were purified, from *in vitro* culture or from single cell suspensions of pooled spleen and lymph nodes, using MACS® cell purification kits (Miltenyi Biotec, Surrey, UK). These kits use beads (MACS beads), linked to antibodies, and magnetic columns [MS (medium) or LS (large) columns were used in this study] to isolate cell populations from mixed cell suspensions. Single cell suspensions were created using the end of a 1ml syringe plunger to force spleen and lymph nodes through a 70µm nylon cell strainer (Becton Dickinson, Oxford, UK). Single cell suspensions were maintained in MACS buffer (PBS, 2mM EDTA, 1% FCS), pelleted by centrifugation at 400g for 5mins and washed once with MACS buffer.

Cells harvested from *in vitro* culture were washed once and re-suspended in MACS buffer. 20µl of anti-CD4- (for Marilyn T cells) or anti-CD8- (for MataHari T cells) MACS beads were added to the washed cells and incubated for 20mins at 4°C. Samples were washed once, re-suspended in 1ml MACS buffer and then added to an MS or LS MACS separation column. The column was washed three times with MACS buffer to remove the negative fraction. The positive fraction was isolated by removing the column from the magnet and flushing with at least 8ml MACS buffer. The positive fraction was washed twice in PBS before being re-suspended in PBS for i.v. injections or culture medium for *in vitro* work.

2.4.2 : Regulatory T cells

To purify CD4⁺CD25⁺ Treg cells, a single cell suspension was made with pooled spleen and lymph nodes from a Rag^{+/-} female Marilyn mouse (as described in the last section). 1µl of anti-CD25-PE was added per sample and incubated for 15 mins at 4°C. Each sample was washed with MACS buffer then 20µl anti-PE microbeads were added for 20mins at 4°C. Samples were each washed and re-suspended in 1ml MACS buffer and added to an MS or LS separation column. The column was washed three times with MACS buffer and the negative fraction collected and used to generate CD25 depleted conventional CD4 T cells. The positive fraction was isolated by removing the column from the magnet and flushing with at least 8ml MACS buffer.

To obtain a more pure population of Treg, the CD25⁺ fraction was stained with 1µl of anti-CD4-PE/Cy5, anti-Vβ6^{FITC} and anti-CD25^{PE} per sample. Cells were then sent to the FACS facility at the Clinical Research Centre, Hammersmith Hospital for sorting. CD4⁺Vβ6⁺CD25⁺ Marilyn nTreg, GFP⁺CD4⁺Vβ6⁺CD25⁺ green Marilyn nTreg or CD4⁺CD25⁺ polyclonal Treg cells were purified to greater than 98% purity using a FACS Aria (Becton Dickinson).

2.4.3 : Antigen presenting cells (APC)

Spleen and/or lymph nodes were diced using a Size 22 scalpel and the resulting pulp was added to a 50ml Falcon tube. 2 mg/ml Collagenase XI and 10 µg/ml DNase in 1ml PBS with 2% FCS was added and incubated on a shaker at 37°C for 30mins. 100µl 0.1M EDTA

was added for a further 5 mins. Then 10µl/spleen anti-CD11c microbeads were added and incubated for 20mins at 4⁰c. Labelled cells were washed twice in MACS buffer and separated on an LS column as previously described. The positive fraction was washed once in culture medium and used for culture. The negative fraction was incubated with anti-CD11b microbeads and separated as described. The CD11b⁺ fraction was used for cell culture and the negative fraction was incubated with anti-B220 microbeads and B220⁺ cells were isolated and used for culture.

2.5 : Proliferation assays

2.5.1 : Peptide specific T cell proliferation assay

Single cell suspensions from spleen and lymph nodes were cultured with complete medium with or without HY peptide and with or without cytokines. On day 3, 0.5µCi/well of tritiated thymidine (New England Nuclear) was added. 16 hours later cells were harvested onto a filter mat and analysed using a Wallac Trilux 1450 Microbeta liquid scintillation and luminescence counter (PerkinElmer, Beaconsfield, UK).

2.5.2 : Mixed Lymphocyte Response Assay

CD11c, CD11b or B220 APC were purified by MACS and plated at a defined concentration in 100µl in a 96 well U-bottomed tissue culture dish (Falcon) using a 200µl pipette. Single cell suspensions of spleen and lymph nodes were cultured with complete medium with or without HY peptide and with or without cytokines. On day 3, 0.5µCi/well of tritiated thymidine (New England Nuclear) was added. 16 hours later cells were harvested onto a filter mat and analysed using a Wallac Trilux 1450 Microbeta liquid scintillation and luminescence counter (PerkinElmer).

2.6 : Flow cytometry analysis

Cells were prepared for Flow Cytometric analysis as follows: Cells were depleted of red blood cells using 2ml of red cell lysis (Qiagen) buffer incubated for 15mins at room

temperature. For the analysis of surface markers, cells were washed and incubated with conjugated antibodies (eBioscience, Hatfield, UK or R&D Systems, Abingdon, UK) for 15mins at 4⁰C in 100µl PBS. The amount of antibody used to detect each marker was determined in preliminary experiments. Cells were washed once with MACS buffer and analysed using a FACSTTM Calibur (Becton Dickinson). For tetramer staining, cells depleted of red blood cells were incubated with tetramer conjugated with PE for 15mins at 37⁰C in 100µl complete medium, followed by staining with anti-CD8^{PerCP} at 4⁰C. Cells were washed once with PBS and analysed. For intracellular cytokine staining, T cells were cultured in complete medium with 0.01µg/ml PMA and 0.05µg/ml Ionomycin, after 2hrs 0.5µg/ml Brefeldin (BD Biosciences, Oxford, UK) was added for 2 hours (hrs). At the end of the incubation, live cells were isolated by layering harvested cells on Ficoll and centrifuging for 20mins at 400g. Live cells were lifted from the top layer of Ficoll and washed once with PBS, before incubation with conjugated cell surface antibodies as described above. After staining the surface markers, cells were washed with MACS buffer. After washing, 0.3ml Fix/Perm (eBioscience) was added per sample and cells were incubated at 4⁰C for 1hr. Cells were washed once with Perm buffer (eBioscience) prior to incubation with 0.5µl/sample conjugated anti-cytokine antibody for 15mins at 4⁰C. Fixed and stained cells were washed once with Perm buffer to remove any excess intracellular antibody and then once with cold PBS before being analysed.

2.7 : *Carboxyfluorescein Succinimidyl Ester* dilution assay

To study proliferation, T cells were isolated from the spleen and lymph node or directly from culture. After purification, using MACS or FACS, as described above, cells were washed three times with PBS and then resuspended well, in 1ml PBS. 1 µM of CFSE (Molecular Probes, Cambridge, UK) was added into cell suspension, and the cells were incubated for 10mins at room temperature. At the end of the incubation cells were washed once with MACS buffer and twice with PBS before being re-suspended in complete medium for *in vitro* culture or in PBS for i.v. injection.

After *in vitro* culture or *in vivo* passage T cells were harvested and stained with antibodies against cell surface markers or intracellular cytokines as previously described. The dilution of CFSE and thus T cell division, was analysed using a FACSTTM Calibur.

2.8 : Antibodies

Monoclonal antibodies were purified from hybridomas that were grown in 500ml of complete medium in 1L roller bottles rotating at 0.02rpm at 37°C for 10 days. Cells were removed from the supernatant by centrifugation (2000 r.p.m., 10 mins, RT). Cell free supernatant was then removed and slowly mixed 1:1 with saturated ammonium sulphate solution (VWR, Lutterworth, UK) then mixed with a magnetic stirrer at 4°C overnight. The resultant precipitate was pelleted by centrifugation at 2000g for 30mins. The pellet was resuspended in 10ml PBS and put in dialysis tubing sealed at both ends. The dialysis tubing was floated in 4L PBS and stirred overnight at 4°C. The PBS was replaced and stirred overnight again. Purified antibody was isolated from the dialysis tubing and centrifuged at 400g for 10mins, to remove any remaining cell particulates. The supernatant was filter sterilised and used i.p. *in vivo* or further purified using a protein AG column (ThermoFisherScientific, UK).

To further purify antibodies, 10ml of supernatant was concentrated using a Centricon YM-10 centrifugal filter, as per manufacturers instructions (Millipore, Southampton, UK). Antibody was purified from 2ml of concentrated supernatant using a Protein A/G spin kit as per the manufacturers instructions (ThermoFisherScientific). The concentration of purified antibody was calculated using a BCATM Protein Assay Kit (ThermoFisherScientific).

Table 5. Hybridomas.

Antibody/Hybridoma	Brief Description	Reference
DTA-1	Rat anti-mouse GITR, IgG2a	145
1D11	Anti-TGFβ	210
PC61	Anti-CD25	211
1B1.3a	Anti-IL-10R	192
GL113	Anti-Galactosidase, control mAb	
YTS169.4	Anti-CD4	212
YTS191	Anti-CD8	213
R46A/2	Anti-IFNγ	100

2.9 : Skin grafting

Skin grafting was conducted using a method previously described by Billingham and Medawar²¹⁴ using tail skin grafted onto the lateral thorax.

Donor tail skin was removed and cut into 1 cm² sections. Recipient mice were anaesthetised using Isoflurane (Halocarbon Products Corp.). When the mice reached a surgical plane of anaesthesia 0.26 µg of Metacam (Boehringer Ingelheim GmbH) was injected s.c. Then the mice were stretched out, to ensure easy access to the surgical site, using elastic bands. The selected area was shaved, washed with 70% alcohol and sprayed with Opsite spray dressing. A piece of skin was removed from the right flank to create a graft bed, any excess blood was removed and a 1 cm² tail skin graft was placed in the graft bed. The graft was covered with muslin soaked with Vaseline (VWR). A plaster was then wrapped around the midriff and graft. Mice were put in a warming chamber at 32°C for the plaster to dry and for the mice to recover.

Plasters were removed 7-10 days after grafting and grafts were inspected every other day.

2.10 : Tumour cells

2.10.1 : MB49

MB49 is a murine bladder carcinoma that was generated *in vitro* by culturing explanted bladder from C57Bl/6J mice with 7,12-Dimethylbenz(a)anthracene (DMBA)²¹⁵.

MB49 cells were stored in liquid nitrogen until needed. When needed, one vial was rapidly thawed and washed in pre-warmed complete culture medium (Described in Section 2.1 :). MB49 cells in 10ml complete culture medium were cultured for 10 days. MB49 cells were maintained in culture and split every 3-4 days then used when needed. To harvest MB49 cells, culture medium was removed and excess cells and protein were removed by washing 3 times with PBS. 2ml of Trypsin solution (Invitrogen) was added to flasks and incubated at RT for 10mins. Cells were then removed from the flask and washed once with complete culture medium and then three times with PBS. For *in vivo* experiments MB49 cells were resuspended at 2.5×10^6 cells/ml in PBS and mice were injected, with an insulin needle, with 0.2ml (5×10^5 cells) s.c. to the dorso-lateral flank unless otherwise stated. The area of

the tumour (mm²) (Length (mm) X Width (mm)) was measured at regular intervals using a pair of digital callipers. Data are presented as means +/- Standard deviations unless otherwise stated and group comparisons were done using the two-tailed Students t-test unless otherwise stated.

2.10.2 : B16F10

B16F10²¹⁶ cells were thawed, maintained and injected in the same way as described for MB49 cells.

2.11 : Polymerase Chain Reaction (PCR)

I am indebted to Dr. Caroline Addey for completing this protocol. To examine the expression of HY genes in MB49 and B16-HY cells, RNA was isolated from MB49, B16-HY, C57BL/6 male spleen or female spleen cells by phenol/chloroform extraction using Trizol (Invitrogen) according to the manufacturer's instructions. RNA from MB49 cells was quantitated and converted to complementary DNA (cDNA) by Reverse Transcription-PCR (RT-PCR) using BRL Superscript Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. cDNA was then used in PCRs with primers specific for the HY genes: For PCR, in a final volume of 25 µl, 2µl cDNA was mixed with 0.4 µM forward primer, 0.4 µM reverse primer, 1 U Taq Polymerase (Invitrogen), 1 X reaction buffer (supplied with Polymerase), 2 mM MgCl₂ and 0.4 mM dNTPs. The following specific primers were used (written 5'-3'):

Dby: Forward: CAGCAAGAGGTCTAGACA. Reverse: TCCTCTGTTGTGGCTACT
Smcy: Forward: TGAGACATAAGAAGCCCC. Reverse: GGCTTAATCCTTGAACTC
Uty: Forward: ACTTCGCCATATCCTCCT. Reverse: GTGCTTTCTGTCTCCTTC

The reaction mixture was incubated at 95°C for 5mins then cycled 30 times through the following programme: 95°C for 1min, 52.5°C for 1min, 72°C for 1min, before a final extension step of 72°C for 7mins. PCR products were analysed by agarose gel electrophoresis on a 1% agarose gel containing Safeview (Invitrogen).

To check for the presence/absence of the dnTGFβRII gene in T cells from dnTGFβRII transgenic mice, DNA was isolated from tail tips by incubation in 50mM Tris (pH8), 2mM NaCl, 10mM EDTA, 1% SDS and 1mg/ml proteinase K for 16hrs followed

by incubation at 100°C for 5mins. Extracts were then washed and diluted 1: 100 before addition to PCR.

2.5 µl was diluted in a final volume of 25 µl with 0.4 µM forward primer, 0.4 µM reverse primer, 1 U Taq Polymerase (Invitrogen) 1 X reaction buffer (supplied with Polymerase), 2 mM MgCl₂ and 0.4 mM dNTPs. The following primers were used (written 5'-3'):

Forward: CCCAACCAACAAGAGCTCAGG

Reverse: TTGGGGTCATGGCAAACCTGTCTC

The reaction mixture was incubated at 95°C for 5mins then cycled 5 times through the following programme: 94°C for 1min, 60°C for 1min, 72°C for 1min, before a final extension step of 72°C for 10 mins. PCR products were analysed by agarose gel electrophoresis on a 1% agarose gel containing Safeview. The PCR product in dnTGFβIIR⁺ samples is 350bp.

2.12 : IL-15 Transgenic Mouse Typing

I am indebted to Dr. Caroline Addey for completing this protocol. IL-15 KO mice were typed using an IL-15 ELISA from R&D Systems following the manufacturer's instructions.

2.13 : Lipofectamine transfection of Phoenix cells

I am indebted to Dr. Caroline Addey for completing this protocol. To transfect MataHari cells with the IL-2 gene, MataHari cells from pooled spleen and lymph nodes were cultured at 2×10^5 cells/well in a 6 well plate with 0.3nM HYUty peptide for three days. Medium was removed 4 hours before transfection, and cells were washed once with PBS. 2ml medium [Complete culture medium, without penicillin (pen) and streptomycin (strep)], was added to each well. Two tubes were prepared: Tube 1 contained 250µl OptiMEM (Serum-Free medium), 3µg MigR1-IL-2, and 1µg helper vector, pCL-Eco. Tube 2 contained 10µl Lipofectamine™ 2000 (Invitrogen) and 250µl OptiMEM. Tube 2 was vortexed and incubated for 5 minutes at 20-25°C. The two solutions were then gently mixed together and incubated for 20mins at 20-25°C. At the end of this incubation, 500µl of the resultant mixture was added to each well of cells and the cells were incubated at 37°C, 5% CO₂, overnight. The medium was then removed and replaced with 5ml of

Complete Culture Medium and cells were incubated for a further 24 hours at 37°C, 5% CO₂. Cells were tested for successful transfection by flow cytometric analysis of GFP expression.

2.14 : Immunofluorescence microscopy

Lymph nodes, MB49 tumours and spleen were removed from MB49-bearing C57BL/6 female mice. Tissues were fixed in 4% Paraformaldehyde and 10% Sucrose for 2 hrs. Tissues were then washed once in PBS before overnight incubation in OCT. Tissues immersed in OCT were flash frozen in cold propanol then transferred to liquid nitrogen and stored at -80°C until needed. For sections, tissues were removed from liquid nitrogen and warmed to -40 °C prior to cutting. 5µm sections were made using a cryostat. Sections were immediately mounted on poly-lysine coated microscope slides. Sections were stored at -20°C until needed.

To analyse the location and number of T cells in the sections, sections were removed from the -20°C freezer and fixed in acetone for 20mins at -20°C. Sections were then washed twice with PBS and incubated with PBS containing 10% v/v FCS, for 20mins at 4°C, to block Fc receptors and reduce non-specific binding. 50µg/ml anti-CD3 (BD) or Isotype antibody/sample was diluted in 100µl PBS and then added directly to the section. Sections were incubated with antibody at RT for 1hr. Sections were washed twice with PBS before a coverslip was mounted using vector shield containing DAPI (Invitrogen). Slides were dried for 1hr at RT before being analysed using a deconvolution microscope (Leica) and mmap software.

2.15 : Analysis of adoptively transferred T cells

Tumour dLNs, ndLNs, spleen and tumours were harvested from mice. The organs were cleaned of excessive fat and tissue before being forced through a cell strainer to create a single cell suspension. Cells to be analysed by flow cytometry were incubated with Red Blood Cell lysis buffer (Qiagen) at RT for 10 mins before being washed once with PBS and stained as described in section 2.6. Cells to be used for culture were washed once in culture medium and plated out at the desired concentrations for each experiment.

Chapter 3 : Endogenous T cell responses against MB49

3.1 : Introduction

3.1.1 : MB49

Minor histocompatibility (H) antigens are the self-peptides derived from polymorphic genes and presented to the immune system via the MHC¹⁸⁷. The molecular composition of minor H antigens share many similarities with that of tumour antigens; both types of antigen are comprised of a limited set of peptide epitopes which derive from variants of self-proteins. Expression of these antigens is often at a low level in normal tissue, resulting in limited cell surface density of pMHC complexes which may be insufficient to trigger T cell activation. T cell responses to both antigen classes are therefore, subject to the same controlling mechanisms and limits. Thus, data obtained from studies on minor H antigen immunity are relevant to tumour immunity.

The male-specific transplantation antigen, HY, is one of the best characterised minor H antigens. Three genes, identified on the Y-chromosome of H2b mice, encode proteins that give rise to the H2-Db- and H2-Ab- restricted peptide epitopes of HY; the H2-Db-restricted peptide epitopes are derived from the *Smcy* and *Uty* genes, while the H2-Ab-restricted peptide epitope is derived from the *Dby* gene^{186, 217}.

The MB49 tumour has been chosen for this study for a number of reasons: It is a murine carcinoma cell line that was generated by incubating bladder tissue, from male C57BL/1crf-a^t mice, with the carcinogen DMBA (7,12-Dimethylbenz(a)anthracene)²¹⁵. Therefore, MB49 is a chemically induced murine bladder carcinoma that, because it was generated from a C57BL/6 male, is H2b⁺ as well as being positive for the three genes that make up the male specific transplantation antigen, HY^{178, 189, 190}.

In addition, unlike many current tumour models using tumours over-expressing 'foreign' proteins such as cOVA²¹⁸ or viral antigens¹³⁰, the HY system employs self tissue-proteins, HY, which are naturally expressed by MB49 cells at a physiological level, thus the results obtained from the HY system are more likely to reflect immune responses against TAA which are largely non-mutated self-proteins. Although MB49 has been shown to express other tumour specific markers for bladder carcinoma, such as the human bladder cancer marker, BCLA-4, the Prostate Cancer Stem Cell Antigen (PSCA) and Six-Transmembrane Epithelial Antigen of the Prostate (STEAP)¹⁸⁹, the primary immune

response in female C57BL/6 mice is against the HY antigen. Additional evidence for the specificity of the female response to the HY antigen, in this system, comes from previous studies that demonstrated that male C57BL/6 mice fail to reject MB49 tumours; suggesting that there are no syngeneic immunogenic antigens expressed by MB49²¹⁹.

Cultured tumour cell lines may change their phenotypes after developing into tumours *in vivo* as variants are selected, by immuno-editing mechanisms (or under pressure of immunosurveillance), that can divide faster, live longer and evade the immune system. As discussed in the Introduction, there are several mechanisms utilised by tumours to suppress immune responses: Generally, tumour cells are poor antigen presenting cells (APC), as they express limited amounts of MHC-I and T cell co-stimulatory molecules. However, tumour cells can express abundant T cell inhibitory molecules including PD-L1¹¹⁵ and B7-H4^{116 117}. The tumour microenvironment also contains immuno-suppressive factors that can be secreted directly from the tumour or from APC and T cells. Immuno-suppressive factors directly secreted from tumours include gangliosides, GM-CSF, IDO¹²⁰, IL-6, IL-10, prostaglandins, TGF β and VEGF²²⁰. IL-10 and TGF β are the two best characterised suppressive cytokines in the tumour microenvironment, as described in Section 1.3 of this thesis.

Currently there is limited published data reporting the expression of MHC-I, MHC-II, co-stimulatory and inhibitory ligands on the surface of the MB49 cell line and the *ex vivo* MB49 tumour. Therefore, together with evaluating the expression of HY antigens by the MB49 cell line and *ex vivo* tumour, it was important to also test the expression of MHC proteins, common tumour-associated cell surface proteins and immuno-suppressive cytokines. The results to these experiments are shown later in Section 3.2.1.

3.1.2 : T cell responses to MB49/HY

Growth of MB49 *in vivo* in C57BL/6 female mice has been tested by intravenous (i.v.), intraperitoneal (i.p.) and subcutaneous (s.c.) injection. Inoculation of MB49 i.v. or i.p. creates metastatic tumours that are not easily quantified and lead to rapid morbidity and mortality^{189, 221}. When administered s.c. to female C57BL/6 mice MB49 is an immunogenic tumour but inoculation of MB49 cells at doses of 7×10^5 and 1×10^6 produces a progressive solid tumour in female C57BL/6 mice¹⁸⁹ and thus demonstrates that the immunogenicity of the tumour is not sufficient for it to be rejected when these high doses of MB49 cells are administered. Consistent with these results, another study showed that

female C57BL/6 mice reject low doses of MB49 (1×10^4) but not high doses (1×10^6)²¹⁹. Moreover, when T cells from female C57BL/6 with large tumours were adoptively transferred to athymic mice those mice were able to reject male skin grafts suggesting that tumour bearing mice do generate a functional immune response to HY antigen from MB49 tumours²¹⁹. Together these results demonstrate that MB49 is highly immunogenic.

Some reports have shown that female C57BL/6 mice do not generate detectable CD8⁺ T cell responses *in vivo* against the Db-Smcy and Db-Uty antigens despite being able to secrete IFN γ in response to both MHC-I antigens and Ab-Dby *in vitro*²¹⁹. However, other reports suggest that female C57BL/6 mice can generate CD8 T cell responses detectable by tetramers¹⁷⁸. This lab is experienced in using tetramers to detect CD8 T cells responses¹⁸⁵ and the reagents are available to test tetramer responses.

Studies in mice have demonstrated that *in vivo* Treg depletion enhances tumour rejection in multiple murine tumour models^{125, 147, 222}. The efficacy of mAb PC61, specific for CD25, is dependent on when it is administered with the best therapeutic results coming from administration 4 days before tumour administration. The effects of PC61 are dependent on a replete immune system with both CD4 and CD8 T cells²²².

In the study described in this thesis, the growth of MB49 tumours was measured and T cell responses, to the tumours, were analysed using tetramers. To investigate the contribution of CD4 and CD8 T cells to the growth of MB49, the CD4 and CD8 subsets of T cells were depleted, individually or in combination, using antibodies (the mAb YTS 191²¹³ is effective at depleting CD8 cells and the YTS 169²¹² antibody can be used to deplete CD4 T cells). To investigate the contribution of Treg to the growth of MB49 female C57BL/6 mice were injected i.p. with CD25 depleting antibody, PC61²¹¹, to remove CD25⁺ T cells. The results of these studies are shown in Section 3.2.2.4.

3.1.3 : Tumour-related Cytokines

Cytokines play a major role in the progression or rejection of tumours and many tumours have been shown to secrete tumourogenic cytokines or to induce surrounding cells to secrete them. A number of knockout mice, transgenic mice or cytokine depleting antibodies are available to study the contribution of IL-10, TGF β , IFN γ , IL-15 and IL-17A to the growth of MB49 tumours.

3.1.3.1 IL-10

IL-10 can modulate T cell responses directly or indirectly via APC⁸¹. Importantly, IL-10 is a key cytokine controlling the development and function of Tr1 cells which are an independent regulatory T cell population, as Tr1 cells do not express Foxp3⁸². IL-10 has been associated with several different forms of cancer including human melanoma²²³⁻²²⁵, small cell lung cancer²²⁶ and renal cell carcinoma²²⁷.

The role of IL-10 in tumour progression has been fiercely debated. There are conflicting reports, some claiming IL-10 inhibits tumour growth²²⁸⁻²³⁰ whilst others claim it promotes tumour growth^{190, 231-233}. Indeed, IL-10 can both inhibit and stimulate CD8 T cells, inhibiting antigen-specific responses in the presence of APC but stimulating responses when combined with IL-2²³⁴.

IL-10 inhibits cellular immune responses and antigen induced proliferation by down regulating MHC- II⁸⁴ and co-stimulatory CD80/86 molecules on monocytes and macrophages⁸⁴⁻⁸⁷. IL-10 inhibits cytokine production by macrophages⁸⁹ as well as inhibiting the ability of APC to prime T cells⁸⁸. IL-10 can also directly inhibit IL-2 production by CD4 T cells⁹⁰. In addition, IL-10 drives the differentiation of the regulatory Tr1 subset of CD4 T cells which in turn inhibit CD4 responses⁸³. However, IL-10 is also pro-inflammatory and may lead to extravasation of T cells and increased numbers of tumour infiltrating lymphocytes.

It has been demonstrated that MB49 tumours, growing in female C57BL/6 mice, induce tumour infiltrating cells to produce IL-10 which in turn inhibits Th1 responses¹⁹⁰. Consistent with this, IL-10 KO mice had prolonged survival after administration of 1×10^6 MB49 cells¹⁹⁰.

Together these studies suggest that IL-10 is a potent cytokine whose involvement in tumour rejection needs to be properly established in relation to adoptive immunotherapy.

To examine the MB49 responses to IL-10, this study used IL-10 knockout mice¹⁹¹, that do not produce any IL-10, and an IL-10R blocking antibody (1B1.3a)¹⁹². The experiments and results are described in Section 3.2.3.1.

3.1.3.2 TGF β

TGF β is an important mediator of suppression which is produced by tumour cells and natural or induced Treg cells⁵⁸. TGF β can convert naïve CD4 T cells to Foxp3-expressing

Treg cells¹⁰⁵. TGF- β is secreted by numerous lung cancer cell lines *in vitro* and can prevent the induction of CD8 mediated responses¹⁰³.

To study the effects of TGF β , a transgenic mouse was created by L. Gorelik *et al.*²⁰⁷, using a gene encoding a dominant negative (dn) form of the TGF β RII²³⁵. T cells from dnTGF β RII are defective in TGF β signalling¹³⁰ and suppression of CD4 and CD8 T cells by TGF β -1, upon stimulation by CD3 and CD28, is abrogated²⁰⁷. Studies in which dnTGF β RII transgenic mice were inoculated i.v. with the poorly immunogenic D5-B16BL/6 melanoma demonstrated that TGF β is critical for the creation of tumour-associated induced Treg which in turn suppress anti-tumour T cell function²³⁶.

A mAb that neutralises TGF β (1D11)²¹⁰ has shown some effectiveness in enhancing anti-tumour responses which are further augmented by the addition of IL-2²³⁷. However, anti-TGF β antibody alone does not inhibit the growth of the BL-6 derived lung epithelial cell line but anti-TGF β does enhance tumour vaccination with a combination of antigen, hepatitis B virus core class II helper peptide, GM-CSF and incomplete Freund's adjuvant²³⁸ indicating that TGF β is involved in the suppression of the immune responses needed for anti-tumour responses.

Both dnTGF β RII transgenic mice and 1D11 mAb were used in this study to test the importance of TGF β for the growth of MB49 tumours. The results of these experiments are shown in Section 3.2.3.5.

3.1.3.3 IFN γ

IFN γ , a signature cytokine for Th1 cells, plays a critical role in anti-tumour immunity¹⁹⁷⁻¹⁹⁹. Neutralisation of IFN γ with the mAb R46/A2 inhibits anti-viral and anti-tumour responses¹⁰⁰. The R46/A2 antibody was used in this study to test the importance of IFN γ in the progress of MB49 tumours. Results are shown in Section 3.2.3.2

3.1.3.4 IL-15

IL-15 is a pleiotropic cytokine that influences both the innate and adaptive immune response. IL-15 plays an important role in the differentiation and survival of NK cells and memory CD8 T cells. IL-15 stimulates DC maturation and NK and CD8 T cell cytotoxicity^{94, 95}. IL-15 promotes the survival and expansion of CD8 T cells^{66, 93} and has been implicated in enhancing the efficacy of adoptively transferred CD8 T cells^{167, 169}. IL-15 has also been

implicated in the *de novo* generation of Treg whilst simultaneously abrogating the effect of Treg on CD4 and CD8 T cell effector function. Together these effects of IL-15 may allow rapid but transient responses against pathogens⁹⁶.

Taking advantage of the availability of human IL-15 (hIL-15) transgenic (Tg) mice¹⁹³ this study investigated the effect of exogenous IL-15 on the development of the MB49 tumour. The results of these investigations are shown in Section 3.2.3.3.

3.1.3.5 IL-17

There is intense debate over whether IL-17 is pro- or anti-tumour, as reviewed by Gopal Murugaiyan and Bhaskar Saha¹⁹⁴: IL-17 promotes cytotoxic T cells which leads to tumour regression, however, IL-17 also promotes angiogenesis and metastasis which leads to tumour growth. Tumour growth leads to hypoxia within the tumour and this stimulates increased angiogenesis which in turn supplies more oxygen and nutrients to the tumour. The enlarged intra-tumour vasculature leads to increased metastasis of tumour cells out of the tumour but it also allows immune cells to infiltrate into the tumour which may enhance tumour rejection.

The majority of IL-17 is derived from Th17 CD4 T cells that secrete IL-17 under the influence of the transcription factor Ror γ ²³⁹ and are generated by the combined actions of TGF β and IL-6²⁴⁰. The frequency of Th17 T cells is increased in several different forms of cancer^{241, 242} and indeed the tumour microenvironment is a source of cytokines, including IL-6, IL-21, IL-23, and TGF β ^{243, 244}, that drive Th17 differentiation.

IL-17A KO¹⁹⁵ mice (on a C57BL/6 background) were used, in this study, to understand whether or not IL-17 is important to the growth of the MB49 tumour *in vivo*. IL-17A KO mice have previously been shown to be more resistant than wild type control to the MC38 tumour¹⁹⁶.

3.2 : Results

3.2.1 : MB49

Since a prolonged *in vitro* culture of MB49 cells could lead to loss of the Y chromosome, these cells were examined, for the expression of HY genes, by RT-PCR: HY gene expression was tested by isolating RNA from either MB49 cells or freshly isolated female B6 splenocytes (as internal negative controls). RNA was amplified by RT-PCR using primers specific for *Uty*, *Smcy*, *Dbp* and *HPRT* genes. As shown in Figure 1A, *Uty*, *Smcy* and *Dbp* genes were clearly present in MB49 cells but not in female B6 spleen cells, indicating that MB49 cells constitutively express all three HY genes encoding the T cell epitopes of interest.

As mentioned in the introduction to this chapter, it has been suggested that cultured tumour cell lines may change their phenotypes after developing into tumours *in vivo*, therefore it was necessary to determine whether the phenotype of MB49 changes *in vivo*. To this end, *in vitro* long term cultured MB49 cells and the cells from an MB49 tumour, freshly isolated from a female C57BL/6 mouse, were analysed for cell surface expression of MHC-I (D^b and K^b), -II (A^b), CD40, CD80 and CD86 molecules.

In vitro cultured MB49 cells expressed a high level of MHC-I, D^b and K^b , but little, if any, MHC-II, A^b , they were positive for CD40 but negative for CD80 and CD86 (Figure 1B).

Ex vivo MB49 cells were isolated from one female mouse 30 days after s.c. inoculation of 5×10^5 MB49 cells. Tumours were dissected and digested with collagenase before staining with antibodies. As shown in Figure 1C, the freshly isolated MB49 cells were found to up-regulate A^b but down-regulate D^b and K^b molecules, in comparison to cultured MB49 cells. Furthermore, fresh isolated MB49 cells had increased CD40, CD80 and CD86 expression (Figure 1C) compared to cultured MB49 cells.

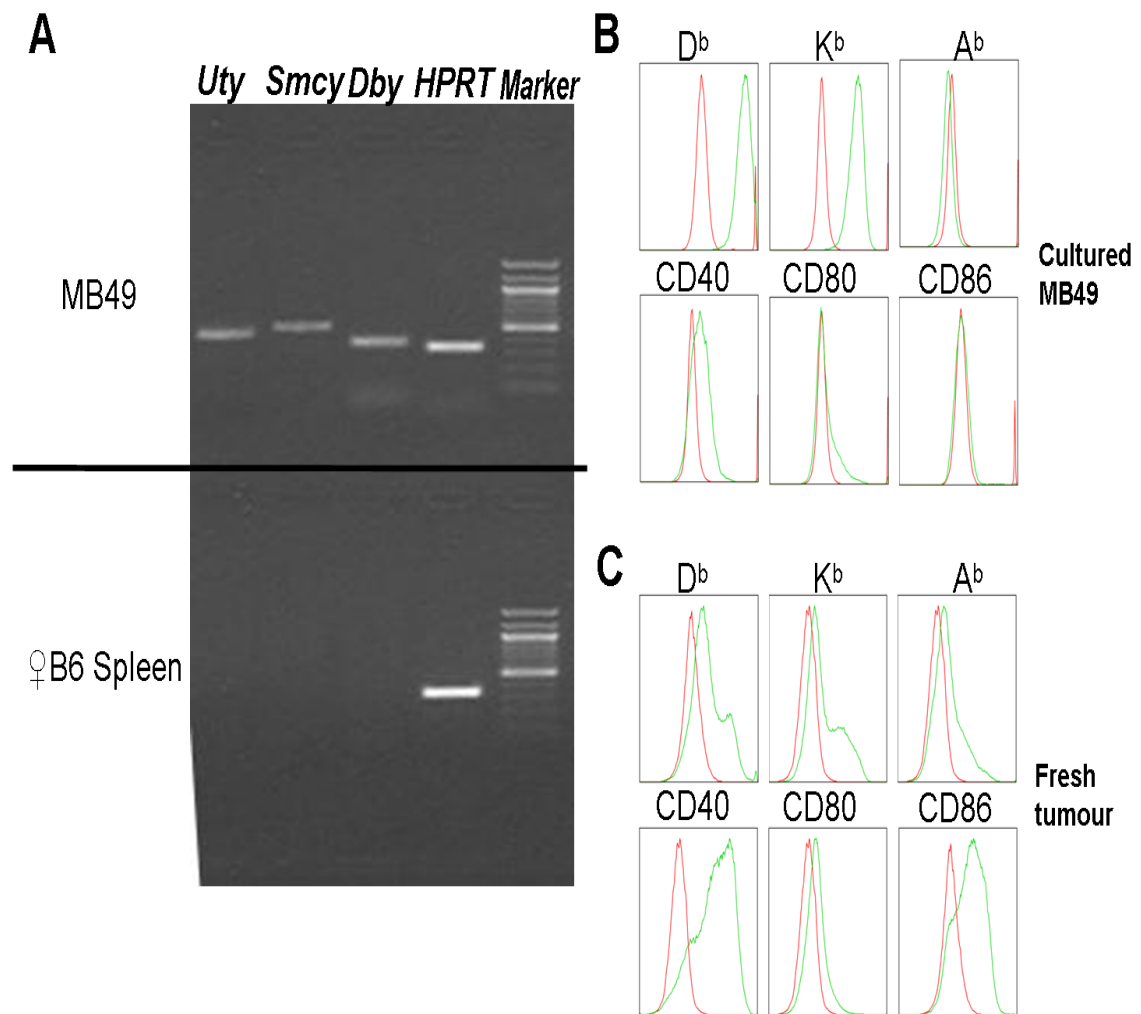


Figure 1. Characterization of the phenotype of MB49 cells

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 *Utv*, *Smcy*, *Dby* and HPRT genes. PCR products were analysed on a 1% Agarose gel. **B** and **C**) Analysis of expression of MHC and co-stimulatory molecules by fresh and cultured MB49 cells. Cultured (**B**) or *ex-vivo*(**C**) MB49 cells were stained with PE- or FITC- conjugated antibodies (—) or isotype controls (—) and analysed by FACS for the expression of MHC Class I (*D^b* and *K^b*), Class II (*A^b*), co-stimulatory molecules (CD40, CD80 and CD86).

Two years after the experiments presented in Figure 1C were conducted, the phenotype of *ex vivo* MB49 was analysed again with two modifications: Firstly, CD45 was introduced as a marker to distinguish tumour cells from tumour-infiltrating lymphocytes; CD45, a lymphocyte common antigen²⁴⁵, is not expressed by MB49 cells which are of epithelial origin²¹⁵. Secondly, more markers were analysed because other co-stimulatory molecules and chemokine receptors had become of interest to this study.

As shown in Figure 2, the freshly isolated MB49 cells were found to be positive for D^b and K^b. *Ex vivo* MB49 expressed a significantly higher level of CCR9 compared to the four other chemokine receptors tested, which were expressed at only very low levels. In addition, *ex vivo* MB49 tumour cells expressed PD-L1 and PD-L2 but a higher level of PD-L1 than PD-L2. 4-1-BB was not expressed, CD86 was expressed at very low levels and levels of GITR-L were low and varied between the two tumour cells analysed.

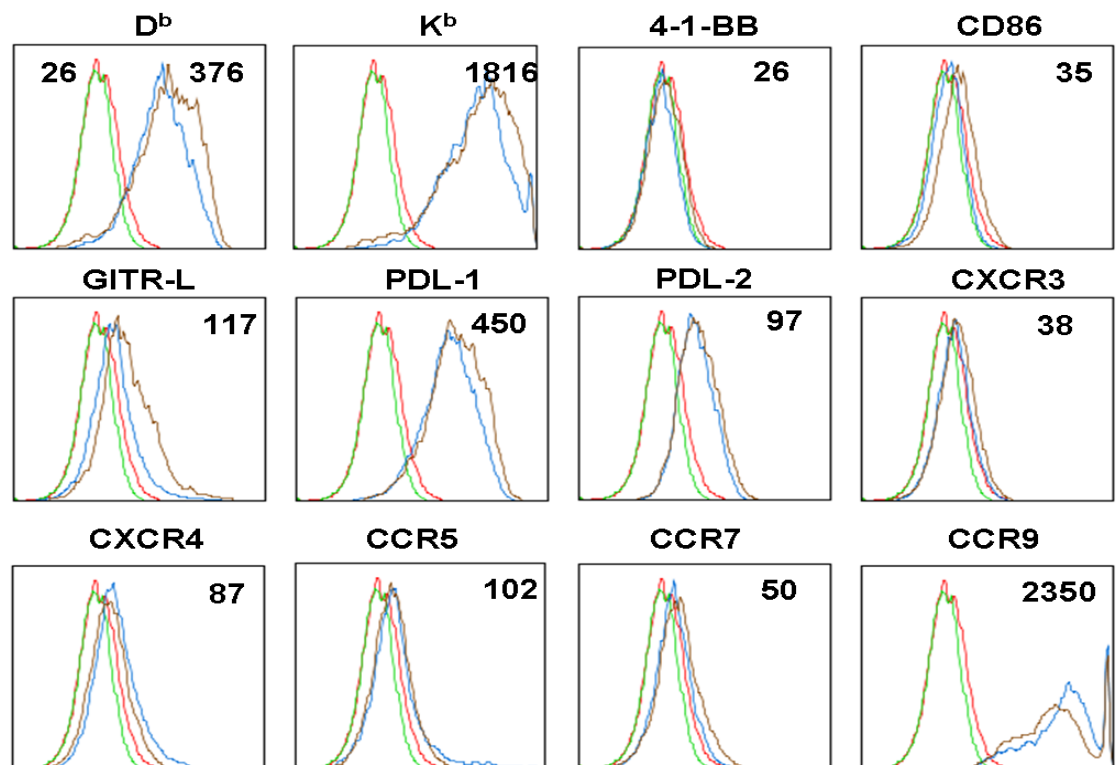


Figure 2. *Ex vivo* MB49 Cell surface markers.

MB49 cells (5×10^5) were s.c. injected to two C57BL/6 female mice. 20 days later, MB49 cells from two tumours were isolated and digested with collagenase before staining with anti-CD45^{FITC} and PE-conjugated antibodies against respective cell surface markers. CD45-negative and -intermediated cells were gated and analysed for cell surface marker expression. Isotype control tumour 1 (—) and tumour 2 (—), PE-conjugated stained tumour 1 (—) and tumour 2 (—). Inserted numbers in each histogram represent mean fluorescent intensity (MFI).

3.2.2 : T cell responses to MB49/HY

3.2.2.1 Growth of MB49 tumour in C57BL/6 mice

It has been previously described that s.c. inoculation of MB49 cells at doses of 7×10^5 and 1×10^6 produced a progressive solid tumour in female C57BL/6 mice ¹⁸⁹. To establish a dose of MB49 cells that would be relevant to this study we titrated the dose of MB49 cells. To this end, 1×10^5 , 3×10^5 , 6×10^5 or 1×10^6 MB49 cells were injected s.c. to the right lateral flank of female C57BL/6. Tumours became visible as small lumps after 3-5 days and after that were measured every other day, where possible, until the mouse had to be culled for ethical reasons as stipulated by the project licence. With a low dose of MB49 cells (1×10^5 cells/mouse), 60% mice developed tumours which were smaller and grew relatively slowly (Figure 3A). With an intermediate dose of MB49 cells (3×10^5 cells/mouse), up to 80% mice developed tumours which were bigger and grew faster (Figure 3B). With a high dose of MB49 cells (6×10^5 and 1×10^6 cells/mouse), 100% mice rapidly developed tumours which were very aggressive, some tumour-bearing mice had to be culled at an early stage due to tumour ulceration, which is one of the end-points of the experiments under our licence (Figure 3C and D).

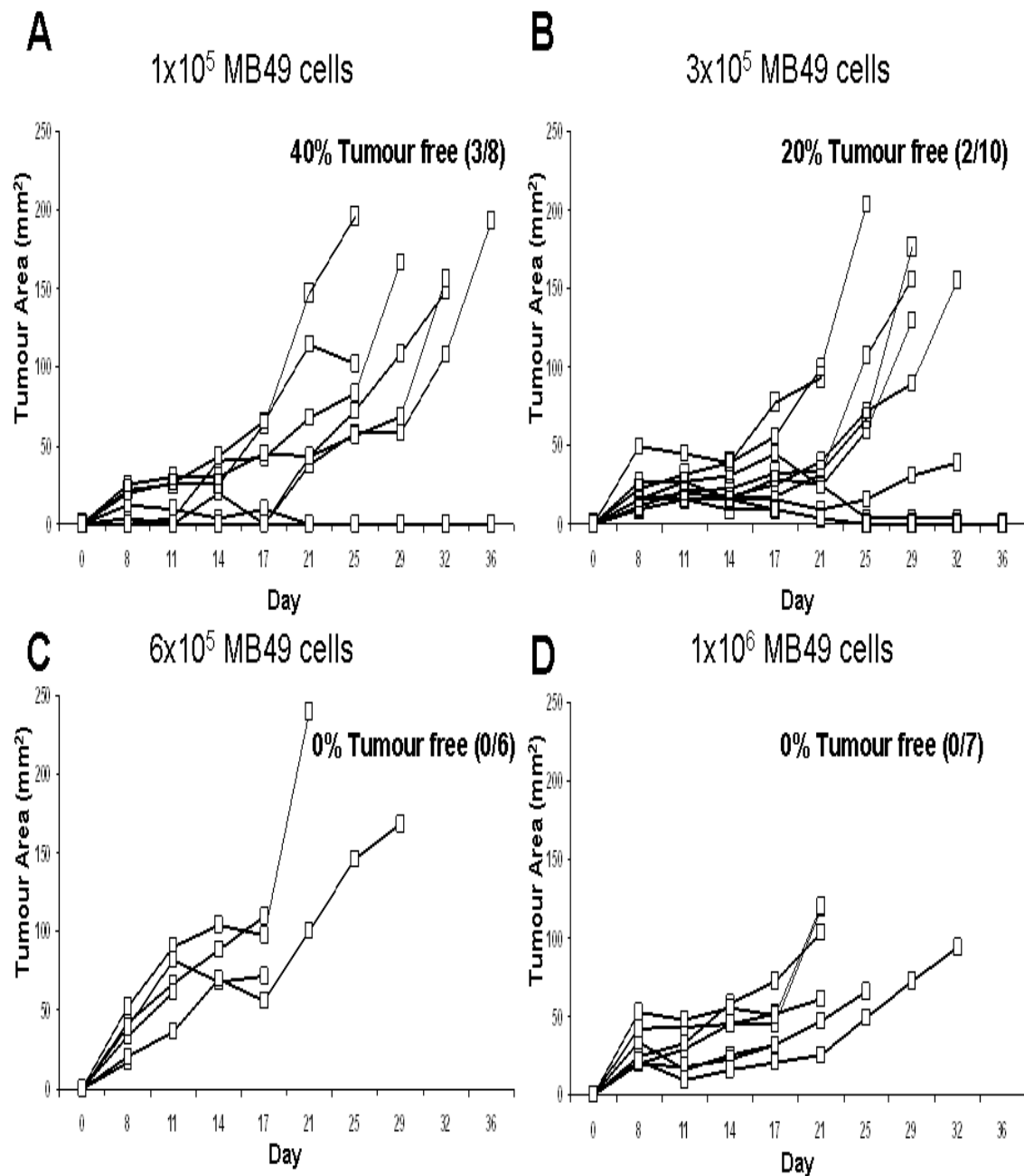


Figure 3. Investigation of growth characteristics of MB49 tumours in C57BL/6 female mice.

Female B6 mice were injected s.c. with 1×10^5 (**A**), 3×10^5 (**B**), 6×10^5 (**C**) or 1×10^6 (**D**) MB49 cells. Tumour area was measured at regular intervals, each line represents an individual mouse.

Although HY has been used as a surrogate tumour antigen on MB49 cells, they may also express other unknown tumour antigens. To determine the relative importance and contribution of HY antigens to anti-MB49 T cell responses, the growth characteristics of MB49 tumours in B6 female and male mice was compared. Due to central and peripheral self-tolerance mechanisms, T cells with HY specificity are absent in the male recipients of MB49 cells, although T cell responses against other unknown MB49 antigens are present. In contrast, female mice harbour normal T cell repertoires against HY and other unidentified tumour antigens expressed by MB49 tumours.

To test the immunogenicity of the MB49 tumours, 1×10^5 and 6×10^5 MB49 cells were injected s.c. to male and female C57BL/6 mice, respectively. As shown in Figure 4, at a dose of 6×10^5 , MB49 tumours grew faster which led to an earlier mortality in male (mean survival time = 12.7d) when compared to female (mean survival time = 22.8d) mice. When the dose of MB49 cells was reduced to 1×10^5 , the difference in terms of mean survival time (MST) between female and male groups appears to become smaller (20.3 vs. 29.3) (Figure 4B).

The correlation between the presence of HY-specific T cells and prolonged survival of tumour-bearing mice indicated that anti-HY T cell responses contribute to control of MB49 tumour *in vivo*.

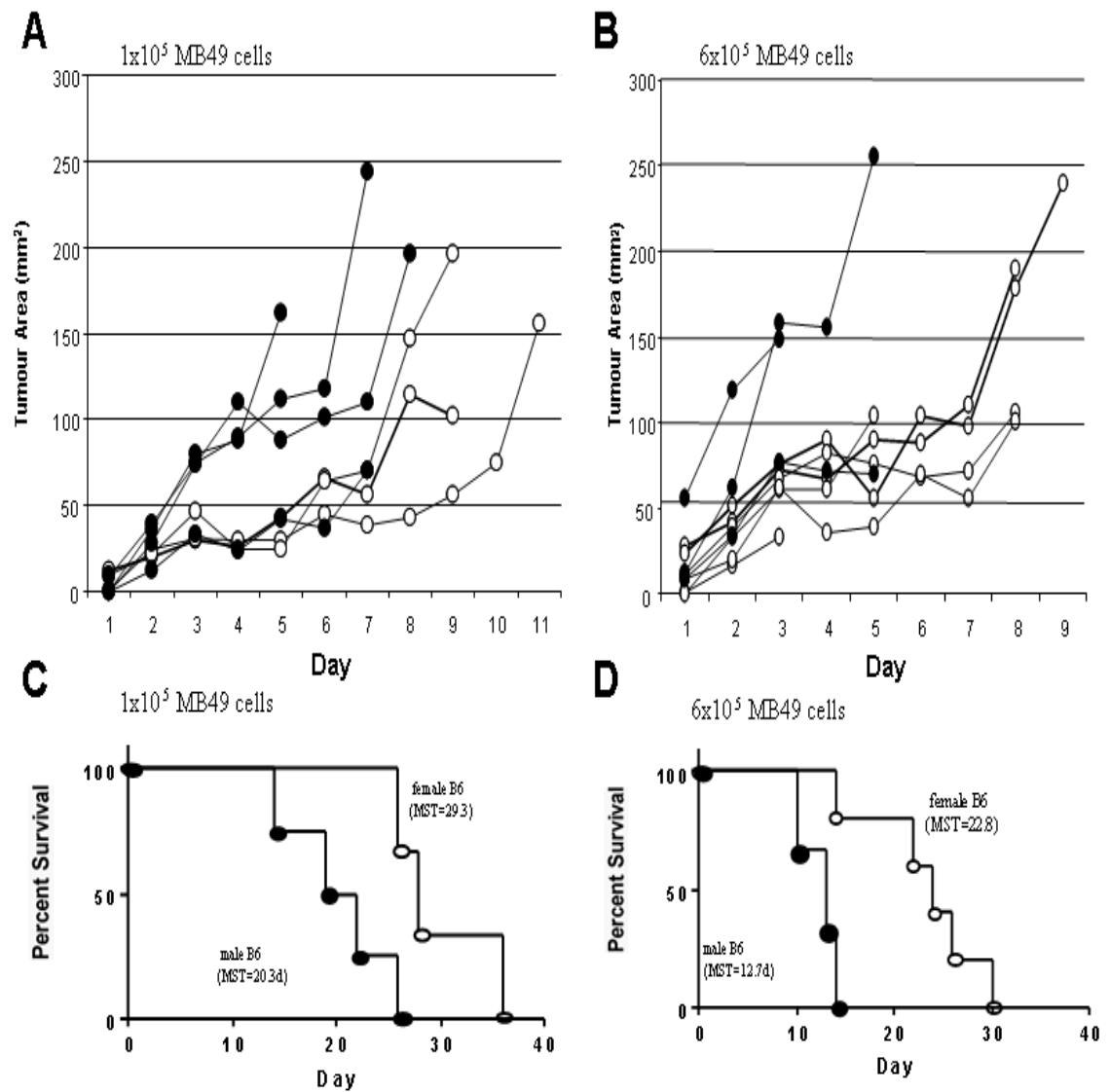


Figure 4. Testing immunogenicity of MB49 cells

A and **C**) Female (○, $n = 3$) or male C57BL/6 mice (●, $n = 4$) were injected s.c. with 1×10^5 MB49 cells. **B** and **D**) Female (○, $n = 6$) or male C57BL/6 mice (●, $n = 3$) were injected s.c. with 6×10^5 MB49 cells. Tumour area was measured at regular intervals. Results are presented as tumour growth (**A** and **B**) and percent survival (**C** and **D**).

3.2.2.2 Antigen presenting cells associated with MB49 tumours

It has been indicated that tumour-derived antigens are persistent in tumour-dLN however, the relative contribution by DC, B cells and macrophages, three individual APC subsets, to antigen presentation has not previously been explored in great detail. To test antigen presentation by DC, B cells and macrophages in the tumour-dLN during the course of tumour growth, three groups of B6 females (two mice/group) were injected s.c. with 5×10^5 MB49 on days -24, -17 or -10. A group of naïve female C57BL/6 was used as a control. On day 0, CD11c⁺ DCs were purified by MACS from tumour-dLN (See Materials and Methods for more details). Purified DCs were then incubated with resting Marilyn CD4⁺ T cells (4×10^5 /well), purified from Rag^{-/-} Marilyn females by MACS positive selection. Co-cultures were incubated for four days and proliferation was measured using a tritiated thymidine incorporation assay.

Marilyn CD4 T cells did not produce a significant response against DCs from control mice, however they responded vigorously to DCs isolated from MB49-bearing mice. A peak CD4 response was seen against DCs isolated from tumour-dLN 17 days after tumour inoculation (Figure 5A).

To directly compare antigen presentation abilities of B cells, macrophages and DCs, individual APC populations were isolated from MB49-dLN of female C57BL/6 mice that had been given 5×10^5 MB49 cells s.c. 17 days previously. 1×10^5 purified B cells, macrophages or DC were cultured with Marilyn CD4⁺ T cells, as described in Figure 6A.

CD11c⁺ DCs were significantly more potent at stimulating Marilyn T cells ($p < 0.0005$) than either macrophages or B cells (Figure 5B), suggesting that they are the most effective APC in the tumour dLNs of MB49 bearing mice.

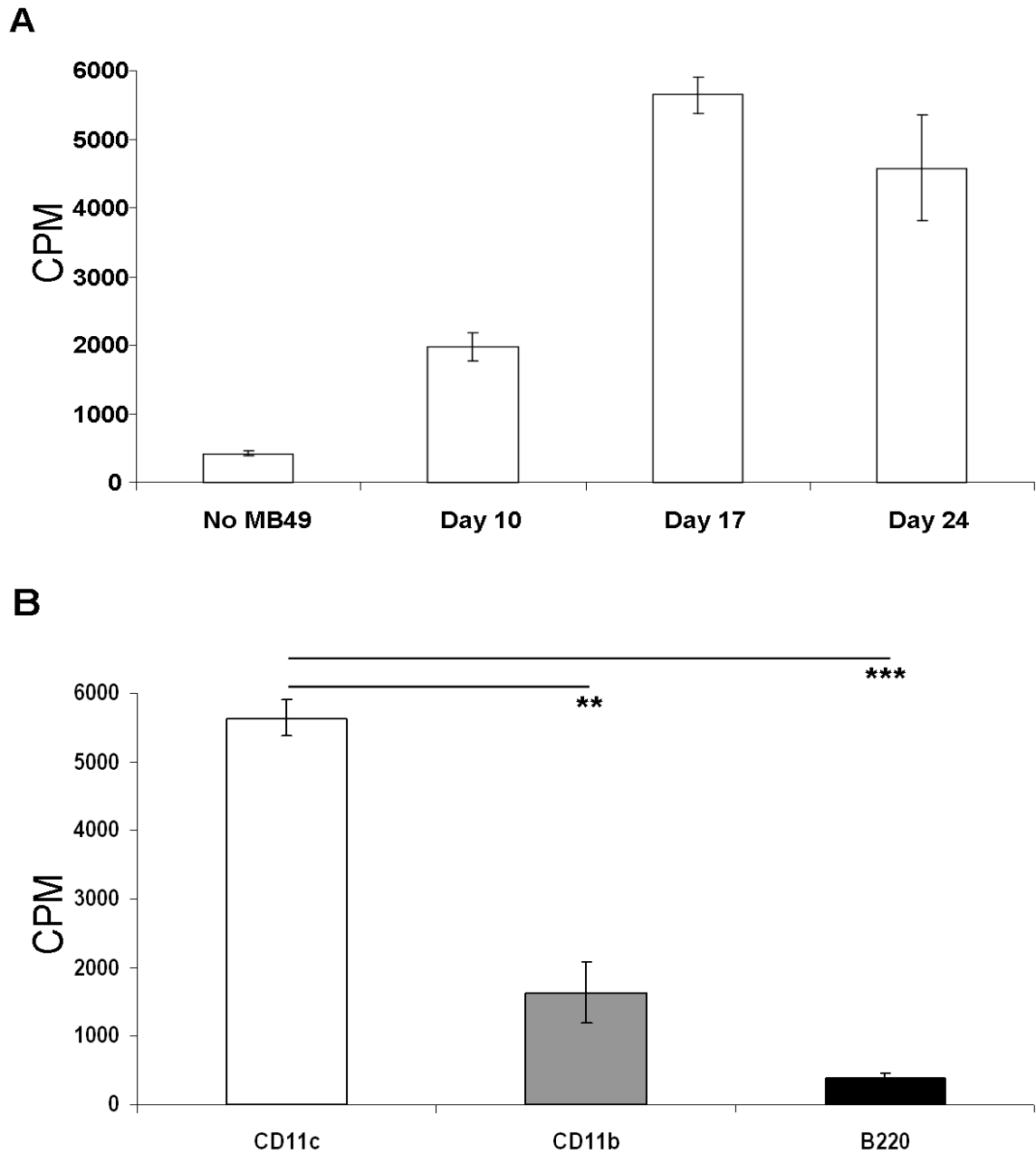


Figure 5. MB49-Dby-A^b antigen presentation by APC subsets

A) On day 0, CD11c⁺ cells were isolated from the tumour draining LN of three groups of female C57BL/6 mice that were given 5×10^5 MB49 cells s.c. on day -24, -17 or -10. CD11c⁺ cells (1×10^5 /well) were then co-cultured with purified Marilyn CD4⁺ T cells (4×10^5 /well) for 3 days in a 96 well U-bottomed plate. Tritiated thymidine (0.5 μ Ci/well) was added on day 3 and proliferation was evaluated 24 hours later as counts per minute (CPM). The results are presented as mean $\pm$ SD.

B) CD11c⁺(□), CD11b⁺(■) or B220⁺(■) APC were isolated from female C57BL/6 that were given 5×10^5 MB49 cells s.c. on day -17 and cultured with Marilyn CD4⁺ T cells as described above. The results are presented as mean $\pm$ SD. CD11b⁺(■) vs. CD11c⁺(□), ** (p<0.0005). CD11c⁺(□) vs. B220⁺(■) *** (p<0.0005).

3.2.2.3 Role of CD4 and CD8 effector T cells in MB49 rejection

Previous studies have demonstrated that Marilyn CD4 and MataHari CD8 T cells recognise MB49-derived HY antigen *in vivo* but only Marilyn CD4 T cells can cause MB49 tumour rejection¹⁷⁸. Generally, CD8 T cells are the major effector cells for killing tumour cells but they require help from CD4 T cells. To examine the relative importance of polyclonal CD4 and CD8 T cells in B6 females, to the rejection of MB49 tumours, an *in vivo* T cell depletion strategy was developed: Female C57BL/6 mice were depleted of CD4, or CD8, or both populations using a single i.p. injection of 1 mg of depleting monoclonal antibodies (clones YTS 191²¹³ for CD8; YTS 169²¹² for CD4). These clones recognise the distinct epitopes of respective target molecules which results in an efficient and a long lasting depletion *in vivo*. Three days after T cell depletion, the mice were injected s.c. with 2×10^4 MB49 cells, which represents a sub-optimal dose of inoculation (i.e., under this dose, the injected MB49 cells do not develop into tumours in naïve B6 females, but do in Rag^{-/-} B6 mice). Tumour area was then measured at regular intervals.

The depletion of CD4 or CD8 T cells abrogates the immune resistance to MB49 tumours and results in faster tumour growth and earlier mortality in comparison to control mice which received no mAb (Figure 6). However, there is no significant difference in terms of MST between anti-CD4-treated (MST=34 days) and anti-CD8-treated groups (MST=33.8 days), suggesting that both cell types play an equally important role in controlling tumour growth. Depletion of both CD4 and CD8 T cells had a synergistic effect, resulting in faster MB49 growth and reduced survival (MST= 29.3 days) than either CD4 or CD8 depletion alone (Figure 6).

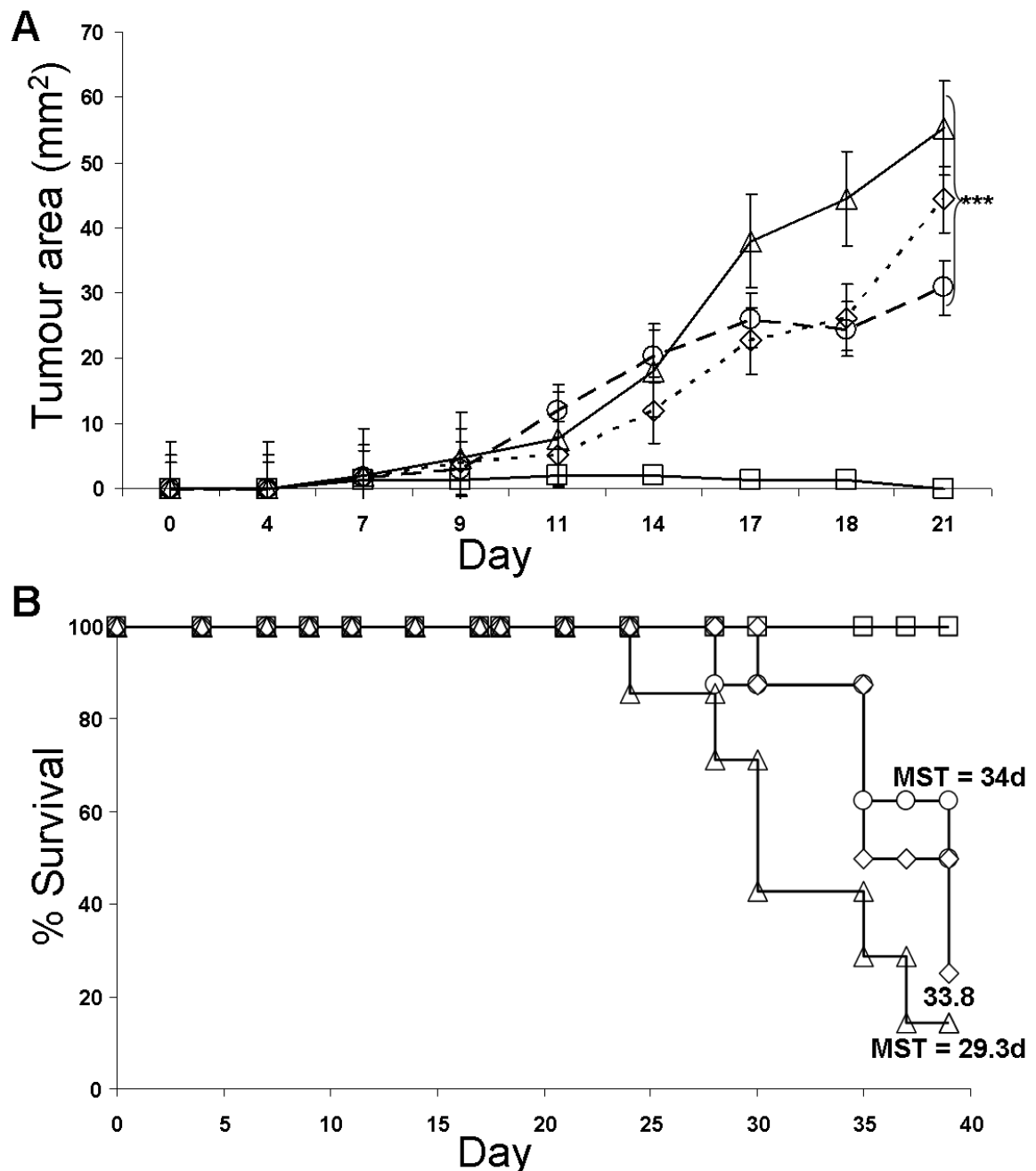


Figure 6. Effect of in vivo depletion of T cell subsets in MB49-bearing mice

A) 2×10^4 MB49 cells were injected s.c. to four groups of female C57BL/6 mice that had been treated with anti-CD4 (○, n=7), anti-CD8 (◇, n=7), or both antibodies (△, n=6) i.p. on day -3 or untreated (□, n=6). **A)** Tumour area was measured, results are presented as mean $\pm$ SEM. ***p<0.0001.

B) MB49 progression was presented as % survival. The s.c. inoculation of B6 mice with MB49 cells does not lead to death. But the mice are recoded as death once they reach one of following end points of experiment. One is the maximum of size 14 mm x 14mm, the other is the development of severe ulceration by tumour. The mean survival time (MST) of individual groups was also indicated.

Depletion of CD4 or CD8 T cells abrogates anti-tumour responses which reiterates their critical role in the endogenous immune response by female C57BL/6 mice against HY expressing MB49 tumours. To further explore cellular events during the course of endogenous T cell responses, tumour dLN and non-draining lymph nodes (ndLN) from C57BL/6 mice with 7, 13 or 21 day-old tumours, or control LN from naïve female C57BL/6 mice were harvested and counted using a haemocytometer. In addition, for flow cytometric analysis, single cell suspensions were 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 regulatory T cells (Treg). The number of each cell type was calculated by multiplying the number of cells counted and the percentage of cells in the FSC:SSC lymphocyte gate and the percentage of cells in the CD4, CD8 or Treg populations.

The total number of lymphocytes was significantly higher in tumour-dLN than that in tumour-ndLN and control LN ($p = 0.05$) from naïve mice at all three time points tested. On day 7 the number of lymphocytes in the tumour dLN was three times higher than in non-draining lymph nodes (6.1×10^6 for dLN compared to 2.1×10^6 for ndLN), suggesting that there is local tumour-antigen dependent T cell expansion (Figure 7A).

On day 7 the numbers of CD4 and CD8 T cells were increased in tumour dLN compared to non-draining and naïve lymph nodes. The number of CD4 T cells increased by at least 1.4 times, from 600×10^3 in naïve lymph nodes and 677×10^3 in non-draining lymph nodes to 930×10^3 in tumour dLN (Figure 7B).

The number of CD8 T cells increased by more than 1.4 times from 475×10^3 in naïve lymph nodes and 672×10^3 in non draining lymph nodes to 968×10^3 in tumour-dLN (Figure 7C).

Regulatory T cell numbers were also increased in tumour dLN, on day 7, by at least 1.4 fold, from 72×10^3 in naïve lymph nodes and 108×10^3 in non draining lymph nodes to 150×10^3 in tumour dLN (Figure 7D).

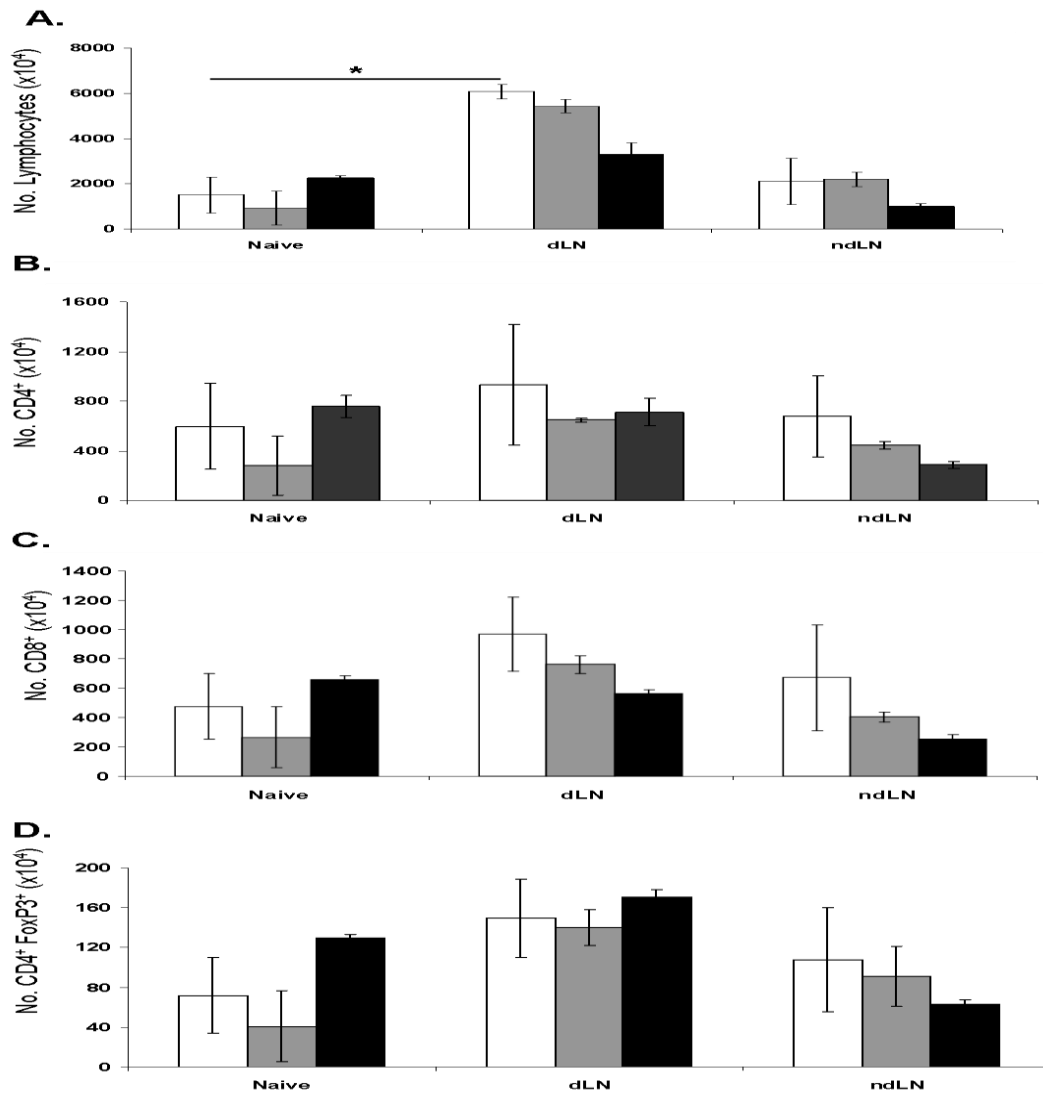


Figure 7. Representation of T cell subsets in the dLN of MB49 bearing female C57BL/6

Female C57BL/6 were injected s.c. with 5×10^5 MB49 cells on day 0. On day 7 (□, n=2), day 13 (■, n=2) and day 21 (■, n=2) peripheral lymph nodes from naïve mice and tumour draining lymph nodes (dLN) or non-draining lymph nodes (ndLN) from MB49 bearing mice were isolated and the number of cells/lymph node were counted. Cells were then stained and analysed by FACS.

A) Total lymphocyte number was calculated by multiplying the number of cells counted by the percentage of cells in the lymphocyte gate. Results are presented as mean ($\pm$ SD). Day 7 naïve vs. day 7 dLN * $p = 0.05$

B) LN were stained with anti-CD4^{FITC} and anti-TCR β ^{PE}. The number of CD4⁺ T cells was calculated by multiplying the number of cells, the percentage of cells in the lymphocyte gate and the percentage of cells in the CD4⁺TCR⁺ population. Results are presented as mean ($\pm$ SD)

C) LN were stained with anti-CD8^{PerCP} and anti-TCR β ^{PE}. The number of CD8⁺ T cells was calculated by multiplying the number of cells, the percentage of cells in the lymphocyte gate and the percentage of cells in the CD8⁺TCR⁺ population. Results are presented as mean ($\pm$ SD)

D) LN were stained with anti-CD4^{PE} then fixed and permeabilised and stained with anti-FoxP3^{FITC}. The number of Treg was calculated by multiplying the number of cells, the percentage of cells in the lymphocyte gate and the percentage of cells in the CD4⁺FoxP3⁺ population. Results are presented as mean ($\pm$ SD)

The analysis of T cell infiltration into the tumour mass requires the ability to distinguish host cells from tumour cells. We decided to take advantage of the availability of enhanced Yellow Fluorescent Protein (eYFP)-transgenic C57BL/6 mice, which constitutively express YFP in all cells, allowing “yellow” host cells to be distinguished from MB49 tumour cells by flow cytometry and by fluorescent microscopy. 5×10^5 MB49 cells were injected s.c. into female eYFP-transgenic C57BL/6 mice. 15 days later, tumours were isolated and single cell suspensions were stained for anti-TCR^{PE} and anti-CD4^{PerCP} or anti-CD8^{PerCP}. Tumour-infiltrating CD4 and CD8 T cells were detected by gating on YFP⁺ lymphocytes then analysing the proportions of CD4⁺TCR⁺ and CD8⁺TCR⁺ T cells. CD4⁺ T cells accounted for 6% and CD8⁺ T cells accounted for 5% of the total number of cells in the tumour (Figure 8A).

To verify the above results, tissue sections from the same tumour were stained with DAPI and anti-CD3^{PE} before being analysed by deconvolution microscopy. As shown in Figure 8B, DAPI⁺YFP⁺ cells represented the host cells within the tumour whereas MB49 tumour cells were DAPI⁺YFP⁻. CD3⁺ T cells were present at high numbers in the surrounding host tissue but at lower numbers within centre of the tumour, indicating that there is a limited infiltration.

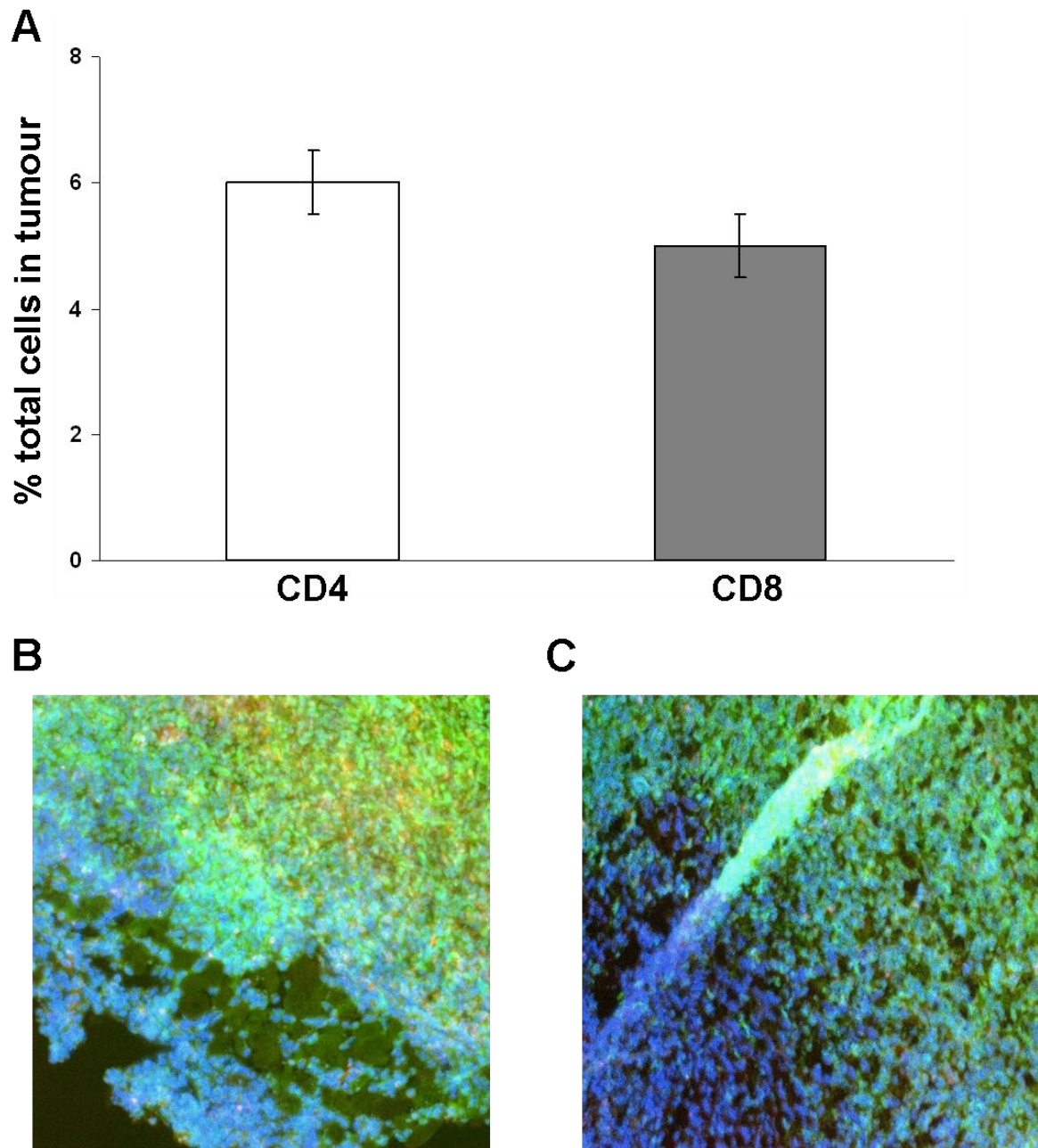


Figure 8. Detection of tumour-infiltrating CD4 and CD8 T cells by FACS and microscopy

A) 5×10^5 MB49 cells were injected s.c. to 4 YFP⁺ female C57BL/6 mice. 15 days later, tumour was isolated and stained with anti-TCR^{PE} and either anti-CD4^{PerCP} or anti-CD8^{PerCP}. The proportion of TCR⁺CD4⁺ or TCR⁺CD8⁺ T cells as the percentage of all tumour-infiltrating cells was determined by FACS analysis.

B and C) 5×10^5 MB49 cells were injected s.c. to a YFP⁺ female C57BL/6 mouse. 15 days later, whole tumour was isolated and embedded in OCT then flash frozen in liquid nitrogen. Sections were cut and stained with DAPI and anti-CD3^{PE} (**B**) or isotype control (**C**). The host CD3⁺ T cells are stained red, the non-T lymphocyte host cells green and tumour cells blue.

As MB49 tumours naturally express male antigens^{178, 189} it was of interest to test if tumour rejection is dominated by anti-HY T cell responses, which can be measured by HY-specific tetramers¹⁸⁵. To detect tetramer⁺CD8⁺ T cell responses, female C57BL/6 mice were injected s.c. with 5×10^5 MB49 cells. Tumour-dLN were then harvested at day 6, 10, 19 and 24. Single cell suspensions were stained with D^b-Uty^{PE} and D^b-Smcy^{APC} tetramers and anti-CD8^{PerCP} and analysed by flow cytometry. No detectable levels of tetramer⁺CD8⁺ T cells were found at any of the time points tested (Figure 9), indicating that systemic HY specific CD8 cell activation did not occur during the course of the primary response to the tumour.

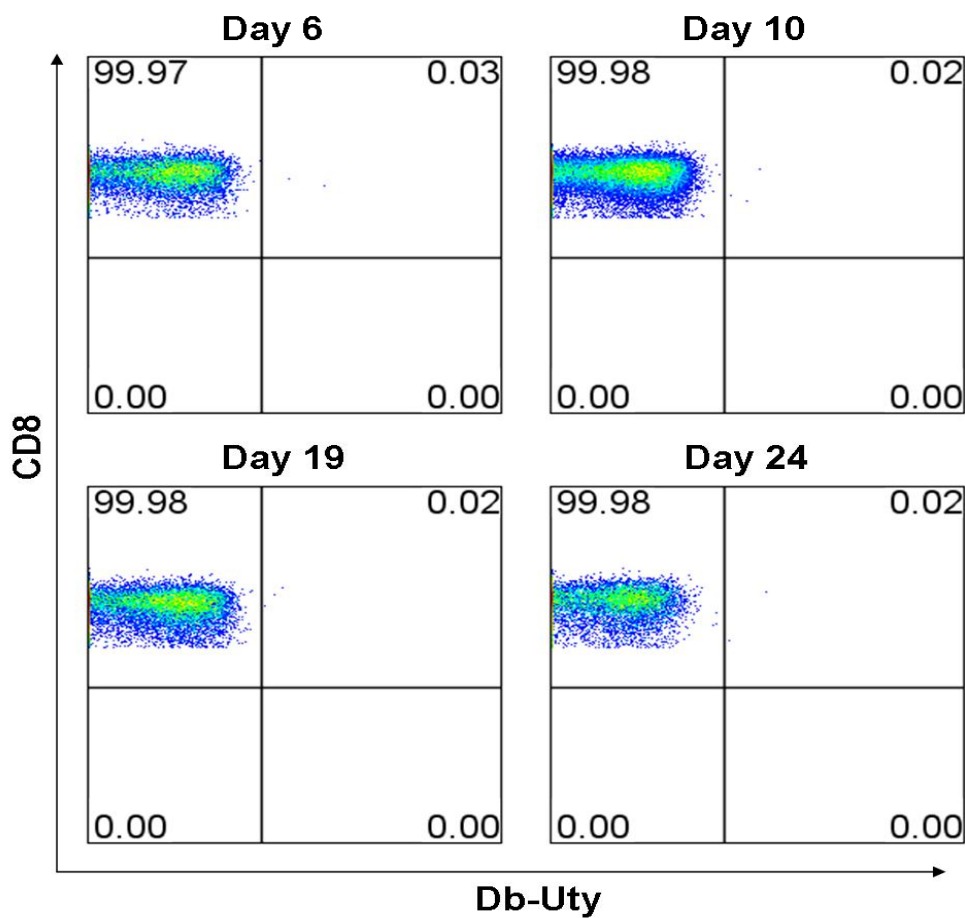


Figure 9. The failure of the induction of HY-specific tetramer+ cells in MB49-bearing mice

Draining lymph nodes were harvested from female C57BL/6 mice that had been injected s.c. with 5×10^5 MB49 cells and stained with D^b-Smcy^{PE}, anti-CD8^{PerCP} and D^b-Uty^{APC} then analysed by FACS. Representative dot plots shown were from three independent experiments. Smcy tetramer data not shown.

3.2.2.4 Role of regulatory T cells in MB49 rejection

Studies in mice have demonstrated that *in vivo* Treg depletion, using PC61 (an anti-CD25 antibody), enhances tumour rejection^{125, 147, 222}. To test whether this is the case in the MB49 system, female C57BL/6 mice were depleted of CD4⁺ or CD25⁺ T cells 3 days before being inoculated s.c. with a sub-optimal dose of MB49 (2 x 10⁴ MB49 cells). Tumour size and survival were measured.

Untreated mice quickly rejected tumours. Mice depleted of CD4⁺ T cells developed tumours more rapidly and had a shorter mean survival time, than mice depleted of CD25⁺ T cells; 34 days vs. 37.3 days respectively (Figure 10). Contrary to published results and predictions, depletion of CD25⁺ T cells caused increased tumour growth and decreased survival compared to untreated controls.

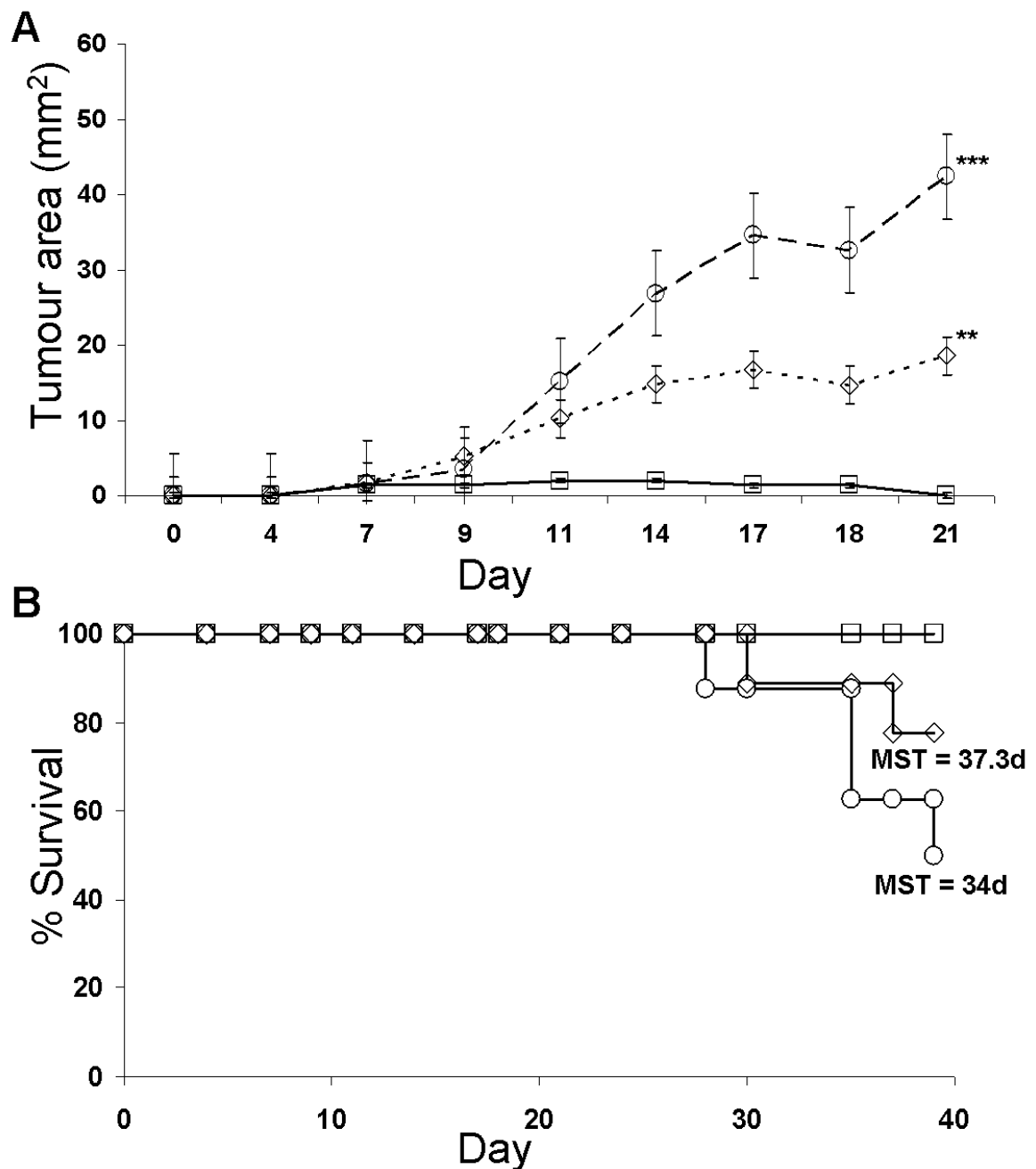


Figure 10. The impact of depletion of Tregs to MB49 growth in vivo

2×10^4 MB49 cells were injected s.c. to female C57BL/6 mice that had been treated with anti-CD4 (○, n=7) or anti-CD25 (◇, n=7) i.p. on day -3 or untreated (□, n=6).

A) Tumour area was measured, results are presented as mean $\pm$ SEM. (○, anti-CD4 vs. □, untreated; *** $p < 0.001$, ◇, anti-CD25 vs. □, untreated; ** $p < 0.001$).

B) MB49 progression was presented as % survival. The s.c. inoculation of B6 mice with MB49 cells does not lead to death. But the mice are recorded as dead once they reach one of following end points of experiment. One is the maximum of size 14 mm x 14mm, the other is the development of severe ulceration by tumour. The mean survival time (MST) of individual groups was also indicated.

3.2.3 : Role of immuno-regulatory cytokines in MB49 rejection

3.2.3.1 IL-10

To test the role of IL-10 in the MB49 system, two strategies were employed. Firstly, IL-10 KO or wild type (WT) female C57BL/6 mice were injected s.c. with 5×10^5 MB49 cells and the kinetics of tumour growth compared. Secondly, anti-IL-10R mAb (1B1) was injected i.p. to one group of tumour-bearing WT mice and tumour growth was compared to untreated controls.

Wild type mice were significantly better at controlling tumour growth when compared to IL-10 KO mice ($p = 0.04$) (Figure 11A). Moreover untreated WT female C57BL/6 mice were significantly better at controlling MB49 tumours than mice treated with an IL-10R blocking antibody ($p = 0.01$) (Figure 11B).

Together these results agree with the reports (described earlier) that suggest that IL-10 is important for tumour rejection however, the exact causes for the anti-tumour effect of IL-10 in this system have not been tested.

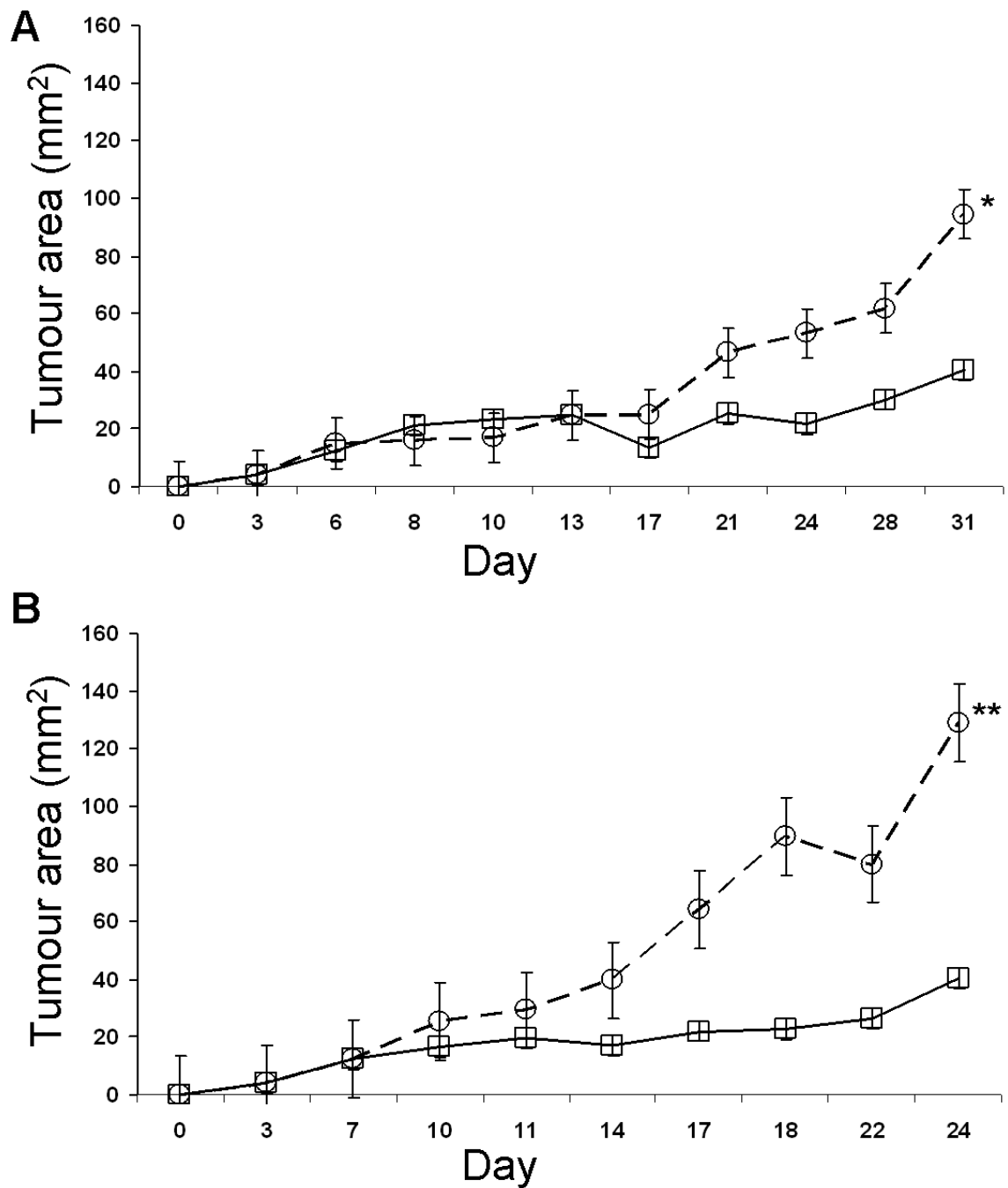


Figure 11. Contribution of IL-10 to MB49 growth in vivo.

A) Wild type ($\square$, $n=4$) or IL-10 KO ($\circ$, $n=4$) female B6 mice were injected s.c. with 5×10^5 MB49 cells. Tumour area was measured at regular intervals, results are presented as mean $\pm$ SD. * $p=0.04$.

B) Wild type female B6 were injected s.c. with 5×10^5 MB49 cells then one group was injected i.p. with anti-IL-10R ($\circ$, $n=3$) and the other left untreated ($\square$, $n=4$). Tumour area was measured at regular intervals, results are presented as mean $\pm$ SD. ** $p=0.01$

3.2.3.2 IFN γ

To test the influence of IFN γ on the growth of MB49, C57BL/6 female mice were divided into two groups of six mice; on day 0, a suboptimal dose of 5×10^4 MB49 cells was injected s.c. to both groups, one group was then given no further treatment whilst the other group was injected i.p. with R46/A2 (an anti-IFN γ antibody that neutralises IFN- γ^{100}). on days -5, -3, 0, 7, 11, 14 and 18. Tumour progress was monitored by measurement of tumour size and mortality.

As shown in Figure 13, *in vivo* neutralisation of IFN γ led to significantly faster tumour growth in WT female C57BL/6 mice compared to untreated females ($p = <0.001$) (Figure 12A). Accelerated tumour growth in R46/A2 treated mice correlates with earlier mortality (Figure 12B). It was therefore concluded that IFN γ is a critical cytokine in anti-MB49 immunity.

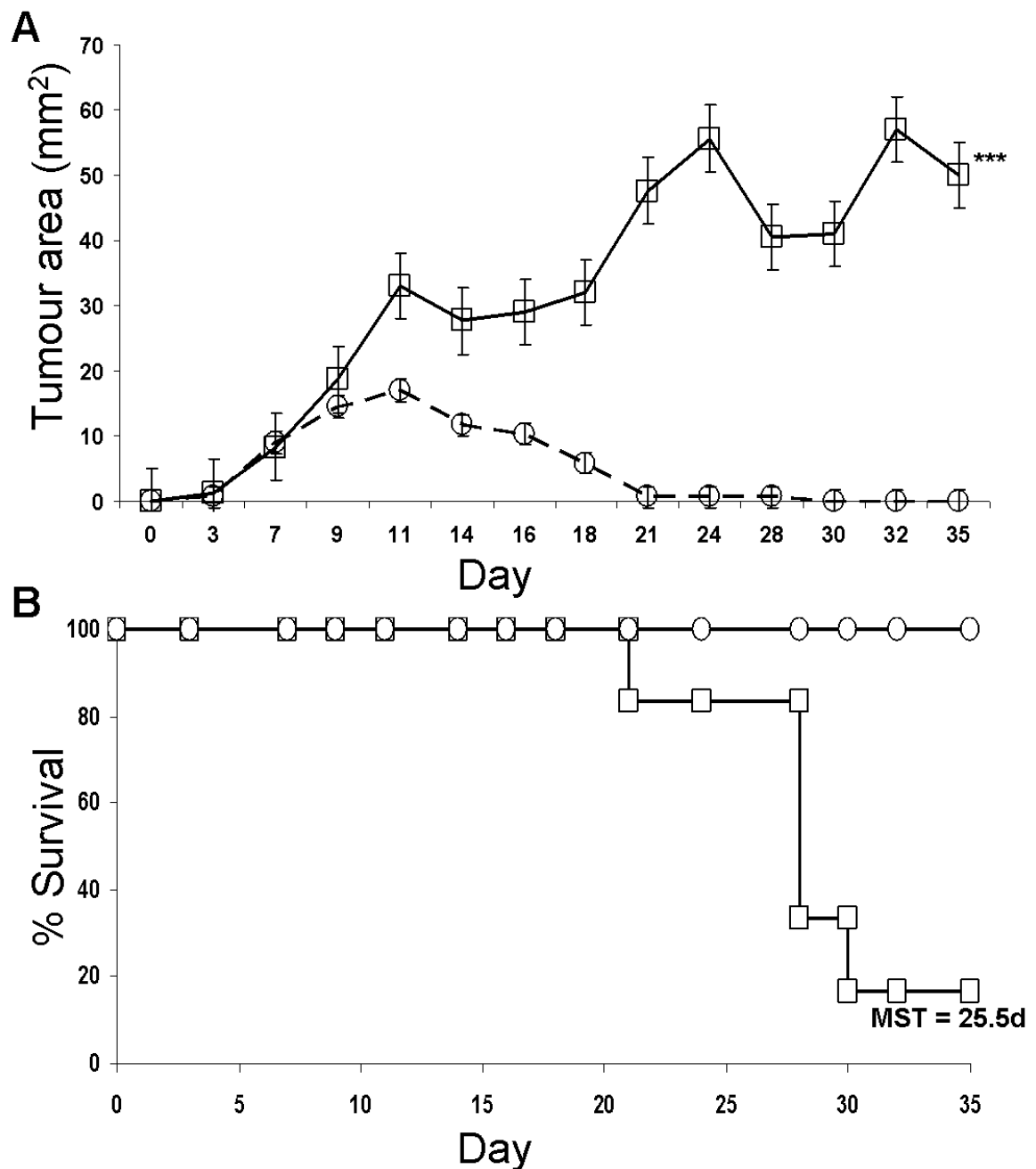


Figure 12. Contribution of IFN γ to MB49 growth

6 female C57BL/6 mice were treated i.p. with anti-IFN γ ($\square$, n=6) on days -5, -3, 0, 7, 11, 14 and 18 and 6 mice were untreated ($\circ$, n=6). On day 0 all 12 mice were given 5×10^4 MB49 cells by s.c. inoculation.

A) Tumour area was measured at regular intervals and results are presented as mean $\pm$ SEM. *** p<0.001.

B) MB49 progression was presented as % survival. The s.c. inoculation of B6 mice with MB49 cells does not lead to death. But the mice are recoded as death once they reach one of following end points of experiment. One is the maximum of size 14 mm x 14mm, the other is the development of severe ulceration by tumour. The mean survival time (MST) of individual groups was also indicated.

3.2.3.3 IL-15

The role of IL-15 in the activation and differentiation of CD8 T cells in viral infection is well established^{66, 93}. However, less information is available in terms of the role of IL-15 in the context of tumour immunity. Taking advantage of the availability of hIL-15 transgenic (Tg) mice, the kinetics of MB49 growth in WT versus Tg mice was compared. IL-15-Tg and WT mice were injected s.c. with 1×10^5 MB49 cells and tumour size was measured at regular intervals. No significant difference was seen between the IL-15-Tg and WT group (Figure 13).

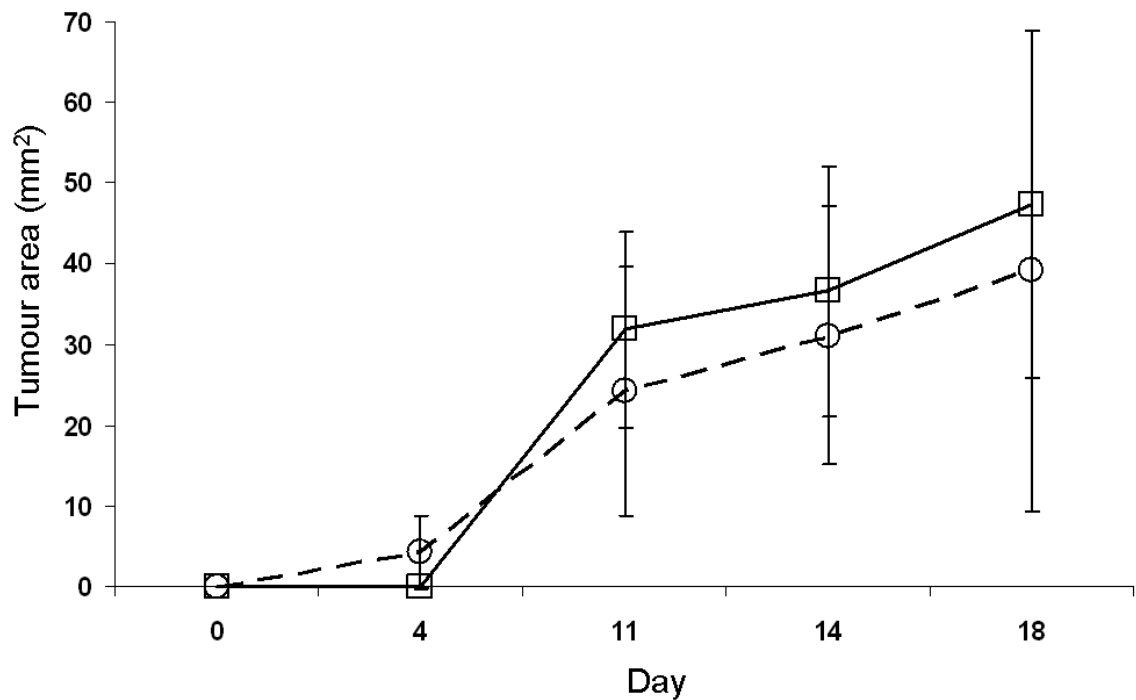


Figure 13. Comparison MB49 growth between WT and IL-15 transgenic mice IL-15-Tg (○, n=3) or wild type (□, n = 4) female C57BL/6 mice were given 1×10^5 MB49 cells s.c. Tumour area was measured at regular intervals and results are presented as mean $\pm$ SD. One representative of three independent experiments is shown.

3.2.3.4 IL-17A

To test the effect of IL-17A on the growth of MB49 tumours, we injected 5×10^5 MB49 cells s.c. to IL-17A KO¹⁹⁵ or WT female C57BL/6 mice and measured the growth of the tumour. IL-17A KO mice showed a small but still significant acceleration in tumour growth ($p = 0.038$) when compared to wild type mice, suggesting that IL-17 inhibits tumour growth.

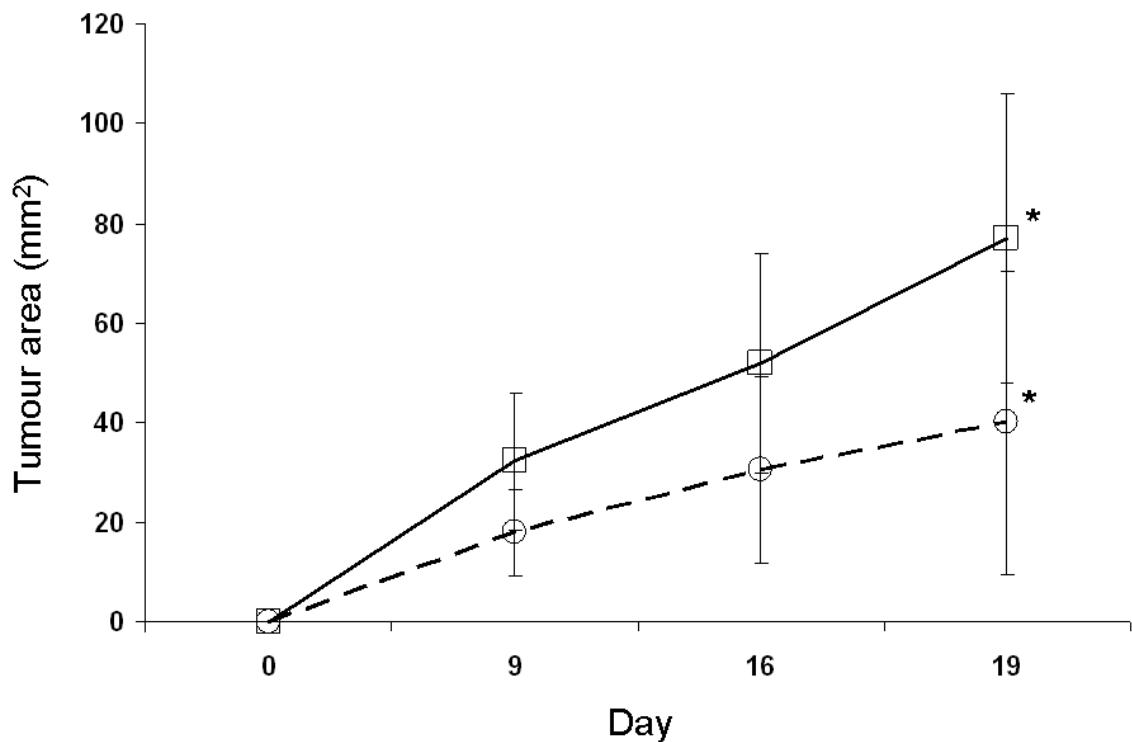


Figure 14. MB49 growth in IL-17A KO mice

Wild type (○, n=8) or IL-17A KO (□, n=6) female C57BL/6 mice were inoculated s.c. with 5×10^5 MB49 cells. Tumour area was measured and results are presented as mean $\pm$ SD

* $p=0.038$.

3.2.3.5 TGFβ

To examine the role of TGFβ in the MB49 system, two different approaches were employed. The first approach was to use the dnTGFβRII-Tg mice which express a dominant negative form of the TGFβ receptor type II (dnTGFβRII) on all T cells. T cells from these Tg mice are therefore defective in TGFβ signalling¹³⁰. When dnTGFβRII-Tg mice were challenged with a low dose of MB49 cells (1×10^5), no tumour development was seen, although the inoculation of WT mice with the same dose of MB49 cells led to tumour growth (Figure 16B), suggesting that dnTGFβRII-Tg mice are completely resistant to low dose MB49 challenge.

On the other hand, dnTGFβRII-Tg mice developed tumours at the same rate as WT mice when a higher dose (1×10^6) of MB49 cells was injected (Figure 15A).

The second approach used to look at the role of TGFβ in the MB49 system was to neutralise TGFβ *in vivo*. C57BL/6 female mice were injected with 1×10^6 MB49 cells s.c. and then, on day 1, 2, 7, 10, 14 and 18, anti-TGFβ neutralising mAb, 1D11²¹⁰, was injected i.p.. Tumour growth in this group was compared to untreated controls. *In vivo* neutralisation of TGFβ had no effect on MB49 growth (Figure 15C).

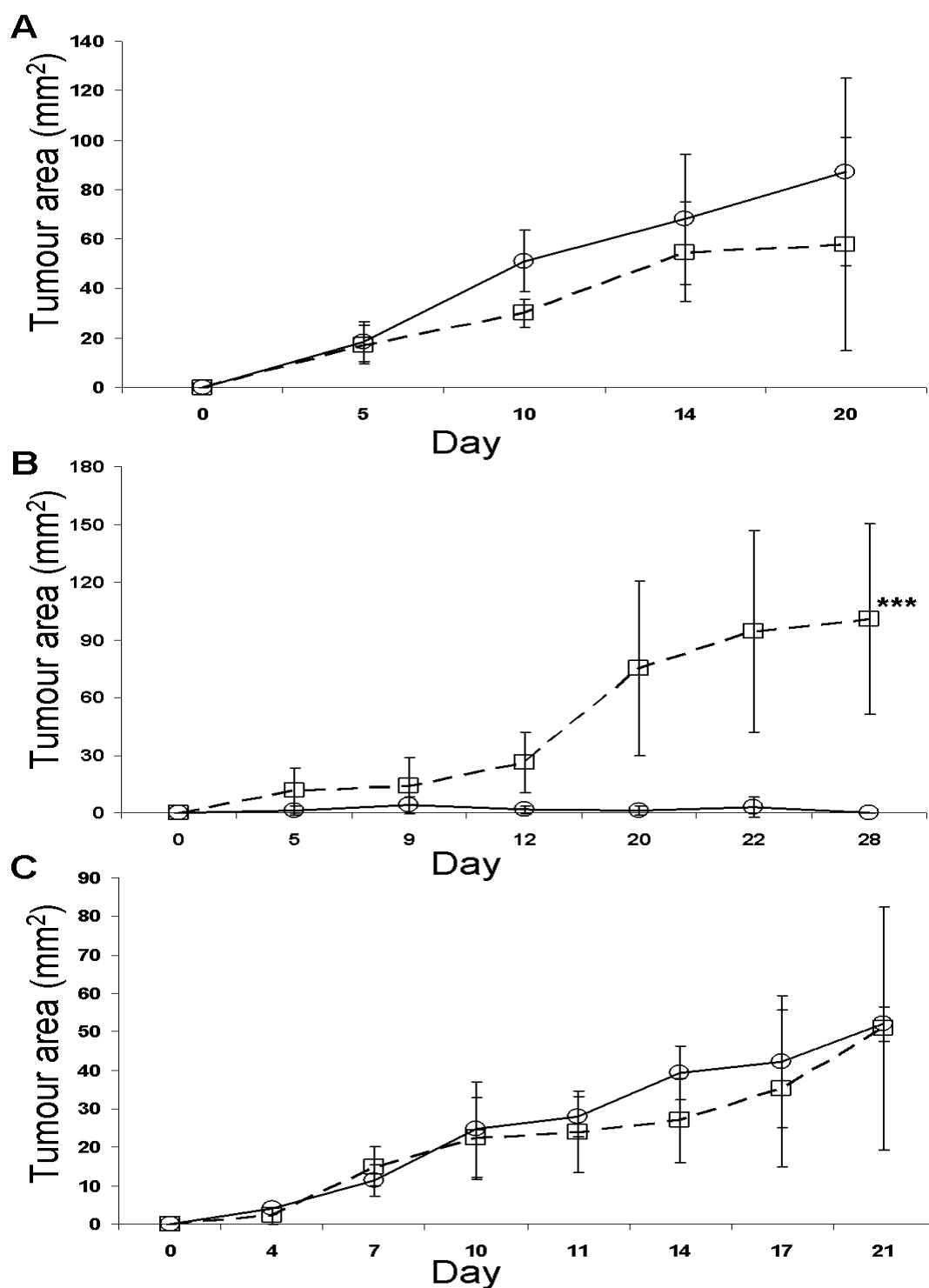


Figure 15. Effect of TGFβ on MB49 growth *in vivo*

A) dnTGFβRII-Tg (○, n=7) or wt (□, n=3) female B6 mice were injected s.c. with 1×10^6 MB49 cells. Tumour area was measured, results are presented as mean $\pm$ SD.

B) dnTGFβRII-Tg (○, n=3) or wt (□, n=6) female B6 mice were injected s.c. with 1×10^5 MB49 cells. Tumour area was measured, results are presented as mean $\pm$ SD. ***p<0.001

C) Female C57BL/6 mice were injected s.c. with 1×10^6 MB49 cells on day 0, and then treated i.p. with anti-TGFβ (○, n=3) on day 1, 2, 7, 10, 14 and 18 or untreated (□, n=6) Tumour area was measured, results are presented as mean $\pm$ SD.

3.3 : Discussion

MB49 is a chemically induced murine bladder carcinoma that naturally expresses HY antigens. Since a prolonged *in vitro* culture could lead to loss of the Y chromosome, MB49 cells were examined for the expression of HY genes by RT-PCR. *Dby*, *Smcy* and *Uty* genes were readily detectable in MB49 cells (Figure 1A.), thus confirming the published results^{178, 189, 190, 215}. In agreement with other reports MB49 expresses class I MHC molecules D^b and K^b, MB49 doesn't express class II MHC, A^b *in vitro*^{178, 246} (Figure 1B).

It was interesting to discover that MB49 cells down regulate MHC I and up regulate MHC II after being passaged *in vivo*. Studies have suggested that MB49 cells up regulate A^b after exposure to IFN γ *in vitro* and *in vivo*^{178, 246} but there have been no reports demonstrating the down regulation of D^b and K^b. In fact exposure to IFN γ has been shown to up regulate D^b and K^b on MB49 cells¹⁷⁸. It is possible that this is an immuno-regulatory mechanism and that MHC Class I cells are selected because of an on going immune response to the tumour.

There are no published results examining the expression of co-stimulatory receptors on MB49 cells *ex vivo* and so it was of great interest to demonstrate the expression of PDL-1 and PDL-2 on the surface of MB49 cells (Figure 2). MB49 can be added to the list of murine tumours and cell lines that express PDL-1¹⁴² and may be useful in studying the effects of the PDL-1/PD-1 signalling pathway to tumour progression¹⁴¹.

The growth of MB49 tumours after inoculation with varying cell numbers closely resembled what has already been published^{189, 190}, with 6×10^5 and 1×10^6 MB49 cells causing a progressively growing tumour. Whereas 3×10^5 and 1×10^5 MB49 cells caused tumours in only 20% and 40% of female C57BL/6 mice respectively (Figure 3).

The growth of MB49 tumours was inhibited in female C57BL/6 but not male C57BL/6 suggesting that previous reports demonstrating a immune response to the HY antigen are correct^{178, 190, 219} (Figure 4).

CD11c⁺ cells in tumour dLN display major histocompatibility/HY peptide complexes and therefore are likely to be the primary APC subset involved in antigen-derived expansion of T cells. Although tumour antigens persist in the dLN of tumour-bearing mice²⁴⁷ it has not been investigated which population of APC are involved in antigen presentation.

This study provides direct evidence for the role of CD11c⁺ cells (Figure 5) in the presentation of tumour antigen in the tumour dLN. Although the MB49 cell line does not express H2A^b *in vitro* it has shown that freshly isolated MB49 tumour cells do¹⁷⁸. This

raises the possibility of direct antigen presentation by tumour cells to CD4 T cells.

Whether freshly isolated MB49 tumour cells would be able to stimulate Marilyn CD4 T cells in the presence of interleukin-2 *in vitro* has not been investigated.

Depletion of CD4 and CD8 T cells caused an increase in the growth of MB49 tumours as well as leading to a related reduction in survival (Figure 6). There was no significant difference between the effects of depleting CD4, CD8 or both subsets of T cells suggesting that they are equally important in controlling the MB49 tumour.

The numbers of Lymphocytes, CD4 and CD8 T cells are elevated in the tumour dLN of MB49 bearing mice. The numbers of all cells peaks at day 7.

The number of FoxP3⁺ regulatory T cells (Treg) is higher in the MB49 dLN than in the ndLN and pLN of control mice. Unlike other T cells, Treg numbers did not decrease over time and were as high on day 21 as they were on day 7 (Figure 7).

This result is consistent with previous reports demonstrating increased Treg frequency in the tumour dLN of mice²⁴⁸ and humans¹²⁸. The consistent number of Treg over the course of 21 days may suggest that dying cells or cells that are moving out of the dLN are being replaced by fresh natural Treg or the conversion of conventional CD4 T cells to induced Treg. It has been demonstrated that cytokines (e.g. TGFβ) and inhibitory molecules in the tumour microenvironment cause iTreg conversion²⁴⁹.

To analyse whether T cells were not only expanded in the tumour dLN but were also infiltrating into the tumour itself we made use of YFP⁺ C57BL/6 female mice which constitutively expresses the YFP protein in all cells. By staining MB49 tumour sections with anti-CD3^{PE} and DAPI it was possible to distinguish between tumour tissue, host tissue and lymphocytes. MB49 tumour tissue was found to be infiltrated by CD3⁺ T cells (Figure 8B). These results were confirmed by FACS that showed that CD4 and CD8 T cells represented 6% and 5% of the total number of cells in the tumour respectively (Figure 8A).

Despite having increased levels of CD8 T cells in the tumour dLN and showing signs of tumour infiltrating T cells the tumour dLN and peripheral blood did not contain any tetramer positive CD8 T cells (Figure 9). This result is in agreement with a previous study that also found low to undetectable frequencies of Uty-tetramer⁺ CD8 T cells in MB49 bearing mice²¹⁹.

Together the observations, that there are CD4 and CD8 T cells infiltrating the tumour and that there is T cell expansion in the tumour dLN, but no systemic CD8 HY response or tumour rejection, leads to the conclusion that tumour derived factors are causing T cell suppression. Indeed, it has been shown that suppressive CD4 and CD8 T

cells can be induced by the tumour microenvironment to secrete TGF β and IL-10 which promotes tumour growth²⁵⁰.

Depletion of CD25⁺ T cells 3 days before MB49 inoculation using the PC61 antibody leads to increased tumour growth in the depleted mice compared to the controls. This result is at odds with the current literature^{125, 147, 222} and may be an anomaly, depletion with PC61 was attempted again in Chapter 4.

The contribution of IL-10, IL-15, IL-17, IFN γ and TGF β to the growth of MB49 tumours was studied.

MB49 tumours in IL-10 KO¹⁹¹ mice were larger and grew faster than in control mice (Figure 11), a result that was confirmed when IL-10 signalling was blocked using an IL-10R mAb¹⁹². Together these results confirm reports that IL-10 inhibits tumour growth²²⁸⁻²³⁰ but is contradictory to studies using IL-10 KO mice¹⁹¹ and even the IL-10 blocking mAb¹⁹². Although I am confident with the results we have observed I am unsure how to explain the apparent contradiction with the results from Halak *et al.* who also used the MB49 tumour and IL-10 KO mice.

Neutralisation of IFN γ with the mAb R46/A2¹⁰⁰ led to large rapidly growing MB49 tumours in all mice tested (Figure 12) confirming numerous reports that IFN γ is critical in anti-tumour responses¹⁹⁷⁻¹⁹⁹.

There was no difference in the growth of MB49 in hIL-15 transgenic (Tg) mice¹⁹³ which demonstrated that over expression of IL-15 alone is not sufficient to cause MB49 tumour rejection (Figure 13). The ability of IL-15 Tg mice to reject MB49 tumours is discussed in chapter 6.

MB49 tumours grew more rapidly and to a larger size in IL-17 KO¹⁹⁵ mice (Figure 14). A result which, in agreement with a study by Kryczek *et al.*¹⁹⁶ which investigated the growth of MC38 tumour in IL-17 KO mice, suggests that IL-17 inhibits tumour growth. However, this result contradicts a study by Wang *et al.*²⁵¹ which shows that the growth of MB49 is inhibited in IL-17 KO mice when compared to WT mice, although they do not state the dose of MB49 used. It may be that the MB49 dose is critical to the growth of MB49 tumour in IL-17 KO mice. However, in support of IL-17 mediated MB49 rejection a study by Vokaer *et al.*²⁵² showed delayed rejection of syngeneic C57BL/6 male skin grafts transplanted to female IL-17 KO mice. The debate about IL-17 being pro or anti tumour remains open¹⁹⁴.

In vivo neutralisation of TGF β did not effect the growth of MB49 tumours when 1×10^6 MB49 cells were injected s.c. A result that is in agreement with previous reports that suggest additional help with IL-2 or vaccination and adjuvant therapy are required to cause

tumour rejection after TGF β neutralisation^{237, 238}. Moreover, dnTGF β RII-Tg mice²⁰⁷ could not control MB49 tumours at the same dose. However, dnTGF β RII-Tg mice did successfully control a lower dose of 1×10^5 MB49 cells. These results suggest that the growth of MB49 tumours is independent of TGF β mediated immune suppression when MB49 is inoculated at high doses, possibly because other immuno-regulatory mechanisms become dominant (i.e. IL-10 suppression/PD-L1/2 expression). At low doses of MB49 tumour, TGF β signalling is critical to MB49 growth suggesting that the immune system is only able to control the tumour if Treg and conventional CD4 and CD8 T cells can respond to TGF β .

Chapter 4 : Treatment of MB49 tumours with immuno-modulatory monoclonal antibodies

4.1 : Introduction

Immunomodulation using monoclonal antibody (mAb) therapy has emerged as a powerful tool to treat a variety of diseases, including cancer. The journey to the clinic for mAb therapy often starts in murine models. Recent studies in mice have revealed the potent anti-tumour effect of a mAb against Glucocorticoid-Induced Tumour necrosis factor Receptor (GITR), DTA-1.

GITR is a member of the Tumour Necrosis Factor Receptor (TNFR) family. It is expressed on T lymphocytes with the primary purpose of protecting T cells from activation-induced cell death^{72, 144, 253}. Engagement of GITR with DTA-1 mAbs or its natural ligand (GITR-L) activates resting T cells and causes up-regulation of CD25, CD69 and expression of IFN γ , IL-2, IL-4 and high levels of IL-10¹⁴⁸. GITR-L is expressed on murine B cells, DC and macrophages¹⁴⁶.

Administration of DTA-1 to mice eliminates established tumours^{147, 149, 200, 201}, although it is unclear whether DTA-1 abrogates Treg suppression or activates CD4 and CD8 effector cells¹²⁶. Under normal conditions, CD4⁺ CD25⁺ FoxP3⁺ regulatory T cells (Treg) constitutively express higher levels of GITR than conventional CD4 and CD8 T cells^{145, 254}. The outcome of GITR signalling on Treg cells appears to be functional inactivation (i.e. abrogation of their inhibitory function)^{145, 255}, whereas ligation of GITR on conventional T cells provides a co-stimulatory signal promoting activation^{148, 256-259}.

Since ligation of GITR by DTA-1 may disable Treg as well as co-stimulating conventional T cells^{146, 260}, DTA-1 is potentially an effective reagent for enhancing T cell immunity against tumours^{126, 147, 149, 150, 261}. Indeed, the effectiveness of DTA-1 at enhancing tumour rejection in murine models is well established, however the mechanisms of action of DTA-1 are currently being debated. Studies arguing that DTA-1 abrogates the inhibitory ability of Treg cells are based largely on *in vitro* data^{145, 254} but, these conclusions have been challenged by a more recent study that suggests that the action of DTA-1 is to make CD25⁻ T cells resistant to CD25⁺ Treg mediated inhibition²⁵⁹.

It is important to bear in mind that T cell behaviour *in vivo* cannot always be predicted by *in vitro* analysis, and few studies have addressed the impact of DTA-1 on Treg cells *in vivo*²⁶². Inactivation^{145, 147, 262}, expansion¹⁵⁰, and depletion²⁶³, or inhibition of migration²⁰⁰ of Treg cells are three possible outcomes of GITR ligation by DTA-1 on Treg

cells *in vivo*. Although it has been proposed¹⁴⁶ and indicated^{200, 263} that DTA-1 depletes Treg *in vivo*, this has not yet been formally demonstrated.

To investigate the effects of DTA-1 *in vivo*, several strategies were tested in this study. The results are described below.

4.2 : Results

As mentioned above, DTA-1 treatment stimulates resting T cells and may cause inactivation^{145, 147, 262}, expansion¹⁵⁰, depletion²⁶³ or inhibition of migration²⁰⁰ of Tregs. This study set out to investigate the mechanisms by which DTA-1 therapy causes tumour rejection in the MB49 model. After confirming that the MB49 tumour model could be used to analyse the mechanisms of DTA-1 action, the effect of DTA-1 on the proportions of T cell subsets, Treg activity and survival and CD8 T cell activity, was examined. The results of these experiments are described below.

4.2.1 : Treatment of established MB49 tumours with DTA-1

Although administration of DTA-1 monoclonal antibodies (mAb) alone is sufficient to cause the rejection of some types of experimental tumours (Meth A and Colon 26¹⁴⁷), this treatment appears to be less effective for other experimental tumours (B16 and RENCA^{147, 150}) unless an additional manipulation, such as DNA vaccination¹⁴⁹, is also included. This suggests that the efficiency of DTA-1 therapy is dependent on the characteristics of the tumour cells used.

The efficiency of DTA-1 therapy has not previously been tested in the MB49 tumour. To assess the susceptibility of MB49 tumours to DTA-1 treatment, 17 female C57BL/6 mice were injected s.c. with 5×10^5 MB49 cells on day 0. These mice were then randomly assigned to three different groups which were given either 50µg of DTA-1 by a single i.p. injection on day 3 ($n = 4$) or day 8 ($n = 9$) or were left untreated ($n = 4$).

Growth of MB49 tumours in the untreated group was rapid, reaching an average size of greater than 100 mm² by day 28. In contrast, DTA-1 treatment, on either day 3 or day 8, caused a significant reduction in tumour growth (Figure 16A, $p < 0.004$) and an increase in survival (Figure 16B) compared to untreated controls. Of note, the timing of administration of DTA-1 may be important for the anti-tumour response: mAb treatment given 8 days after tumour inoculation appeared to be more effective than mAb treatment given 3 days after inoculation; day 3 treated mice had larger tumours (Figure 16A, $p = 0.04$) and reduced survival (Figure 16B, MST; 47.5 days vs. 49.4 days) compared to the mice treated on day 8.

These data confirmed that the DTA-1 antibody was able to effect rejection of MB49 tumours and thus this system could be used to further investigate the mechanisms of DTA-1 mediated tumour rejection.

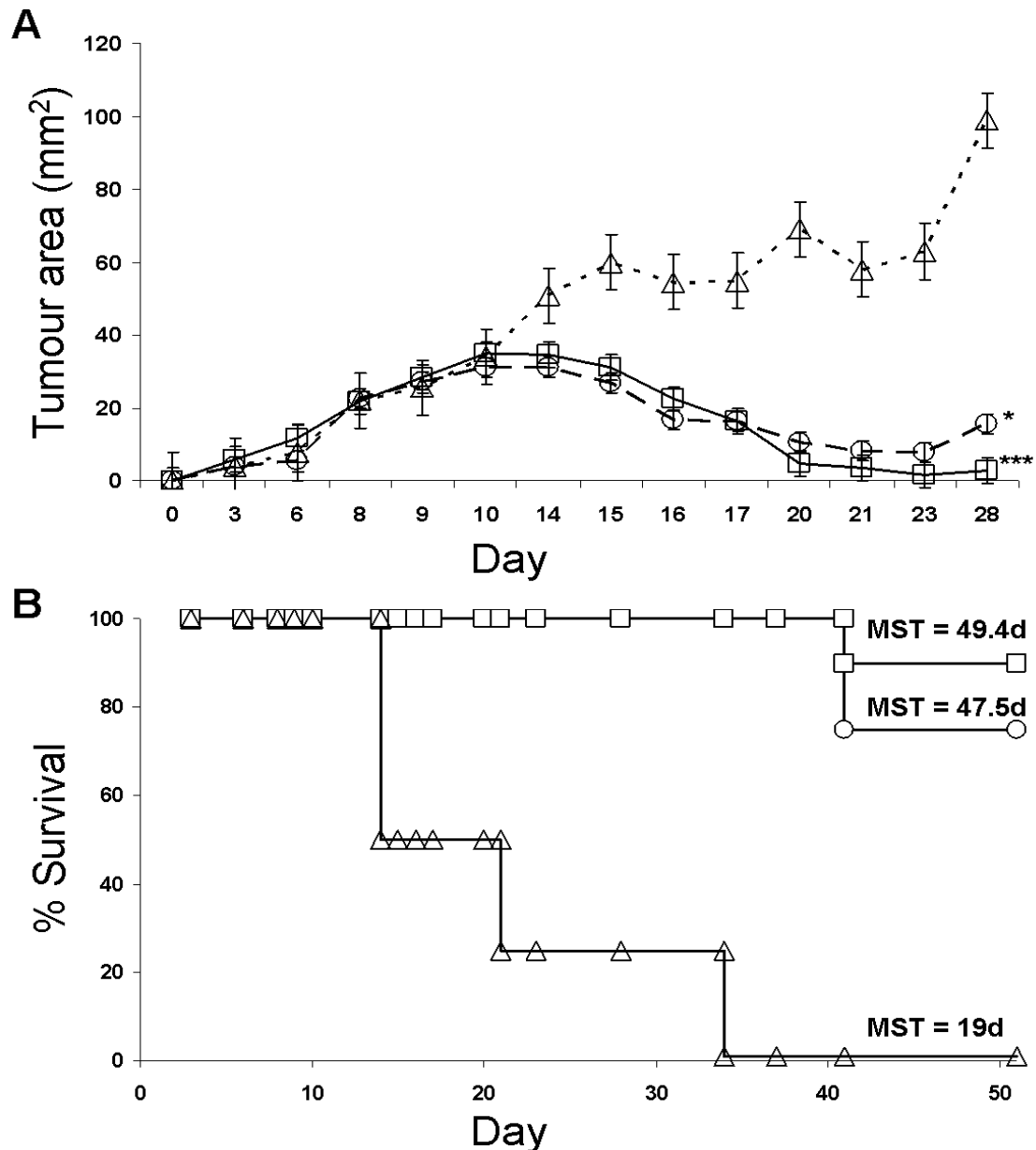


Figure 16. DTA-1 effectively treats MB49 tumours

A) A total of 17 B6 female mice were injected with 5×10^5 MB49 cells s.c. Mice were randomly divided into three groups: one was treated with 50 μ g of DTA-1 i.p. on day 3 (○, $n = 4$), one on day 8 (□, $n = 9$) the other group was not treated (Δ, $n = 4$). Tumour area was measured, the results are presented as the mean $\pm$ SD. *** $p < 0.004$ (control, Δ vs. day 8, □), * $p = 0.04$ (day 3, ○ vs. day 8, □).

B) MB49 progression is presented as % survival. The s.c. inoculation of B6 mice with MB49 cells does not lead to death. But the mice are recoded as death once they reach one of following end points of experiment. One is the maximum of size 14 mm x 14mm, the other is the development of severe ulceration by tumour. The mean survival time (MST) of individual groups was also indicated. One representative of three independent experiments is shown.

4.2.2 : The effect of DTA-1 on the proportions of T cell subsets

The proportions of T cell subsets were analysed, in mice treated with DTA-1, to see if DTA-1 caused any significant changes, in these populations, which could explain the anti-tumour effect of DTA-1. Naïve female C57BL/6 mice were injected i.p. with 50µg of DTA-1 or an isotype control antibody (GL113) and after 5 days, peripheral blood was collected from the lateral tail vein and depleted of RBCs, before staining with anti-FoxP3^{FITC}, anti-CD8^{PerCP} and anti-CD4^{APC}. The percentage of CD4⁺ CD8⁺, and CD4⁺FoxP3⁺ T cells in the peripheral blood lymphocytes (PBL) was analysed by flow cytometry. The second analysis was carried out on day 11 but peripheral LN cells, rather than PBL, were analysed.

The proportion of CD4, CD8 T cells and Treg cells did not change significantly in the peripheral LNs of mice treated with DTA-1 (Figure 17B), however there was a noticeable, but not significant, reduction in the percentage of Treg (from 8% to 6%), in the blood of DTA-1 treated mice (Figure 17A).

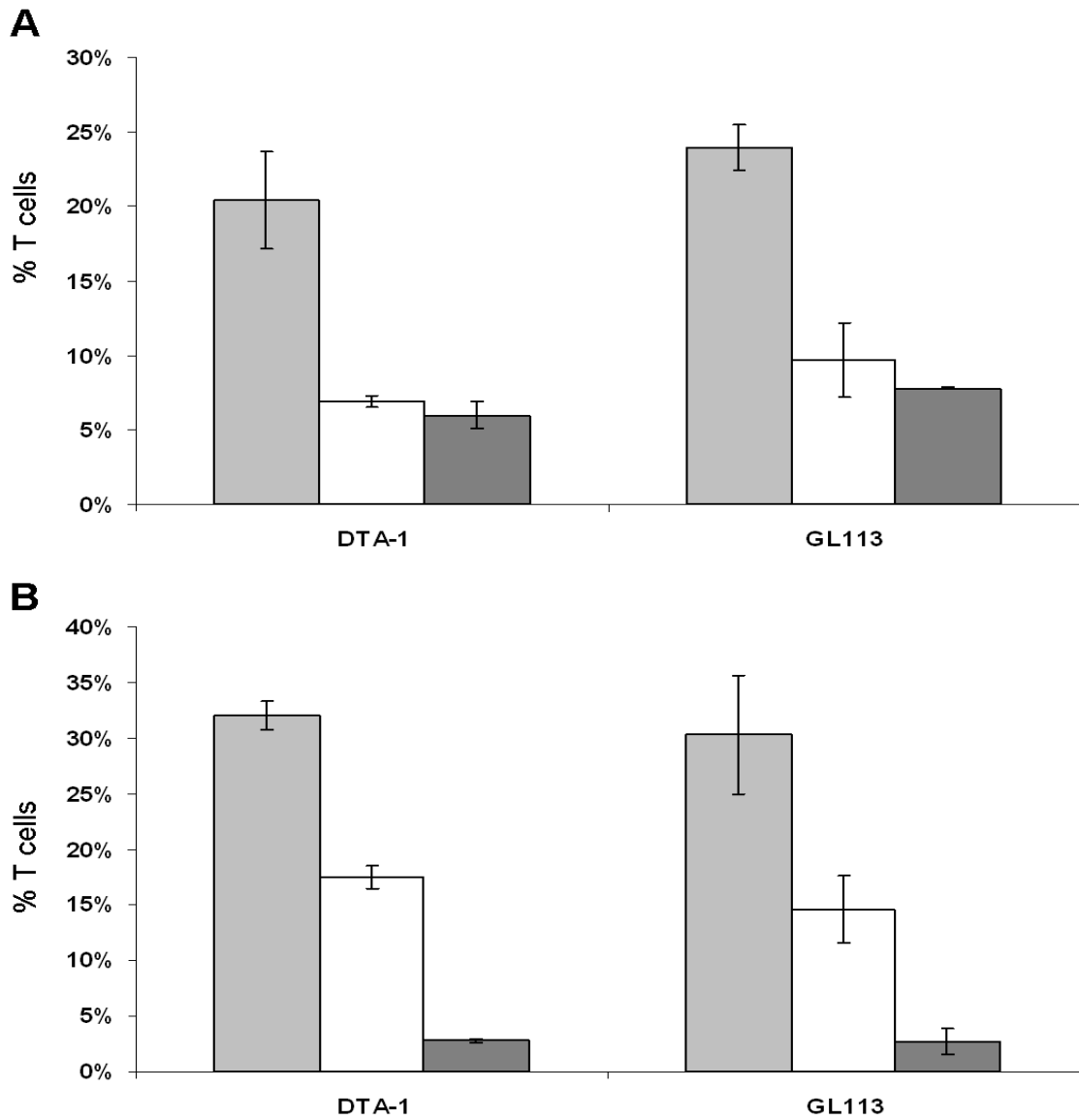


Figure 17. Effect of DTA-1 treatment on T lymphocyte populations and subsets.

A) Female C57BL/6 mice (n=2) were injected with DTA-1 or GL113 i.p., 5 days later PBL was taken and depleted of red blood cells and stained with anti-FoxP3^{FITC}, anti-CD8^{PerCP} and anti-CD4^{APC}. The percentages of CD4⁺ (■), CD8⁺ (□) and CD4⁺Foxp3⁺ T cells (■) as a fraction of the total lymphocyte population are presented.

B) Peripheral lymph nodes (pLN) from the same mice as described in A, were taken 11 days after DTA-1 or GL113 treatment and stained with anti-FoxP3^{FITC}, anti-CD8^{PerCP} and anti-CD4^{APC}. The percentages of CD4⁺ T cells (■), CD8⁺ T cells (□) and Treg (■) as a fraction of the total lymphocyte population are presented.

4.2.3 : The effect of DTA-1 on Treg activity

DTA-1-mediated GITR signalling on Treg cells abrogates their suppressive activities *in vitro*^{145, 254}. However, because T cell behaviour *in vivo* cannot be predicted by analysing *in vitro* T cell co-cultures, we evaluated the inhibitory capacities of Treg cells isolated *ex vivo* from either untreated or DTA-1-treated mice, one day after DTA-1 administration. In a preliminary experiment we injected anti-GITR^{PE} i.v. to a female GFP-FoxP3 C57BL/6 mouse and were able to demonstrate that CD4⁺GFP⁺ Treg preferentially bound anti-GITR for up to 2 days²⁶⁴. In a standard *in vitro* suppression assay, Treg cells isolated from DTA-1-treated mice were as potent as those from untreated mice at inhibiting the response of conventional CD4 T cells (isolated from untreated mice) to anti-CD3 stimulation (Figure 18). Likewise, Treg cells from control antibody (GL113)-treated mice showed similar suppressive activity to those from untreated or DTA-1-treated mice (data not shown). Thus, activation of GITR signalling on Treg cells *in vivo* does not lead to their functional inactivation. This suggests that there are additional interactions occurring *in vivo*, to maintain the Tregs suppressive activities or abrogate the inhibiting effect of DTA-1, that do not occur in the *in vitro* assays described in other studies^{145, 254}. The results of this study are consistent with the findings of Ramirez-Montagut *et al.*¹⁵⁰.

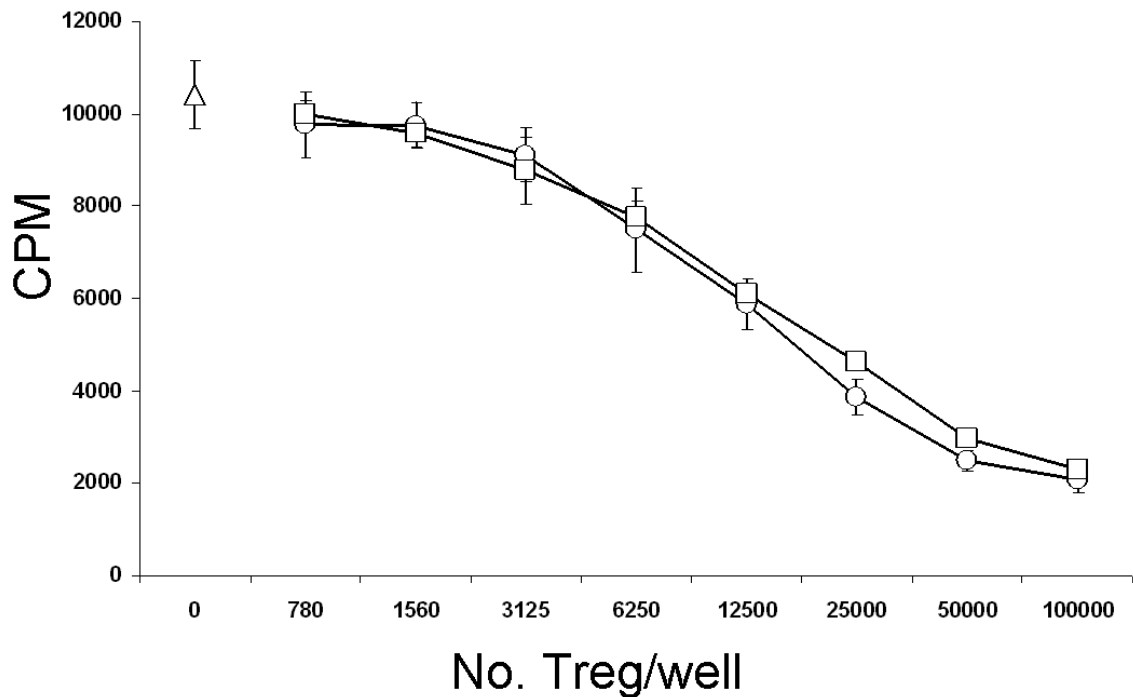


Figure 18. Normal suppressive function of Treg cells from DTA-1 treated mice.

Two groups of B6 mice (3 mice/group) were untreated or treated with anti-GITR by i.p. injection of 50 μ g of DTA-1. 24 h later, Treg ($CD4^+CD25^+$) and conventional CD4 T cells ($CD4^+CD25^-$) were purified from pooled LN and spleen cells by FACS sorting.

Conventional CD4 T cells (1×10^5 /well) from the untreated group were cultured with anti-CD3 (0.05 μ g/ml) and accessory cells (T cell-depleted and irradiated B6 spleen cells, 2×10^5 /well) in the absence (Δ) or presence of two times serially diluted Treg (from 1×10^5 /well to 780 /well) from either untreated ($\square$) or DTA-1-treated mice ($\circ$) in 96-round bottomed plates for 3 days. Tritiated thymidine (0.5 μ Ci/well) was added to each well for the last 12 h. One representative of two independent experiments is shown.

4.2.4 : The effect of DTA-1 on Treg survival

Treg cells constitutively express higher levels of GITR than conventional T cells. It is thought that this may make them susceptible to deletion. To determine the effect of DTA-1 on Treg survival (something that has been suggested to be affected by DTA-1 and may contribute to the anti-tumour effect of DTA-1), intra-tumoural accumulation of Treg cells was analysed: Single cell suspensions of MB49 tumours isolated from untreated and DTA-1-treated mice were stained with anti-CD4^{PerCP} followed by intracellular staining with anti-Foxp3^{FITC}. The percentage of CD4⁺Foxp3⁺ cells in MB49 tumours from individual mice is shown in Figure 19A. The percentage of Treg in MB49 tumours is significantly decreased by DTA-1 treatment, declining from 10% in untreated mice to 6% in DTA-1 treated mice ($p=0.026$)(Figure 19A), which is consistent with other reports²⁰⁰. Figure 19A also shows that the depletion of Treg cells by DTA-1 is more extensive in tumours than in the dLN.

It is also speculated that the environment of a growing MB49 tumour might preferentially increase GITR expression on tumour-infiltrating Treg cells, rendering them more susceptible to depletion. To test this, Foxp3/GFP mice were injected s.c. with 5×10^5 MB49 cells, 8 days later the expression of GITR by Treg cells in the MB49 tumour was compared with those in dLN. As shown in Figure 19B, there was a 4-fold increase in GITR expression on MB49-infiltrating Treg cells (Mean fluorescent intensity increased from 590 in tumour draining LN to 2182 in tumour; $p < 0.0003$), which is likely to increase sensitivity to DTA-1-induced depletion.

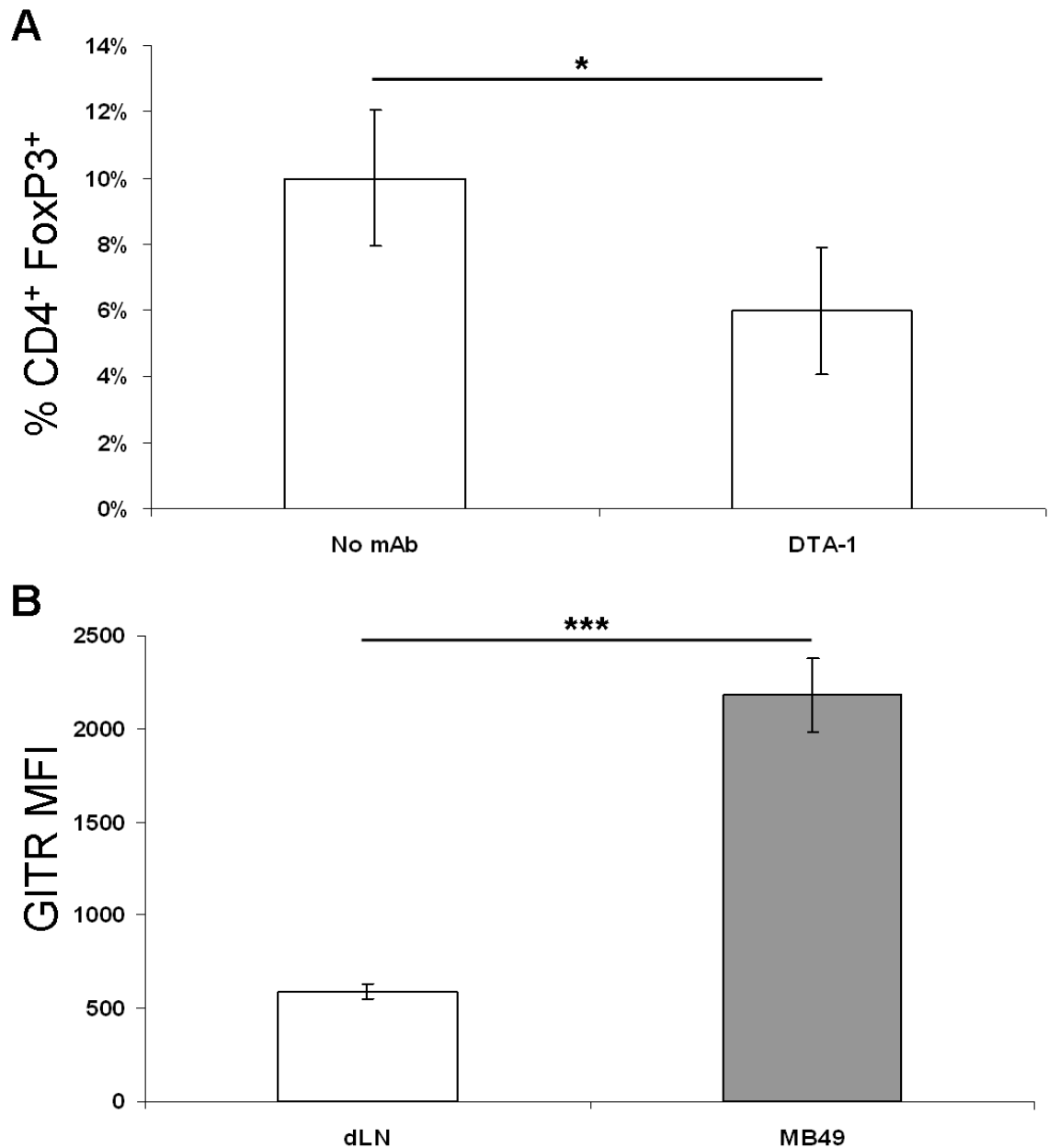


Figure 19. DTA-1 preferentially depletes GITR^{hi} Treg in MB49 tumours.

A) On day 0, C57BL/6 female mice (n=8) were s.c. inoculated with 5×10^5 MB49 cells. Half the mice were injected i.p. with DTA-1 on day 5 and 9, and half of them received no mAb treatment. On day 15, all mice were culled and MB49 tumours were isolated and stained with anti-CD4^{PerCP} followed by intracellular staining with anti-Foxp3^{FITC}. The percentage of lymphocytes that were CD4⁺FoxP3⁺ is presented (n=4). * $p = 0.026$

B) Female FoxP3-GFP C57BL/6 mice (n=4) were injected with 5×10^5 MB49 s.c. On day 8 tumour draining lymph nodes (dLN) and MB49 tumours (n=4) were isolated and stained with anti-GITR^{PE} and anti-CD4^{PerCP} and analysed by FACS. The mean fluorescent intensity (MFI) of GITR expressed by CD4⁺ FoxP3-GFP⁺ lymphocytes is presented. *** $p = 0.0003$.

If one of the actions of DTA-1 is to deplete Treg cells *in vivo*, it would be interesting to compare DTA-1 with PC61, a standard anti-CD25 depleting mAb. A single injection of PC61 was very effective in causing the regression of MB49 tumours with 100% (5/5) of mice tumour-free by day 20, when it was administered to female C57BL/6 mice 2 days before MB49 inoculation (Figure 20A, ○). However, the same treatment protocol using DTA-1 had a limited impact on tumour growth (Figure 20A, □), with only 25% (1/4) of mice tumour-free at day 20.

This difference could be explained by the observation that PC61 was more effective at depleting Treg than DTA-1 *in vivo* (data not shown). On the other hand, DTA-1 treatment was very efficient at causing the rejection of established MB49 tumours (Figure 16 A and B) whereas PC61 therapy had no effect (Figure 20B, ○, 0% tumour-free mice). The anti-tumour activity of DTA-1 was completely lost if PC61 was co-injected with DTA-1 on day 8 (Figure 20B, △, 0% tumour-free mice).

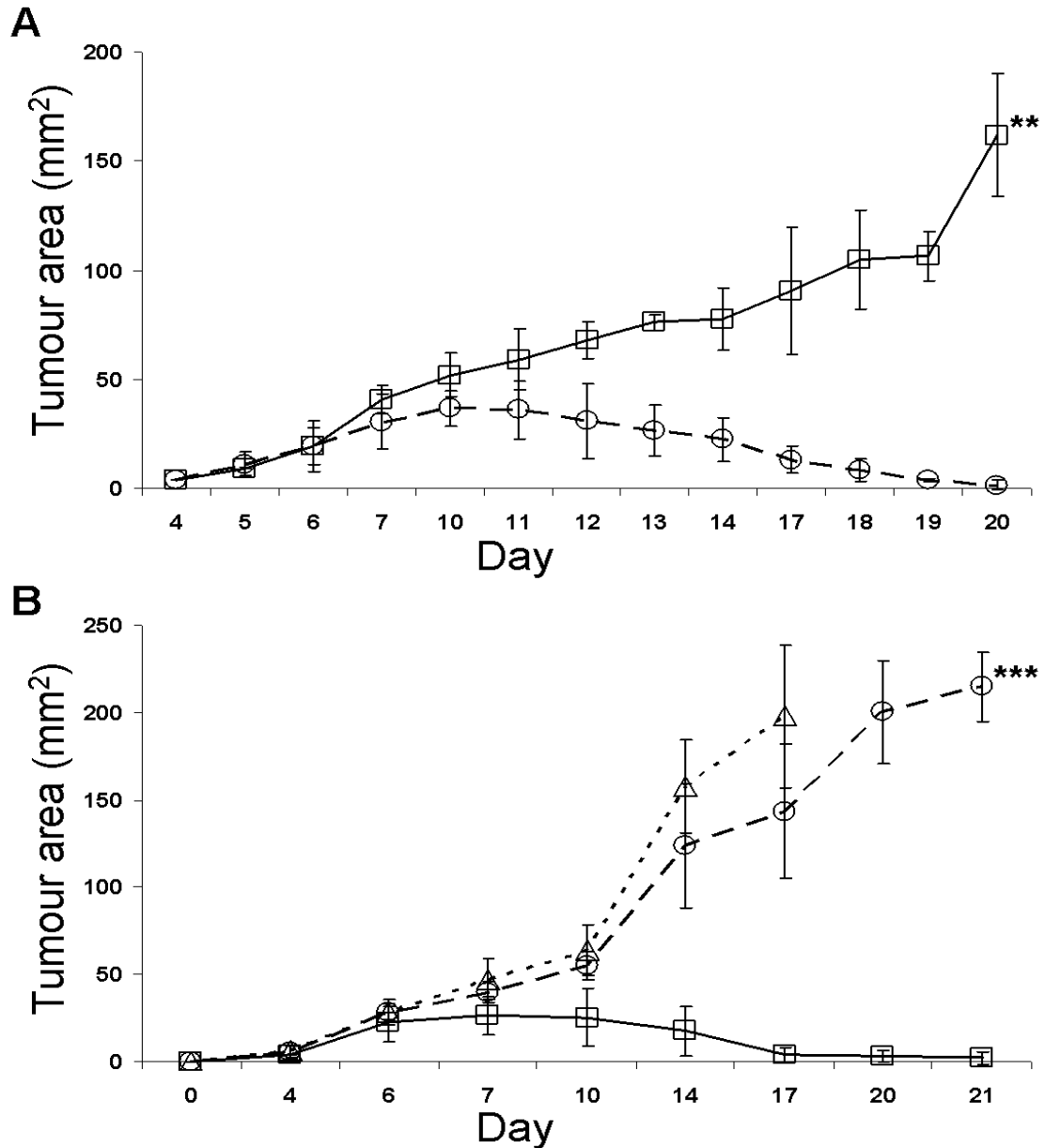


Figure 20. Comparison of DTA-1 and PC61 anti tumour efficacy

A) Two groups of B6 female mice were treated with 50µg of PC61 (○, n=5) or DTA-1 mAb (□, n=4) by i.p. injection. 2 days later, all mice were injected s.c. with 5×10^5 MB49 cells. The growth of MB49 tumours was monitored by measuring tumour size. Data is presented as the mean $\pm$ SD, **p = >> 0.0001

B) A total of 16 B6 female mice were injected s.c. with 5×10^5 MB49 cells. 8 days later, the mice were randomly divided into three groups and treated with 50µg of DTA-1 (□, n=6), 50µg of PC61 (○, n=5), or 50µg of DTA-1 mixed with 50µg of PC61 (△, n=5) by i.p. injection. The growth of MB49 tumours was monitored by measuring tumour size every other day. Data is presented as the mean $\pm$ SD ***p = >> 0.0001.

4.2.5 : The effect of DTA-1 on CD8 T cell activity

Another potential mechanism of DTA-1 mediated tumour rejection is the augmentation of CD8 T cell effector functions. DTA-1-mediated GITR signalling has been shown to enhance vaccine-induced CD8 tetramer responses against B16F10 melanomas¹⁴⁹. A basic measure of CD8 effector activity is their proliferation in response to stimuli. To test the CD8 co-stimulatory potential of DTA-1 mAb *in vitro*, MataHari CD8 T cells were stimulated with cognate peptide in the absence or presence of DTA-1. As shown in (Figure 21), the presence of DTA-1 mAb failed to augment the proliferation of MataHari CD8 T cells in response to peptide, indicating that DTA-1 is not co-stimulatory to CD8 T cells.

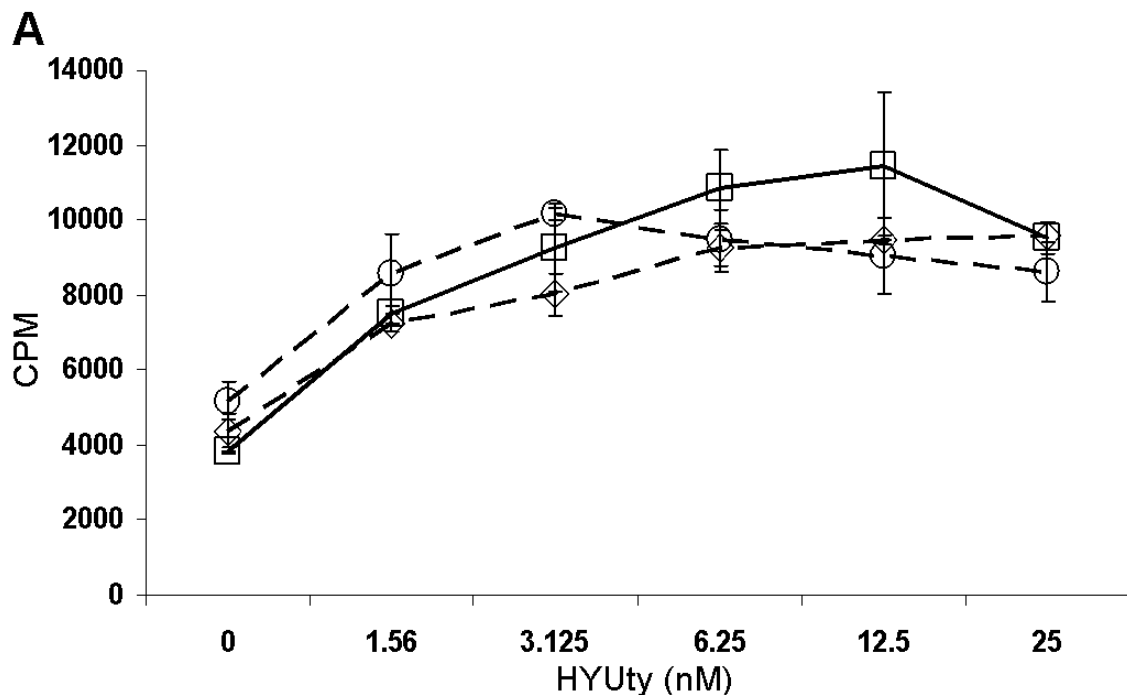


Figure 21. *In vitro* response of activated CD8 T cells stimulated with DTA-1

A) 1×10^5 Spleen and LN from Rag^{-/-} MataHari were cultured in a 96 well U bottom plate for 4 days with HYUty peptide in the absence (□) or presence of 1:300 DTA-1 (○) or 1:1000 DTA-1 (◇). Tritiated thymidine (0.5 μ Ci/well) was added on day 3 and proliferation was measured on day 4.

4.3 : Discussion

Although DTA-1 has been proposed¹⁴⁶ and indicated²⁶³ to deplete GITR expressing-Treg cells *in vivo*, a formal demonstration has never been made. This chapter provides evidence showing that DTA-1 is a depleting mAb causing a moderate reduction of endogenous polyclonal Treg in tumour free mice (Figure 17A) and a significant reduction in MB49 tumours (Figure 19A).

In agreement with previously published results¹⁴⁷ DTA-1 therapy is extremely effective at causing complete tumour rejection and prolonged tumour free survival (Figure 16). These results suggest that MB49 can be added to the list of tumours that do respond to GITR therapy, which includes the B16 melanoma²⁰⁰ and MethA¹⁴⁷, and it would be of interest to further study the phenotype of tumours that are susceptible to DTA-1 therapy.

Administration of DTA-1 i.p. to WT C57BL/6 female mice did not significantly change the ratio of B cells (data not shown) or T cells in either peripheral blood (Figure 17A) or lymph nodes (Figure 17B). However there was a noticeable albeit non-significant reduction in the percentages of Treg in the peripheral blood. GITR is only expressed on CD4 and CD8 T cells after activation but is constitutively expressed at higher levels on Treg¹⁴⁵ and therefore it is possible that DTA-1 binds only to GITR on Treg cells in the naïve mouse. The reduction of Treg from 8% to 6% (Figure 17A) suggests that DTA-1 is not causing activation and expansion of Treg, or CD4 and CD8 T cells, but is instead causing depletion of Treg. It has also been shown that DTA-1 can activate Natural Killer T cells²⁶⁵ although we have not assessed their involvement in this study.

The small reduction of Treg demonstrated in Figure 17 may be caused by antibody mediated depletion or it could potentially be caused by deactivation of Treg. If DTA-1 abrogated Treg function and led to a subsequent reduction in autocrine signalling via the TGF β and IL-10 pathways then the generation of Treg would also be reduced. To test this hypothesis the *ex vivo* function of Treg was tested .

Tregs were isolated 24h after DTA-1 treatment to allow sufficient time for GITR binding and signal transduction. Isolated CD4⁺CD25⁺ T cells were found to be functional Treg cells with similar suppressive activity as those from untreated mice (Figure 18) which is consistent with other reports^{150, 200} and supports the hypothesis that DTA-1 does not

abrogate the suppressive function of Treg. These findings suggest that the reduction of Treg in the peripheral blood demonstrated in Figure 17 is not due to the abrogation of Treg function and may in fact be due to antibody mediated depletion. Having tested the effect of DTA-1 on naïve populations and on Treg function it was then of interest to look at the effect of DTA-1 therapy on Treg in the MB49 tumour.

Mice that had been treated twice with DTA-1, on day 5 and 9 after MB49 tumour showed significantly reduced ratios of tumour infiltrating Tregs when compared to control mice. It is possible that the reduction in Treg within the tumour may be because of inhibition of Treg migration to the tumour or because of antibody mediated depletion. In support of antibody mediated depletion DTA-1 has an IgG2b isotype which is shared by a large number of *in vivo* depleting mAb including those with specificity for CD4 (GK1.5⁵⁰), CD8 (YTS169²¹³) and Thy1.2 (30H12²⁶⁶). However, a recent study from (Cohen/Wolchok; 2010) suggests that DTA-1 inhibits migration of Treg into B16 melanomas and leads to loss of FoxP3 expression, but not suppressive function.

CD8⁺ MataHari T cells cultured with their cognate peptide with or without two concentrations of DTA-1 did not show any difference in their proliferative capacity (Figure 21). This result conflicts with previous results demonstrating that DTA-1 stimulates CD8⁺ T cells *in vitro*²⁵⁷. However the study by Ronchetti *et al.* incubated polyclonal T cells with CD3, CD28 and DTA-1 and not with cognate peptide. It is possible that the type of signal through the TCR is critical to the response to DTA-1.

Because one of the actions of DTA-1 is to deplete Treg cells *in vivo*, it was of interest to compare DTA-1 with PC61, a standard anti-CD25 depleting mAb. A single injection of PC61 has been shown to be effective at reducing tumour progression in several murine tumours^{80, 147, 222}. In agreement with previous studies PC-61²¹¹ caused the rejection of tumours in 100% of female C57BL/6 mice when it was administered i.p. 2 days before MB49 inoculation. Conversely DTA-1 treatment had limited impact on MB49 tumour growth (Figure 20A). This difference could be explained by the observation that PC61 was more potent than DTA-1 at depleting Treg *in vivo* (data not shown). On the other hand, DTA-1 treatment was very efficient at causing the rejection of established MB49 tumours that were 8 days old whereas PC61 therapy had no effect (Figure 20B). It is possible that CD25-expressing effector T cells with anti-MB49 activity are depleted by PC61 treatment. Moreover, in support of this, the anti-tumour activity of DTA-1 was completely lost if PC61 was injected with DTA-1 on day 8 (Figure 20B).

I owe a great deal of thanks to Shaima Begom, Dr. Caroline Addey, Matthew White, Prof. Julian Dyson and Dr. Jian-Guo Chai, for their help in the preparation of this chapter and the paper that we published together using these results. The paper is published under the title ‘Depletion of regulatory T cells by the anti-GITR mAb as a novel mechanism for cancer immunotherapy’ in the Journal of Cancer Immunology and Immunotherapy²⁶⁴, a copy of this paper is attached in Appendix A.

Chapter 5 : Adoptive Transfer of CD4⁺ T cells

5.1 : Introduction

Adoptive cell transfer of *ex vivo* expanded lymphocytes promises to be a successful therapy for cancer. Nevertheless, because of the suppressive environment that surrounds tumours it is clear that additional methods, that will improve T cell activation, persistence *in vivo* following transfer and anti-tumour activity, are still needed.

CD4 T cells are critical for anti-tumour responses against a variety of murine tumours¹⁷⁰⁻¹⁷³. This study has shown that CD4 T cells are critical for MB49 tumour rejection, as CD4 depletion leads to larger tumours and increased mortality (Chapter 1). Several murine studies have suggested that CD4 T cells are effective at causing tumour rejection^{172, 174, 178}. Moreover, two of these studies find that CD4 T cells are more effective than CD8 T cells at causing tumour rejection even when adoptively transferred into immuno-deficient mice and they suggest that CD4 T cells can directly kill MHC-II negative tumours^{172, 178}. The ability of CD4 T cells to contribute to tumour rejection is thought to be largely based on their ability to secrete IFN γ ^{171, 174, 175}.

Despite the ability of CD4 T cells to cause tumour rejection alone or in combination with other treatments, the field of CD4 adoptive immunotherapy has not advanced very far. Basic^{168, 170, 176} and clinical studies^{13, 16, 19} have concentrated on the adoptive transfer of CD8 T cells alone or in some cases with CD4 T cells^{170, 173} or CD4 peptide vaccines¹⁷⁷.

In this study CD4 T cells from Marilyn mice were used for adoptive transfer experiments. Marilyn CD4 T cells express the Tg TCR, encoding the VJ segments TCR α (V α 1.1-J α 35) and TCR β (V β 6-J β 2.3), which recognises the HY/A^b/Dby peptide (NAGFNSNRANSSRSS) presented in the context of the H2-A^b²⁶⁷ MHC-II molecule. All T cells in the peripheral blood, spleen and lymph nodes of female Marilyn Rag^{-/-} mice are V β 6⁺ CD4⁺ transgenic T cells. Marilyn CD4 T cells have been shown to be effective at causing the rejection of MB49 tumours in Rag^{-/-} C57BL/6 mice¹⁷⁸. To test how effective naïve and activated Marilyn T cells were in immuno-competent mice, and at what dose, experiments were carried out in this study in which Marilyn T cells were adoptively transferred to WT female C57BL/6 mice with MB49 tumours. The results are discussed after the introduction.

The adoptive transfer of T cells is often confounded by the suppressive conditions surrounding the tumour, the presence of Treg and the lack of co-stimulatory cytokines. To enhance adoptive T cell transfer, many murine studies have used sub-lethal irradiation of between 4 – 8Gy¹⁵⁹⁻¹⁶¹. Irradiation reduces the number of suppressive Tregs, increases antigen shedding from tumours and increases the amount of ‘space’ and cytokine availability by depleting competing T cells, all of which combined mean that adoptively transferred T cells have better anti-tumour potential.

As previously described in Chapter 3, IL-17A has the potential to mediate tumour rejection. One of the main sources of IL-17A is Th17 CD4⁺ T cells that secrete IL-17A under the influence of the transcription factor Ror γ ²³⁹ and are generated by the combined actions of TGF β and IL-6²⁴⁰. The frequency of Th17 T cells is increased in several different forms of human and murine cancer^{241, 242, 268} and indeed the tumour microenvironment is a source of cytokines that drive Th17 differentiation including IL-6, IL-21, IL-23, and TGF β ^{243, 244}.

Th17 cells produce pro-inflammatory cytokines including IL-17, IL-6, and G-CSF and have been associated with inducing experimental autoimmunity including autoimmune encephalitis^{179, 269} (EAE) and collagen induced arthritis. The importance of Th17 cells in tumour immunity has only recently been investigated, with one study demonstrating that tumour specific Th17 cells are more effective than Th1 cells at destroying established B16 melanomas¹⁸⁰.

An *in vitro* culture protocol was designed, during this study, in order to generate large numbers of Th17 cells for adoptive transfer. Th17 cells generated, using this protocol, were then used in adoptive transfer experiments. The results of which are described later in this chapter.

5.2 : Characterisation of Marilyn T cells

To study the adoptive transfer of CD4⁺ T cells, Marilyn CD4⁺ T cells were utilised as tumour-specific effector cells for cancer immunotherapy.

Before using Marilyn T cells *in vivo* it was necessary to investigate their phenotype and responsiveness to cognate peptides. For the experiments described in this chapter Marilyn cells were identified by flow cytometry using anti-V β 6 and anti-CD4 and/or anti-Thy1.1/1.2. Peripheral lymph node cells from a Rag2^{-/-} Marilyn female mouse were stained with anti-V β 6^{PE}, anti-CD4^{PerCP} and anti-CD62L^{APC} for flow cytometric analysis. It was

found that virtually all T lymphocytes co-expressed CD4 and V β 6 (Figure 22A, left panel), indicating a monoclonal CD4 T cell population was present. The majority of gated CD4⁺V β 6⁺ cells also expressed a high level of CD62L, suggesting that they are naïve T cells (Figure 22A, right panel). When spleen and pooled LN cells from a Rag2^{-/-} Marilyn mouse were cultured with HY/A^b/Db_y peptide, cells proliferated in a peptide dose-dependent manner, demonstrating that Marilyn CD4 T cells express a functional transgenic TCR (Figure 22B).

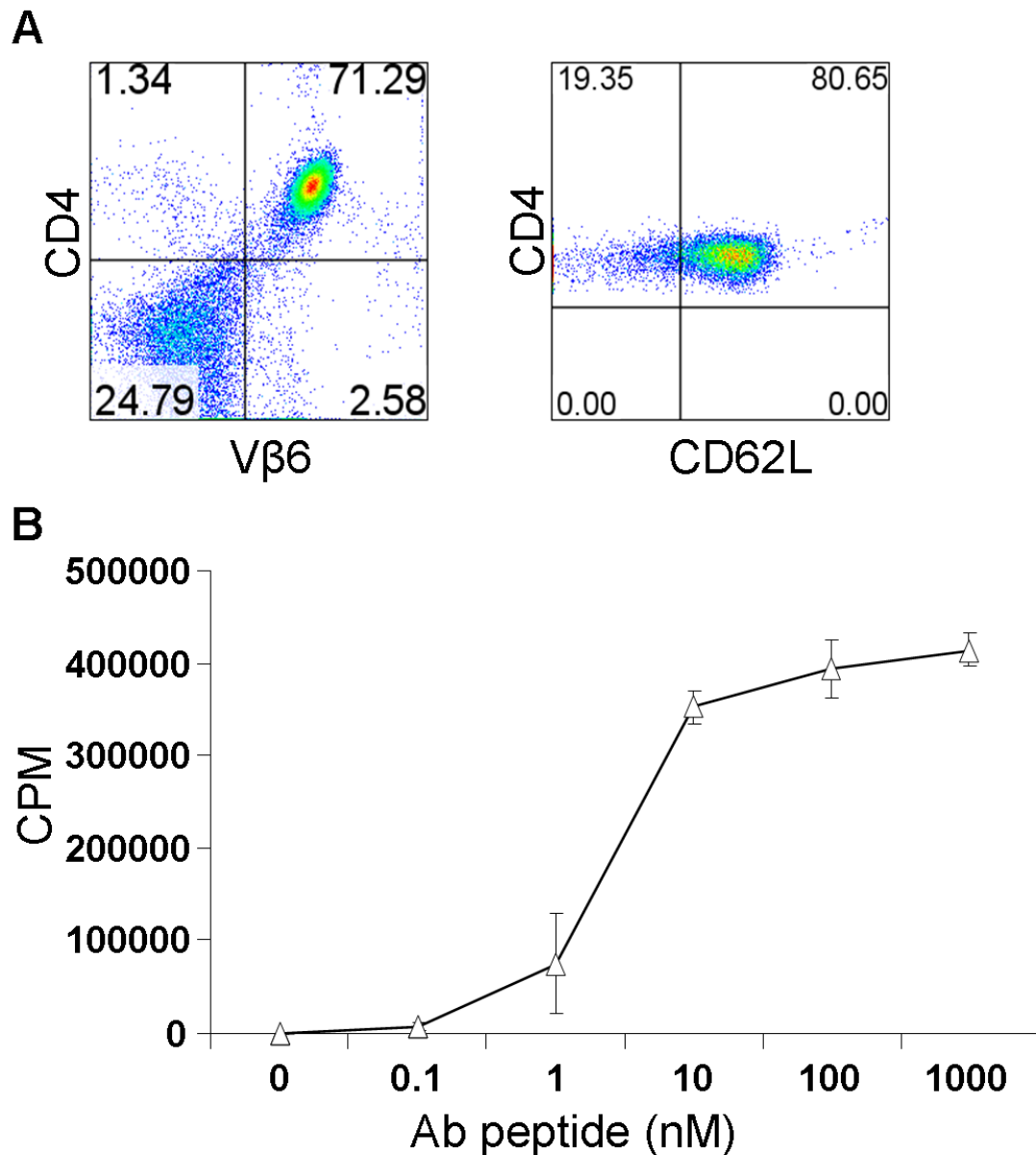


Figure 22. Characterisation of Marilyn T cells and response to antigen

A) Peripheral lymph node (LN) cells from a naïve Marilyn Rag2^{-/-} female mouse were stained with anti-Vβ6^{PE}, anti-CD4^{PerCP} and anti-CD62L^{APC} and the FACS data presented as a density plot.

B) Spleen and pooled peripheral LN cells (2x10⁴/well) from a naïve Marilyn Rag2^{-/-} female mouse were stimulated with the indicated concentrations of HY/A^b/Dby peptide. Tritiated thymidine was added at 48 hours (0.5 μCi/well) and results are expressed as the mean ±SD of the counts per minute (CPM)

5.3 : Treatment of MB49-bearing mice with adoptively transferred Marilyn CD4 T cells

Marilyn CD4 T cells have previously been shown to be effective at causing the rejection of MB49 tumours¹⁷⁸. However, those studies were exclusively performed using Rag^{-/-} B6 females as recipients of CD4 T cells. To test how effective naïve and activated Marilyn T cells are, at various doses, in a more physiological situation, naïve or activated Marilyn T cells were adoptively transferred to WT female C57BL/6 mice with MB49 tumours.

Female C57BL/6 mice were injected i.v. with naïve 0.31×10^6 or 2.5×10^6 pooled spleen and lymph node cells from Rag^{-/-} Marilyn female mice, on the same day the mice were inoculated s.c. with 5×10^5 MB49 cells. Tumour growth in mice treated with 2.5×10^6 adoptively transferred naïve Marilyn T cells, was significantly slower (tumours reaching an average of $39 \pm 14 \text{ mm}^2$ by day 19) than in control mice ($104 \pm 26 \text{ mm}^2$ at day 19) ($p = >0.01$), or mice that had been treated with 0.31×10^6 Marilyn T cells ($89 \pm 15 \text{ mm}^2$ at day 19) (Figure 23A). Although the adoptive transfer of Marilyn T cells slowed tumour growth in a dose dependant manner, it did not cause complete tumour rejection in any of the treated mice even at a higher dose of 5×10^6 Marilyn cell/mouse (data not shown).

In an attempt to increase the efficacy of adoptively transferred Marilyn T cells against MB49 tumours, T cells were activated *in vitro* before adoptive transfer. The spleen and lymph nodes from Rag^{+/-} Marilyn female mice were isolated and cultured for three days with 100nM HY-Dby peptide. After three days cells were harvested, and the T cells purified. T cells (0.5×10^6 or 2×10^6) were then adoptively transferred into female C57BL/6 mice which were then injected s.c. with 5×10^5 MB49 cells. The adoptive transfer of either 0.5×10^6 or 2×10^6 activated Marilyn T cells significantly reduced the growth of the MB49 tumour, within the time frame of this experiment (21 days), when compared to controls ($p > 0.01$), from $119 \pm 50 \text{ mm}^2$ in untreated mice, to $14 \pm 12 \text{ mm}^2$ and $12 \pm 12 \text{ mm}^2$ in mice treated with 2×10^6 and 0.5×10^6 Marilyn T cells, respectively (Figure 23B). The adoptive transfer of activated Marilyn CD4 T cells caused tumour free survival up to day 31 in 1/3 mice treated with 2×10^6 T cells and 2/4 mice treated with 0.5×10^6 T cells (data not shown).

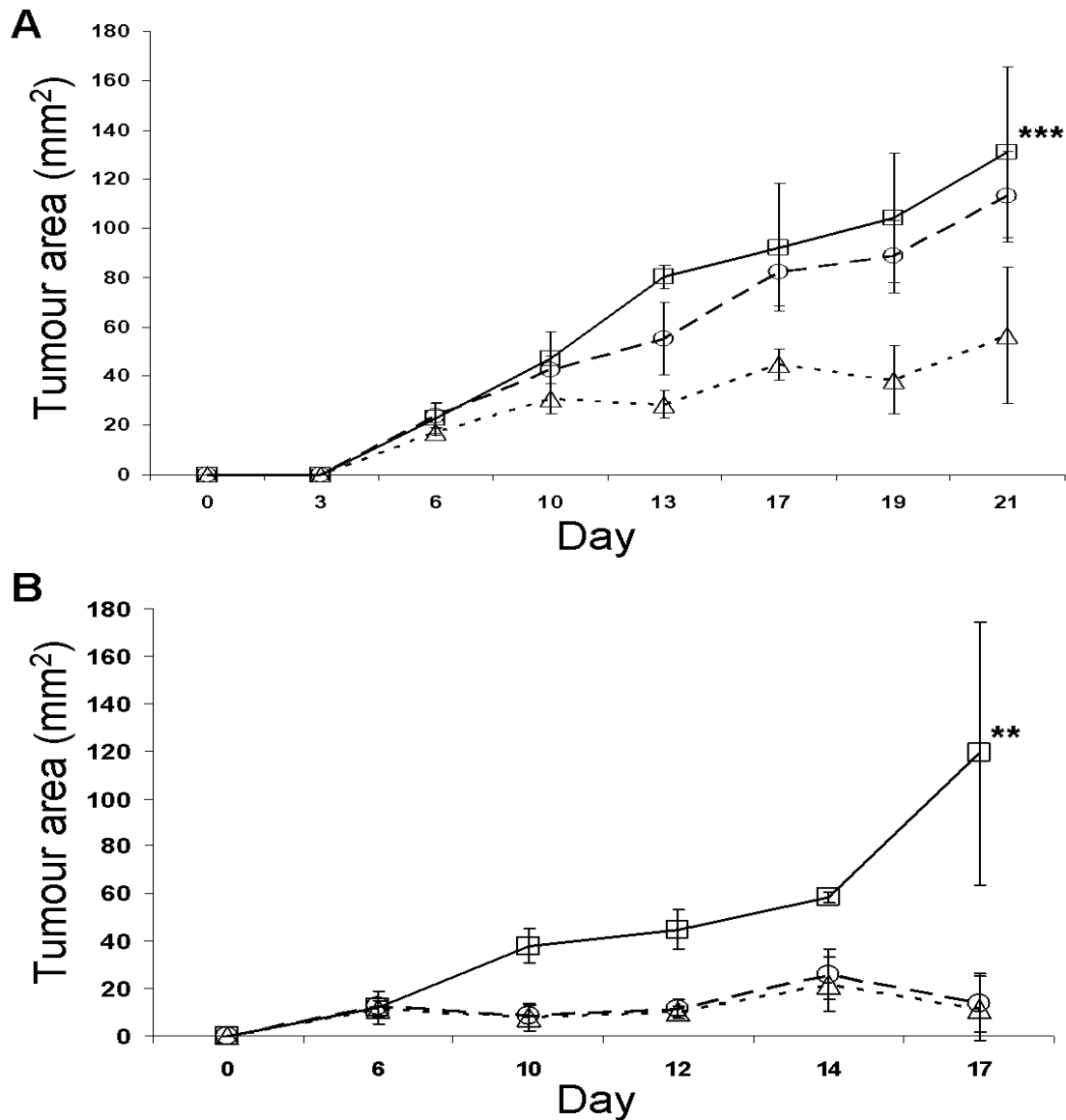


Figure 23. Comparison of the effect of the adoptive transfer of naïve or activated Marilyn T cells on MB49 tumour growth

A) Female C57BL/6 mice were not treated (□, n=3) or injected i.v. with 2.5×10^6 (△, n=4) or 0.31×10^6 (○, n=5) cells pooled from spleen and lymph nodes from Rag^{-/-} Marilyn female mice. On the same day, 5×10^5 MB49 cells were injected s.c. to all mice. Tumour size was measured. Data is presented as mean $\pm$ SD. *** $p = <0.01$ (□-control vs. △- 2.5×10^6 Marilyn i.v.)

B) Female C57BL/6 mice were not treated (□, n=3) or injected i.v. with 0.5×10^6 (△, n=4) or 2×10^6 (○, n=3) in vitro activated Marilyn CD4 T cells which were purified from 3 days cultures containing Marilyn spleen cells and 100 nM HYDby peptide. On the same day of T cell transfer, 5×10^5 MB49 cells were injected s.c. to all mice. Tumour area was measured as Width x Length at regular intervals. Data is presented as mean $\pm$ SD. ** $p = 0.01$ (□-control vs. ○- 2×10^6 Marilyn i.v.)

To better understand the underlying cellular mechanisms responsible for this successful therapy, based on Marilyn CD4 transfer, we decided to monitor the fate of donor CD4 T cells *in vivo*. To this end, Thy1.1⁺ Marilyn CD4 T cells were labelled with CFSE and adoptively transferred into Thy1.2 C57BL/6 mice that had been inoculated, s.c. with 5 x 10⁵ MB49 cells, 7 days previously. Eight days after the adoptive transfer, the tumour dLN, ndLN and tumour were harvested and the expansion and proliferation of the adoptively transferred Marilyn T cells was analysed by flow cytometry.

Donor Marilyn CD4 T cells were found to have accumulated in the tumour dLN as well as in ndLN. A small but significant proportion of donor T cells were also present in MB49 tumours. For example, donor CD4 T cells made up 0.6 ± 0.1 % in the tumour dLN, 0.2 ± 0.02 % in the ndLN and 0.03 ± 0.001 % in the tumour (Figure 24A and B). In mice adoptively transferred with Marilyn T cells but without MB49 tumours, the frequency of donor CD4 T cells in pLN was 0.1%.

When the CFSE dilution profile of donor CD4 T cells was analysed, it was found that Marilyn T cells in the dLN divided faster than those in the ndLN or in pLN of control mice. Only 8 ± 3 % of Marilyn T cells in the dLN were undivided compared to 39 ± 13 % in the ndLN, and 99% in pLN of control mice (Figure 24 C and D).

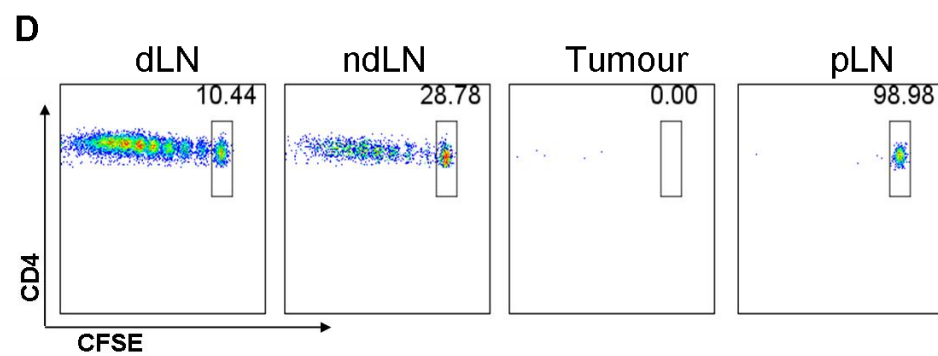
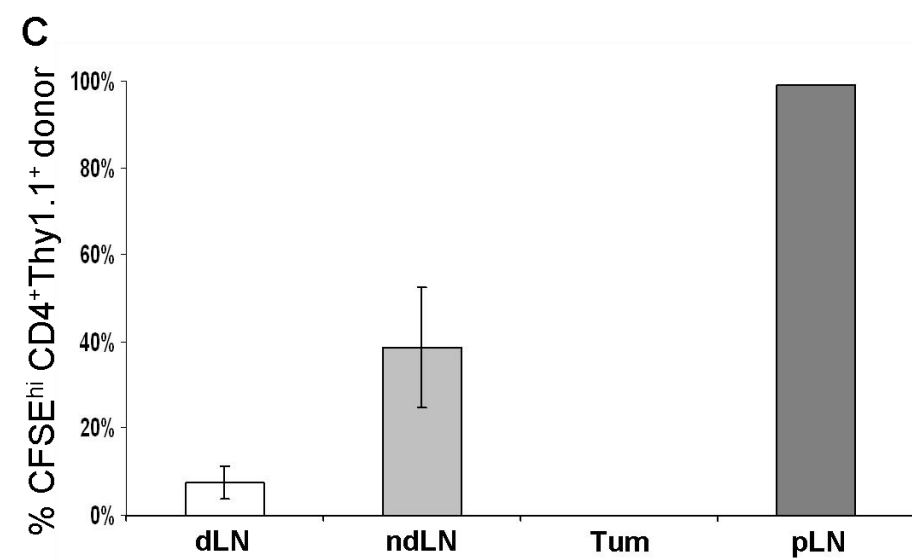
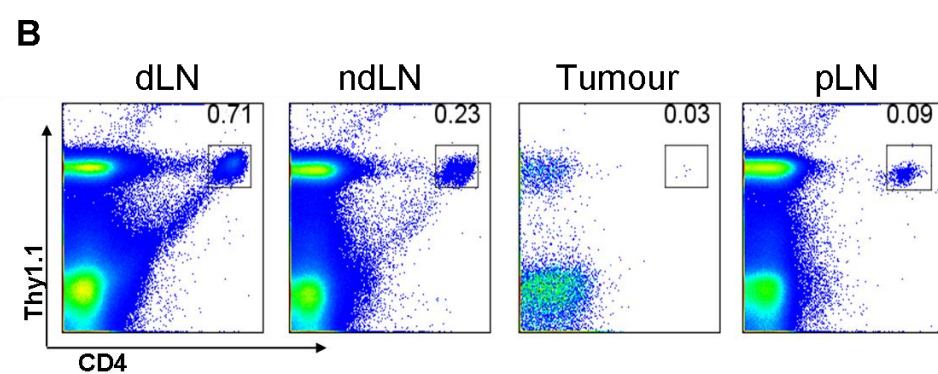
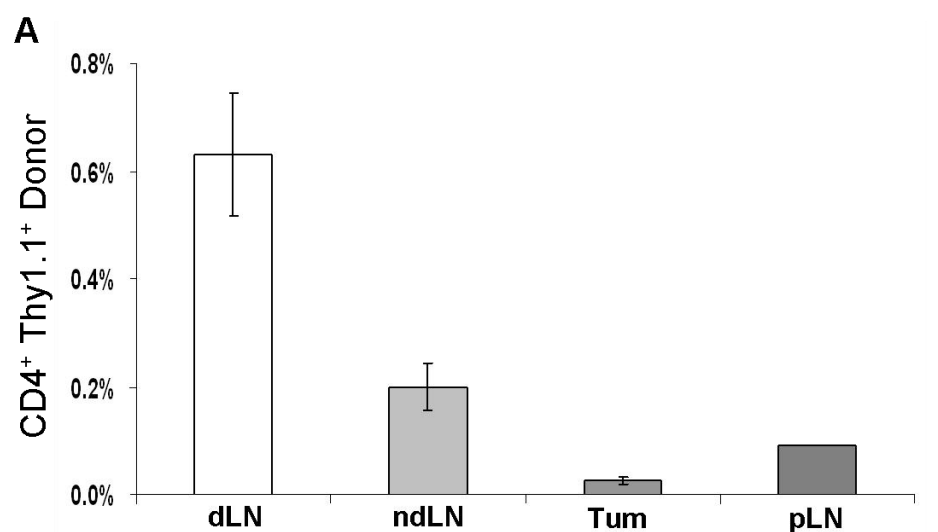


Figure 24. Representation of donor CD4 T cells in various tissues of tumour-bearing mice.

On day 0, two Thy1.2 C57BL/6 female mice were s.c. inoculated with 5×10^5 MB49 cells. 7 days later 2×10^6 CFSE labelled Thy1.1 Marilyn T cells were adoptively transferred to control mice (n=1) and MB49 tumour bearing mice (n=2). 8 days later tumour dLN (□, n=2), ndLN (■, n=2), tumour (■, n=2) and control pLN (■, n=1) were harvested and prepared for FACS.

A) The cells from different tissues were stained with anti-Thy1.1^{PerCP} and anti-CD4^{APC}. The cells were gated on the small lymphocyte gate and the percentage of CD4⁺Thy1.1⁺ cells were presented as the mean±SD from two individual mice.

B) One representative density plot is shown.

C) The cells from different tissues were stained anti-Thy1.1^{PerCP} and anti-CD4^{APC}. The cells were gated on the small lymphocyte gate and the donor cells were identified by being CD4⁺Thy1.1⁺. The percentage of donor cells that remained CFSE^{hi} were designated undivided and are presented as the mean ± SD from two individual mice. **D)** One representative density plot is shown.

From the previous experiments it was evident that Marilyn T cells were proliferating and expanding in response to MB49 tumour antigen however, the anti-tumour capacity was not enough to completely clear the tumour in 100% of mice. To define whether Marilyn T cells were being inhibited by tumour-related mechanisms, the capacity of Marilyn T cells to divide in response to tumour versus male skin grafts was compared. The hypothesis was that the tumour microenvironment is immuno-suppressive but that of the allograft is not.

Thy1.2⁺ female C57BL/6 mice were transplanted with syngeneic male skin grafts or inoculated s.c. with MB49 cells and then injected i.v. with CFSE labelled Thy1.1⁺ Marilyn T cells. T cell expansion and CFSE dilution in graft dLN (gdLN) and tumour dLN (tdLN) was then compared by flow cytometry.

The results of these experiments were that there were more donor T cells present in the gdLN (0.6% ± 0.3%) than in the tdLN (0.14% ± 0.08%) (Figure 25 A and B). A significantly higher proportion of the donor T cells was CD44^{high} in the gdLN (90% ± 1%) compared to in the tdLN (69% ± 11%; p = 0.006) (Figure 25C and D). The frequency of undivided CFSE^{high} T cells was significantly higher in the tdLN (10% ± 3.7%) than in the gdLN (3% ± 1%; p = 0.004) (Figure 25E and F). These results were consistent with the hypothesis of the experiment.

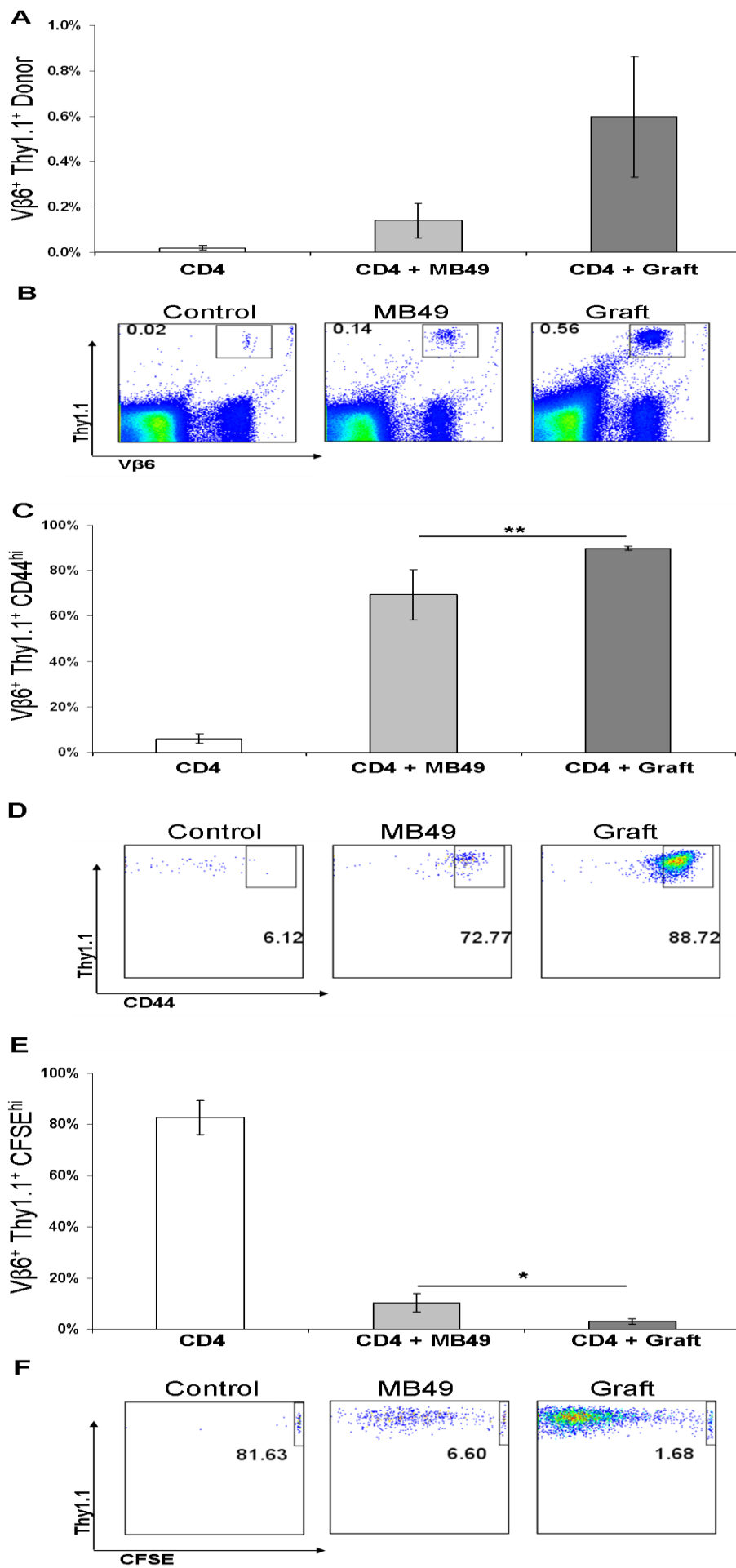


Figure 25. Comparison of donor CD4 T cell response to male skin grafts and MB49 tumours.

Thy1.2 female B6 mice were either not treated (□, n=4) or injected s.c. with 5×10^5 MB49 cells on both sides of flanks (■, n=6) or transplanted with syngeneic male skin grafts (▣, n=3). 7 days later, 4.6×10^6 CFSE labelled CD4⁺ Marilyn T cells were injected i.v. 5 days later, peripheral lymph nodes, tumour or graft draining lymph nodes, MB49 tumours and skin grafts were harvested and stained, anti-Vβ6^{PE}, anti-Thy1.1^{PerCP} and anti-CD44^{APC} and analysed by FACS.

- A)** Percentage of donor T cells in pLN, tumour-dLN and graft-dLN lymph nodes are represented as mean ± SD.
- B)** Density plots of one representative example are shown.
- C)** Percentage of CD44^{high} donor T cells in pLN, tumour-dLN and graft-dLN lymph nodes are represented as mean ± SD. ** $p = 0.006$.
- D)** Density plots of one representative example are shown.
- E)** Percentage of CFSE^{hi} undivided donor T cells in pLN, tumour-dLN and graft-dLN lymph nodes are represented as mean ± SD. ** $p = 0.004$.
- F)** Density plots of one representative example are shown.

5.4 : A combination therapy using Marilyn CD4 T cells and pre-conditioning of hosts.

Adoptive T cell therapy using naïve and activated HY-specific Marilyn T cells slows the growth of the MB49 tumour in a dose dependent manner (Figure 23) however, the tumour does seem to exert some immunosuppressive effect (Figure 25) which may explain why Marilyn T cell therapy does not cause complete remission or prolonged tumour-free survival in 100% of mice treated. It is likely that additional manipulations are needed to boost the anti-tumour effects of Marilyn T cells to overcome tumour mediated suppression and cause complete tumour remission and prolonged survival in all animals treated.

Sub-lethal irradiation has been shown to enhance the efficacy of adoptively transferred T cells by; reducing the number of Treg that can inhibit anti-tumour responses; increasing the availability of cytokines required for the proliferation and survival of adoptively transferred cells and; increased antigen presentation via antigen shedding from dying tumour cells¹⁵⁹⁻¹⁶¹.

To study the effect of irradiation on the adoptive transfer of Marilyn T cells to mice with established MB49 tumours, female C57BL/6 mice were injected s.c. with 5×10^5 MB49 cells. Seven days after injection, established tumours, with an average area of $22 \pm 9 \text{ mm}^2$, had developed. On day 7, mice were sub-lethally irradiated with 5Gy and then 1×10^6 Marilyn T cells, from pooled spleen and lymph nodes of Rag^{-/-} Marilyn female mice, were adoptively transferred by i.v. injection. A control group were inoculated with MB49 cells but were not irradiate and did not receive Marilyn T cells.

Tumours, in the group that was irradiated and received Marilyn T cells, continued to grow to an average size of $43 \pm 14 \text{ mm}^2$ on day 15. But after day 15, MB49 tumours began to regress, and by day 27 were significantly smaller than tumours in the untreated group (Figure 26A, $p = 0.004$). At the end of the experiment (day 34) all mice (7/7) in the treated group were tumour free (data not shown). Treatment correlated with long term survival, with 100% of the treated group living until the end of the experiment compared to a mean survival time of just 18.8 days for the untreated group (Figure 26B).

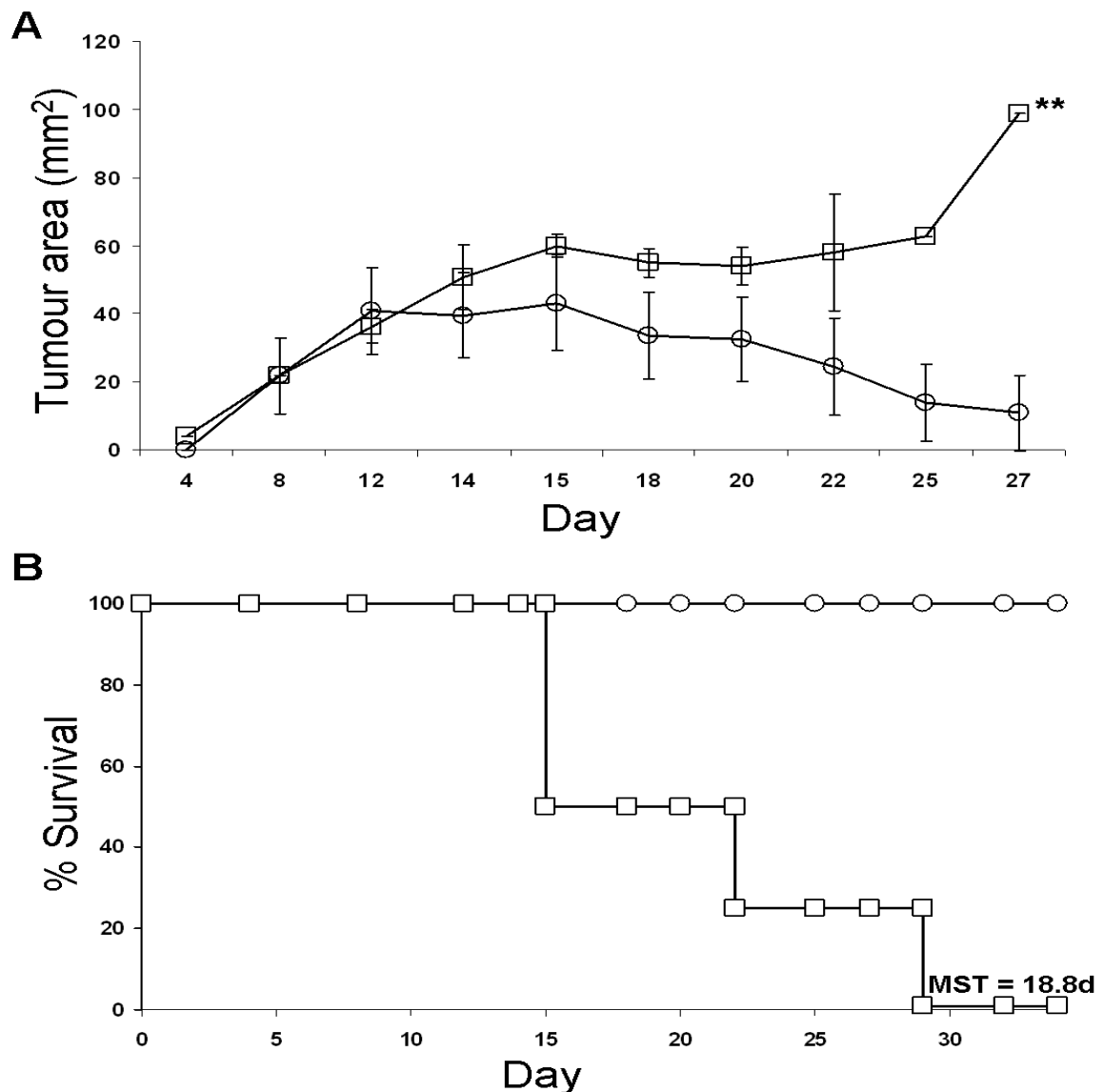


Figure 26. Combinational therapy with irradiation and CD4 T cell transfer causes the complete rejection of established MB49 tumours.

A) Female C57BL/6 mice were injected with 5×10^5 MB49 cells s.c. Seven days later mice were irradiated with 5Gy and then received 1×10^6 Marilyn CD4 T cells, purified from the pooled spleen and lymph nodes of Rag^{-/-} Marilyn female mice, by i.v. injection (○, n=7) or were left untreated (□, n=4). Tumour area was measured at regular intervals. Data are presented as mean $\pm$ SD, ** $p = 0.004$.

B) MB49 progression is presented as % survival. The s.c. inoculation of B6 mice with MB49 cells does not lead to death. But the mice are recoded as death once they reach one of following end points of the experiment. One is the maximum of tumour size with 14 mm x 14 mm, the other is the development of severe ulceration by tumour. The mean survival time (MST) of individual groups is also indicated.

At the end of the irradiation-adoptive transfer experiment (day 34), MB49-dLN and spleen were harvested from three MB49-free, treated mice and the production of IFN γ and phenotype of the donor Marilyn T cells were analysed.

11% of donor Marilyn T cells in the MB49-dLN and 29% of donor Marilyn T cells in the spleen of treated mice, produced IFN γ following a short period in culture with PMA and Ionomycin (Figure 27A and B).

The analysis of the phenotype of adoptively transferred T cells revealed that 40% of gated donor CD4 T cells in MB49-dLN were Central Memory (CM) cells (CD62L^{hi} CD44^{hi}) and 35% of donor CD4 T cells were Effector Memory (EM) cells (CD62L^{lo} CD44^{hi}) (Figure 28A and B). The expression of chemokine receptors by adoptively transferred T cells was also analysed; the only chemokine receptor that was up-regulated on the adoptively transferred T cells was CCR9 which was expressed on more than 12% of donor T cells (Figure 28C and D).

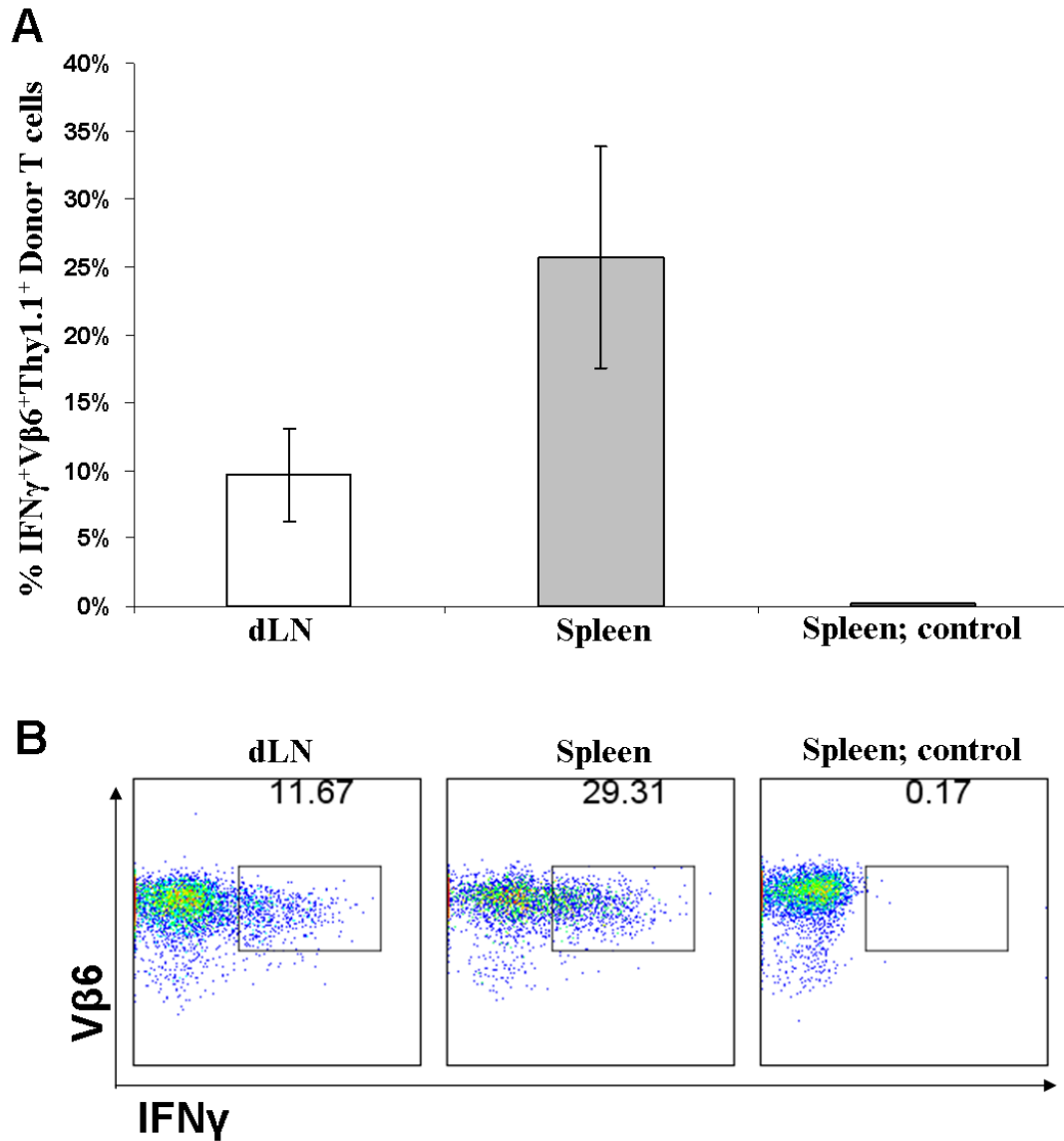


Figure 27. Adoptively transferred Marilyn cells were differentiated to Th1-like effectors in MB49-bearing mice after pre-conditioning with 5Gy irradiation.

Thy1.2⁺ female C57BL/6 mice were inoculated s.c. with 5×10^5 MB49 cells. 7 days later, mice were irradiated with 5Gy and injected i.v. with 1×10^6 Thy1.1⁺ Marilyn CD4 T cells. At day 34, 1×10^6 spleen or dLN cells from three individual tumour-free mice were cultured with 4×10^6 male Rag^{-/-} spleen cells in 24-well plates. 5 days later, PMA (50 ng/ml), Ionomycin (500 ng/ml) and Brefeldin (1 μ g/ml) were added to 24-well plates. 4 hours later, the cells were harvested and the dead cells removed using Ficoll. After washing, the cells were surface stained with anti-V β 6^{PE}, anti-CD4^{FITC} and anti-Thy1.1^{PerCP}. Cells were then fixed and permeabilised before being stained with anti-IFN γ ^{APC}.

A) The frequency of IFN γ -producing donor CD4 T cells recovered from MB49-dLN and spleen of rejected and control mice is shown.

B) Density plots of one representative example are shown.

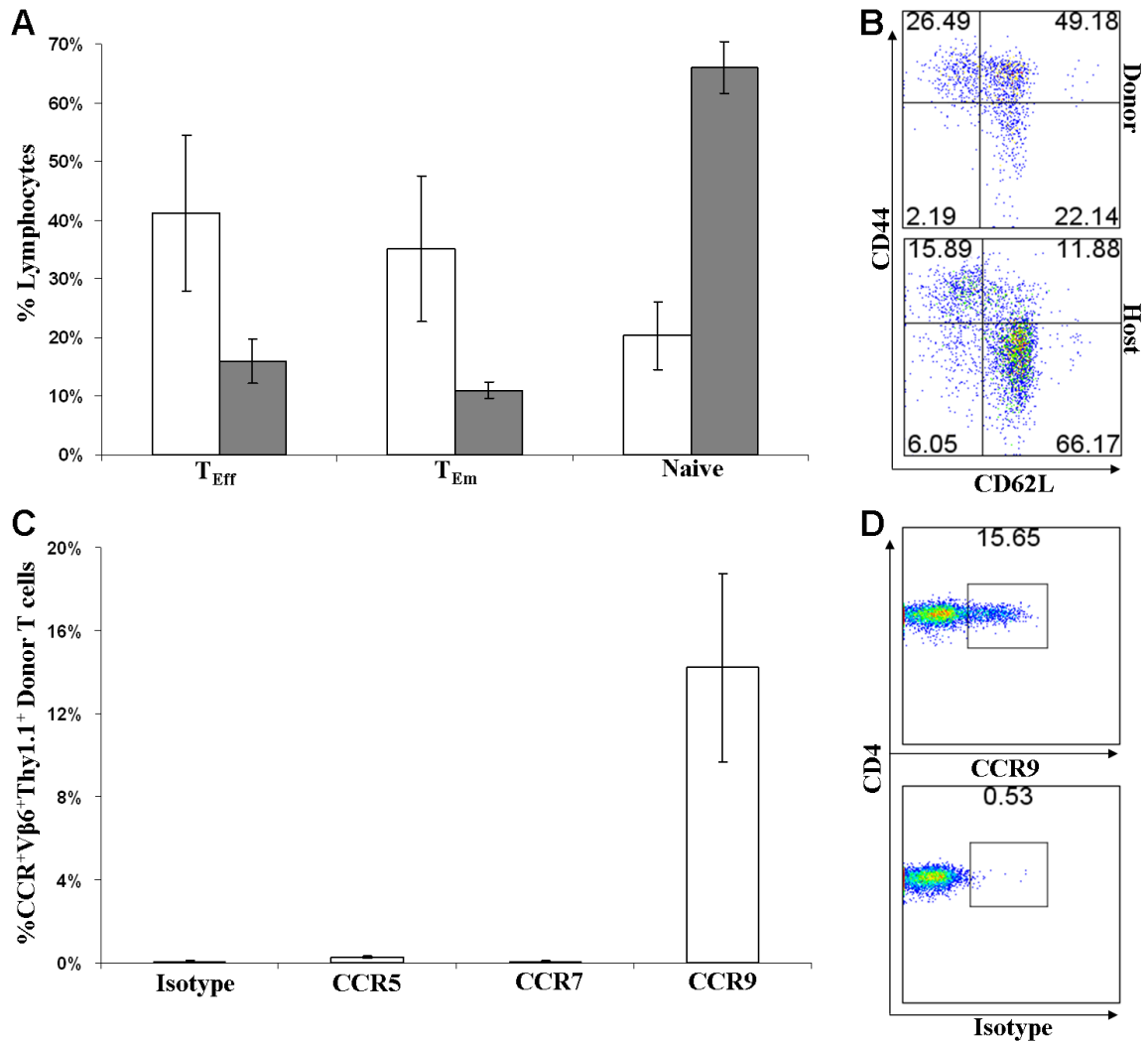


Figure 28. Phenotype of adoptively transferred Marilyn T cells following pre-conditioning with 5Gy irradiation

As described in Figure 5, Thy1.2⁺ female C57BL/6 were inoculated s.c. with 5×10^5 MB49 cells. 7 days later, mice were irradiated with 5Gy and injected i.v. with 1×10^6 Thy1.1⁺ Marilyn CD4 T cells. dLN cells from three tumour-free mice were harvested and analysed individually on day 34.

A) dLN cells were stained with anti-Vβ6^{FITC}, anti-CD62L^{PE}, anti-CD44^{APC} and anti-Thy1.1^{PerCP}. Proportions of naïve (CD62L^{hi} CD44^{lo}), CM (CD62L^{hi} CD44^{hi}), and EM (CD62L^{lo} CD44^{hi}) subsets within host T cells (Thy1.1⁻, ■) or donor T cells (Thy1.1⁺, □) was analysed.

B) Density plots of one representative example are shown.

C) dLN cells were stained with anti-Vβ6^{FITC}, Isotype^{PE} or three individual PE-conjugated mAb including anti-CCR5, anti-CCR7 and anti-CCR9 and anti-Thy1.1^{PerCP}. The percentage of donor T cells expressing respective CCR receptors is presented (n=3).

D) Density plots of one representative example are shown.

5.5 : Differentiation of Th17 cells from naïve Marilyn CD4 T cells *in vitro*

To explore the anti-tumour efficacy of Th17 cells in the MB49 model, a variety of *in vitro* conditions were tested in order to create an optimal culture system whereby a large number of tumour-specific Th17 cells could be produced for adoptive immunotherapy. Several conditions were tested, including neutralisation of IL-2 with mAb (S4B6)²⁷⁰ because IL-2 has been shown to inhibit the development of Th17 cells²⁶⁸.

We confirmed that neither IL-6 or TGF β alone can induce Th17 cells from naïve Marilyn CD4 T cells after activation with HYDby peptide and that Th17 development requires a combination of both cytokines. In addition, IL-23 did not appear to expand differentiated Th17 cells. More importantly we demonstrated, by two different approaches, that IL-2 inhibits Th17 development. Firstly, the presence of neutralising anti-IL-2 mAb (clone S4B6) greatly enhanced Th17 development (Figure 29A and B) and secondly, addition of exogenous IL-2 into culture profoundly inhibited Th17 differentiation (data not shown).

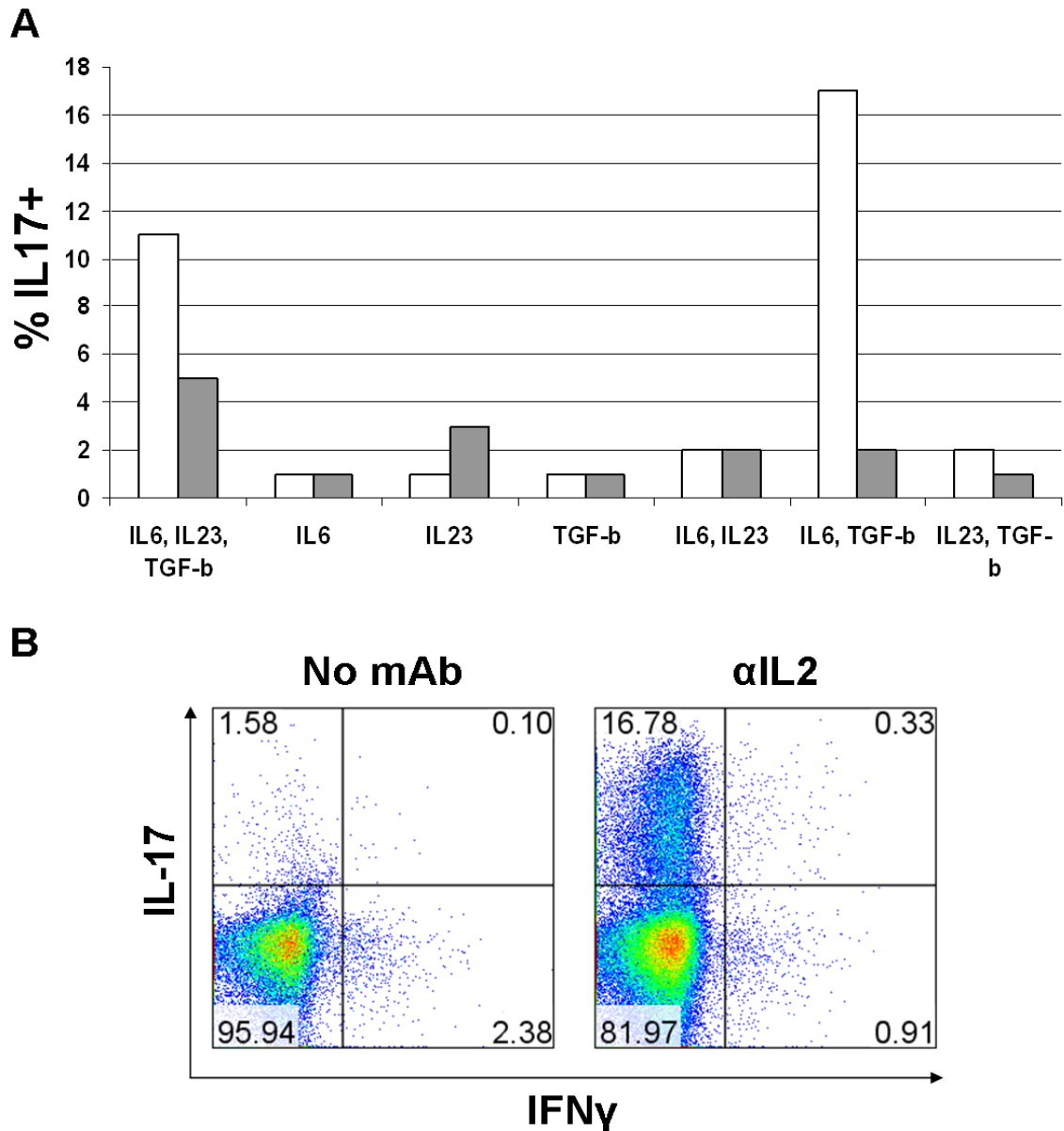


Figure 29. IL-2 inhibits in vitro Th17 differentiation from naive Marilyn CD4 T cells following peptide stimulation in the presence of IL-6 and TGFβ.

A) Splenocytes and lymph node cells (2×10^5 /well) from Rag2^{-/-} Marilyn female mice were stimulated with HYDby peptide (150 nM) plus the cocktail of Th17- polarising cytokines (TGFβ, IL-6 or IL-23, 2ng/ml) in the presence (□) or absence of anti-IL-2 (■) (clone S4B6, 1:100 dilution of hybridoma supernatant) in a 96 well plate for 6 days. The cells were stimulated with PMA and Ionomycin in the presence of monensin for 4 hours. Dead cells were removed using Ficoll and viable cells were stained with anti-Vβ6^{FITC} and anti-CD4^{PerCP}, fixed and permeabilised then stained with anti-IL-17^{PE} and anti-IFNγ^{APC} and analysed by FACS. Gated Vβ6⁺CD4⁺ cells were analysed for IL-17 and IFNγ production.

B) Representative dot plots show the percentage of IL-17-producing and IFNγ-producing cells within gated Vβ6⁺CD4⁺ population.

5.6 : Adoptive therapy using *in vitro* polarised Th17-like Marilyn CD4 T cells.

Using the *in vitro* Th17 and Th1 conditioning regimen described above, Th17-like Marilyn CD4 T cells were adoptively transferred into C57BL/6 mice that had established MB49 tumours. Similar to HYDby-activated Marilyn CD4 T cells, *in vitro* differentiated “Th17-like” Marilyn CD4 T cells were also capable of eliminating established MB49 tumours, with no difference in terms of kinetic of regression (Figure 30). Unfortunately, due to time constraints this was the only experiment of this type performed.

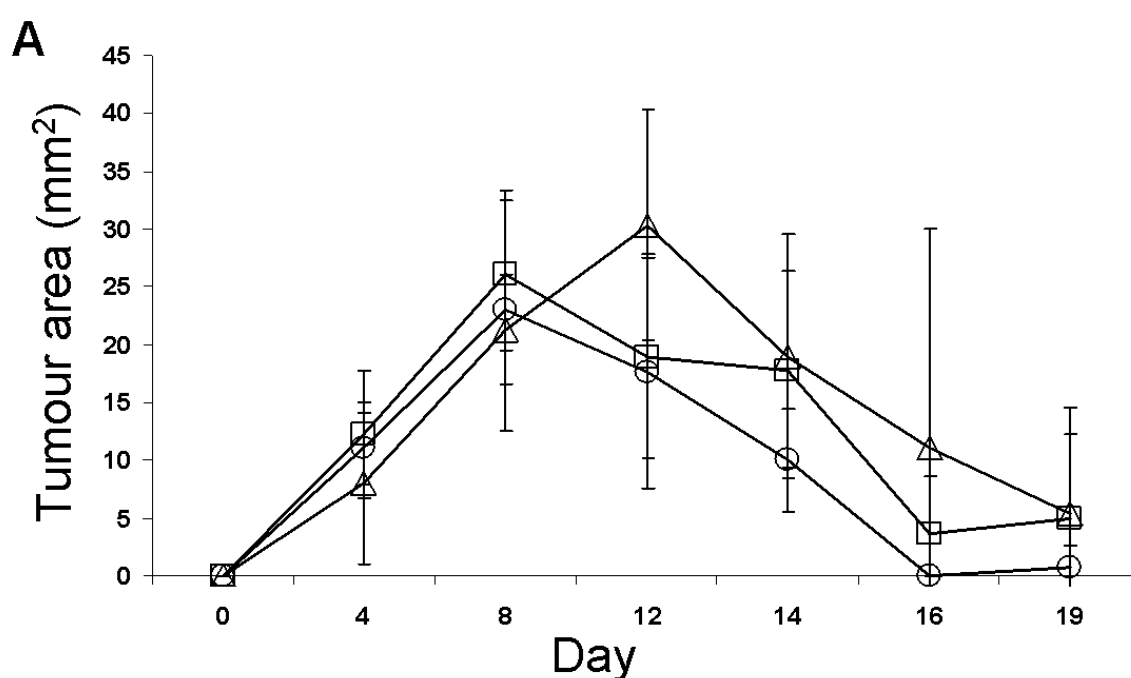


Figure 30. Adoptive transfer of Th17 conditioned Marilyn T cells leads to the regression of established MB49 tumours.

A) Three groups of B6 female mice were injected s.c. with 5×10^5 MB49 cells. 4 days later the mice were split into three groups and adoptively transferred with 1.2×10^6 Th1 (○, n=5), Th17 (△, n=3) or naïve (□, n=5) Marilyn T cells. The growth of MB49 tumours was monitored by measuring tumour size. Data is presented as the mean $\pm$ SD.

5.7 : Discussion

As Marilyn CD4 T cells were adoptively transferred to treat MB49 tumours, it was of importance to characterize their phenotype and function. FACS analysis confirmed that Rag2^{-/-} Marilyn female mice harbour monoclonal naïve CD4 T cells that express the transgenic TCR (Figure 22) and proliferate *in vitro* in response to A^b-Dby peptide with a peak response at 100-1000nM (Figure 22B) which confirms previous results^{178, 206}.

Adoptive transfer of naïve Marilyn cells inhibited the growth of MB49 tumours in a dose dependent manner. High doses (2.5×10^6) of Marilyn T cells inhibited the growth of the tumour in all mice tested, however treatment did not cause tumour rejection (Figure 23A). Marilyn T cells that had been activated *in vitro* for 3 days before being adoptively transferred to female C57BL/6 mice were more effective at controlling subsequent tumour growth but, again, did not successfully clear the tumour (Figure 23B).

Together these results suggest that Marilyn T cells are effective at reducing the growth of MB49 tumours in female C57BL/6 WT mice. However, whereas a previous study in Rag^{-/-} female C57BL/6 mice demonstrated that 8×10^5 naïve Marilyn cells were sufficient to cause the rejection of 1×10^5 MB49 cells injected s.c. one day previously¹⁷⁸, these results suggest that a much higher number (5×10^6) is required in immuno-competent mice. Immunosuppression by Treg, which are present in immuno-competent mice but not Rag^{-/-} mice, is the likely reason. The interaction between Treg and the MB49 tumour are discussed in Chapter 7.

Activating Marilyn T cells *in vitro* before adoptive transfer may increase infiltration into the tumour, a hypothesis that is corroborated by a study from O. Lantz *et al.*²⁷¹. Using Marilyn cells, they describe a direct relation between the strength of Marilyn cell priming *in vivo* and their subsequent ability to infiltrate into Dby expressing MCA-101 tumours. Priming *in vitro* decreases the expression of CD62L and increases expression of chemokine receptors, a combination of changes that is required for infiltration of T cells into the periphery and into the tumour mass.

Adoptively transferred naïve Marilyn cells proliferate and expand in MB49 tdLN and migrate into the tumour mass. Proliferation and accumulation in ndLN is less significant than in dLN (Figure 24). All Marilyn cells within the MB49 tumour had undergone more than 7 cell division, which is the limit for detection using CFSE. This result was in line with previous studies²⁷¹.

To further investigate whether Marilyn cells were being inhibited by the MB49 tumour, their response against MB49 tumour and syngeneic male skin grafts were compared. It was found that Marilyn T cells proliferate more robustly in response to male skin grafts than to MB49 tumours. The frequency of Marilyn cells in the gdLN was higher than in tdLN, these cells expressed higher levels of the activation marker CD44 and the number of undivided Marilyn cells was lower (Figure 25). Both MB49 tumour and male skin grafts express the HY antigen, although MB49 tumours, because they are larger than grafts, probably release more antigen. Despite this Marilyn T cells respond better to antigen in the gdLN. Together these results suggest that inhibition of the immune response by MB49 tumours extends to the tdLN as well as being directly associated with the tumour microenvironment.

Marilyn T cell therapy does not cause complete remission of MB49 tumours. Preconditioning recipients with sub-lethal irradiation is a commonly used regimen to enhance adoptive transfer¹⁵⁹⁻¹⁶¹. 5Gy of irradiation enhanced the therapeutic potential of Marilyn cells transferred to tumour bearing mice. So much so, that rather than administering Marilyn T cells then tumour on the same day, it was possible to inoculate C57BL/6 female mice with MB49 cells 7 days before treatment.

In agreement with previously published results, established tumours were rejected in mice treated with 5Gy irradiation followed by adoptive transfer of Marilyn cells (Figure 26). This experiment demonstrated the importance of pre-conditioning in eliciting therapeutic responses from adoptive transfer therapy.

FACS analysis of the donor T cells in the dLN and spleen of treated mice revealed that up to 29% of the cells expressed IFN γ showing that they were Th1 T cells (Figure 27B). Furthermore, up to 50% of donor T cells expressed a T cell effector phenotype, with up to 35% expressing a T central memory phenotype (Figure 28 A and B).

Interestingly, when donor T cells were examined for the expression of chemokine receptors, only CCR9 was expressed at high levels (Figure 28 C and D). The ligand for CCR9 is CCL25 and CCR9 KO mice have reduced numbers of T cells in the lamina propria of the small intestine^{272, 273}. CCR9 is critical for T cell migration to the small intestine and its expression on Marilyn T cells may be essential for their response to the murine bladder carcinoma. It would be interesting to study whether CCR9 KO Marilyn T cells are impaired for their ability to cause MB49 tumour rejection.

In agreement with previous studies TGF β and IL-6 are required but IL-2 is inhibitory for Th17 cell differentiation *in vitro*^{268, 274} (Figure 29). Adoptively transferred Th17 polarised cells have been shown to be effective at destroying established B16

melanomas¹⁸⁰. Using IL-6, TGF β and anti-IL-2 represents a unique system to polarise Th17 cells for adoptive transfer. However subsequent adoptive transfer of Th17 T cells did not control tumours any better than naïve or Th1 T cells, in this experiment all mice treated with Marilyn cells rejected MB49 tumours (Figure 30).

Chapter 6 : Adoptive Transfer of CD8+ T cells

6.1 : Introduction

Because of their powerful *in vitro* and *in vivo* killing activities, CD8⁺ T cells have been utilised in adoptive transfer experiments for treating murine tumours^{168, 176, 275}. As described in Chapter 1, the successful treatment of established B16 tumours by adoptive transfer of TCR transgenic pmel1 (specific for the gp-100₂₅₋₃₃ peptide) cells requires a combination of the following four essential elements¹⁵³: 1) 5Gy irradiation (or 9Gy following bone marrow transplantation)¹⁵⁷ prior to CD8 transfer; 2) adoptive transfer of pmel1 cells; 3) gp100₂₅₋₃₃ vaccination after cell transfer, and 4) co-administration of IL-2¹⁵³, IL-15¹⁶⁷ or IL-15 plus IL-21²⁷⁶.

CD8 T cell responses against the H2D^b-restricted HYUty peptide, expressed by MB49 cells, were studied using CD8 T cells from MataHari mice that express a Tg TCR specific for the HYUty peptide (WMHHNMDLI) and restricted by the H2-D^b MHC-I molecule²⁰⁵. It has been previously described that MataHari CD8 T cells do not cause the rejection of MB49 tumours¹⁷⁸ however, in that study, immuno-deficient Rag^{-/-} B6 female mice were used as recipients of MataHari CD8 T cells. The behaviour of T cells in lymphopenic Rag^{-/-} hosts is quite different to that in WT recipients²⁷⁷⁻²⁷⁹. Therefore, in this study, to evaluate their anti-tumour efficacy, MataHari T cells were adoptively transferred into MB49-bearing WT mice.

In order to better understand the behaviour of adoptively transferred MataHari T cells *in vivo*, their phenotype and functionality was further characterised *in vitro*.

To increase the activation and survival of adoptively transferred MataHari CD8 T cells, we pre-conditioned the host by using a low dose of irradiation (5Gy), and included vaccination strategies, as these additional manipulations appear to be essential for the success of CD8-based immunotherapy in the pmel-B16/F10 model¹⁵³.

In an attempt to further enhance the effectiveness of these CD8 cells, *in vitro* activated rather than resting MataHari CD8 T cells were utilised for adoptive transfer. In addition, MataHari CD8 T cells co-expressing Tg human-IL-15¹⁹³, or MataHari CD8 T cells transduced with human IL-2, were also tested.

6.2 : Characterisation of MataHari T cells in vitro

Before using MataHari T cells *in vivo*, it was necessary to investigate their phenotype and responsiveness to cognate peptides.

When peripheral lymph node cells from a Rag2^{-/-} MataHari female mouse were stained with anti-Vβ8.3^{PE}, anti-CD8^{PerCP} and anti-CD62L^{APC} we found that there were two populations of T cells-expressing transgenic Vβ8.3. 5% of lymphocytes co-expressed CD8 and Vβ8.3 and 7% co-expressed CD4 and Vβ8.3 (Figure 31A, left panel). The majority of gated CD8⁺Vβ8.3⁺ cells expressed a high level of CD62L, suggesting that they are naïve CD8 T cells (Figure 31A, right panel) whereas the majority of the CD4⁺Vβ8.3⁺ cells expressed low levels of CD62L suggesting that they are anergic T cells which may have cross reactivity with self/environmental antigen.

When spleen and pooled LN cells from a Rag2^{-/-} MataHari mouse were cultured with HYUty peptide, proliferation was observed in a peptide dose-dependent manner, demonstrating that the Tg TCR is functional (Figure 31B).

To distinguish which population, (i.e, CD8⁺Vβ8.3⁺ or CD4⁺Vβ8.3⁺) was responsible for peptide-induced proliferation, we labelled spleen and pooled LN cells from a Rag2^{-/-} MataHari mouse with CFSE before culturing them with HYUty peptide. We observed that the CD8⁺Vβ8.3⁺ cells (Figure 31C, bottom panel) but not the CD4⁺Vβ8.3⁺ cells (Figure 31C, top panel) proliferated, demonstrating that only CD8⁺Vβ8.3⁺ cells were able to recognise cognate peptide, thus representing functional MataHari T cells.

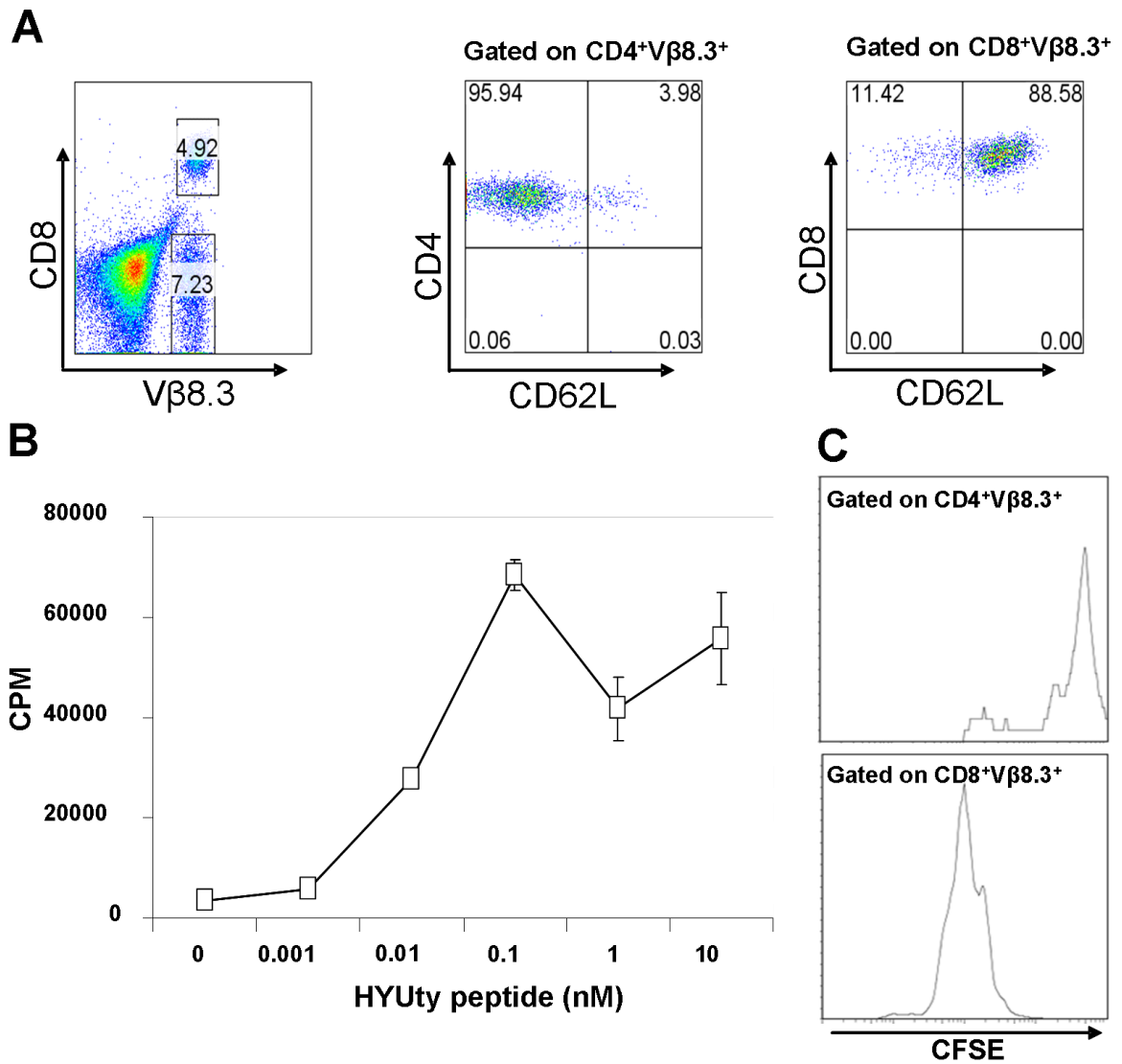


Figure 31. Characteristic of MataHari T cells in vitro.

A) Peripheral LN cells from a naïve MataHari Rag2^{-/-} female mouse were stained with anti-Vβ8.3^{FITC}, anti-CD62L^{PE}, anti-CD8^{PerCP} and anti-CD4^{APC}. CD4⁺Vβ8.3⁺ (middle panel) and CD8⁺Vβ8.3⁺ (right panel) T cells were gated and analysed for CD62L expression.

B) Spleen and pooled peripheral LN cells (2x10⁴/well) from a naïve MataHari Rag2^{-/-} female mouse were stimulated with the indicated concentrations of HYUty peptide.

Tritiated thymidine (0.5 μCi/well) was added at 48 hours and the cells were harvested 24 hours later. Results are presented as the mean CPM ±SD.

C) Spleen and LN from MataHari Rag2^{-/-} female mouse were labelled with CFSE and then cultured with HYUty peptide for 3 days. Cells were harvested and stained with anti-Vβ8.3^{PE}, anti-CD8^{PerCP} and anti-CD4^{APC}. The dilution of CFSE was analysed after gating for CD8⁺Vβ8.3⁺ T cells (bottom panel) and CD4⁺Vβ8.3⁺ T cells (top panel).

6.3 : Adoptive therapy with MataHari T cells with or without additional manipulations

To test whether or not adoptively transferred naïve MataHari T cells can cause MB49 tumour rejection in WT female C57BL/6 mice, 2×10^6 CD8 T cells purified from pooled spleen and lymph nodes of MataHari Rag^{-/-} female mice, were injected i.v. into female C57BL/6 mice which were subsequently inoculated s.c., two days later, with 3×10^5 MB49 cells. No difference in tumour growth was observed between the MataHari treated group and the control group (no CD8 transfer) (Figure 32A).

In an attempt to activate donor MataHari T cells *in vivo*, we introduced a vaccination protocol. WT B6 females were inoculated s.c. with MB49 cells 24 hours before i.v. injection of purified MataHari CD8 T cells and s.c. vaccination with HYUty peptide, with or without Complete Freund's Adjuvant (CFA). The combination of CD8 transfer and vaccination with peptide plus CFA was more effective at inhibiting tumour growth than either no CD8 transfer ($p = 0.002$) or treatment with peptide without CFA ($p = 0.017$) (Figure 32B).

To test whether or not irradiation can be used to enhance the anti-tumour responses of MataHari T cells, female C57BL/6 mice were inoculated s.c. with 6×10^5 MB49 cells. Two days later one group was irradiated with 5Gy and adoptively transferred with 2×10^6 CD8 MataHari T cells whilst the other (control) group just received the adoptively transferred CD8 MataHari T cells. There was no observable difference in tumour growth between the two groups (Figure 32C).

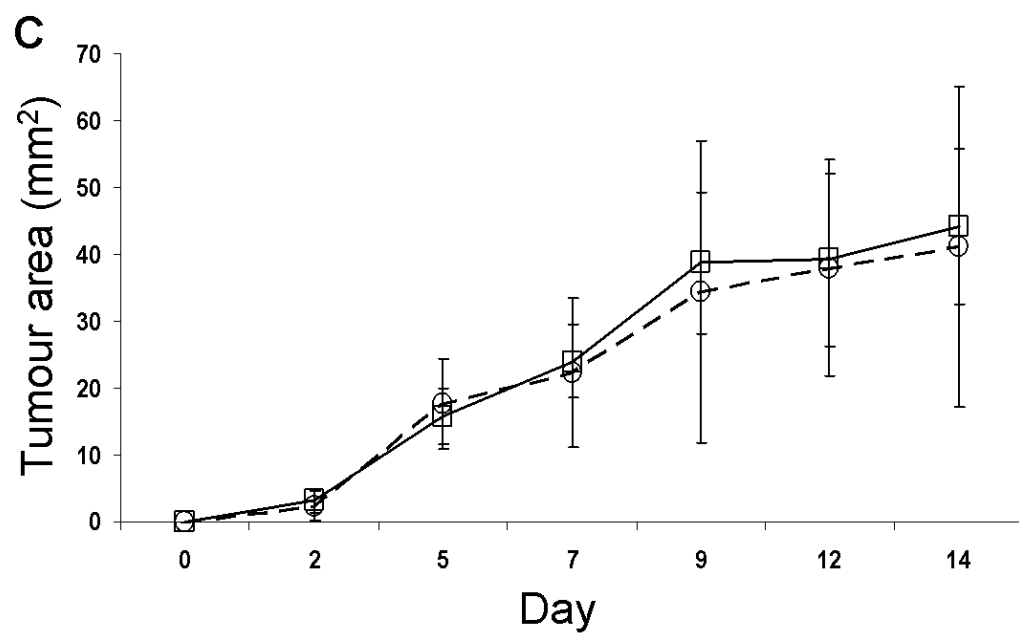
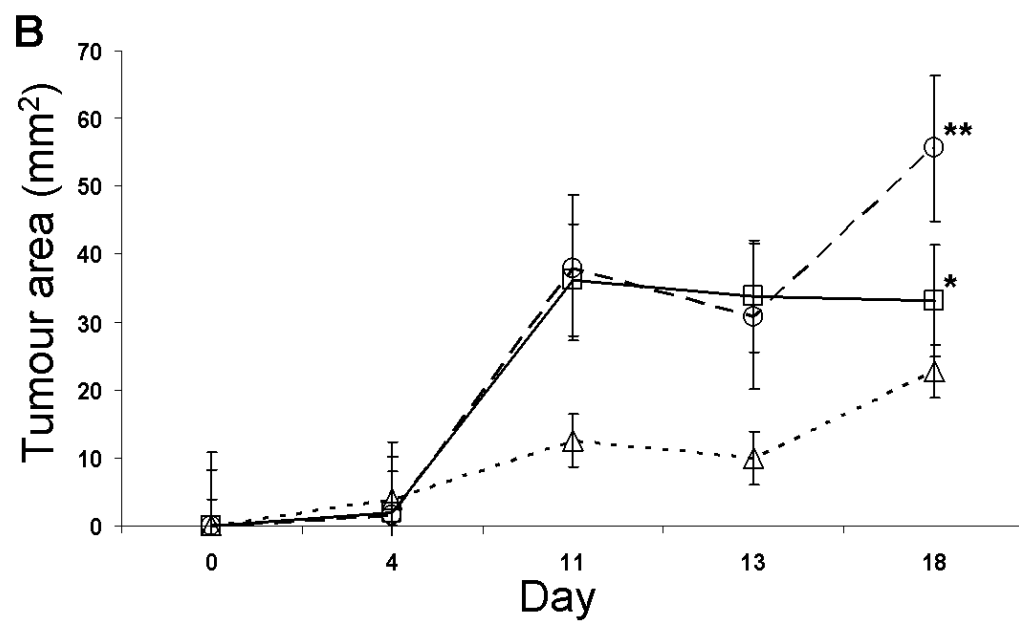
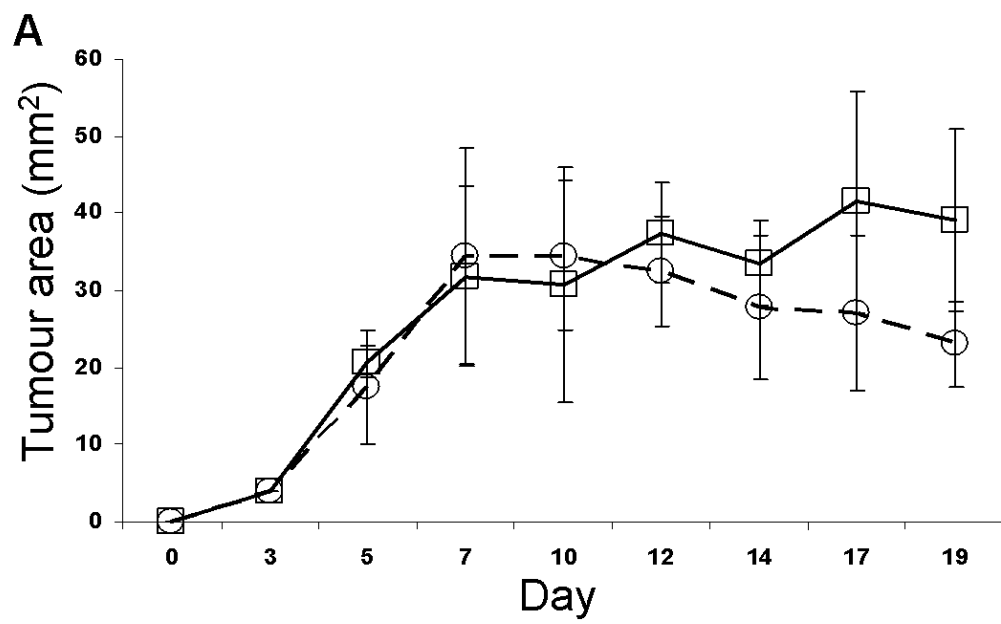


Figure 32. Vaccination but not irradiation boosts anti-tumour responses mediated by adoptively transferred MataHari CD8 T cells.

A) 8 Female C57BL/6 mice were given 2×10^6 CD8⁺ MataHari T cells i.v. (○, n=4) or were untreated (□, n=4). Two days later, the mice in both groups were inoculated s.c. with 3×10^5 MB49 cells. Tumour area was measured as Width x Length at regular intervals. Data is presented as mean ± SD.

B) At day 0, 16 Female C57BL/6 mice were given 5×10^5 MB49 cells s.c.. At day 1, the mice were then randomly split into three groups. Two groups of mice were adoptively transferred with 2.1×10^6 MataHari CD8⁺ T cells (○ and △), and the third group (□) of mice were not. At day 5, two groups of mice received CD8 T cells were either left unvaccinated (○, n=5) or vaccinated (△, n=5) with HYUty peptide plus CFA by s.c. injection. Vaccines were made by mixing 100 µl PBS containing 100 nM HYUty peptide with 100 µl CFA. The third group (no CD8 transfer) also received the same vaccination at day 5 (□, n=6). Tumour area was measured as Width x Length at regular intervals. Data are presented as mean ± SEM. (□, peptide and CFA vs. △, peptide, CFA and MataHari; * $p = 0.017$), (○, untreated vs. △, peptide, CFA and MataHari; ** $p = 0.002$).

C) 10 Female B6 were inoculated s.c. with 6×10^5 MB49 cells. Two days later the mice were split into two groups, one was irradiated with 5Gy (○, n=5) and the other was not (□, n=5), then both groups were injected i.v. with 2×10^6 MataHari CD8⁺ T cells. Tumour area was measured as Width x Length at regular intervals. Data are presented as mean as mean ± SD.

6.4 : Adoptive therapy using in vitro activated MataHari T cells

In an attempt to increase the efficacy of adoptively transferred CD8 T cells against MB49 tumours, MataHari T cells were activated *in vitro* with 0.1 nM HYUty peptide in the presence or absence of exogenous IL-2, for 1 or 2 days. These cells were subsequently purified before being adoptively transferred (1×10^6 cells or 3×10^6 cells) to female C57BL/6 mice which had been inoculated s.c., with 5×10^5 MB49 cells, 3 days earlier. There was no significant reduction in tumour growth irrespective of how the T cells were activated or how many were transferred.

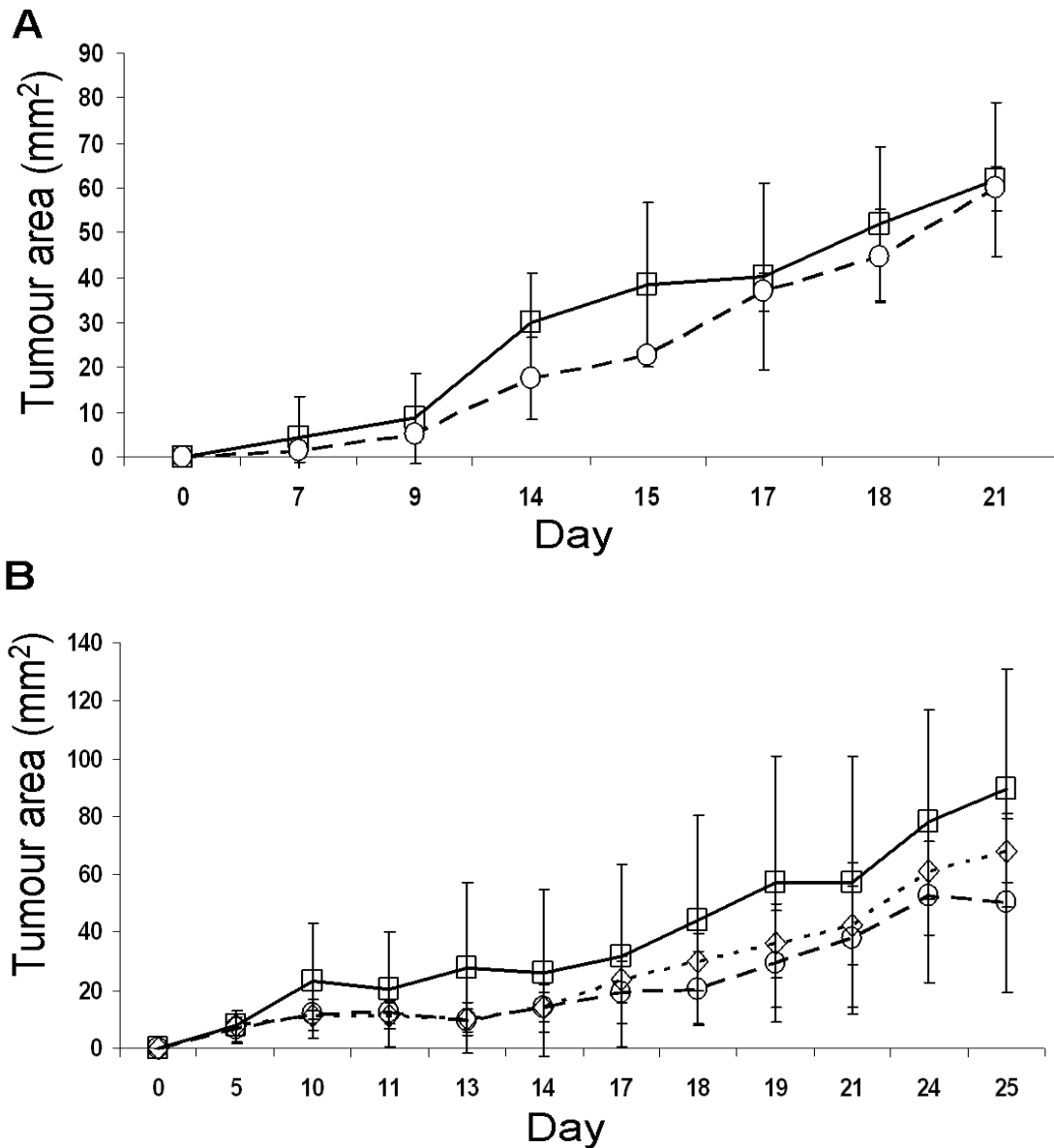


Figure 33. Adoptively transferred MataHari CD8 T cells which have been activated in vitro failed to control the growth of MB49 tumours.

A) Rag1^{-/-} MataHari spleen and LN cells were cultured with 0.1 nM HYUty for 1 or 2 days, CD8 T cells were purified and adoptively transferred to female B6 that were inoculated s.c. with 5 x 10⁵ MB49 on the same day of T cell transfer. Two doses of purified CD8 T cells with different activation stages were used [1 x 10⁶ from 1 day culture (□), 3 x 10⁶ from 2 days culture (○)].

B) Rag1^{-/-} MataHari spleen and LN cells were cultured with 0.1 nM HYUty in the presence of different concentration of hIL-2 (10 or 50 U/ml) for 3 days, CD8 T cells were purified and adoptively transferred to female B6 that had been inoculated s.c. with 5 x 10⁵ MB49 3 days previously [(10U/ml, □, n=2, 5x10⁵ cells/mouse), (50 IU/ml, ○, n=3, 1x10⁶ cells/mouse), (50U/ml, ◇, n=4, 2x10⁶ cells/mouse)].

6.5 : Adoptive therapy using hIL-15 Tg MataHari T cells

In tumour-bearing hosts, the induction of CD8 T cell tolerance and anergy is very common, representing one of the major challenges to successful immunotherapy¹⁵¹. A promising strategy to optimise adoptive T cell immunotherapy is to use genetically modified, antigen-specific CD8 T cells¹⁵². One genetic modification strategy is to genetically engineer T cells to express specific cytokines; in tumour models in which the genes encoding IL-2^{11, 12, 280} or IL-15^{167, 169} have been transferred into T cells, the T cell responses are significantly enhanced.

To test whether the over-expression of Tg hIL-15 in MataHari CD8⁺ T cells would enhance their capacity to control MB49 tumours, we compared the anti-MB49 activities between CD8 T cells expressing MataHari TCR alone and those co-expressing MataHari TCR and the hIL-15 transgene. Double Tg mice were created by mating Rag2^{-/-} MataHari females with hIL-15-Tg males to produce F1 mice. The expression of the MataHari TCR was screened, by analysis of V β 8.3 in PBL, by flow cytometry, and the expression of hIL-15 was examined by hIL-15 ELISA to determine the concentration of hIL-15 in mouse serum. From F1 females, Rag2^{+/-} MataHari females with and without co-expressing IL-15-Tg were selected as donor mice to provide single Tg or double Tg CD8 T cells.

Before using double Tg CD8 T cells *in vivo*, we wanted to investigate whether the expression of hIL-15 gene would alter their phenotype and responsiveness to cognate peptides. We compared the expression of CD62L, V β 8.3, CD8 and CD44 and the proliferation of PLN cells from a single Tg (MataHari) and a double Tg female B6 mouse in response to cognate HYUty peptide. When spleen and pooled LN cells were cultured with HYUty peptide, proliferation was observed in a peptide dose-dependent manner in both types of T cells. Proliferation was slightly but not significantly lower in double Tg T cells in response to a range from 0.1 to 100 nM HYUty peptide (Figure 34A). Although single and double Tg mice had similar proportions of V β 8.3⁺ CD8⁺ T cells (data not shown), they displayed different frequencies of naïve, EM and CM T cells. In the single Tg mice, the vast majority (73%) of gated CD8⁺V β 8.3⁺ T cells were naïve (CD44^{lo}CD62L^{hi}), with a minor fraction of CM (8%) and EM (7%) phenotype (Figure 34B, left panel). In contrast, in the double-Tg mice, the frequency of CM and EM cells was increased whereas that of naïve cells was reduced (Figure 34B, right panel). Taken together, these results suggest that although single- and double-Tg MataHari CD8 T cells express a functional Tg

TCR capable of recognising cognate peptide, they harbour a different proportion of naïve, CM and EM subsets.

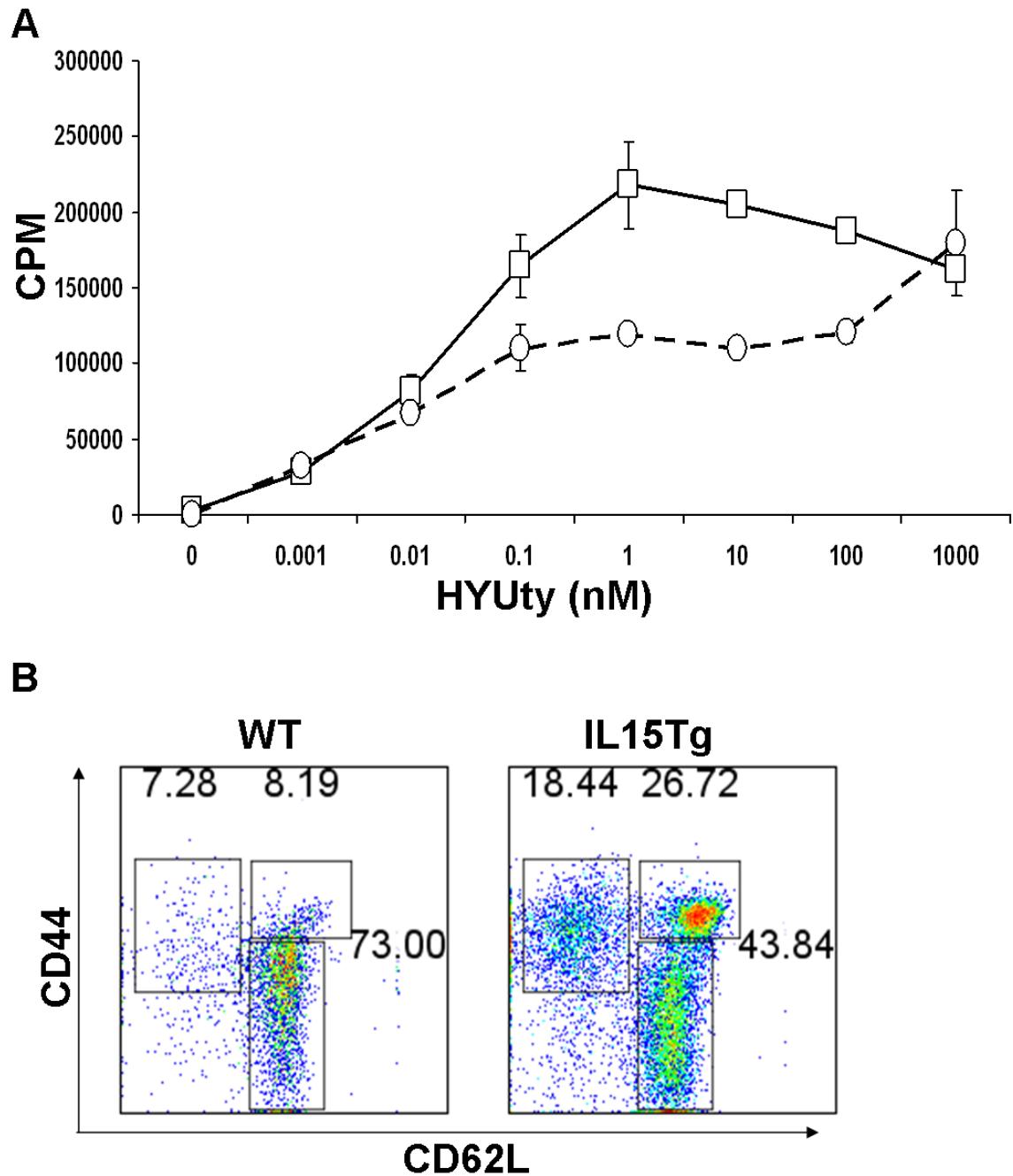


Figure 34. Comparison of peptide responses and phenotypes between MataHari and MataHari IL-15Tg CD8 T cells.

A) Spleen and LN cells (2×10^5 /well) from single-Tg (□) or double-Tg mice (○) were stimulated with HYUTY peptide at variable concentrations for 3 days. Tritiated thymidine (0.5 μ Ci/well) was added at 48 hrs, 24hrs later the cells were harvested and proliferation was measured. Data are presented as mean $\pm$ SD.

B) Lymph nodes from single-Tg or double-Tg mice were stained with anti-CD62L^{FITC}, anti-V β 8.3^{PE}, anti-CD8^{PerCP} and anti-CD44^{APC}. CD8⁺V β 8.3⁺ MataHari T cells were gated and analysed for CD44 and CD62L expression.

To test whether or not double-Tg CD8 T cells would be more potent than single-Tg CD8 T cells at rejecting MB49 tumour, 10×10^6 purified CD8 T cells, from single-or double-Tg mice, were injected i.v. into female C57BL/6 mice which were then inoculated with 3×10^5 MB49 cells s.c. on the same day as T cell transfer. Early tumour growth was significantly inhibited in mice adoptively transferred with double-Tg CD8 compared to mice with single-Tg CD8 (Day 11; $p = 0.02$). However, no long term difference in tumour growth, between the groups, was recorded (Figure 35A).

In an attempt to increase the anti-MB49 efficacy of adoptively transferred double-Tg CD8 T cells, we activated the T cells *in vitro* before transfer: The spleen and LN cells from double-Tg mice were stimulated with 0.3 nM HYUty peptide for 3 days before CD8 T cells were purified and adoptively transferred to female C57BL/6 mice (which were then inoculated s.c. with 4×10^5 MB49 cells on the same day as T cell transfer). Early tumour growth was significantly inhibited in mice adoptively transferred with activated double-Tg CD8 compared to mice with activated single-Tg CD8 (Day 11; $p = 0.04$). However, activated double-Tg T cells, did not control long term tumour growth any better than single-Tg T cells (Figure 35B).

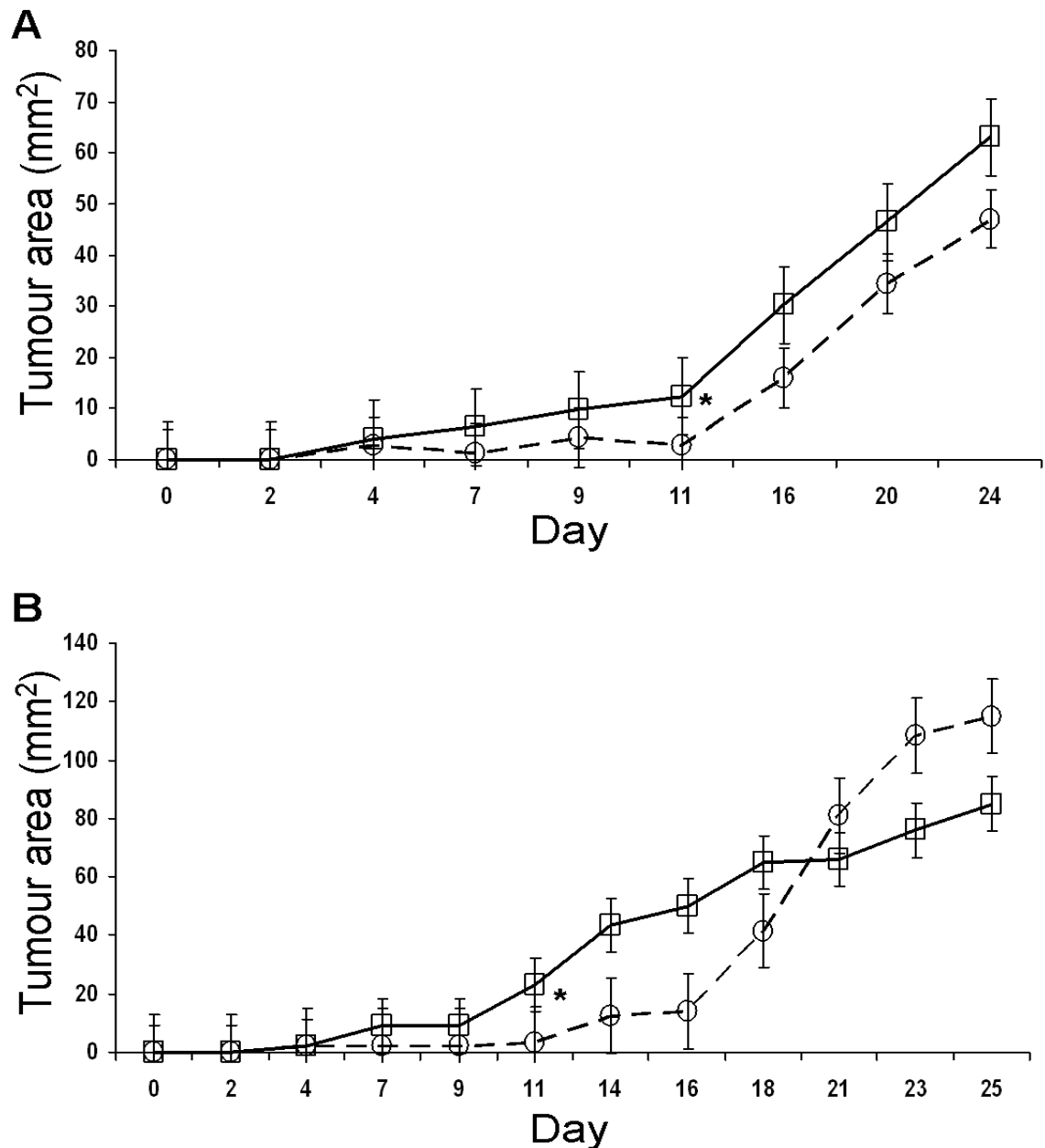


Figure 35. Adoptive therapy using resting or *in vitro* activated single-Tg or double-Tg CD8 T cells.

A) 10×10^6 CD8⁺ cells purified from either single-Tg (□, n=3) or double-Tg mice (○, n=3) were adoptively transferred to female C57BL/6 mice which were then inoculated s.c. with 3×10^5 MB49 cells on the same day of T cell transfer. Tumour area was measured as Width x Length at regular intervals. Data is presented as mean± SEM. Day 11; $p = 0.02$

B) Spleen and LN cells of single- or double-Tg mice were stimulated with 0.3 nM HYUty peptide in 24 well plates. 3 days later, viable CD8⁺ T cells were purified by MACS and 5×10^6 single-Tg CD8⁺ (□, n=2) or 10×10^6 double-Tg CD8 T cells (○, n=2) were adoptively transferred to female C57BL/6 which were then inoculated s.c. with 4×10^5 MB49 cells. Tumour area was measured as Width x Length at regular intervals. Data is presented as mean ± SEM. Day 11; $p = 0.04$.

To follow the fate of donor MataHari CD8 cells in MB49-bearing mice, Thy1.1⁺ single- or double-Tg CD8 T cells were adoptively transferred to Thy1.2⁺ WT C57BL/6 mice that were s.c. inoculated with 5×10^5 MB49 cells. After 11 days the tdLN and tumour tissues were harvested and the frequency and absolute numbers of the adoptively transferred donor CD8 T cells was analysed. The absolute number of tumour-infiltrated double-Tg CD8 T cells was significantly higher than that of tumour-infiltrated single-Tg CD8 T cells (Figure 36 A and B, $p = 0.025$). However, the number of donor T cells recovered from draining lymph nodes of the two groups was similar (Figure 36 C and D).

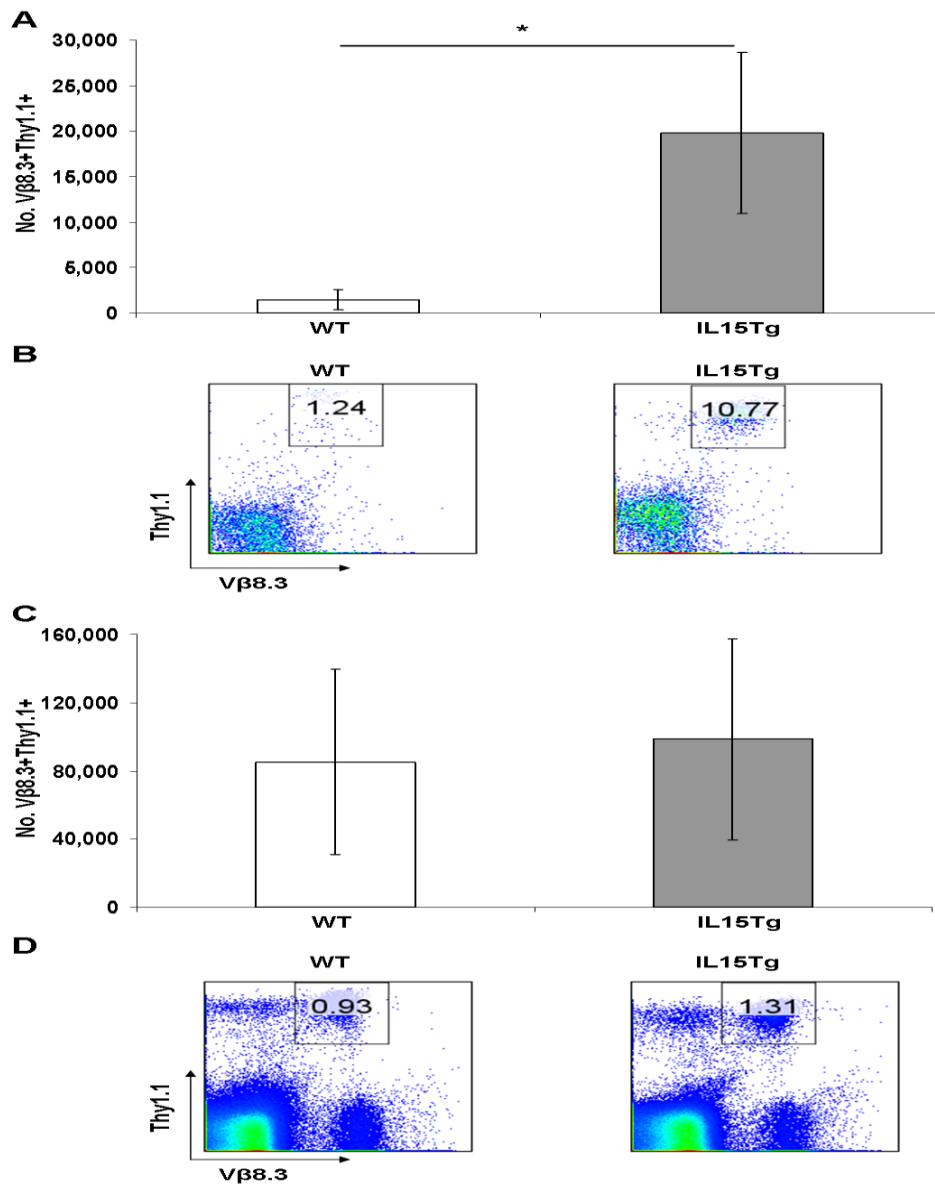


Figure 36. For accumulation in MB49 tissues, double-Tg CD8 T cells are more potent than single-Tg CD8 T cells

6×10^6 naïve single- or double-Tg CD8 T cells (Thy1.1) were injected i.v. to female C57BL/6 mice (Thy1.2) which were inoculated s.c. with 5×10^5 MB49 cells on the same day of T cell transfer. 11 days later, tumour and tumour-dLN were harvested. The cells were counted and then stained with anti-Vβ8.3^{PE}, anti-Thy1.1^{PerCP} and anti-CD44^{APC}. The small lymphocyte gate used to gate lymphocytes was also applied to the MB49 tissues.

A) The absolute number of double-Tg (□, n = 4) or single-Tg CD8 T cells (■, n = 4) recovered from the tumours was calculated by the total number cells/tumour x % Thy1.1⁺ Vβ8.3⁺ donor cells. Data are presented as mean ± SD. * $p=0.025$.

B) The frequency of tumour-infiltrated donor CD8 T cells is shown by one representative density plot.

C) The absolute number of double-Tg (□, n = 4) or single-Tg CD8 T cells (■, n = 4) recovered from tumour-dLN was calculated by the total number cells/tumour x % Thy1.1⁺ Vβ8.3⁺ donor cells. Data are presented as mean ± SD.

D) The frequency of donor CD8 T cells in MB49-dLN is shown by one representative density plot.

6.6 : A combinational therapy using double-Tg CD8 T cell transfer and host preconditioning

Despite the increased frequency and higher absolute number of tumour-infiltrated donor double-Tg CD8 T cells, MB49 tumours are not rejected. Therefore it is likely that additional manipulations are needed to boost the anti-tumour effects of double-Tg CD8 T cells. Sub-lethal irradiation has been shown to synergise with IL-15 to enhance the cytotoxicity and proliferation of adoptively transferred pmel CD8 T cells¹⁶⁶. Moreover, we have shown in Chapter 5, that sub-lethal irradiation enhances the anti-tumour effects of adoptively transferred Marilyn cells. To study the effect of irradiation on the anti-MB49 capacity of adoptively transferred double-Tg CD8 T cells, female C57BL/6 mice were irradiated with 5Gy before i.v. adoptive transfer of double-Tg CD8 T cells and s.c. MB49 challenge.

The results of two independent experiments are shown below. In the first experiment, MB49 tumours in the control mice (irradiated but no CD8 transfer) grew faster than in mice that had been treated with double-Tg CD8 T cells. Furthermore MB49 tumours reached an average size of $64 \pm 8 \text{ mm}^2$ by day 18 in control mice compared to significantly smaller ($p = 0.019$) tumours ($39 \pm 14 \text{ mm}^2$ by day 18) in mice treated with double-Tg CD8 T cells (Figure 37A). A similar result was observed in the second experiment, MB49 tumours in the control group were larger and grew to $80 \pm 22 \text{ mm}^2$ by day 17, whereas tumours in mice that had been treated with double-Tg CD8 T cells were significantly smaller ($p = 0.013$) reaching a size of $38 \pm 14 \text{ mm}^2$ by day 17 (Figure 37B).

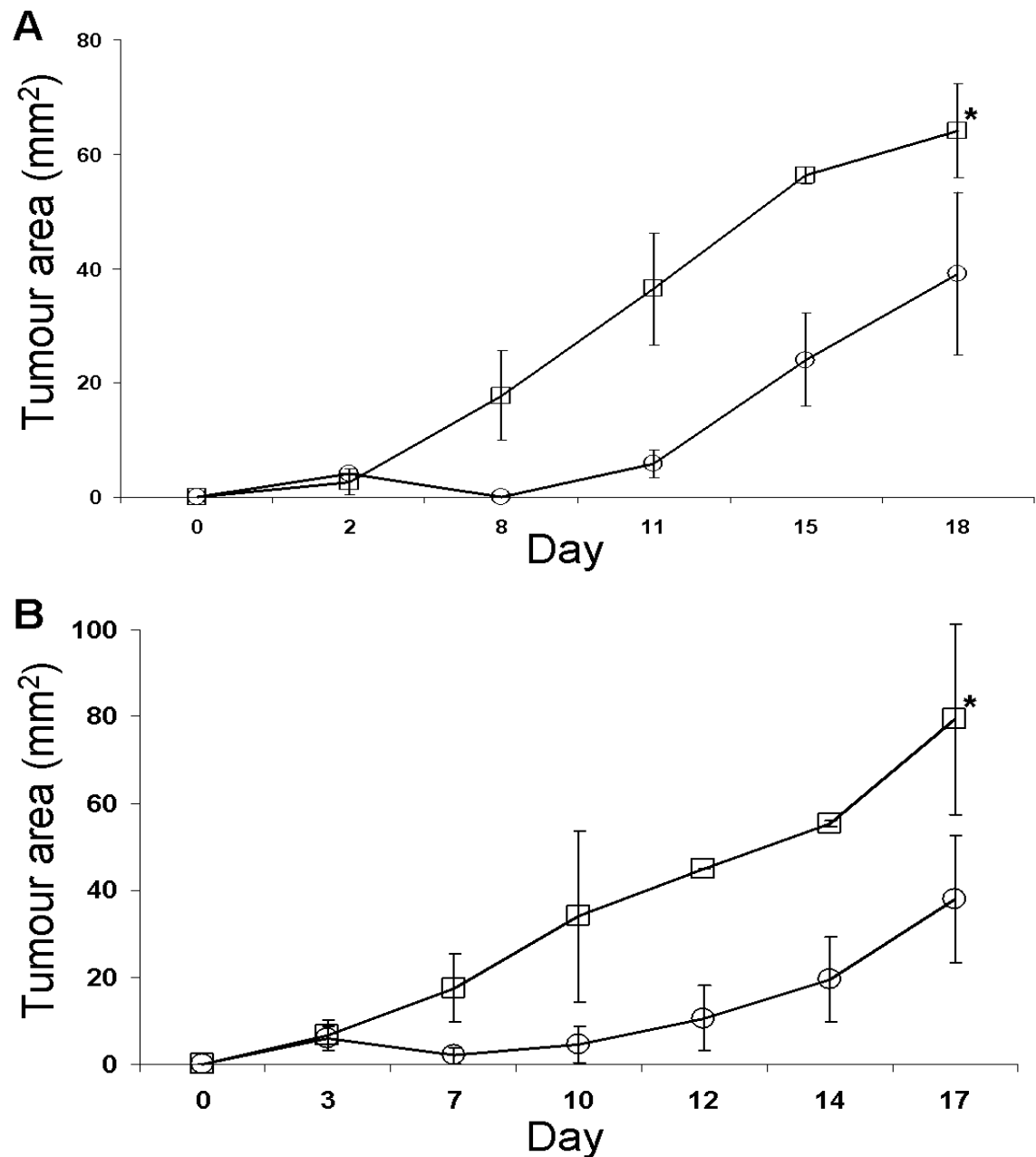


Figure 37. Effective adoptive immunotherapy with double-Tg CD8 T cells requires pre-conditioning of host with 5Gy irradiation.

A) 7 female C57BL/6 mice were irradiated with 5Gy, of which 4 mice ($\circ$, $n=4$) were given 3.7×10^6 double-Tg CD8 T cells by i.v injection, the remaining 3 mice were not ($\square$, $n=3$). Then all mice were inoculated s.c. with 4×10^5 MB49 cells on the same day of CD8 transfer. Tumour area was measured at regular intervals. Data are presented as mean $\pm$ SD. * $p = 0.019$.

B) 7 female C57BL/6 mice were irradiated with 5Gy. Of which, 5 mice ($\circ$, $n = 5$) were treated with 6.6×10^6 double-Tg CD8 T cells i.v, two remaining mice were untreated ($\square$, $n = 2$). Then all mice were inoculated s.c. with 4×10^5 MB49 cells. Tumour area was measured at regular intervals. Data are presented as mean $\pm$ SD. * $p = 0.013$

6.7 : The use of genetically modified MataHari CD8 T cells to treat MB49 tumour.

Having found that antigen specificity of CD8 T cells is necessary but not sufficient for clearing tumours, we decided to use genetic modification approaches to enhance anti-tumour CD8 responses. To this end, we engineered retroviral constructs encoding the human IL-2 gene (hIL-2). MataHari CD8 T cells were activated with HYUty peptide prior to transduction with the hIL-2 encoding retrovirus. The expression of human IL-2 by MataHari CD8 T cells was verified by co-expression of GFP from the viral vector.

To test whether hIL-2-transduced MataHari CD8 T cells could cause MB49 tumour rejection, 10×10^6 hIL-2-transduced MataHari CD8 T cells were injected i.v. into female C57BL/6 mice that had been irradiated with 5Gy. One day after T cell transfer, the mice were inoculated s.c. with 5×10^5 MB49 cells. Control mice were irradiated and inoculated s.c. with 5×10^5 MB49 cells but were not injected with CD8 T cells.

MB49 tumours in the control mice were significantly larger, reaching an average size of $60 \pm 6 \text{ mm}^2$ by day 13, than in treated mice ($p > 0.0001$) whose tumours measured an average of $6 \pm 6 \text{ mm}^2$ (Figure 38A).

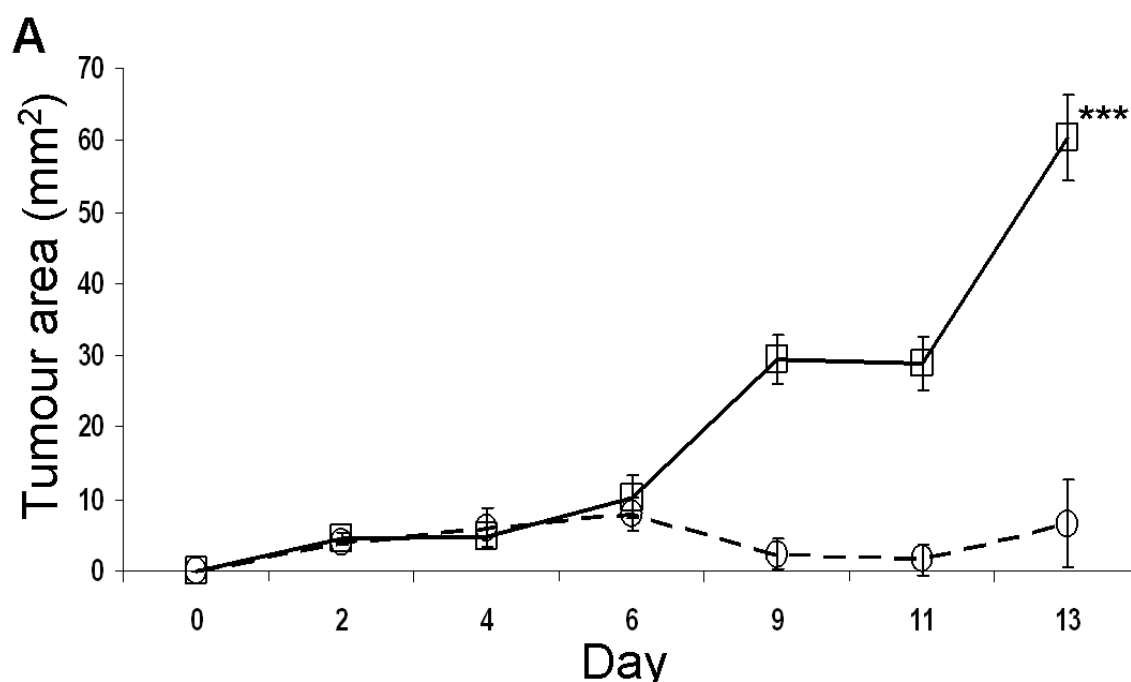


Figure 38. Irradiation followed by adoptive transfer of IL-2-transduced MataHari T cells efficiently controls MB49 tumours

A total of 13 WT female C57BL/6 were irradiated with 5Gy. Twenty four hours later, five mice were injected i.v. with 10×10^6 IL-2-transduced MataHari T cells ($\circ$, $n = 5$), and 8 mice were not ($\square$, $n=8$). All mice were inoculated s.c. with 5×10^5 MB49 on the same day of CD8 transfer. Tumour area was measured as Width x Length at regular intervals. Data are presented as mean $\pm$ SD. $p > 0.0001$.

6.8 : Discussion.

As MataHari CD8 T cells would be utilised as effectors in treating MB49 tumours by adoptive transfer, it was of importance to characterize their phenotype and function. FACS analysis showed that Rag2^{-/-} MataHari female mice have two populations of T cells- expressing transgenic V β 8.3, 5% of lymphocytes co-expressed CD8 and V β 8.3 and 7% co-expressed CD4 and V β 8.3 (Figure 31A, left panel). This unexpected observation does not correlate with the originally derived transgenic MataHari which expressed a monoclonal population of CD8⁺V β 8.3⁺ T cells^{178, 205}. However, the majority of gated CD8⁺V β 8.3⁺ cells expressed a high level of CD62L, suggesting that they are naïve CD8 T cells (Figure 31A, right panel) whereas the majority of CD4⁺V β 8.3⁺ cells expressed low levels of CD62L

suggesting that they are anergic T cells which may have cross reactivity with self/environmental antigen.

When spleen and pooled LN cells from a Rag2^{-/-} MataHari mouse were cultured with HYUty peptide, a strong proliferation was observed in a peptide dose-dependent manner, demonstrating that the transgenic TCR is functional (Figure 31B).

To distinguish which population, (i.e, CD8⁺Vβ8.3⁺ or CD4⁺Vβ8.3⁺) is responsible for peptide-induced proliferation, we labelled spleen and pooled LN cells from a Rag2^{-/-} MataHari mouse with CFSE before culturing them with HYUty peptide. We observed that the CD8⁺Vβ8.3⁺ cells (Figure 31C, bottom panel) but not the CD4⁺Vβ8.3⁺ cells (Figure 31C, top panel) showed CFSE dilution, demonstrating that only CD8⁺Vβ8.3⁺ cells are able to recognise cognate peptide, thus representing functional MataHari T cells.

Adoptive transfer of naïve MataHari cells inhibited the growth of MB49 tumours when combined with a vaccine of complete freunds adjuvant mixed with HYUty peptide (Figure 32B). Naïve MataHari cells alone or after irradiation did not successfully clear the tumour (Figure 32A and C). MataHari T cells that had been activated *in vitro* before being adoptively transferred to female C57BL/6 mice did not successfully inhibit MB49 tumour growth (Figure 33A and B).

Together these results suggest that MataHari cells are only effective at reducing the growth of MB49 tumours in female C57BL/6 WT mice when they are boosted by vaccination. This confirms results from a previous study in Rag^{-/-} female C57BL/6 mice that demonstrated that 1 x 10⁶ naïve MataHari cells did not cause the rejection of 1 x10⁵ MB49 cells injected s.c. one day previously¹⁷⁸, these results suggest that additional help is required in immuno-deficient and immuno-competent mice. These results differ from those of many studies that have found that the adoptive transfer of tumour reactive CD8 T cells inhibits tumour growth^{168, 176, 275}. MataHari T cells efficiently kill MB49 cells *in vitro*¹⁷⁸ but are ineffective *in vivo*. This may be because of tumour mediated suppression²⁸¹, exhaustion by chronic antigen stimulation²⁸², anergy²⁸³ or FAS/FAS-L mediated activation induced cell death²⁸⁴.

To try and boost the anti-tumour capacity of MataHari T cells, MataHari mice were crossed with IL-15 transgenic mice¹⁹³. IL-15 was chosen because it can abrogate the effects of AICD^{193, 285} and Treg mediated suppression on conventional CD4 and CD8 T cells⁹⁶, it helps long term survival of CD8 memory cells^{91, 92} and increases anti tumour activity¹⁶⁷.

MataHari IL-15 double transgenic CD8 T cells were used as effectors in treating MB49 tumours by adoptive transfer. FACS analysis showed that double-Tg female mice

had increased fractions of effector memory (EM) and central memory (CM) cells, whereas T cells in single transgenic mice were predominantly naïve cell (Figure 34B), which is in agreement with previously published results¹⁶⁷. Proliferation in response to cognate peptide by both single and double transgenic was comparable (Figure 34A).

Adoptive therapy using naïve (Figure 35A) and activated (Figure 35B) double transgenic T cells inhibited early growth of MB49 tumours when compared to single transgenic cells. However by the end of the experiment tumour sizes in both groups were similar, suggesting double transgenic T cells are marginally better at controlling MB49 tumours than single transgenic cells. *In vitro* activation of single and double transgenic cells did not improve tumour therapy.

When tumours from treated mice were analysed it was found that double transgenic cells were present at higher numbers than single transgenic cells (Figure 36A and B). However the proportions of single and double transgenic cells in the tdLN were very similar (Figure 36C and D), suggesting that double transgenic cells had an increased capacity to infiltrate into the tumour. In a similar study, Klebanoff *et al.* used pmel T cells crossed with IL-15 Tg mice to show that IL-15 reduced the growth of B16 melanomas. This study demonstrated that IL-15Tg T cells were better at controlling B16 melanomas than either pmel or pmel expressing IL-2¹⁶⁷.

Double transgenic T cell therapy does not cause complete remission of MB49 tumours. Preconditioning recipients with sub-lethal irradiation is a commonly used regimen to enhance adoptive transfer. 5Gy of irradiation significantly enhanced the therapeutic potential of double transgenic cells transferred to tumour bearing mice (Figure 37A and B). These results corroborate previous findings suggesting that pre-conditioning with irradiation synergises with IL-15 to abrogate CD8 T cell tolerance¹⁶⁶.

In a separate experiment, 5Gy of irradiation followed by adoptive transfer of hIL-2 transduced Matahari CD8 T cells was even more effective at preventing the growth of MB49 tumours than double transgenic cells (Figure 38). This is in agreement with several experiments that have used IL-2 to augment *in vivo* anti-tumour responses^{167, 286, 287}.

Together the results in this chapter demonstrated that MataHari cells are incapable of causing tumour rejection alone despite being able to recognise tumour antigen. In this sense MataHari cells represent an anergic and tolerant CD8 T cell population similar to those found in tumour bearing patients. Therefore, the extra manipulations that are needed to activate MataHari cells to become tumour effector cells may well be representative of those found in a clinical setting. Here we describe that a strong TCR signal by vaccination,

or a combination of pre-conditioning by irradiation followed by transfer of T cells expressing exogenous IL-2 or IL-15 are effective at causing tumour rejection.

Chapter 7 : Treg and MB49

7.1 : Introduction

The primary function of natural CD4⁺ CD25⁺ Foxp3⁺ regulatory T (nTreg) cells is to control self-reactive peripheral T cells that escape thymic deletion^{57, 60, 61}. As many tumour associated antigens are aberrantly expressed normal self-proteins²⁸⁸, the activity of self reactive nTreg cells is proposed to inhibit tumour-specific T-cell responses. Indeed, *in vivo* depletion of nTreg cells improves anti-tumour responses and enhances the efficiency of therapeutic cancer vaccines^{125, 126}. Expansion of nTreg cells is often correlated with tumour growth^{151, 289} but several key questions regarding tumour-driven nTreg expansion remain unanswered.

Firstly, do tumour antigens, which are altered self-proteins, activate endogenous polyclonal nTreg cells? Furthermore, does tumour-derived transforming growth factor- β (TGF- β) expand activated Treg cells in an antigen-independent manner²⁹⁰? And do activated nTreg cells, which can suppress anti-tumour immunity non-specifically *in vivo*, become expanded by tumour?

Secondly, do nTreg cells expand solely in response to the tumour and what is the relative contribution of antigen-specific and non-specific expansion to the overall pool of nTreg cells? In addition, is there a difference in the *in vivo* migration of nTreg cells expanded by these two distinct pathways?

Finally, do murine tumours, other than the non-immunogenic B16 tumours, expand polyclonal nTreg cells via a TGF- β -dependent mechanism and does²⁹⁰ this mechanism apply to other immunogenic tumours and to tumour-specific nTreg cells?

The responses of tumour-antigen specific nTreg *in vivo* has only been examined following vaccination to viral components^{291, 292} and little is known about nTreg expansion in response to the tumour.

To address these questions, tumour-specific nTreg cells purified from female Rag2^{+/-} Marilyn mice or polyclonal nTregs purified from WT B6 by FACS sorting were injected intravenously (i.v.) into tumour-bearing wild-type (WT) B6 mice. WT C57BL/6 mice were inoculated with MB49 (as described previously) or the HY-negative B16 melanoma, or B16 melanoma that had been transfected with the Dby gene to produce an HY-positive variant (B16/HY). By comparing the nTreg reactions to all three tumours the relative contributions of non-specific and HY-specific nTreg-cell expansion by tumours can be investigated.

7.2 : Adoptive transfer of CD25 depleted Marilyn T cells

CD25-depleted but not undepleted Marilyn CD4 T cells are potent responders *in vivo*. To compare *in vivo* anti-HY responses by Marilyn CD4 T cells with or without nTreg cells, CD4 cells purified from Rag 2^{+/-} Thy-1.1⁺ Marilyn females were either depleted of CD25-expressing cells or not. After CFSE labelling, CD25 replete and CD25 depleted Marilyn T cells were adoptively transferred into four groups of Thy-1.2⁺ C57BL/6 female mice. Next MB49 cells were injected subcutaneously into the right flank of two groups of recipients of each donor population.

Five days after tumour inoculation, the representation of donor cells in various tissues of the individual mice in all four groups was analysed.

Donor cells in the group which received both CD25 depleted CD4 and MB49 cells mounted a robust response with an eight-fold expansion in the dLN, ($0.545 \pm 0.01\%$ vs. $0.065 \pm 0.02\%$ in the control group that received CD25 depleted CD4 alone, $p < 0.01$), and a significant accumulation in the tumour ($3.55 \pm 0.27\%$)(Figure 39A). In addition, proliferating donor cells (Thy-1.1⁺ V β 6⁺) in dLN significantly increased CD44 expression after progressive cell divisions whereas those in peripheral LN (inguinal, pLN) of the tumour free control group did not ($86.7 \pm 0.3\%$ vs. $5.98 \pm 1.6\%$, $p < 0.01$)(Figure 39B, C and D, right panels). In contrast, the donor cells in the group given both CD25 intact CD4 T cells and MB49 cells (Figure 39C) underwent a poor response with a limited accumulation in the dLN ($0.01 \pm 0.01\%$ vs. $0.055 \pm 0.01\%$ in tumour-free control group, $p > 0.05$) and tumour ($0.06 \pm 0.01\%$) (Figure 39A) which could be the result of either failure of activation or accelerated activation-induced cell death in the presence of CD25⁺ nTreg cells. As $> 90\%$ of CD25 replete donor cells lost CFSE but up-regulated CD44 (Figure 39C, right panel), it is likely that all donor cells were recruited into the anti-MB49 response but most of them were eventually eliminated during the course of the response. Taken together, these results clearly indicate that CD25⁺ nTreg cells have a huge impact on determining whether a CD4 response would be successful, because a minor population of Marilyn nTreg cells co-transferred with a majority of conventional Marilyn CD4 T cells is associated with a substantially reduced response to MB49.

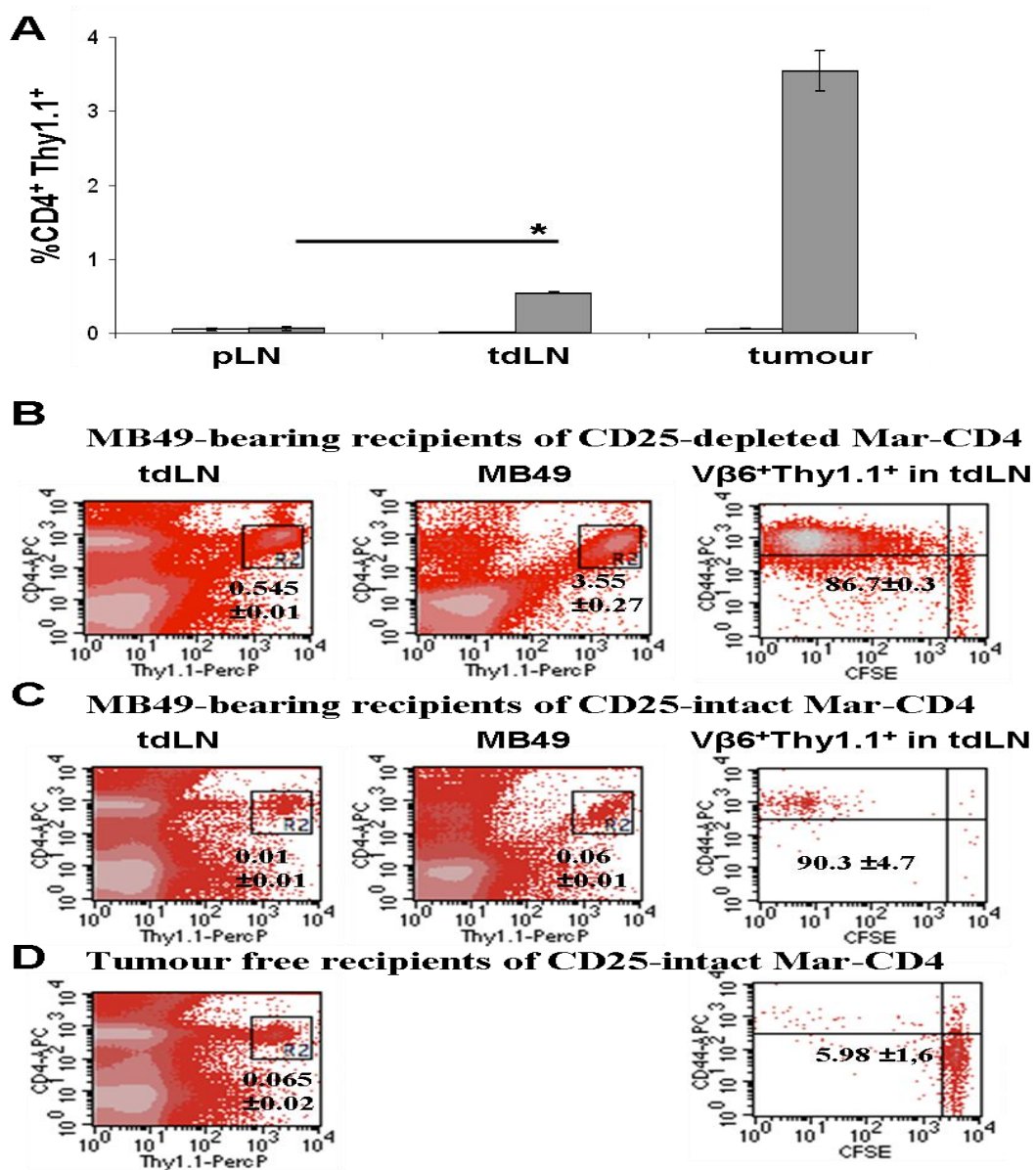


Figure 39. Comparison of anti-MB49 responses by CD25-depleted and CD25 intact Marilyn CD4 T cells.

A) Four groups of Thy-1.2 B6 females (three or four mice/group) were adoptively transferred with 1.5×10^6 CFSE-labelled CD25-depleted (■) or CD25-intact (□) Thy-1.1 Marilyn CD4 T cells by i.v. injection. Next day two groups of mice only were inoculated s.c. with 0.5×10^6 MB49 cells. Five days after MB49 inoculation, all groups were analysed by staining of dLN and MB49 or pLN cells with anti-Vβ6^{PE}, anti-Thy-1.1^{PerCP} and anti-CD4^{APC}. The mean frequency $\pm$ SD is presented. * CD25-depleted, tdLN vs CD25-depleted pLN; $p = <0.01$.

B, C, D) The donor cells in lymph nodes (LN) and MB49 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) was acquired for each sample (**B, C**, right hand side of panel). One representative experiment of three is shown.

7.3 : MB49 expands Marilyn but not B6 natural Treg cells in vivo.

A small nTreg-cell population has a significant impact on a much larger CD4 T-cell population of the same specificity, therefore, it is possible that MB49 can preferentially expand nTreg cells. MB49-induced nTreg-cell expansion can be either antigen driven or not, or a combination of both, because some tumour cell lines can expand nTreg cells in an antigen-independent fashion²⁹⁰. As MB49 variants lacking expression of HY are not available, the relative contribution of antigen non-specific expansion cannot be determined. To overcome this limitation, we used the alternative strategy of transferring polyclonal nTreg cells from Thy1.1⁺ female C57BL/6 mice to MB49 bearing female B6 Thy-1.2 mice. If MB49 can induce nTreg cells to proliferate non-specifically, then expansion of B6 nTreg cells from this source would be seen.

To test antigen dependent expansion of nTreg, CD25 replete Thy1.1⁺ Marilyn nTreg cells were adoptively transferred into two groups of Thy1.2⁺ C57BL/6 females, one group was given MB49 inoculation the next day. As shown in Figure 40A and B, following MB49 challenge, Marilyn nTreg cells underwent an eight-fold expansion in the dLN when compared to tumour free control (0.0207 ± 0.0111 vs. $0.0026 \pm 0.0013\%$ in tumour-free control group, $p < 0.05$). Furthermore, the degree of donor nTreg cells expansion was higher when absolute numbers of donor cells recovered from the two groups was compared (data not shown). Marilyn nTreg cells also showed a significant loss of Foxp3 expression in the dLN of MB49 bearing mice ($84.83 \pm 0.08\%$ vs. $96.55 \pm 3.45\%$ in control group) (Figure 40B, top right panel).

To test antigen independent expansion of polyclonal B6 nTreg, cells were adoptively transferred into two groups of Thy1.2⁺ C57BL/6 females, one group was given MB49 inoculation the next day. There was no expansion of B6 nTreg cells in response to MB49 in the tdLN, based on the measurement of both frequency (Figure 40A and B) and absolute cell number (data not shown), suggesting that MB49 tumour can't non-specifically expand nTreg cells.

The frequency of donor Marilyn nTreg cells that had migrated to the tumour was higher ($0.5821 \pm 0.3011\%$) than that of B6 nTreg ($0.0069 \pm 0.0069\%$)(Figure 40C) But only one-third of tumour-infiltrated donor Marilyn nTreg cells maintained Foxp3 expression ($33.93 \pm 10.49\%$ in MB49 vs. $84.83 \pm 0.08\%$ in dLN)(Figure 40D, top right panel)

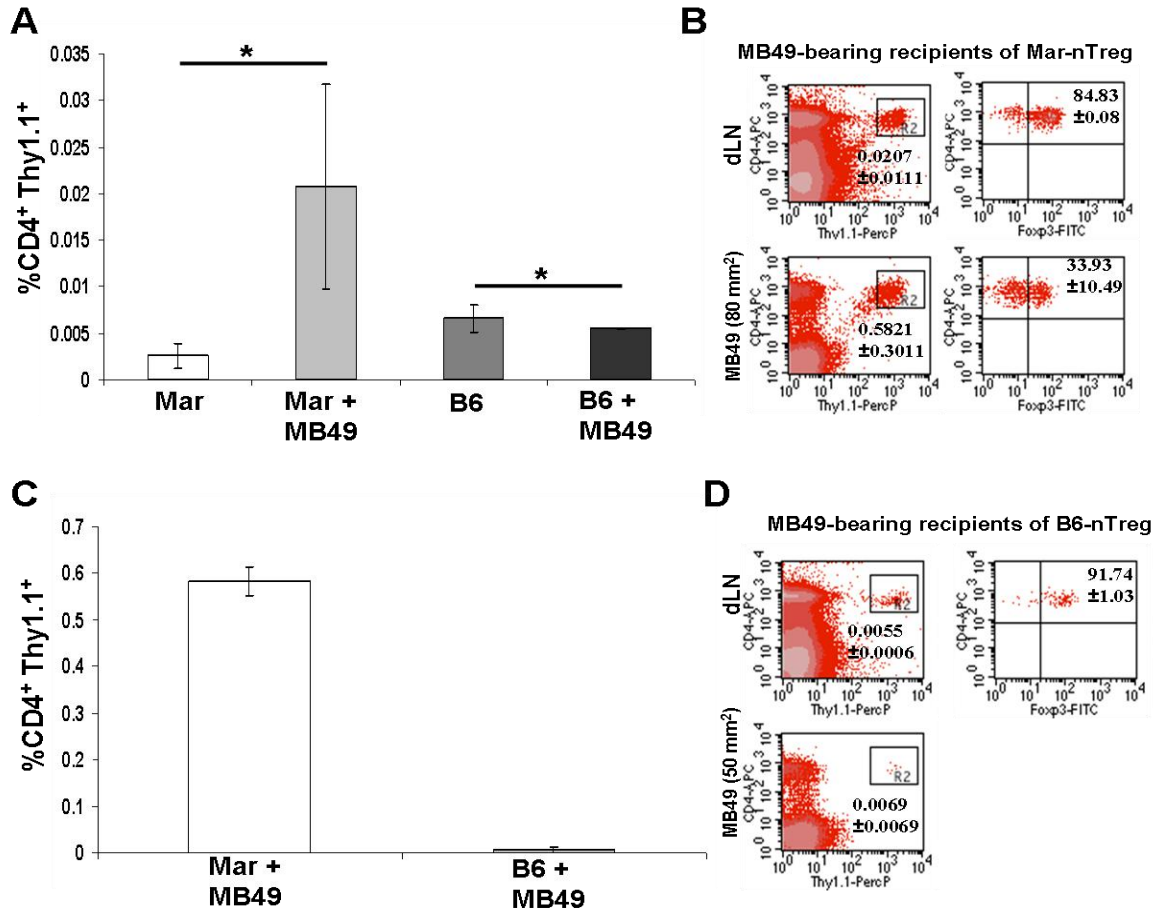


Figure 40. MB49 expands Marilyn but not B6 natural regulatory T (nTreg) cells *in vivo*

Four groups of Thy1.2⁺ C57BL/6 females (three mice/group) were transferred with 1×10^5 Thy-1.1 Marilyn nTreg or Thy-1.1 B6 nTreg cells of which two groups of mice were given MB49 cells the next day but the other two groups were not. 11 days after MB49 inoculation, analysis was performed by staining the cells with anti-V β 6^{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.

A) The frequency of Marilyn nTreg or B6 Treg in the tumour draining lymph nodes of treated mice or peripheral lymph nodes of untreated mice is presented as mean $\pm$ SD. * Marilyn vs. Marilyn + MB49; $p < 0.001$. * B6 vs. B6 + MB49; $p < 0.001$.

C) The frequency of Marilyn nTreg or B6 nTreg in the tumour mass of treated mice is presented as mean $\pm$ SD.

B and D) representative dot plots of nTreg in tdLN and tumour. One representative experiment of four is shown. A total of 2 million events was acquired for each sample. For the MB49 samples, tumour size is also indicated.

7.4 : Characterisation of HY expressing B16 line

Antigen-specific and non-specific nTreg-cell expansion by tumours could be directly compared using antigen-positive and antigen-negative variants of the same tumour cell line. As an HY-negative, mutant MB49 cell line is not available and a knockout of the HY Dby gene from MB49 cells is technically challenging, an HY-expressing variant of an HY-negative tumour cell line was produced. The widely used melanoma cell line B16¹⁷⁸ was transfected with the HYDby gene and a stable HY-expressing clone was obtained (B16/HY). Although Dby expression by B16/HY was lower than MB49, it was significantly higher than B16 ($p < 0.05$) (Figure 41A). Dby expression by B16/HY was maintained following tumour development (Figure 41B), suggesting that the expression of the transgene is stable *in vivo*.

Adoptively transferred, CFSE-labelled Marilyn conventional CD4 T cells proliferated in dLN in response to B16/HY but there was no activation of donor cells in B16-bearing mice (Figure 41C), or in tumour-free controls (data not shown). Donor T cells were also detectable in B16/HY tumours (Figure 41D). These *in vivo* T-cell recognition assays conclusively demonstrated that transduced Dby genes in B16/HY were functional.

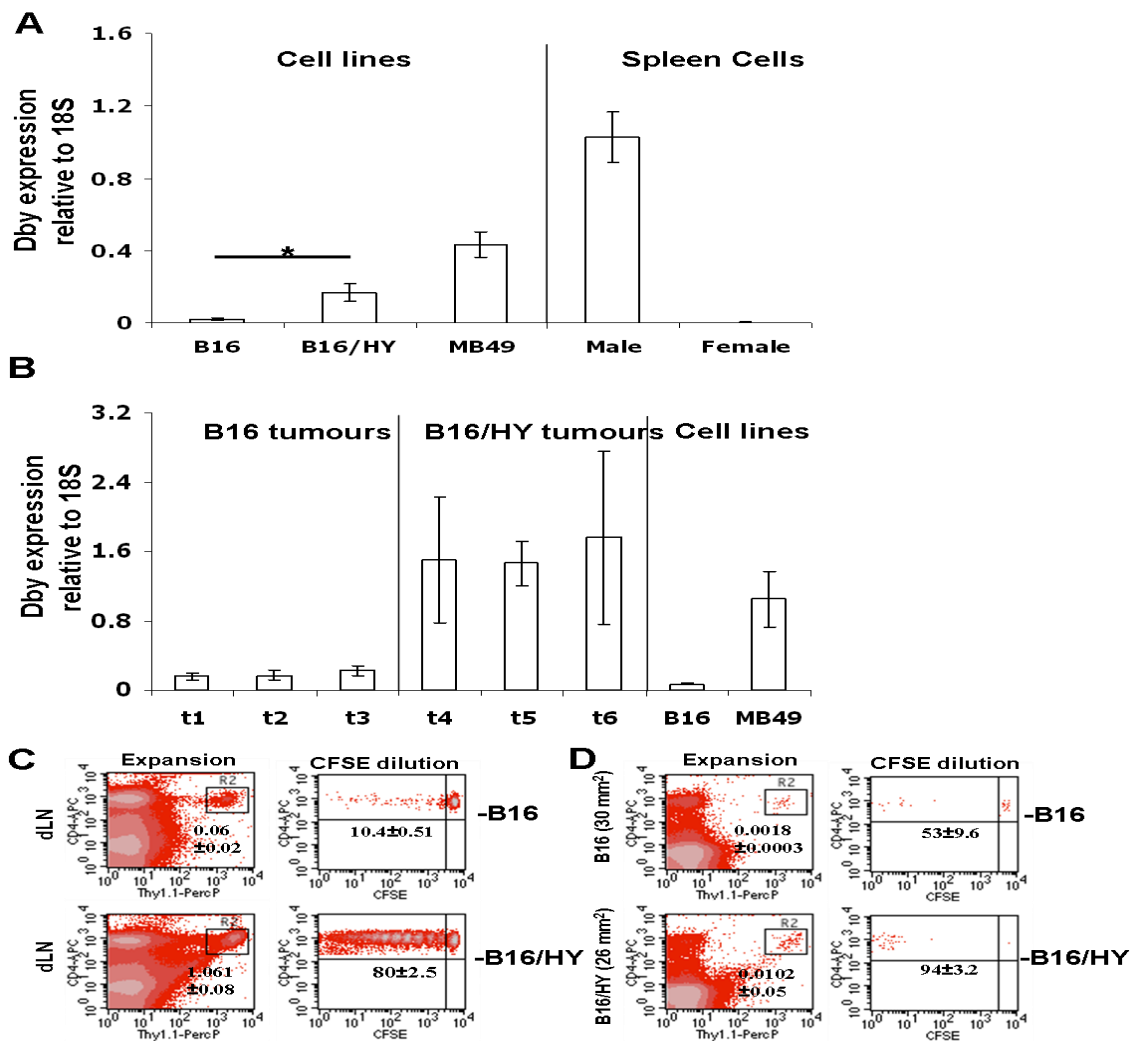


Figure 41. Characterization of HY-expressing B16 cell line (B16/HY)

A) B16, B16/HY and MB49 cells were compared for Dby expression by reverse transcription–polymerase chain reaction (RT-PCR) with male or female B6 spleen cells used as internal controls. One representative experiment of three is shown. Mean Dby expression is presented $\pm$ SD. * B16 vs. B16/HY; $p < 0.05$

B) Two groups of B6 females (three mice/group) were inoculated subcutaneously (s.c.) with 5×10^5 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.

Three groups of Thy-1.2 B6 females (three mice/group) were adoptively transferred with 3×10^6 CFSE-labelled Thy-1.1 Marilyn CD4 T cells. Next day the mice in two groups (d, e) were inoculated s.c. with 5×10^5 B16 or B16/HY cells, respectively. Thirteen days after tumour inoculation, analysis of dLN (**C**) or tumour (**D**) was conducted by staining the cells from various tissues with anti-V β 6^{PE}, anti-Thy-1.1^{PerCP} and anti-CD4^{APC}. The donor cells were identified by co-expression of Thy-1.1 and CD4 (R2). The CFSE dilution by donor cells is presented as CFSE versus CD4. One representative experiment of two is shown. A total of 2 million events was acquired for each sample. For the B16 and B16/HY samples, tumour size is also indicated.

7.5 : Comparison of the relative contribution by non-specific and ag-specific nTreg cell expansion

Thy1.1⁺CD4⁺Vβ6⁺CD25⁺GFP⁺ cells from (Foxp3/GFP knockin x Marilyn) F1 females were sorted by FACS, and adoptively transferred into three groups of Thy1.2⁺ females, then two of the groups were injected s.c. with either B16 or B16/HY cells the next day.

The expansion and Foxp3 expression by donor Treg cells was analysed 11 days after tumour inoculation.

Expansion of Marilyn nTregGFP cells in B16/HY tumour bearing mice was more efficient than in mice with the B16 tumour, 3.8-fold more donor cells accumulated in dLN of B16/HY than in dLN of B16 ($0.0381 \pm 0.0092\%$ vs. $0.0101 \pm 0.0005\%$, $p < 0.05$)(Figure 42A and B). Furthermore, the absolute donor Treg-cell numbers recovered from B16/ HY-bearing mice was significantly higher than those from B16-bearing mice (data not shown). However, B16 tumour appeared to be able to expand Marilyn nTreg cells non-specifically, as the frequency of donor Treg cells detected in dLN of B16-bearing mice was higher than those in pLN of tumour-free control mice ($0.0101 \pm 0.0005\%$, Figure 42, top left panel vs. 0.0013 ± 0.000 , Figure 42A and B).

Marilyn nTreg^{GFP} cells were present in B16/HY tumours ($0.2585 \pm 0.066\%$, Figure 42D, bottom left panel) but were absent from B16 tumours ($0.000 \pm 0.000\%$, Figure 42C and D), indicating that the additional step of migration of nTreg cells to the tumour is antigen dependent.

Finally, Foxp3 expression by nTreg cells *in vivo* appears to be unstable: following antigen recognition, expanded Treg cells can lose Foxp3 expression. Loss of Foxp3 by tumour-infiltrated Treg cells is greater ($77.68 \pm 0.02\%$, Figure 42D, bottom right panel) than that by those in dLN ($91.37 \pm 0.08\%$, Figure 42B, bottom right panel).

To increase sensitivity Rag2^{-/-} C57BL/6 females were used as recipients of Marilyn Treg cells. To make a proper comparison, we also modified the FACS acquisition strategy by collecting the same number of events within a live lymphocyte gate, allowing a more precise measurement of the frequencies of donor Treg cells between the samples. HY-specific green Treg cells were transferred to two groups of Rag2^{-/-} C57BL/6 females, which were inoculated with either B16 or B16/HY cells on the same day. Twelve days later, FACS analysis was performed to compare the frequency of donor Treg cells in dLN, spleen and tumour tissues of individual mice between two groups. Unlike the low recovery

of donor Treg cells in dLN of B16/HY-bearing WT B6 females (0.04%, Figure 42B, bottom left panel), a much higher proportion was present in dLN of B16/HY-bearing Rag2^{-/-} B6 females (15%, Figure 42F, bottom left panel). A significant fraction of activated donor Treg cells also accumulated in both spleen (data not shown) and B16/HY tumours (2.7%, Figure 42F, bottom right panel). In B16-bearing Rag2^{-/-} B6 females, however, the frequency of donor Treg cells was profoundly decreased in the dLN (0.43%, Figure 42F, top left panel, 97% reduction) presumably because of the lack of HY antigens. Consequently, donor Treg cells were barely detectable in the B16 tumour mass (0.02%, Figure 42F, top right panel, 99% reduction). Taken together, these results demonstrate that both antigen-specific and non-specific proliferation of Marilyn nTreg cells contributes to the expansion by HY-expressing B16 tumours, but the presence of antigen not only amplifies the expansion in dLN but is also required for expanded Treg cells to migrate into tumour. A fraction of Treg cells were shown to lose Foxp3 following *in vivo* activation.

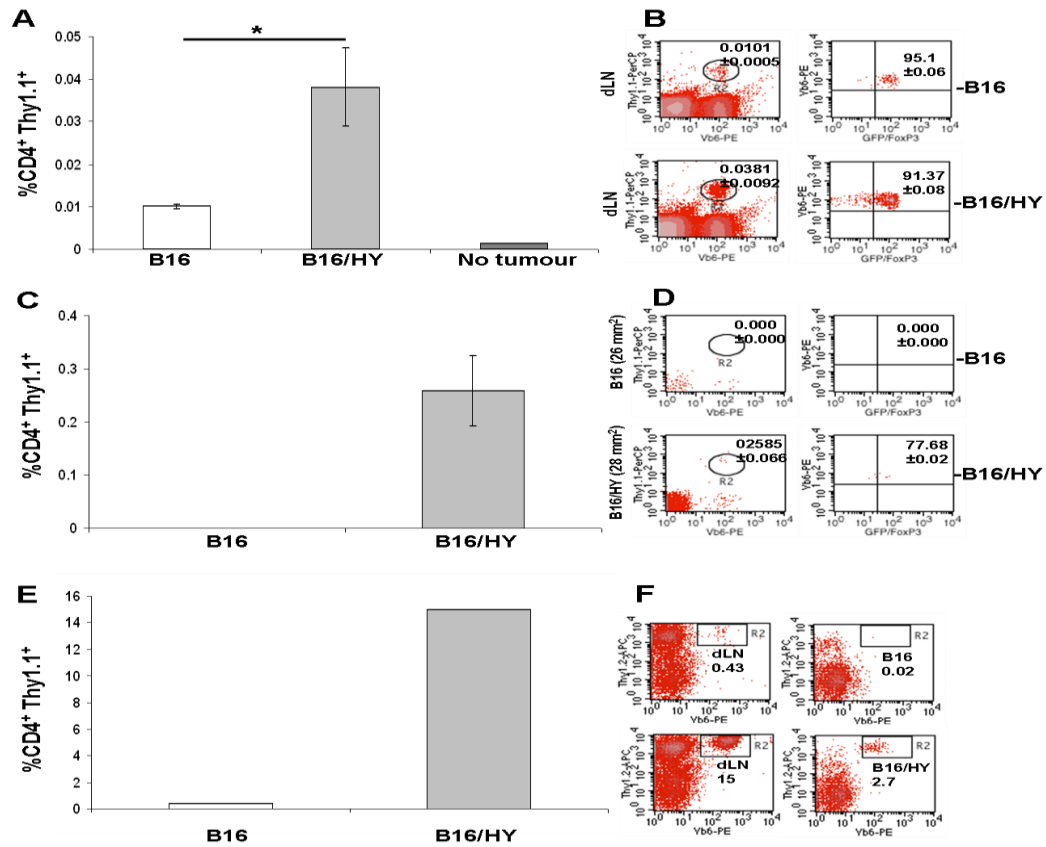


Figure 42. Comparison of the relative contribution by non-specific and antigen-specific natural regulatory T (nTreg) cell expansion.

Three groups of Thy-1.2 B6 females (three mice/group) were injected i.v. with 1×10^5 Thy-1.1 Marilyn nTreg cells co-expressing Foxp3/GFP (Mar-nTreg^{GFP}), of two groups of mice were s.c. inoculated with B16 cells or B16/HY cells (5×10^5) next day. Eleven days later, cells from tdLN or tumour tissues were stained with anti-Vβ6^{PE}, anti-Thy-1.1^{PerCP} and anti-CD4^{APC}. Donor cells were identified by co-expression of Thy-1.1 and Vβ6 (R2) and their Foxp3 expression is presented as Vβ6 versus GFP.

A) dLN was analysed for donor Mar-nTreg^{GFP} and the results are presented as mean $\pm$ SD. * B16 vs. B16/HY; $p < 0.05$. **B)** representative dot plots are shown.

C) Tumour was analysed for donor Marilyn nTreg and the results are presented as mean $\pm$ SD.

D) representative dot plots are shown, One representative experiment of two is shown. A total of 2 million events was acquired for each sample. For the B16 and B16/HY samples, tumour size is also indicated.

E and F) Two groups of Rag2^{-/-} B6 females (four mice/group) were transferred with 2×10^4 Thy-1.2 Mar-nTreg^{GFP} and were also inoculated s.c. with either 1×10^5 B16 or B16/HY cells on the same day. After 12 days, analysis was performed by staining the draining lymph nodes and B16 or B16/HY tumour tissues with anti-Vβ6^{PE}, anti-CD4^{PerCP}, and anti-Thy-1.2^{APC}. Donor Treg cells were identified by co-expression of Thy-1.2 and Vβ6 (R2). FACS dot plots of one representative mouse of each group are shown. An equal number of events in the live lymphocyte gate were acquired for the paired samples (2×10^4 for dLN, 5×10^4 for spleen and 5×10^3 for B16 and B16/HY). One representative experiment of two is shown.

E) dLN was analysed for donor Mar-nTreg^{GFP} and the results are presented as mean $\pm$ SD. * B16 vs. B16/HY; $p < 0.05$. **F)** representative dot plots are shown.

7.6 : Expansion of Marilyn nTreg cells by MB49 is TGF- β dependent.

The MB49 cell line constitutively expresses TGF- β , revealed by intracellular staining (Figure 43A). This cytokine may play an essential role in tumour-induced expansion of Marilyn nTreg cells. To explore this, Thy1.2⁺Marilyn nTreg cells co-expressing the dominant negative TGF- β receptor II (Mar-nTreg^{DN} cells) were transferred to two groups of Thy1.1⁺ C57BL/6 females, one group was inoculated with MB49 cells next day. Twelve days after cell transfer, the mice were analysed for the presence of donor cells. There was no difference in terms of the proportion of donor cells in pLN of tumour-free control ($0.0005 \pm 0.000\%$, Figure 43B) and dLN of MB49-bearing mice ($0.0002 \pm 0.0001\%$, Figure 43C, right panel). Donor cells were barely detectable in ndLN (Figure 43C, middle panel) and MB49 (Figure 43C, right panel). The defect in TGF- β signal transduction on nTreg cells abrogated their expansion in response to MB49 indicating that nTreg expansion by MB49 appears to be TGF- β dependent. It is unclear whether the data shown may result from a developmental defect of nTreg cells expressing the dnTGF- β RII, which may prevent appropriate activation in response to antigen.

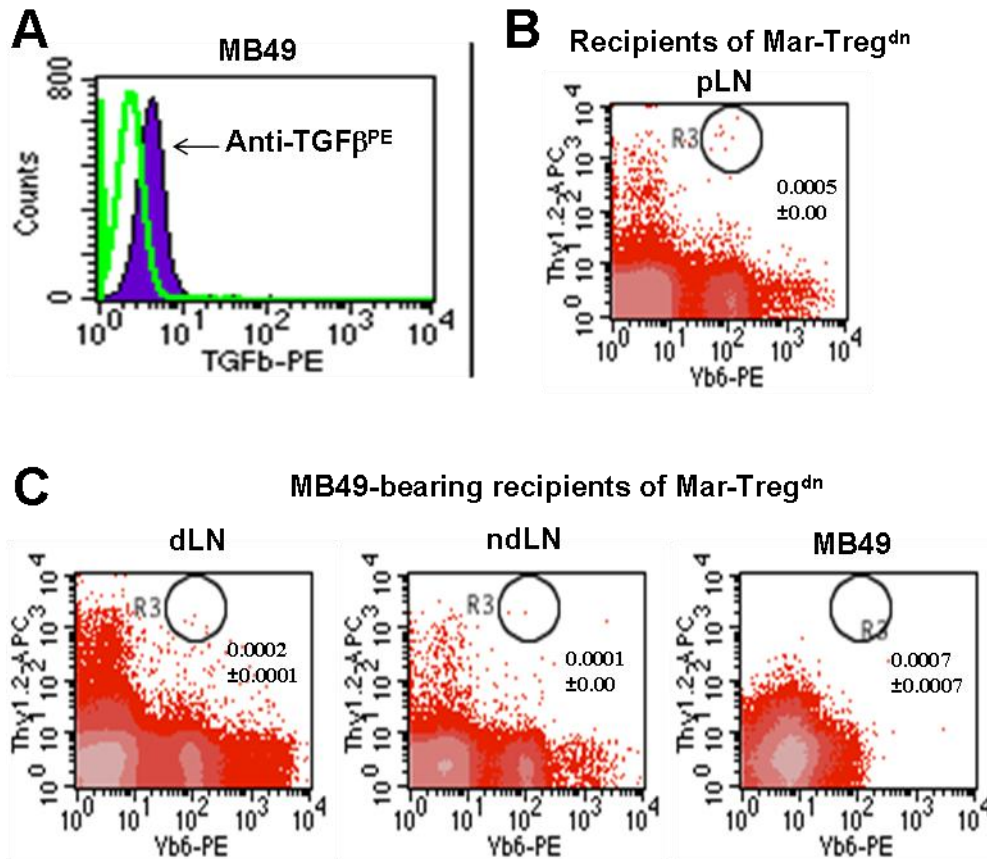


Figure 43. Expansion of Marilyn natural regulatory T (nTreg) cells by MB49 is dependent on transforming growth factor- β (TGF- β).

A) MB49 cells were cultured with 10% fetal calf serum RPMI-1640 in the presence of 1 μ g/ml brefeldin A for 4 hr. After fixing and permeabilizing, the cells were stained for anti-TGF- β ^{PE} (filled) or isotype control (open).

B and C) Two groups of Thy-1.1 B6 females (three mice/group) were transferred with 1×10^5 Thy-1.2 Marilyn nTreg cells co-expressing dnTGF- β RII (Mar-nTreg^{dn}), of which one group of mice received MB49 cells next day (**C**). Eleven days after tumour inoculation, analysis was conducted by staining the cells with anti-V β 6^{PE}, anti-CD4^{PerCP} and anti-Thy-1.2^{APC}, before intracellular staining with anti-Foxp3^{FITC}. The donor cells were identified by co-expression of Thy-1.2 and V β 6 (R3). A total of 2 million events was acquired for each sample.

7.7 : Comparison of Marilyn Treg with or without dnTGF- β *in vivo* and *in vitro*.

To determine the functionality of dnTGF β RII Marilyn Treg cells *in vivo*, we performed co-transfer experiments using Rag2^{-/-} C57BL/6 females as recipients. To this end HY-specific conventional CD4 T cells alone (CD4⁺V β 6⁺CD25⁻CD44^{low} sorted from Thy1.1⁺ Rag2^{-/-} Marilyn females), HY-specific Treg cells alone (CD4⁺V β 6⁺CD25⁺ sorted from Thy1.2⁺ Rag2^{+/-} Marilyn females), HY-specific and dnTGF β RII -expressing Treg cells alone (CD4⁺V β 6⁺CD25⁺ sorted from Thy-1.2⁺dnTGF β RII⁺ Rag2^{+/-} Marilyn females), or a mixture of an equal number of conventional and each Treg-cell population were adoptively transferred into five groups of Rag2^{-/-} B6 females which were given MB49 inoculation on the same day. Analysis was conducted 12 days later. In the absence of donor Treg cells, Marilyn CD4 T cells underwent a robust expansion in response to MB49 tumours with a significant accumulation of donor cells (V β 6⁺ CD4⁺ Thy-1.1⁺) in dLN (50%, Figure 44B, top left panel) and the MB49 tumour mass (16%, Figure 44B, top left panel) . However, this HY-derived systemic activation of donor Marilyn responders was inhibited by co-transferred Marilyn Treg cells with 50% suppression in dLN (Figure 44B, middle left panel) and 50% suppression in MB49 (Figure 44B, middle right panel). Interestingly, dnTGF β RII-expressing Marilyn Treg cells showed a partial suppression in dLN (20% suppression, Figure 44B, bottom right panel), but they failed to inhibit the accumulation of donor responders in MB49 tissue (Figure 44B, bottom right panel). For the suppression of accumulation of donor responder cells in dLN, both types of Marilyn Treg cells were effective regarding the analysis of both frequency (Figure 44A) and absolute cell numbers (data not shown), although overall Treg cells without dnTGF β RII are more potent inhibitors than their dnTGF β RII-expressing counterparts. In the presence of donor responder cells, the frequency of Marilyn Treg cells in dLN was significantly higher than that of dnTGF β RII-positive Treg cells (Figure 44C). Moreover, in the absence of responder cells, MB49-induced expansion of Marilyn Treg cells (20%) was significantly greater than that of dnTGF- β RII-expressing Marilyn Treg cells (4%) (Figure 44D and E).

We also conducted a standard *in vitro* co-culture to determine whether dnTGF β RII-expressing Marilyn Treg cells are functional. Although Marilyn dnTGF β RII⁺ CD4⁺ CD25⁺ cells were apparently anergic, they were also less suppressive than WT Marilyn CD4⁺ CD25⁺ cells, as at a 1 : 10 ratio of Treg cells to responder cells, no inhibition was achieved (Figure 45). In contrast, Marilyn CD4⁺ CD25⁺ cells can readily induced a complete suppression even at 1 : 1 ratio, as shown previously¹⁸³.

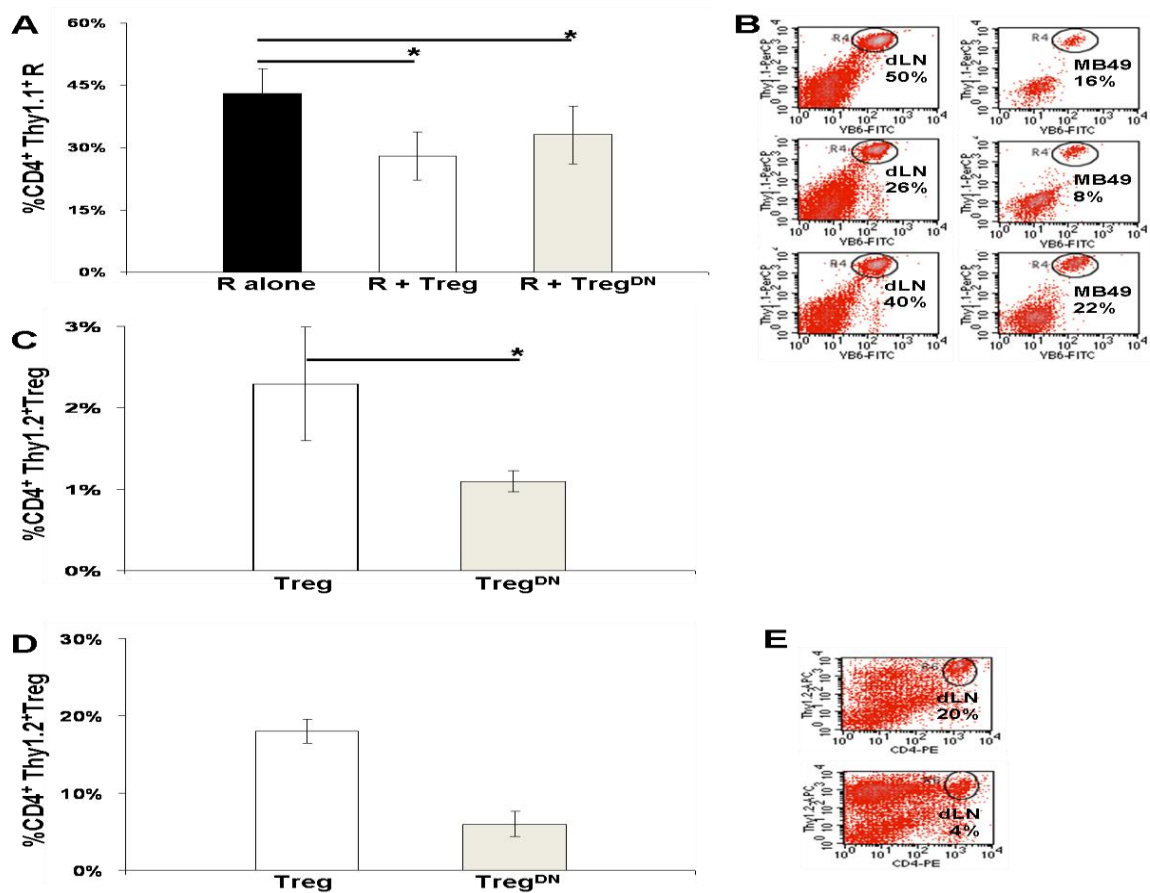


Figure 44. Comparison of Marilyn regulatory T (Treg) cells with or without dnTGF- β RII *in vivo* and *in vitro*

Five groups of Rag2^{-/-} B6 females (four mice/group) were transferred with 2×10^4 Thy-1.1 Marilyn CD4 T cells alone, 2×10^4 Thy-1.2 Marilyn Treg cells alone, 2×10^4 Thy-1.2 Marilyn Treg cells co-expressing dnTGF- β RII alone, or Thy-1.1 Marilyn CD4 mixed with either Thy-1.2 Marilyn nTreg cells or Thy-1.2 Marilyn Treg cells co-expressing dnTGF- β RII. The mice in all groups were also inoculated subcutaneously with 1×10^5 MB49 cells on the same day. Twelve days later, analysis was performed by staining dLN, spleen and MB49 tissues with anti-V β 6^{FITC}, anti-CD4^{PE}, anti-Thy-1.1^{PerCP} and anti-Thy-1.2^{APC}. The donor responder and donor Treg cells were identified by co-expression of Thy-1.1 and V β 6 (R4) and Thy-1.2 and CD4 (R6), respectively.

A and B) The representation of donor responder cells in dLN of three groups, responders alone (R alone), responders plus Marilyn Treg cells (R+Treg), and responders plus dnTGF- β RII-expressing Marilyn Treg cells (R+Treg^{dn}), results are displayed as mean frequency $\pm$ SD.

C) Frequency of donor Treg cells in draining lymph nodes (dLN) of two co-transfer groups responders plus Marilyn Treg cells (Treg), and responders plus dnTGF- β RII-expressing Marilyn Treg cells (Treg^{dn}).

D) The representation of donor Treg cells in dLN of two groups [Marilyn Treg cells alone (Treg), and dnTGF- β RII-expressing Marilyn Treg cells alone (Treg^{dn})]. results are displayed as mean frequency $\pm$ SD.

E) One representative experiment of two is shown. A total of 0.5 million events was acquired for each sample.

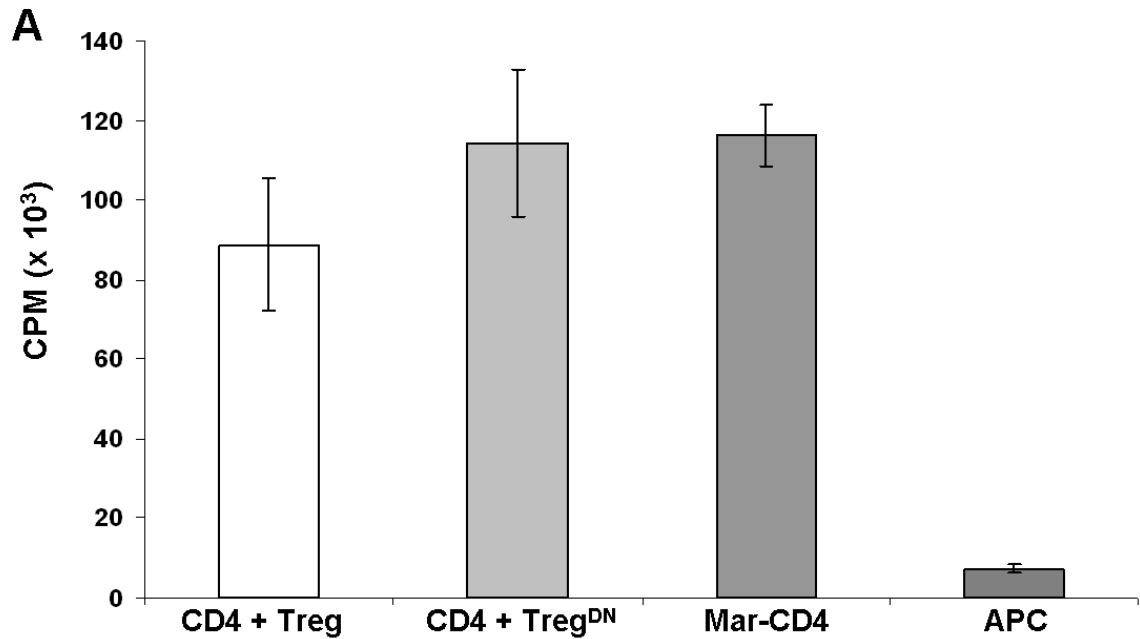


Figure 45. Mar-Treg^{DN} In vitro suppression assay

Marilyn CD4⁺CD25⁻ responder T cells (1 x 10⁴/well) were incubated with either WT Marilyn CD4⁺CD25⁺ (Treg) or dnTGF-βRII⁺ Marilyn CD4⁺CD25⁺ (Treg^{DN}) cells (1 x 10³/well) together with 50nm HYDby peptide in the presence of antigen-presenting cells (5 x 10⁴/well, T-cell-depleted B6 female spleen cells) in round-bottomed 96-well plates for 3 days. Thymidine was added to each well for the last 12 hr. One representative experiment of two is shown.

7.8 : Marilyn CD4 T cells co-expressing transgenic dominant negative TGFβ receptor II are resistant to the induction of Th17 and iTreg cells in vitro.

Marilyn CD4 T cells-expressing transgenic dnTGFβRII also endogenously express an intact TGFβ receptor which can respond to TGFβ. To examine whether endogenous TGFβ-receptors could maintain TGFβ signalling in the presence of transgenic dnTGFβRII in Marilyn CD4 T cells, we have used *in vitro* peptide culture systems of inducing Th17 and Treg cells, as the development of both subsets is dependent on TGFβ^{293, 294}.

In the model for generating iTreg cells, spleen and LN cells from dnTGFβRII-negative or dnTGFβRII-positive Marilyn females were stimulated with cognate HYDby peptide plus rhu-IL-2 in the absence or presence of rhu-TGFβ1. We found that up to 28% of dnTGFβRII-negative Marilyn CD4 T cells were differentiated into Foxp3-expressing iTreg cells following TCR, IL-2 and TGFβ signalling (bottom of Figure 46B). In contrast, development of iTreg cells was profoundly inhibited when dnTGFβRII-positive Marilyn cells were used (top of Figure 46B). Induction of Foxp3 expression in dnTGFβRII-expressing Marilyn CD4 T cells was not rescued by increasing the concentration of TGFβ (Figure 46A).

In the Th17 model, spleen and LN cells from dn TGFβRII-negative or dn TGFβRII-positive Marilyn females were stimulated with cognate HYDby peptide and IL-6 with or without TGFβ. Up to 13% of dn TGFβRII-negative Marilyn CD4 T cells were differentiated into IL-17-producing Th17 cells following TCR, IL6 and TGFβ signalling (bottom of Figure 46D). Th17 development was blocked when dnTGFβRII⁺ Marilyn spleen cells were stimulated under the same condition (top of Figure 46D). Th17 differentiation was not restored in dnTGFβRII-expressing Marilyn CD4 T cells when the concentration of TGFβ was increased to 10 ng/ml (Figure 46C).

These results from two independent *in vitro* culture systems demonstrated that a) intact TGFβ signalling in T cells is needed for Th17 differentiation and iTreg development, and b) the expression of endogenous intact TGFβ receptors is insufficient to restore TGFβ signalling by competing with dnTGFβRII which are over-expressed on Marilyn CD4 T cells.

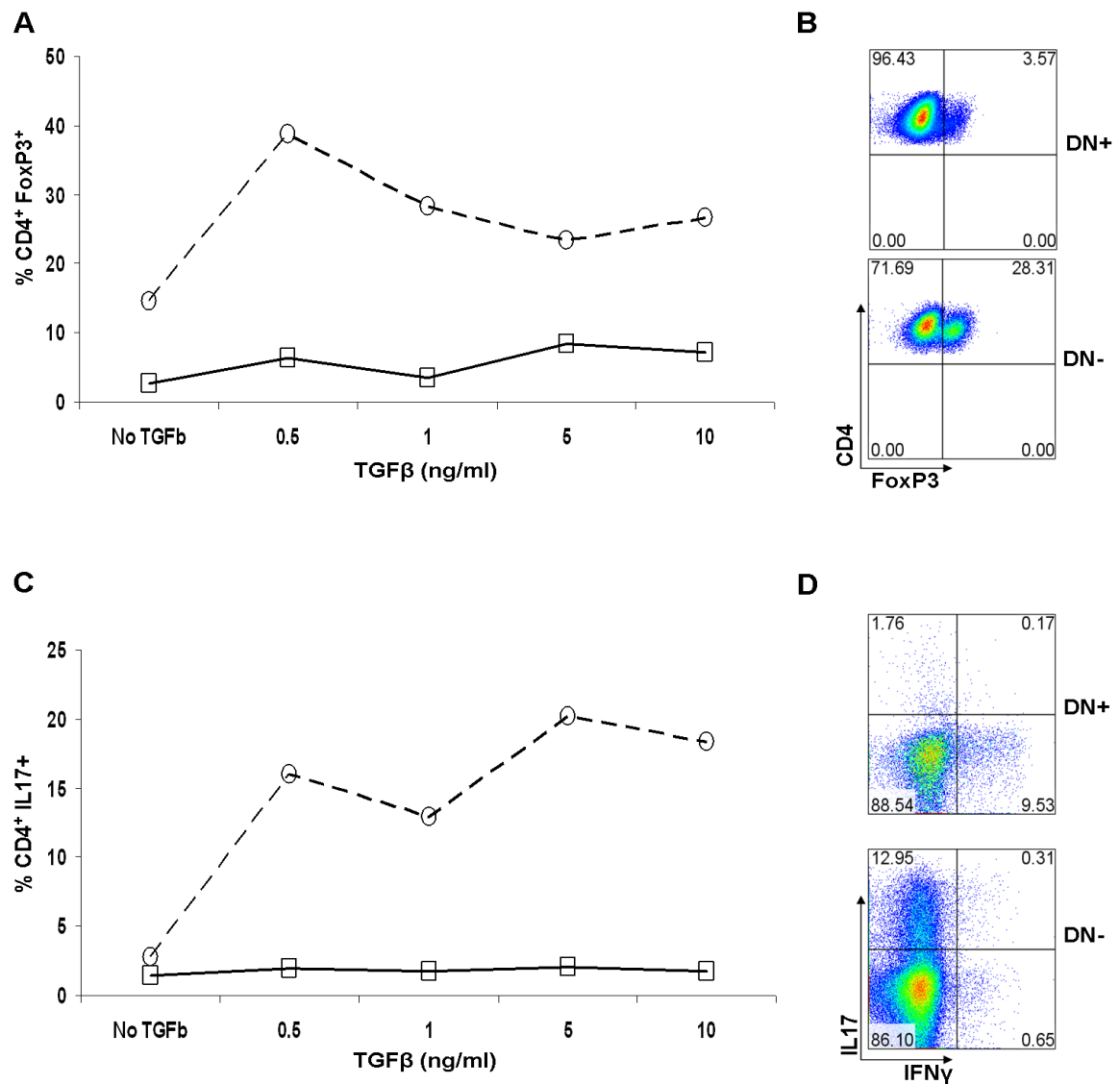


Figure 46. Induction of Th17 and iTreg cells from Marilyn CD4 T cells with or without dnTGFβRII in vitro.

A and B). 2×10^5 of dnTGFβRII-positive (DN⁺, □) or dnTGFβRII-negative (DN⁻, ○) Marilyn spleen and LN cells were cultured with 1nM HYDby peptide plus 20 U/ml of IL-2 in the absence or presence of TGFβ in 96-well plates. After 6 days culture, the cells were harvested and stained with anti-Vβ6^{FITC} and anti-CD4^{PerCP}. Then cells were fixed and stained with anti-FoxP3^{FITC}. Gated CD4⁺Vβ6⁺ T cells were further analysed for the expression of Foxp3.

C and D) 2×10^5 dnTGFβRII-positive (DN⁺, □) or dnTGFβRII-negative (DN⁻, ○) Marilyn spleen and LN cells were cultured with 150 nM of HYDby peptide plus 1ng/ml of IL6 in the absence or presence of TGFβ in 96 well plates. After 6 days the cells were stimulated with PMA (50 ng/ml), Ionomycin (500 ng/ml) and Brefaldin (1 μg/ml) for 4hrs, then harvested and stained with anti-Vβ6^{FITC} and anti-CD4^{PerCP}. After fixation and permeabilization, the cells were stained with anti-IL-17^{PE} and anti-IFNγ^{APC}. Gated CD4⁺Vβ6⁺ cells were further analysed for the intracellular production of IL-17 and IFNγ.

7.9 : Discussion

This chapter establishes an *in vivo* model to study the fate of tumour antigen specific natural Treg (nTreg) cells in response to tumour. There are several unique features to this model. Firstly, the surrogate HY can be expressed either endogenously (MB49) or exogenously (B16/HY). Importantly, this gene transfer does not necessarily result in over expression, indeed the B16/HY line expresses a low level of HY, which is stable both *in vitro* and *in vivo* (Figure 41).

Second, unlike other studies in which the detection of transferred nTreg cells in tumour-bearing mice relies on a vaccination to viral components^{291, 292}, we were able to show the expansion of antigen specific nTreg cells by tumour without vaccination.

Third, unlike other investigations in which the detection of nTreg cells is limited to spleen^{291, 292}, we were able to detect the progeny of antigen-specific nTreg cells in both tumour tissues and tumour draining lymph nodes (tdLN).

The first observation of this study is that the induction of non-specific nTreg-cell expansion is dependent on the tumour cell lines used. This conclusion was based on the results from two sets of adoptive transfer experiments. When B16 and MB49 tumours were compared for their capacities for inducing non-specific nTreg expansion, we found that adoptively transferred polyclonal nTreg cells underwent expansion in dLN of B16-bearing mice (data not shown) but they failed to do so in MB49-bearing mice (Figure 40A), indicating that B16 but not MB49 tumours are able to expand nTreg cells non-specifically.

In the second adoptive transfer experiment, we found that the expansion of HY-specific nTreg cells could be induced by both HY-negative B16 and HY-positive B16 tumours *in vivo* (Figure 42), indicating that B16 is capable of expanding nTreg cells non-specifically. Because MB49 cells constitutively express HY and an HY-negative MB49 cell line is not available, MB49-induced antigen specific nTreg-cell expansion in the presence or absence of HY cannot be assessed. Although we have not used HY-knockout MB49 cells, it is highly unlikely that MB49 is able to induce non-specific nTreg-cell expansion, as MB49 cannot expand polyclonal nTreg cells *in vivo* (Figure 40C). The conclusion that B16 but not MB49 can expand Treg cells non-specifically helps to explain why B16-bearing mice display a significant increase of percentage of nTreg cells in dLN²⁹⁰ whereas MB49-bearing mice do not (data not shown). However, the weaker expansion of

Treg cells in WT female B6 recipients does not represent the most sensitive model system to examine antigen non-specific Treg-cell expansion by tumours. To make a conclusive demonstration, tumour-bearing Rag2^{-/-} recipients of Treg cells should be used in future studies. Whether the capacity of inducing antigen-non-specific nTreg-cell expansion is restricted to spontaneous tumour cell lines remains to be determined. In addition, B16 cells are less immunogenic than MB49 cells. Whether there is a correlation between immunogenicity and capacity of inducing non-specific nTreg-cell expansion also needs to be explored further. Interestingly, there was a significant reduction of Foxp3 expression by a fraction of donor Treg cells in response to HY antigens from MB49 (Figure 40) and B16/HY tumours (Figure 42). Loss of Foxp3 was more marked in tumour-infiltrating donor Treg cells than in dLN. In addition, we show that development of Foxp3-negative donor cells (called ex-Treg cells) represents differentiation from green Treg cells and is not the result of the expansion of contaminating conventional CD4 T cells. The second observation is that although both antigen specific and non-specific expansion contribute to the overall pool of adoptively transferred nTreg cells with defined antigen specificity, nTreg cells expanded through these two pathways display distinct tissue distribution. Although several pairs of antigen-negative and antigen positive tumour cell lines are available, direct comparison of the contributions from antigen-specific and non-specific expansion has not previously been examined. Consequently, there is very limited information regarding the behaviour of nTreg cells expanded by these two pathways. Our current investigation provides a detailed comparison showing that the expansion of nTreg cells with defined antigen specificity in the presence of antigen is more potent than that in the absence of antigen. It is not surprising, as the expansion of Marilyn nTreg cells by B16/HY results from a combination of two pathways. Importantly, we have shown for the first time that nTreg cells undergoing antigen-specific expansion can migrate to tumours where nTreg cells undergoing non-specific expansion cannot (Figure 42). Hence, unlike expansion, migration of nTreg cells into tumours is strictly dependent on the presence of antigen.

The third observation is that TGF β produced by MB49 cells is a key factor promoting expansion of Marilyn nTreg cells (Figure 43). This conclusion is based on the results showing that the presence of intact TGF β signalling on Marilyn Treg cells appears to be essential for their expansion in response to MB49 tumours (Figure 44). A similar strategy has been used by Zitvogel and colleagues to dissect the role of TGF β in the induction of expansion of polyclonal nTreg cells by B16 tumours *in vivo*, but their study was restricted to endogenous polyclonal nTreg cells²⁹⁰. In this respect, our study offers an

example showing that TGF β is required for the expansion of tumour-antigen-specific, monoclonal nTreg cells (Figure 44).

A caveat to this conclusion is the potential for a developmental defect of Treg cells expressing the dnTGF β RII which may prevent appropriate activation in response to antigen. To address this concern, we compared dnTGF β RII-negative and dnTGF β RII-positive Marilyn Treg cells both *in vivo* and *in vitro*. *In vitro*, dnTGF β RII -expressing Marilyn Treg cells are clearly anergic and have weak suppressive activity (Figure 45). Moreover, *in vivo*, dnTGF β RII RII-positive Marilyn Treg cells can inhibit anti-HY T-cell responses, although they are less potent compared with Marilyn Treg cells (Figure 44A).

Finally, dnTGF β RII-positive Treg cells expand less efficiently than dnTGF β RII-negative Treg cells in response to HY antigens from MB49 tumours regardless of the presence or absence of co-transferred conventional Marilyn CD4 T cells (Figure 44D).

Additional *in vivo* TGF β neutralization experiments are needed to confirm the requirement for TGF β signalling for *in vivo* expansion of nTreg cells. Although the MB49 cell line can make TGF β (Figure 43), we have not examined whether growing MB49 tumours would produce TGF β . In this regard, it has been reported that B16 tumours induce a subset of DC in dLN to produce active TGF β , which then expands polyclonal nTreg cells²⁹⁰. In addition, tumour-specific Treg cells are also able to induce tumour resident immature DC to produce TGF β ²⁹⁵. Apart from TGF β , indoleamine 2,3-dioxygenase is another molecule that can directly contribute to Treg-cell expansion²⁹⁶.

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Summary

This work has established the MB49 tumour as a relevant murine model for the study of anti-tumour immune responses. MB49 cells create an immunogenic tumour that is rejected at low doses in female C57BL/6 mice because it expresses the male minor antigen HY.

Cells from MB49 tumours express the inhibitory ligands PD-L1 and PD-L2, and down regulate MHC Class I. Both of these features are hallmarks of cancer and its ability to evade the immune system¹⁰⁸. Further study to examine how these traits affect the immunogenicity of MB49 tumours could prove valuable.

By using both IL-10KO mice and anti-IL-10R mAb we have demonstrated that the growth of the MB49 tumour is inhibited by IL-10 *in vivo*. This confirms previous reports which studied B16 melanoma and murine mammary adenocarcinomas genetically engineered to over express IL-10²²⁸⁻²³⁰, but in a more physiologically relevant system. Future studies need to address the apparent contradictory effect of IL-10 in the context of cancer.

Anti-GITR therapy is extremely effective at causing tumour rejection; it was the only therapy in this study that consistently caused complete tumour rejection. This confirms numerous previous murine studies^{147, 149, 200, 201} demonstrating that anti-GITR therapy causes tumour rejection in several different tumours. It is not surprising that Tolerx Inc. are currently conducting Phase I trials with TRX518, humanised anti-human GITR²⁹⁸, for malignant melanomas. However, the timing of anti-GITR therapy is crucial, and it remains to be seen whether it will be effective in patients with late stage melanomas.

Tumour antigen specific Marilyn CD4 cells become activated, proliferate vigorously and infiltrate MB49 tumours, but despite this they only slow tumour progress and do not cause tumour rejection. It is reported that they cause tumour rejection in Rag^{-/-} mice¹⁷⁸ which suggests that in immuno-competent mice suppression by T regs or competition from other T cells inhibits the CD4 anti-tumour response. Pre-conditioning the mice by irradiation greatly enhanced the anti-tumour efficacy of adoptively transferred Marilyn cells, presumably because of reduction in Treg suppression and T cell competition.

Adoptive transfer of tumour antigen specific MataHari CD8 cells was only effective when mice were preconditioned by irradiation and donor cells secreted

exogenous IL-15. These results suggest that myeloablative pre-conditioning is essential before the adoptive transfer of antigen specific anti-tumour T cells.

The induction of non-specific nTreg-cell expansion is also shown as not representing a common rule for all tumour lines. By making a side-by-side comparison of antigen-specific and non-specific nTreg cell expansion, we provide an example where the relative contribution by two pathways can be evaluated. Moreover, for the first time, distinct nTreg-cell migration patterns are uncovered, depending on whether expansion is antigen dependent or independent. These mechanisms that are responsible for nTreg-cell expansion by tumours have implications for understanding and manipulating tumour immune evasion.

Lessons like these from murine models are directly relevant to adoptive immunotherapy for human cancer and the rapid pace of research in the coming years will drive improvements in the survival rates for cancer patients.

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Appendix A

Depletion of regulatory T cells by anti-GITR mAb as a novel mechanism for cancer immunotherapy

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Abstract In vitro, engagement of GITR on Treg cells by the agonistic anti-GITR mAb, DTA-1, appears to abrogate their suppressive function. The consequence of in vivo engagement of GITR by DTA-1 is, however, less clear. In this study, we show that Treg cells isolated from DTA-1-treated mice were as potent as those from untreated mice in suppressing conventional CD4 T cells in vitro, indicating that in vivo GITR ligation does not disable Treg cells. Treatment of Foxp3/GFP knock-in mice with DTA-1 led to a selective reduction of circulating Treg cells, suggesting that DTA-1 is a depleting mAb which preferentially targets Treg cells. In tumour-bearing mice, DTA-1-mediated depletion of Treg cells was most marked in tumours but not in tumour-draining lymph node. These features were confirmed in an adoptive transfer model using tumour antigen-specific Treg cells. Interestingly, Treg cells detected in tumour tissues expressed much higher levels of GITR than those in tumour-draining lymph nodes, indicating that the efficiency of depletion might be correlated with the level of GITR expression. Finally, in vivo labelling of GITR in naive or tumour-bearing mice demonstrated that Treg cells constitutively expressed higher levels of GITR than conventional T cells, independent of location and activation state, consistent with the preferential in vivo depletion of Tregs by DTA-1. Thus, depletion of

Treg cells represents a previously unrecognised in vivo activity of DTA-1 which has important implications for the application of anti-GITR antibodies in cancer immunotherapy.

Keywords Anti-GITR · Regulatory T cells · Depletion · HY · Tumour · Therapy

Abbreviations

dLN	Tumour-draining lymph node
pLN	Peripheral LN
nTreg	Natural CD4 ⁺ CD25 ⁺ Foxp3 ⁺ regulatory T cells
Mar	Marilyn mice
PBL	Peripheral blood lymphocytes
MFI	Mean florescence intensity
Foxp3/GFP	Foxp3/GFP knock-in mice

Introduction

Glucocorticoid-induced tumour necrosis factor receptor (GITR), a member of the tumour necrosis factor receptor (TNFR) family is expressed on T lymphocytes with the primary function of protection from activation-induced cell death [1–3]. Under steady state conditions, CD4⁺CD25⁺Foxp3⁺ regulatory (Treg) cells constitutively express higher levels of GITR than conventional CD4 and CD8 T cells [4, 5]. The outcome of GITR signalling on Treg cells appears to be functional inactivation (i.e. neutralisation of inhibitory function) [4–6], whereas ligation of GITR on conventional T cells provides a co-stimulatory signal promoting activation [7–11].

Since ligation of GITR by DTA-1, an agonistic anti-GITR mAb, disables Treg whilst co-stimulating conventional T cells [12, 13], DTA-1 is an attractive reagent for enhancing T cell immunity against tumour cells [14–18]. Although the effectiveness of DTA-1 in cancer immunotherapy is well

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established, the mechanisms of action of DTA-1 are complicated by following factors. Firstly, the assumption that DTA-1 disarms Treg cells is based largely, if not exclusively, on in vitro data [4, 5], however, this conclusion has been challenged by a subsequent investigation [11]. Using co-cultures of Treg and conventional CD4 T cells from WT and GITR-deficient mice, Shevach and colleagues showed that GITR engagement on CD25^{-ve} but not CD25^{+ve} T cells is required for neutralising suppression, and the action of DTA-1 is to confer on CD25⁻ T cells resistance to CD25^{+ve} Treg-mediated inhibition, rather than by direct inactivation of Treg cells [11, 12]. Secondly, T cell behaviour in vivo cannot always be predicated on in vitro analysis, and few studies have addressed the impact of DTA-1 on Treg cells in vivo [19]. Inactivation [4, 15, 19], expansion [16] and depletion of Treg cells [20] are three possible outcomes of GITR ligation by DTA-1 on Treg cells. Although it has been proposed [12] and indicated [20] that DTA-1 depletes Tregs in vivo, a formal demonstration has not been made. Thirdly, expression of GITR on Treg has been largely, if not exclusively, investigated in vitro by comparing the pattern of expression before and after activation [4, 5, 11, 21]. In vivo, there is competition between target cells to bind injected antibody effectively lowering the number of molecules bound, in comparison with in vitro studies where an excess of conjugated antibody is used. Evaluation of GITR expression in real time is vital to understand the in vivo mechanisms of DTA-1 action.

To investigate in vivo GITR expression and determine whether DTA-1 selectively targets Treg cells for depletion, several strategies were adopted including the use of Foxp3/GFP knock-in mice [22] and adoptive transfer of tumour-antigen specific Treg cells [23]. Furthermore, a staining protocol enabling in vivo evaluation of GITR expression was developed.

Materials and methods

Mice

Thy1.2 C57BL/6 (B6) and CBA mice were purchased from Harlan Olac (Bicester, UK), and bred in the Central Biological Services Unit at Hammersmith Campus, Imperial College London. TCR-transgenic Marilyn [23] and Foxp3/GFP knock-in [22] mice were kindly provided by Dr. O Lantz and Dr. B Malissen, respectively. Marilyn mice used in this study were Thy1.1⁺ and Rag2^{+/-}.

Tumour cell line, inoculation and measurement of tumour growth

MB49 is a chemically induced murine bladder carcinoma derived from a B6 male mouse [24]. MB49 cells were

maintained in RPMI 1640 Medium (Gibco, UK) supplemented with 10% FCS, 10 mM HEPES, penicillin (100 IU/ml) and streptomycin (100 µg/ml), 5×10^{-5} M 2-ME and 2 mM L-glutamine. 5×10^5 MB49 cells were injected subcutaneously on the right flank of B6 and Foxp3/GFP knock-in mice. Tumour growth was measured every other day and recorded as longest dimension $\times$ shortest dimension in millimetres squared (mm²).

mAbs and treatment

DTA-1 is rat IgG2b mAb specific for mouse GITR [4]. GL-113 is rat IgG1 recognising β -galactosidase [25], and PC61 is rat IgG1 against mouse CD25 [26]. mAbs were partially purified from hybridoma culture supernatants by precipitation in 50% ammonium sulphate, and were further purified using protein G column. mAb treatment was conducted by intraperitoneal (i.p.) injection.

Purification of Treg and conventional CD4 T cells

To purify HY-specific Treg cells, spleen and pooled LN cells from Rag2^{+/-} Marilyn females (Thy1.1) were incubated with anti-B220- and anti-CD8-Dynalbeads (Invitrogen, UK). After depletion of B and CD8 cells, the remaining cells were stained with anti-V β 6^{FTTC}, anti-CD25^{PE} and anti-CD4^{PE/Cy5} before sorting for CD4⁺CD25⁺V β 6⁺ cells (HY-specific Treg cells). To isolate polyclonal Treg and conventional CD4 T cells from B6 mice, similar procedure was used except that anti-V β 6^{FTTC} was omitted. FACS sorted CD4⁺CD25⁺ and CD4⁺CD25⁻ cells were used as polyclonal Treg and conventional CD4 cells, respectively.

In vitro suppression assay

This was conducted as described previously [27]. In brief, conventional CD4 T cells, alone or mixed with Treg cells at various ratios, were stimulated with anti-CD3 mAb in the presence of accessory cells (irradiated T cell-depleted B6 spleen cells) in round-bottomed 96-well plates for 3 days. Thymidine was added to each well for the last 12 h and proliferation was measured by thymidine uptake.

Adoptive T cell transfer

FACS-sorted Marilyn Treg cells (Thy1.1⁺) were adoptively transferred to Thy1.2⁺ B6 female recipients by intravenous (i.v.) injection (1×10^5 /mouse).

FACS analysis

To detect donor cells (Thy1.1 Marilyn Treg) in recipient mice (Thy1.2 B6), the cells from MB49 and its draining

Fig. 1 DTA-1 is highly effective in treating established tumours. **a, b** A single injection of DTA-1 results in the regression of established MB49 tumours. A total of 11 B6 female mice were s.c. inoculated with 5×10^5 MB49 cells. 8 days later, the mice were randomly divided into two groups: one group (B, $n = 6$) was treated with 50 μg of DTA-1 mAb by i.p. injection and the other group was not (A, $n = 5$). The growth of MB49 tumours was monitored by measuring tumour size every other day. One representative of three independent experiments is shown. **c–f** Regression of MB49 is correlated with increased accumulation of tumour-infiltrating CD4 and CD8 T cells but decreased accumulation of Treg cells. Untreated and DTA-1-treated mice (3 mice/group) were prepared as described in A and B. At day 15, MB49 tumours isolated from untreated (C and E) and DTA-1-treated mice (D and F) were stained with anti-CD4^{PerCP} and anti-CD8^{APC} (C and D), or anti-CD4^{PerCP} followed by intracellular staining with anti-Foxp3^{FITC} (E and F). Percentage of CD4⁺, CD8⁺, or CD4⁺Foxp3⁺ cells in MB49 from individual mice in each group is shown as mean $\pm$ SEM. One representative of two independent experiments is shown

lymph node were stained with anti-V β 6^{PE}, anti-Thy1.1^{PerCP} and anti-CD4^{APC}. Donor cells were identified by co-expression of Thy1.1 and V β 6. To measure the percentage of T cell subsets in MB49, the cells from MB49 tissues were stained with anti-CD4^{PerCP} and anti-CD8^{APC}, or anti-CD4^{APC} before intracellular anti-Foxp3^{FITC} staining. Treg cells in Foxp3/GFP mice were identified by co-expression of CD4 and GFP.

In vivo GITR staining

This was conducted by i.v. injection of Foxp3/GFP mice with 5 μg of PE-conjugated DTA-1. GITR expression in T cell subsets was analysed by FACS after CD4 and CD8 staining, but without a further staining of GITR.

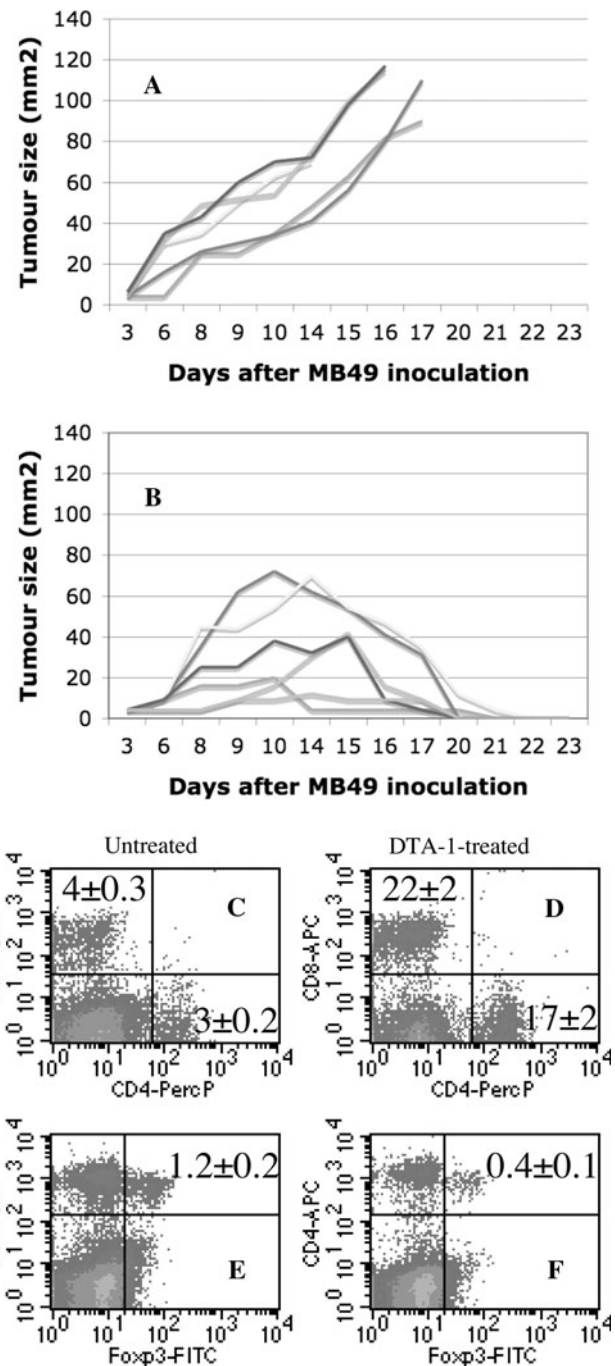
Statistical analysis

This was performed using a two-sided Student's *t* test and GraphPad Prism software (version 3.02).

Results

Rejection of established MB49 tumours by a single dose of DTA-1 treatment

Although administration of DTA-1 alone is sufficient to reject some types of experimental tumours (Meth A and Colon 26 [15]), such a treatment appears to be less effective for other tumours (B16 and RENCA [15, 16]) unless an additional manipulation such as DNA vaccination [17] is included, suggesting that efficiency of DTA-1 therapy is dependent on the characteristics of transplantable tumour. Thus, the starting point of this investigation was to assess the susceptibility of MB49 tumours (murine bladder carcinoma naturally expressing HY) [24] to DTA-1 therapy.



Like other tumour cell lines such as B16 and RENCA [16], MB49 does not express GITR (not shown). On day 0, a total of 11 B6 females were subcutaneously inoculated with 5×10^5 MB49 cells, at day 8, 6 randomly chosen mice were given 50 μg of DTA-1 by i.p. injection and the remaining 5 mice were not. Growth of MB49 tumours in the untreated group was very aggressive reaching a volume $>100 \text{ mm}^2$ by day 17 (Fig. 1a). In contrast, DTA-1 was very effective in treating established MB49 tumours with 100% rejection rate (Fig. 1b). A control rat IgG (GL-113)

[25] showed no effect on MB49 growth (not shown). When the proportion of infiltrating T cell populations ($CD4^+$, $CD8^+$ and $Foxp3^+CD4^+$) within the MB49 tumours of untreated (Fig. 1c, e) and DTA-1 treated groups (Fig. 1d, f) was compared, we found that DTA-1 treatment was associated with an increased accumulation of CD4 and CD8 T cells but a reduction of Treg cells. Therefore, MB49 can be added to a list of tumours which can be rejected by DTA-1. Given, MB49 tumours are very aggressive and resistant to HY vaccination [28], complete rejection of established tumours following a single dose of DTA-1 administration is very encouraging.

Treg cells purified from DTA-1-treated mice are competent suppressive cells

In vitro, DTA-1-mediated GITR signalling on Treg cells abrogates their suppressive activities [4, 5]. As T cell behaviour in vivo cannot be predicated on the in vitro behaviour of T cell co-cultures, we evaluated the inhibitory capacities of Treg cells isolated ex vivo from either untreated or DTA-1-treated mice 1 day after mAb administration. In a standard in vitro suppression assay, Treg cells from DTA-1-treated mice were as potent as those from untreated mice in inhibiting conventional CD4 T cells (from untreated mice) in response to anti-CD3 stimulation (Fig. 2). Likewise, Treg cells from GL-113-treated mice showed similar suppressive activity to those from untreated

or DTA-1-treated mice (not shown). Thus, in vivo GITR signalling on Treg cells does not lead to their functional inactivation. This observation is consistent with another report [16].

DTA-1 treatment in vivo leads to a partial depletion of Treg cells

Although early studies indicated that DTA-1 (rat IgG2b) is a non-depleting mAb for Treg cells [4, 15, 19], we repeatedly observed a significant reduction (up to 40%) of circulating $CD4^+CD25^+$ cells in B6 mice following a single injection of DTA-1, raising the possibility that DTA-1 is a depleting mAb in vivo. To provide a conclusive demonstration, we used $Foxp3/GFP$ knock-in mice in which Treg and non-Treg cells can be reliably distinguished by GFP expression [22]. 3 days after DTA-1 treatment, the proportion of $CD4^+GFP^+$ cells in PBL decreased from 0.89% (untreated) to 0.63% (representing a 30% reduction, $p < 0.05$), whereas that of the $CD4^+GFP^-$ population of cells remained unchanged (from 15.3 to 15.1%, left and middle panels of Fig. 3a). However, in the depletion of Treg cells, DTA-1 was less efficient than PC61, a widely used anti-CD25 depleting mAb [26] which resulted in a 52% reduction of circulating Treg cells (right panel of Fig. 3a). The proportion of Treg and non-Treg in the PBL of $Foxp3/GFP$ mice did not change following GL-113 treatment (not shown).

Next, we examined whether DTA-1 treatment would deplete polyclonal Treg cells in tumour-bearing mice. Two groups of $Foxp3/GFP$ knock-in mice were injected s.c. with MB49 cells at day -8, one group was given DTA-1 at day 0 and the other was not. 3 days after DTA-1 administration, each MB49 tumour and its draining LN (dLN) were analysed for the presence of $CD4^+GFP^+$ cells. Following i.p. injection of DTA-1, there was a 44% (reduced from 0.9 to 0.5%, $p < 0.05$) and 28% (from 3.12 to 2.24%) decrease of Treg cells in MB49 tumours and dLN, respectively (Fig. 3b). Treatment of MB49-bearing mice with GL-113 failed to alter the pattern of Treg accumulation in MB49 or dLN (not shown). Data shown in Fig. 3b indicate that DTA-1 depletes tumour-resident Treg cells. However, DTA-1 treatment also increases the proportion of tumour-infiltrating effector T cells (Fig. 1c, d), the decrease of Treg cells might reflect an increase in another cell population. We therefore compared the absolute number of tumour-infiltrated Treg cells from untreated and DTA-1-treated mice. As shown in Fig. 3c, there was a significant reduction of tumour-resident Treg cells following DTA-1 treatment, strongly suggesting that DTA-1 can preferentially deplete tumour-infiltrating Treg cells.

To investigate whether the results obtained from the measurement of polyclonal Tregs in tumour-bearing mice

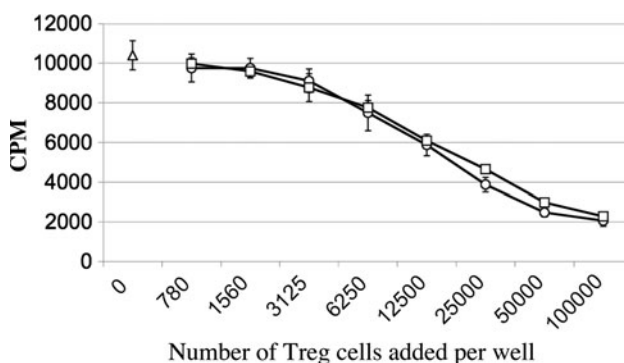
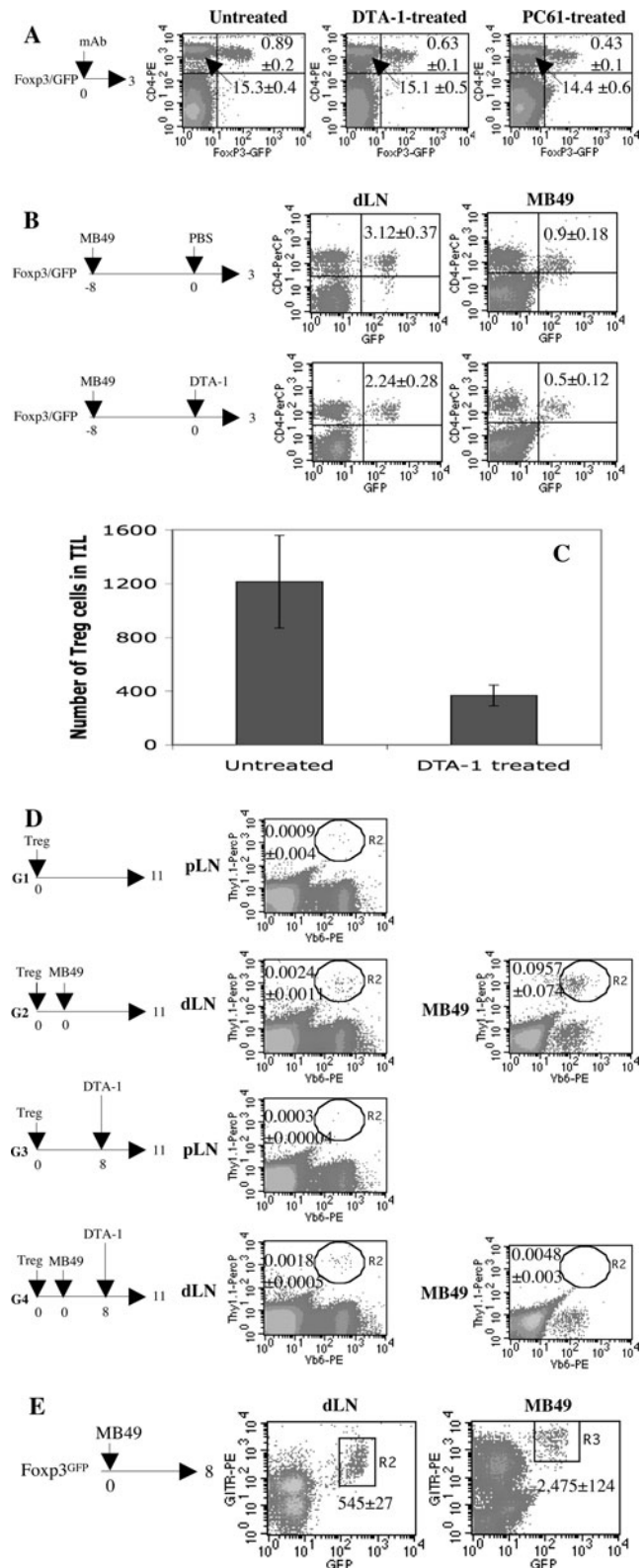


Fig. 2 Treg cells isolated from DTA-1 treated mice are competent suppressive cells in vitro. Two groups of B6 mice (3 mice/group) were untreated or treated with anti-GITR by i.p. injection of 50 μ g of DTA-1. 24 h later, Treg ($CD4^+CD25^+$) and conventional CD4 T cells ($CD4^+CD25^-$) were purified from pooled LN and spleen cells of each group by FACS sorting. Conventional CD4 T cells (1×10^5 /well) from the untreated group were cultured with anti-CD3 (0.05 μ g/ml) and accessory cells (T cell-depleted and irradiated B6 spleen cells, 2×10^5 /well) in the absence (open triangles) or presence of various numbers (two time serial diluted from 1×10^5 /well to 780 /well) of nTregs from either untreated (open squares) or DTA-1-treated group mice (open circles) in 96-round bottomed plates for 3 days. Thymidine (0.5 μ Ci/well) was added to each well for the last 12 h. One representative of two independent experiments is shown

Fig. 3 DTA-1 is a depleting mAb in vivo. **a** A single injection of DTA-1 mAb leads to a reduction of the percentage of circulating CD4⁺Foxp3⁺ cells. Three groups of Foxp3/GFP knock-in mice (4 mice/group) were untreated or treated with 25 µg of DTA-1 or PC61 by i.p. injection. 3 days later, peripheral blood lymphocyte (PBL) samples were collected from individual mice in each group and stained with anti-CD4^{PE}. Percentage of CD4⁺GFP⁺ and CD4⁺GFP⁺ cells in PBL is shown as mean ± SEM. One representative of three independent experiments is shown. **b** DTA-1 preferentially depletes tumour-resident Treg cells. Two groups of Foxp3/GFP knock-in mice (4 mice/group) were s.c. inoculated with 5×10^5 MB49 cells on day -8. The mice were untreated or treated with 50 µg of DTA-1 by i.p. injection on day 0. 3 days later, dLN and MB49 tumours from individual mice in each group were stained with anti-CD4^{PerCP}. Percentage of GFP⁺CD4⁺ cells is shown as mean ± SEM. One representative of two independent experiments is shown. **c** The number of tumour-resident Treg cells was profoundly reduced following DTA-1 treatment. Two groups of Foxp3/GFP knock-in mice (4 mice/group) were s.c. inoculated with 5×10^5 MB49 cells on day -8. The mice were untreated or treated with 50 µg of DTA-1 by i.p. injection on day 0. 3 days later, MB49 tumours from individual mice in each group were stained with anti-CD4^{PerCP}. The absolute tumour-infiltrated Treg cell numbers were determined by total tumour cells × % of small lymphocyte gate × % of CD4⁺GFP⁺ cells within gated tumour-infiltrated lymphocyte (TIL). **d** Accumulation of adoptively transferred HY-specific Treg cells in MB49 tumours is profoundly impaired following DTA-1 treatment. HY-specific Treg cells (Thy1.1⁺) were adoptively transferred to four groups of Thy1.2⁺ B6 females (4 mice/group) by i.v. injection (1×10^5 /mouse). On the same day, the mice in G2 and G4 were inoculated s.c. with MB49 cells (5×10^5 /mouse) and the mice in G1 and G3 were not. On day 8, the mice in G3 and G4 were treated with 50 µg of DTA-1 by i.p. injection, and the mice in G1 and G2 were not. On day 11, dLN (pLN for G1 and G3) and MB49 tumours from individual mice in each group were stained with anti-Vβ6^{PE}, anti-Thy1.1^{PerCP} and anti-CD4^{APC} followed by intracellular staining of anti-Foxp3^{FITC}. Percentage of Treg cells of donor origin (Thy1.1⁺Vβ6⁺) is shown as mean ± SEM. One representative of two independent experiments is shown. **e** Tumour-infiltrating Treg cells express higher levels of GITR than those in dLN. Foxp3/GFP knock-in mice ($n = 4$) were s.c. injected with 5×10^5 MB49 cells on day 0. 8 days later, MB49 and dLN cells from individual mice were stained with anti-GITR^{PE} and anti-CD4^{PerCP}. The MFI of GITR by gated GFP⁺GITR⁺ cells in dLN and MB49 is shown as mean ± SEM. One representative of four independent experiments is shown

would be applicable to monoclonal Treg cells, we adoptively transferred HY-specific Treg cells (Thy1.1⁺CD4⁺Vβ6⁺CD25⁺ cells sorted from Rag2^{-/-} Marilyn females) to four groups of Thy1.2 B6 mice which were injected with MB49 cells (G2 and G4) or not (G1 and G3) on the same day of Treg cell transfer, and given DTA-1 (G3 and G4) or not (G1 and G2) on day 8 (Fig. 3d). The impact of DTA-1 treatment on monoclonal Treg cells was determined by comparing the proportion of donor cells in MB49 and dLN between G2 and G4. DTA-1 treatment led to 95% reduction in the accumulation of Marilyn Treg cells in the MB49 tumour (decreased from 0.0957% in G2 to 0.0048% in G4, $p < 0.001$) (Fig. 3d), whereas depletion in tumour dLN was less marked (0.0024 vs. 0.0018%). However, GL-113 treatment did not influence Marilyn Treg cells in response to MB49 tumours (not shown).



In the same experiment, we also measured the changes in proportions of host Treg cells (Thy1.1⁺CD4⁺Foxp3⁺) in tumour tissues with or without DTA-1 treatment (S-Fig. 1).

Comparing the proportion of host Treg cells within the MB49 tumours between G2 and G4 revealed that a single injection of DTA-1 resulted in 38% reduction of recipient tumour-resident Treg cells (0.993–0.575%, $p < 0.05$). Thus, the results shown in Fig. 3b using Foxp3/GFP knock-in mice were reproducible using WT B6 mice.

Figure 3b–d shows that depletion of polyclonal or monoclonal Treg cells by DTA-1 is more extensive in tumours than in the dLN. We speculated that the environment of a growing MB49 tumour might preferentially increase GITR expression on tumour-infiltrating Treg cells, rendering them more susceptible to depletion. To test this, Foxp3/GFP mice were inoculated with MB49 cells, and 8 days later the expression of GITR by Treg cells in the MB49 tumour was compared with those in dLN. As shown in Fig. 3e, there was a 4.5-fold increase in GITR expression on MB49-infiltrating Treg cells (MFI 545 ± 27 tumour draining LN, MFI 2475 ± 124 tumour; $p < 0.05$), which is likely to increase sensitivity to DTA-1-induced depletion.

Treg cells are primary cellular targets of DTA-1 in vivo

In vitro staining of PBL from Foxp3/GFP knock-in mice with anti-GITR^{PE}, anti-CD4^{PerCP} and anti-CD8^{APC} clearly demonstrated that Treg cells expressed higher levels of GITR than conventional CD4 and CD8 cells (top of Fig. 4a).

Whether GITR expression in vivo is similar to that seen in vitro is unclear. We therefore performed a pilot experiment to test the feasibility of “in vivo” staining. CBA mice were injected i.v. with 20 µg of anti-CD4^{PE}, and PBL samples collected at various time points were stained with anti-CD3^{FITC} in vitro, before analysing for CD4⁺CD3⁺ cells. Cells co-expressing CD4 and CD3 were detectable as early as 18 min after injection, and were still present after 22 h, by which time CD4 molecules were down-regulated, presumably due to antibody-mediated internalisation (S-Fig. 2B).

Having verified this in vivo staining protocol, we applied it to determine the cellular targets of DTA-1. Foxp3/GFP mice were injected i.v. with 5 µg of DTA-1^{PE}, and 15 h later the PBL samples were stained with anti-CD4^{PerCP} and anti-CD8^{APC} to allow identification of Foxp3/GFP positive from Foxp3/GFP negative cells in each population. MFI of GITR by Treg cells was threefold higher than that of conventional CD4 and CD8 T cells (bottom panel of Fig. 5a), indicating that in vivo, DTA-1 antibodies primarily bind to Treg cells which have abundant surface GITR molecules compared with non-Treg cells (top panels of Fig. 4a).

Although Treg cells rather than conventional T cells are the primary targets of DTA-1 under normal conditions, it is not known whether this would be found in tumour-bearing mice. To address this question, we injected 5 µg of DTA-1^{PE}

into Foxp3/GFP mice inoculated with MB49 cells 8 days previously. 6 h later, we analysed GITR expression by CD4⁺GFP⁺, CD4⁺GFP[−] or CD8⁺GFP[−] subsets in spleen, dLN and MB49 tumours (Fig. 4b). Several observations can be made. Firstly, Treg cells within MB49 tumours displayed a 2.1-fold higher GITR expression than those in dLN, thus confirming the in vitro staining results shown in Fig. 3d. Secondly, like Treg cells, conventional CD4 and CD8 T cells present in the MB49 tumour also showed higher MFI of GITR than those in dLN. This observation indicates that either the tumour microenvironment can induce and maintain GITR expression on infiltrating T cell subsets or those T cells with higher GITR expression are able to preferentially migrate into the tumour. Finally, GITR expression by Treg cells was consistently higher than that by conventional CD4 or CD8 T cells, regardless of tissue of origin, suggesting that Treg cells constitutively express higher levels of GITR in vivo.

As shown in Fig. 4b, splenic Treg cells appear to express higher GITR than tumour-resident Treg cells (223 vs. 184 of MFI), raising the possibility that splenic Treg might be the primary target of DTA-1. To explore this, we compared changes of Treg cells in spleen and tumour after DTA-1 treatment. A moderate decrease of splenic Treg cell number in MB49-bearing mice was observed following DTA-1 therapy (Fig. 4c), however, the reduction of tumour-resident Treg cells was more significant in the same experiment (Fig. 3c). This disparity may be related to the experimental conditions including antibody dose (5 vs. 50 µg) and duration of treatment (6 vs. 72 h). In addition, we speculate that i.v. delivered DTA-1^{PE} mAb might reach the spleen before entering tumour tissue. Moreover, some mAb will be consumed within the spleen by binding to GITR-expressing cells, thus reducing the amount of mAb available for entry into other tissues. Thus, the local concentration of mAb in the spleen is likely to be higher than that in tumour, as a result, Treg cells in the spleen show higher GITR expression than those in the tumours.

DTA-1 but not PC61 therapy can control established MB49 tumours

If DTA-1 can deplete Treg cells in vivo, it would be of interest to compare DTA-1 with PC61, a standard anti-CD25 depleting mAb. A single injection of PC61 was very effective in treating MB49 tumours when administered to B6 females 2 days before MB49 inoculation [Fig. 5a, 100% (5/5) tumour-free mice]. However, the same treatment protocol using DTA-1 had limited impact on tumour growth [Fig. 5B, 20% (1/5) tumour-free mice]. This difference could be explained by our observations showing PC61 was more potent than DTA-1 in depleting Treg in vivo (Fig. 3a). On the other hand, DTA-1 treatment was very

Fig. 4 Treg cells constitutively express higher levels of GITR than conventional CD4 T cells in vivo. **a** Treg cells are primary targets of DTA-1. *Top panels*, the PBL from a group of Foxp3/GFP knock-in mice ($n = 3$) were stained with anti-GITR^{PE}, anti-CD4^{PerCP} and anti-CD8^{APC}. The expression of GITR by GFP⁺CD4⁺, GFP⁺CD4⁺ and GFP⁺CD8⁺ T cells from individual mice was presented as histograms. MFI of GITR is shown as mean $\pm$ SEM. *Bottom panels*, a group of Foxp3/GFP knock-in mice were given 5 μ g of anti-GITR^{PE} (clone DTA-1) by i.v. injection. 15 h later, the PBL from individual mice were stained with anti-CD4^{PerCP} and anti-CD8^{APC}. MFI of GITR by GFP⁺CD4⁺, GFP⁺CD4⁺ and GFP⁺CD8⁺ T cells is presented as mean $\pm$ SEM. One representative of three independent experiments is shown.

b Tumour-infiltrating Treg, CD4 and CD8 T cells express higher levels of GITR than those in dLN. A group of Foxp3/GFP knock-in mice ($n = 4$) were s.c. inoculated with MB49 cells (5×10^5 /mouse) 8 days previously. On day 0, the mice were i.v. injected with 5 μ g of DTA-1^{PE}. 6 h later, spleen, dLN and MB49 tumour cells from individual mice were stained with anti-CD4^{PerCP} and anti-CD8^{APC}. MFI of GITR by GFP⁺CD4⁺, GFP⁺CD4⁺ and GFP⁺CD8⁺ T cells is presented as mean $\pm$ SEM. One representative of two independent experiments is shown.

c The number of splenic Treg cells was moderately reduced following DTA-1 treatment. Two groups of Foxp3/GFP knock-in mice (4 mice/group) were s.c. inoculated with 5×10^5 MB49 cells on day -8. The mice were untreated or treated with 50 μ g of DTA-1 by i.p. injection on day 0. 3 days later, MB49 tumours from individual mice in each group were stained with anti-CD4^{PerCP}. The absolute spleen-resident Treg cell numbers were determined by total spleen cells $\times$ % of lymphocyte gate $\times$ % of CD4⁺GFP⁺ cells within gated lymphocytes

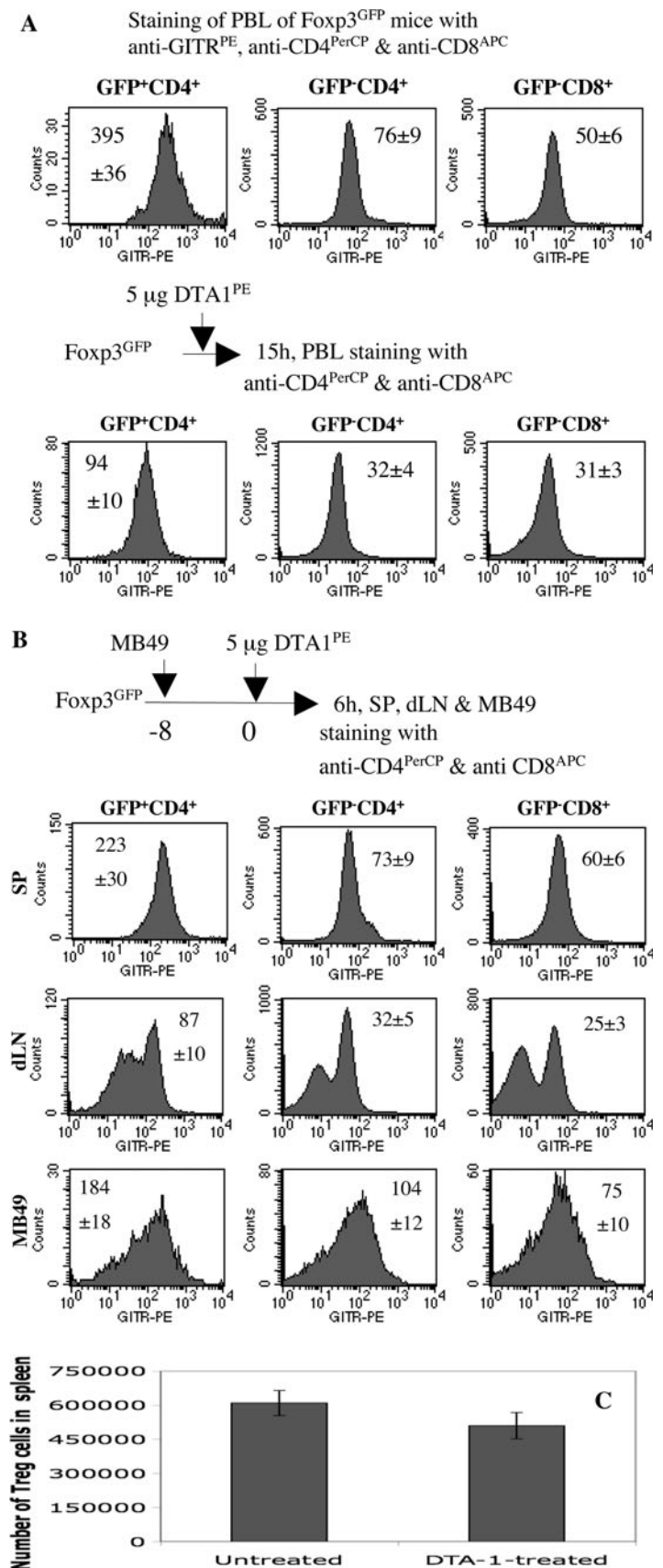


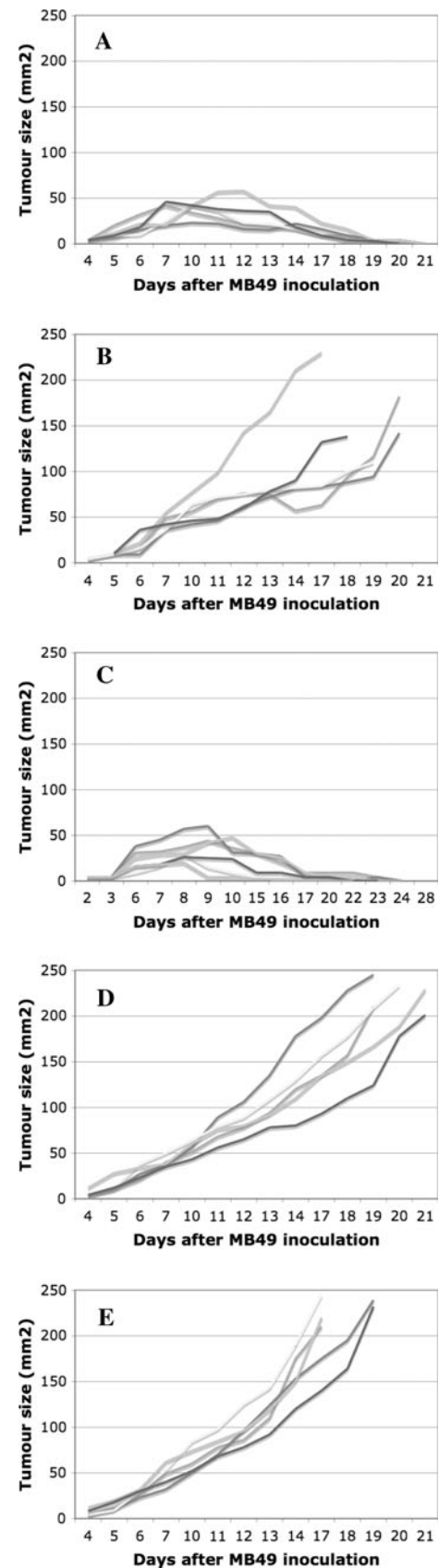
Fig. 5 Comparison of DTA-1 and PC61 for their anti-MB49 activities **in vivo**. **a, b.** Two groups of B6 female mice (5 mice/group) were treated with 50 μ g of PC61 (A) or DTA-1 mAb (B) by i.p. injection. 2 days later, all mice were s.c. inoculated with 5×10^5 MB49 cells. The growth of MB49 tumours was monitored by measuring tumour size every other day. **c–e** A total of 16 B6 female mice were s.c. inoculated with 5×10^5 MB49 cells. 8 days later, the mice were randomly divided into three groups: group one (C, $n = 6$) was treated with 50 μ g of DTA-1 mAb by i.p. injection, group two (D, $n = 5$) was treated with 50 μ g of PC61 mAb by i.p. injection, and group three (E, $n = 5$) was treated with 50 μ g of DTA-1 and 50 μ g of PC61 by i.p. injection. The growth of MB49 tumours was monitored by measuring tumour size every other day

efficient at rejecting established MB49 tumours [Figs. 1b, 5c, 100% (6/6) tumour-free mice] whereas PC61 therapy had no effect (Fig. 5d, 0% tumour-free mice). We speculate that CD25-expressing anti-MB49 effector T cells were depleted by PC61 treatment. In support of this, we were able to show that anti-tumour activity by DTA-1 was completely lost if PC61 was co-injected with DTA-1 on day 8 (Fig. 5e, 0% tumour-free mice). Since DTA-1 leads to partial Treg depletion and timing of DTA-1 administration is the key factor determining the outcome of cancer therapy, we conclude that Treg depletion is one mechanism but cannot be regarded as the only one responsible for cancer therapy by DTA-1, other mechanisms such as co-stimulation of T cells might be in operation.

Discussion

CD4⁺CD25⁺ cells isolated from mice given DTA-1 24 h previously were found to be functional Treg cells with similar suppressive activity as those from untreated mice (Fig. 2), suggesting that *in vivo* GITR signalling does not disable Treg cells. This conclusion is in line with a report showing that Treg cells purified from either rat IgG-treated or DTA-1-treated mice are equally efficient in suppressing allo-MLR [16]. However, only a single ratio of Treg to responder (1:1) was tested in MLR [16], whereas our assay covered a wide range of ratio from 1:1 to 1:128. Since maximum Treg depletion occurred 3 days after DTA-1 administration (Fig. 3a), Tregs were isolated at 24 h after DTA-1 treatment allowing sufficient time for GITR binding and signal transduction (Fig. 4).

As injected DTA-1 mAb preferentially binds to Treg cells (Fig. 4b) but does not inactivate them (Fig. 2), we consider that depletion of Treg cells is an alternative consequence of GITR binding. In support of this, DTA-1 is IgG2b, an isotype shared by a large panel of *in vivo* depleting mAb with specificity for CD4 (GK1.5 and YTS 191), CD8 (YTS169 and 2.43) and Thy1.2 (30H12). Although



DTA-1 has been proposed [12] and indicated [20] to deplete GITR expressing-Treg cells in vivo, a formal demonstration is lacking. In this study we provide evidence showing that DTA-1 is a depleting mAb causing a moderate but significant reduction of endogenous polyclonal Tregs in tumour-free (Fig. 3a) and tumour-bearing Foxp3/GFP knock-in mice (Fig. 3b, c). The most remarkable Treg depletion following DTA-1 treatment is illustrated in adoptive transfer experiments using HY-specific Treg cells (Fig. 3d). Importantly, the reduction of donor Treg cells is more complete in MB49 tissue than dLN (Fig. 3d) and correlates with the level of GITR expression by Treg cells in these two tissues (Fig. 3e).

Discrepancies between our data and other studies [15] regarding the depleting potential of DTA-1 may reflect differences between mouse strains (BALB/c vs. B6) and the cells used for analysis (spleen cells vs. PBL) and the use of different markers (CD25 vs. Foxp3) to identify Treg cells. It becomes increasingly clear that not all CD25⁺ are Treg cells and not all Treg cells are necessarily CD25⁺ [29].

Why DTA-1 can only induce a partial Treg depletion in B6 mice is unclear. One reason is likely to be the nature of this particular clone. In this respect, it would be of interest to compare DTA-1 with other anti-GITR clones of IgG2b isotype for efficiency of Treg depletion. In addition, the current treatment regime might be suboptimal, given a single i.p. injection with 50 µg of mAb. Furthermore, Treg cells are composed of distinct subsets which might display different sensitivity to DTA-1-triggered depletion.

Another key element determining efficiency of depletion is the level of expression of the target molecule on the cell surface. There is a clear link between the degree of depletion and the level of GITR expression. Firstly, Treg cells constitutively express higher levels of GITR than conventional CD4 T cells under steady-state conditions (Fig. 4a). Thus DTA-1 seems to selectively target Treg but spare the conventional CD4 T cells (Fig. 3a). The degree of Treg depletion following DTA-1 treatment is moderate in naïve mice, which is perhaps due to relatively low target molecule expression. Secondly, GITR expression on Treg cells is itself variable with the highest level observed on tumour-resident Tregs and lower levels seen on Treg cells from tumour-draining LN (Fig. 3e). Thus, Treg cells in tumour but not those in the dLN are likely to be depleted in the presence of DTA-1 in vivo (Fig. 3b, c). These observations could explain why the percentage of tumour-resident Treg cells but not those within dLN were significantly reduced following DTA-1 administration, as reported [15] and shown in Fig. 1. Finally, timing of DTA-1 treatment determining the efficiency of cancer therapy (Fig. 5) [15, 17], as mAb given on day 8 after tumour inoculation is more effective than that on day 1. A potential interpretation for this observation is that Treg cells in the tumour reach the peak

of GITR expression on day 8 (Figs. 3e, 4b), perhaps making them optimally sensitive to DTA-1-mediated depletion (Fig. 3c, d). Like GITR, other Treg-related molecules including CD103, ICOS, PDL1 and OX40 were found to be up-regulated preferentially by Treg cells detected in MB49 mass, compared with those in dLN (data not shown). These results are consistent with two recent reports showing that vast majority of tumour-infiltrating Treg cells are OX40^{high} [30] and GITR^{high} [31]. However, this comparison was made with splenic Treg cells from tumour-free naïve mice [30] rather than Treg cells from dLN of tumour-bearing mice. In this respect, our data are more informative as GITR expression was assessed on both MB49 tumour-infiltrating Treg cells and those in the tumour dLN.

An alternative interpretation for the reduction of Treg cells (95% reduction, from 0.48 to 0.02%) in tumour tissue following DTA-1 treatment is altered Treg migration. If this hypothesis is correct, one would expect that reduced Treg representation in tumour mass should correlate with increased Treg accumulation in other lymphoid tissues such as dLN and ndLN. However, we observed that the decreased proportion of adoptively transferred Marilyn Treg cells was also evident, but to a less extent, in dLN (64% reduction, from 0.28 to 0.10%) and ndLN (40% reduction, from 0.15 to 0.09%) (S-Fig. 3A), making this explanation unlikely. In addition, the reduction of donor Treg cells in dLN was also evident when absolute cell number was compared between untreated and DTA-1-treated groups (S-Fig. 3B). Nevertheless, LN and tumour tissues are different microenvironments with distinct structures and capacity to recruit Treg cells. The contributions of depletion and altered migration to the reduced accumulation of Tregs in tumour tissues following DTA-1 therapy needs further investigation.

Treg cells promote tumour progression by suppressing anti-tumour immunity via different mechanisms [32, 33]. Treg cells are recruited into the tumour mass because they not only recognise tumour-associated antigens but also normal self-antigens released from the tumour [34]. In addition, tumours are able to induce a subset of DC to produce TGFβ [35] or IDO [36], which trigger endogenous Treg cell expansion and activation. Although the initiation of Treg activation requires TCR engagement, once activated, Treg cells can inhibit T cell responses non-specifically via bystander suppression, thus the action of Treg cells can be both antigen specific and non-specific [37]. In vivo Treg cells can target various populations such as CD8 T cells, APC and innate immune cells including NKT and NK cells via cell-to-cell contact (using CTLA-4 or membrane bound TGFβ) or production of inhibitory cytokines such as TGFβ and IL10 [37]. Treg cells prevent CD8 T cells from differentiation into cytolytic effector cells in vivo without affecting their proliferation or IFNγ production [38, 39].

In addition, Treg cells can exhibit their suppression in the tumour microenvironment. For example, tumour-infiltrating Treg cells can kill anti-tumour effector cells such as CD8 and NK cells locally by releasing granzyme B and perforin [40].

We have not explored the mechanisms of Treg depletion by DTA-1 in great detail. Both expression of FcR and complement by the host appear to be required for optimal Ab-mediated depletion. It has been difficult to determine the relative contributions of T cell costimulation and Treg depletion by DTA-1. In this regard, it would be of interest to compare between DTA-1 and the soluble GITR-L for their effects, as the latter can only deliver co-stimulatory signals without the involvement of Treg depletion. Also, 2F8 [41], is another agonist anti-GITR mAb which is unable to deplete or disable Treg cells in vivo, presumably because of the isotype (rat IgG2a). It has been demonstrated recently that 2F8 treatment significantly accelerates the onset of diabetes in NOD mice by promoting T cell activation without affecting Treg cells [41].

It has been well documented that T cell costimulation by DTA-1 is another key mechanism for cancer immunotherapy [7–11]. We observed that in vivo, GITR signalling enhanced allo-recognition by conventional CD4 T cells, and amplified anti-MB49 responses by adoptively transferred Marilyn CD4 T cells (manuscript in preparation). In this study, we also verified whether successful cancer immunotherapy using DTA-1 could be extended to the aggressive HY⁺H2^{b+} MB49 tumour. Given HY vaccination fails to control MB49 growth [28], a complete regression of established MB49 tumours following a single DTA-1 administration is very impressive. As reported [15] and shown in Fig. 1, the proportion of Treg cells in rejecting tumour tissues is dramatically reduced, which appears to be a direct consequence of depletion. Intra-tumour injection of DTA-1 [15] or depleting anti-CD4 mAb [42] results in regression of established tumours, highlighting the impact of local removal of Treg cells on therapeutic outcome.

The current study has two implications. Firstly, the consequence of an in vivo therapeutic antibody treatment can be down-regulation (7D4, anti-CD25), blockade or functional inactivation (YTS 177, non-depleting anti-CD4), co-stimulation (37.51, anti-CD28) or depletion of the target T cell population. Apart from antibody specificity and isotype, outcome depends on cellular targets, expression level of targeted molecules, tissue distribution and modulation under physiological or pathological circumstances. The results of antibody therapy need to be carefully interpreted, as for example, a putative blocking antibody (MR1, anti-CD40L) shown in earlier studies [43], was found to be depleting by a subsequent investigation [44]. Secondly, for the identification of the cellular targets of an antibody and investigation of their expression pattern under different

conditions, “in vivo” staining by injection of conjugated antibodies has the advantage of allowing expression to be monitored in real time.

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Appendix B

The roles of antigen-specificity, responsiveness to transforming growth factor- β and antigen-presenting cell subsets in tumour-induced expansion of regulatory T cells

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Summary

In this study we investigated the impact of several factors on the expansion of natural regulatory T (nTreg) cells by tumours, including antigen specificity, transforming growth factor- β (TGF- β) signalling and the antigen-presenting cell subsets responsible for expansion. We found that antigen non-specific expansion of nTreg cells is tumour cell line-dependent. Although both antigen-specific and non-specific pathways can contribute to expansion, the migration of activated nTreg cells to tumour tissues is strictly antigen-dependent. Intact TGF- β signalling on nTreg cells is also essential for tumour-induced expansion. Finally, for stimulation of resting antigen-specific CD4 T cells, CD11c⁺ cells purified from tumour-draining lymph nodes were more potent than CD11b⁺ cells, suggesting that dendritic cells are the key antigen-presenting cell subset involved in cross-presentation of tumour antigens. This study not only provides an *in vivo* system in which cross-talk between nTreg cells and tumours can be explored but also reveals novel aspects of tumour immune evasion.

Keywords: adoptive transfer; Foxp3; immune evasion; regulatory T cells; tumour

Introduction

The primary function of natural CD4⁺ CD25⁺ Foxp3⁺ regulatory T (nTreg) cells is to control self-reactive peripheral T cells that escape thymic deletion, as suggested by early studies using neonatal thymectomy or depletion using anti-CD25 antibodies,^{1,2} and verified by recent investigations using Foxp3^{DTR} mice.³ As many tumour-associated antigens are aberrantly expressed normal self-proteins,⁴ the activity of self-reactive nTreg cells is also proposed to mediate an inhibitory influence over tumour-specific T-cell responses. Indeed, *in vivo* depletion of nTreg cells improves tumour immunosurveillance and enhances the efficiency of therapeutic cancer vaccines.^{5,6}

Expansion of nTreg cells is often correlated with tumour growth,^{7,8} but several key questions regarding tumour-driven nTreg expansion remain unanswered. First, many tumour antigens are altered self-proteins and

nTreg cells are highly self-reactive, it is possible that tumours can activate endogenous polyclonal nTreg cells. Furthermore, tumour-derived transforming growth factor- β (TGF- β) can further expand activated Treg cells in an antigen-independent manner.⁹ Moreover, activated nTreg cells can suppress anti-tumour immunity non-specifically *in vivo*. Hence, polyclonal nTreg cells non-specifically expanded by tumour could make a significant impact on tumour progression. Although several spontaneous tumour lines can induce endogenous polyclonal nTreg cells to proliferate in tumour-draining lymph node (dLN),⁹ it is unclear whether non-specific expansion of nTreg would also be mediated by carcinogen-induced tumour lines. Second, the responses of tumour-antigen-specific nTreg cells *in vivo* have only been examined following viral vaccination,^{10,11} and little is known about nTreg expansion solely in response to the tumour. Tumour-specific nTreg cells are able to proliferate in

Abbreviations: dLN, draining lymph node; dnTGF β RII, dominant negative TGF- β receptor type II; Mar, Marilyn; nTreg, natural CD4⁺ CD25⁺ Foxp3⁺ regulatory T cells; Mar-nTreg, nTreg purified from Rag2^{+/-} Marilyn mice; Mar-nTreg^{dn}, nTreg purified from (dnTGF β RII $\times$ Marilyn) F₁ mice; Mar-nTreg^{GFP}, nTreg purified from (Foxp3/GFP knockin $\times$ Marilyn) F₁ mice; ndLN, non-draining lymph node; pLN, peripheral lymph node; TGF- β , transforming growth factor- β .

response to a growing tumour, the relative contributions of antigen-specific and non-specific expansion to the overall pool of nTreg cells has not been investigated. In addition, the *in vivo* migration of nTreg cells expanded by these two distinct pathways has not been explored. Third, although non-immunogenic B16 tumours expand polyclonal nTreg cells via a TGF- β -dependent mechanism,⁹ whether this mechanism applies to other immunogenic tumours and to tumour-specific nTreg cells has not been evaluated. Finally, although it has been suggested that cross-presentation of tumour antigens operates continuously in dLN,¹² the precise antigen-presenting cell subset(s) involved has not been identified.

To address these issues, we have employed adoptive transfer experiments in which nTreg cells (polyclonal or tumour-specific monoclonal) were injected intravenously (i.v.) into tumour-bearing wild-type (WT) B6 mice. The male-specific minor histocompatibility antigen, HY¹³ is used as a surrogate tumour antigen. HY is naturally expressed by the chemically induced B6 male bladder MB49 carcinoma, an immunogenic but nevertheless aggressive tumour.¹⁴ The HY-negative B16 melanoma cell line was transfected with the *Dby* gene to produce an HY-positive variant (B16/HY). Hence, the relative contributions of non-specific and HY-specific nTreg-cell expansion by tumours can be investigated. Tumour-specific nTreg cells for adoptive transfer are purified from female Rag2^{+/-} HY TCR-transgenic Marilyn mice.¹⁵ The availability of Marilyn mice has facilitated analysis of HY-specific CD4 responses and immunoregulation.¹⁶⁻¹⁸ Recently, the HY system has been employed to explore T-cell responses against tumours.^{19,20} For example, B6 recipients of Rag2^{+/-} Marilyn CD4 T cells and murine fibrosarcoma cells transfected with HY *Dby* complementary DNA (cDNA) have been used to analyse intra-tumour CD4 T-cell accumulation.¹⁹ However, the responses of HY-specific nTreg cells in tumour-bearing mice have not been explored.

In this study, we aimed to address the following questions. Whether the induction of non-specific nTreg-cell expansion is tumour cell line dependent. What are the relative contributions of antigen-specific versus non-specific expansion of nTreg cells by tumours. Is expansion of tumour-antigen-specific nTreg cells by tumours also dependent on TGF- β ? Which subset(s) of antigen-presenting cells in tumour-draining LN is involved in cross-presentation of tumour antigen?

Materials and methods

Tumour cell lines

MB49 is a chemically induced B6 bladder carcinoma¹⁴ that constitutively expresses the endogenous HY genes. B16 is a murine melanoma cell line that does not express HY.²⁰ Both lines were maintained in 10% RPMI-1640

supplemented with fetal calf serum (10%), antibiotics, HEPES, glutamine and 2-mercaptoethanol.

Generation of B16/HY cell line

B16 cells were plated out at 2×10^5 cells/well in six-well plates and after 24 hr were transfected using Lipofectamine 2000 (Invitrogen, Paisley, UK) and 2 mg pcDNA3.1/*Zeo-Dby* following the manufacturer's guidelines. After 48 hr, cells were diluted 1 : 10 into selection medium containing 1 mg/ml Zeocin. Resistant colonies were picked at 2–3 weeks, expanded and *Dby* expression was measured by quantitative reverse transcription-polymerase chain reaction (RT-PCR).

Mice

Thy-1.2 B6, and Thy-1.1 B6 mice were purchased from Harlan Laboratories (Bicester, UK) and the Jackson Laboratory (Bar Harbor, ME), respectively. Marilyn mice¹⁵ were obtained from Dr O. Lantz (Institut Curie, Paris, France) were originally on Rag2^{-/-} Thy-1.2⁺ background. Thy-1.2⁺, Thy-1.1⁺ and Thy-1.1⁺ Thy-1.2⁺ Marilyn mice on either Rag2^{+/-} or Rag2^{-/-} background were subsequently produced after crossing with Thy-1.1 B6 mice. Either dnTGF β R1I-transgenic²¹ or Foxp3/green fluorescent protein (GFP) knock-in²² mice on B6 background, kindly provided by Dr R Flavell (Yale University School of Medicine, New Haven, CT) and Dr B Malissen (Centre d'Immunologie de Marseille-Luminy, Marseille, France), respectively, were mated with Rag2^{-/-} Marilyn mice to produce F₁ mice, from which double-transgenic nTreg cells (Mar-nTreg^{dn} or Mar-nTreg^{GFP}) were purified. Although WT B6 females were used as recipients in a majority of experiments, Rag2^{-/-} B6 mice were used as recipients in some adoptive transfer experiments.

Purification of nTreg and conventional CD4 T cells

This was conducted as described previously.¹⁶ In brief, enriched CD25⁺ cells were obtained by magnetic antibody cell sorting (MACS) positive selection (anti-CD25^{PE} or anti-CD25^{APC} followed by anti-phycoerythrin (PE) or anti-allophycocyanin (APC) -microbeads), and then were sorted on a BD FACS Aria after staining. We sorted CD4⁺ CD25⁺ cells for polyclonal nTreg cells, CD4⁺ V β 6⁺ CD25⁺ cells for Marilyn nTreg cells (Mar-nTreg or Mar-nTreg^{dn}), and CD4⁺ V β 6⁺ CD25⁺ GFP⁺ cells for green Marilyn nTreg cells (Mar-nTreg^{GFP}). The combination of MACS enrichment and fluorescence-activated cell sorting (FACS) sorting is efficient for obtaining nTreg cells with a high purity and a good yield. Conventional CD4 T cells were purified by MACS positive selection. CD25-depleted CD4 T cells were prepared by MACS positive selection after depletion of

CD25-expressing cells (anti-CD25^{PE} staining followed by anti-PE-microbeads).

Adoptive cell transfer and tumour inoculation

Most adoptive transfer experiments were conducted by i.v. injection of Thy-1.1⁺ conventional CD4 [carboxyfluorescein succinimidyl ester (CFSE) -labelled, CD25-intact or depleted] or Thy-1.1⁺ nTreg cells (monoclonal or polyclonal) into Thy-1.2 B6 females. CFSE labelling was conducted as described previously.^{16,23} Tumour cells (MB49, B16 or B16/HY) were inoculated subcutaneously (s.c.; 5×10^5) 1 day after T-cell transfer.

Analysis of expansion and accumulation of donor T cells in various tissues

Single-cell suspensions from dLN, ndLN and tumour tissues were stained with anti-V β 6^{PE} or anti-TCR^{PE}, anti-Thy-1.1^{PercP} or anti-CD4^{PercP} and anti-CD4^{APC} or anti-CD44^{APC}. Donor cells were identified by gating of Thy-1.1⁺ CD4⁺, Thy-1.1⁺ V β 6⁺ or CD4⁺ Thy-1.2⁺ before further analysis of CFSE/CD44 profiles or Foxp3 expression. To produce a physiologically relevant model, WT (rather than Rag-deficient) mice were used as recipients and relatively small populations (10^5) of donor nTreg cells were transferred. To obtain a reliable representation of the small cohorts of donor nTreg cells, two million events were acquired, except for Rag2^{-/-} B6 recipients where 0.5 million events were acquired because donor cells undergo homeostatic expansion). Acquisition of the same number of events for each sample enables reliable comparison of the percentages of donor Treg cells between the samples. Analysis of gated lymphocytes was performed using CELLQUEST software (version 3.3, Becton Dickinson UK Ltd, Oxford, UK).

Quantitative RT-PCR

RNA (DNase I treated) and first-strand cDNA were prepared using the RNAqueous-4PCR and High Capacity cDNA synthesis kits (Applied Biosystems, Warrington, UK). Relative concentrations of 18S ribosomal RNA and *Dby* transcripts were determined using Power SYBR PCR master mix (Applied Biosystems), and performing the reactions on the 7900HT Fast Real-Time PCR system (Applied Biosystems). Primer pairs were assessed by gel electrophoresis and melting curve analysis, to ensure that only specific products were generated. No product was obtained when female cDNA was tested with *Dby*-specific primers. The primer sequences were as follows: 18S forward, 5'-CCGCAGCTAGGAATAATGGAAT-3'; 18S reverse, 5'-CGAACCTCCGACTTTCGTTCT-3'; *Dby* forward, 5'-GAAAGTAGTATGGGTTGAAAGAAC-3'; *Dby* reverse, 5'-CTCCTCTCGATCTTTCTGTGAT-3'.

Test samples (cell lines, tumour tissues and spleen cells) were assayed in triplicate. Expression values were determined from standard curves generated using dilutions of male cDNA and plotting cycle threshold values against log quantity. Normalized *Dby* expression was calculated by dividing *Dby* quantity by the corresponding 18S quantity.

Purification of antigen-presenting cell subsets from LN cells

Pooled LN were chopped into small pieces and incubated in a mixture containing collagenase XI and DNase. After digestion, cell suspensions were prepared from which CD11c⁺ cells were isolated by MACS after incubating with anti-CD11c microbeads. The CD11c-negative fractions were subsequently incubated with anti-CD11b microbeads to isolate CD11b⁺ cells. Finally, B220⁺ cells were positively selected from the CD11b-negative fractions.

Proliferation assay

This was conducted as described previously.^{16,23}

Statistical analysis

Data are presented as means $\pm$ SEM. Group comparisons were conducted using the two-tailed Student's *t*-test or a two-tailed non-parametric Mann-Whitney *U*-test, depending on the number of samples. *P*-values of < 0.05 are considered statistically significant.

Results

CD25-depleted but not CD25-intact Marilyn CD4 T cells are potent responders *in vivo*

To compare *in vivo* anti-HY responses by Marilyn CD4 T cells with or without nTreg cells, CD4 cells purified from Rag 2^{+/-} Thy-1.1⁺ Marilyn females were either depleted CD25-expressing cells or not. After CFSE labelling, these two donor cell populations were adoptively transferred into a total of four groups of Thy-1.2 females. Next day MB49 cells were injected subcutaneously into the right flank of two groups of recipients of each donor population. Five days after tumour inoculation, we analysed the representation of donor cells in various tissues of the individual mice in all four groups.

As shown in Fig. 1(a), the donor cells in the group which received both CD25-depleted CD4 and MB49 cells, mounted a robust response with an eight-fold expansion in the dLN ($0.545 \pm 0.01\%$ versus $0.065 \pm 0.02\%$ in the tumour-free control group, $P < 0.01$), and a significant accumulation in the tumour ($3.55 \pm 0.27\%$). In addition, proliferating donor cells (Thy-1.1⁺ V β 6⁺) in dLN significantly increased CD44 expression following progression

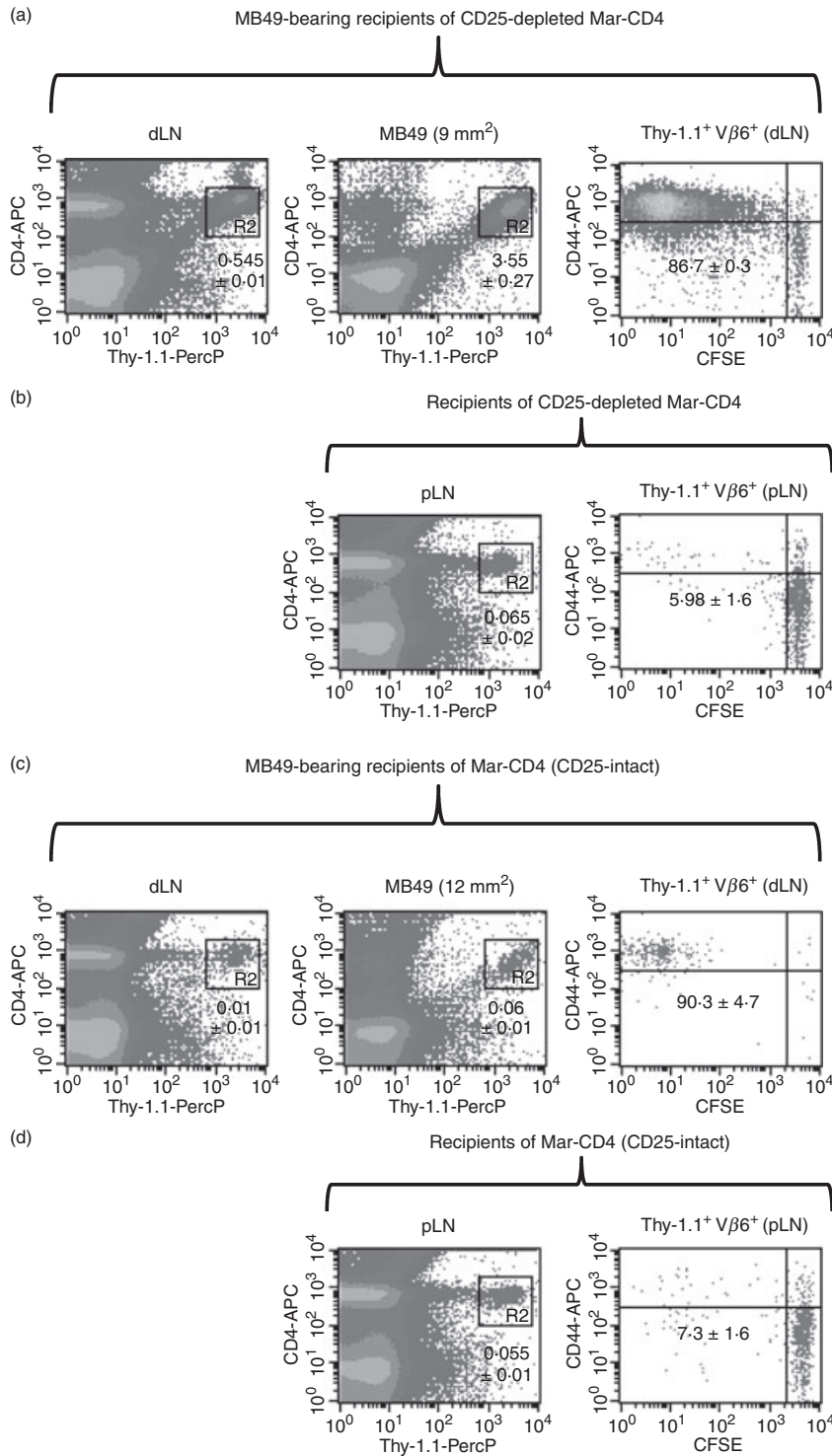


Figure 1. Comparison of anti-MB49 responses by CD25-depleted and CD25-intact Marilyn CD4 T cells. Four groups of Thy-1.2 B6 females (three or four mice/group) were adoptively transferred with 1.5×10^6 CFSE-labelled CD25-depleted- (a, b) or CD25-intact Thy-1.1 Marilyn CD4 T cells (c, d) by intravenous injection. Next day two groups of mice only were inoculated subcutaneously with 0.5×10^6 MB49 cells (a, c). Five days after MB49 inoculation, all groups were analysed by staining of dLN and MB49 (a, c) or pLN (b, d) cells with anti-V $\beta 6^{\text{PE}}$, anti-Thy-1.1 $^{\text{PerCP}}$ and anti-CD4 $^{\text{APC}}$. The donor cells in lymph nodes (LN) and MB49 were identified by co-expression of Thy-1.1 and CD4. In addition, LN samples were also stained with anti-V $\beta 6^{\text{PE}}$, anti-Thy-1.1 $^{\text{PerCP}}$ and anti-CD44 $^{\text{APC}}$. The donor cells were identified by co-expression of Thy-1.1 and V $\beta 6$ and their CFSE profiles were presented as CFSE versus CD44. A total of 2 million events (no gate) was acquired for each sample. For MB49 samples, tumour size is also indicated. One representative experiment of three is shown.

of cell division whereas those in peripheral LN (inguinal, pLN) of the tumour-free control group did not ($86.7 \pm 0.3\%$ versus $5.98 \pm 1.6\%$, $P < 0.01$).

In contrast, the donor cells in the group given both CD25-intact CD4 T cells and MB49 cells (Fig. 1c), underwent a poor response with a limited accumulation in the dLN ($0.01 \pm 0.01\%$ versus $0.055 \pm 0.01\%$ in

tumour-free control group, $P > 0.05$) and tumour ($0.06 \pm 0.01\%$), which could be the result of either failure of activation or accelerated activation-induced cell death in the presence of CD25 $^{+}$ nTreg cells. As $> 90\%$ of CD25-intact donor cells lost CFSE but up-regulated CD44 (Fig. 1c), it is likely that all donor cells were recruited into the anti-MB49 response but most of them

were eventually eliminated during the course of the response.

Taken together, these results clearly indicate that CD25⁺ nTreg cells have a huge impact on determining whether a CD4 response would be successful, because a minor population of Marilyn nTreg cells co-transferred with a majority of conventional Marilyn CD4 T cells is associated with a substantially reduced response to MB49.

To provide direct evidence that HY-specific nTreg cells can impact the progress of MB49 tumours, we compared tumour growth in B6 female mice with or without adoptively transferred Marilyn Treg cells (1×10^5 /mouse). As shown in Fig. S1(b), the presence of adoptively transferred HY-specific nTreg cells not only led to an accelerated MB49 growth, but also to increased mortality (25%, 2/8). In contrast, inoculated MB49 cells with an optimal dose (5×10^5 cells/mouse) into the right flank of B6 females in the absence of transferred Marilyn Treg cells develop into tumours within 12 days, which usually do not kill the hosts (i.e. 0% mortality) (Fig. S1a). These results were reproduced in a second Treg transfer and MB49 challenge experiment in which the data are presented as survival of tumour-bearing mice (Fig. S1c). The impact of the transferred HY-specific nTreg cells on tumour progress is therefore of biological significance. To further explore this, we repeated the above experiment in the second tumour model using HY/*Dby*-transduced B16 cells. Injection of B6 female mice with a suboptimal dose of B16/HY cells (2×10^5) led to a slow tumour growth (Fig. S2a), which was significantly enhanced in the presence of transferred Marilyn Treg cells (Fig. S2b). Similar results were also observed when the percentage of survival was used as readout (Fig. S2c). Collectively, these experiments indicate that in this adoptive transfer model, tumour-antigen-specific Treg cells are capable of suppressing anti-tumour T-cell responses *in vivo*.

MB49 tumour expands HY-specific but not polyclonal nTreg cells

How might the small nTreg-cell population have such a striking impact on a major CD4 T-cell population of the same specificity? We hypothesized that MB49 could preferentially expand nTreg cells. To test this, Thy-1.1 Marilyn nTreg cells were adoptively transferred into two groups of Thy-1.2 B6 females, of which one group was given MB49 inoculation next day.

As shown in Fig. 2(b), following MB49 challenge, donor nTreg cells underwent an eight-fold expansion in dLN (0.0207 ± 0.0111 versus $0.0026 \pm 0.0013\%$ in tumour-free control group, $P < 0.05$). Interestingly, expanded donor Treg cells also showed a significant loss of Foxp3 expression ($84.83 \pm 0.08\%$ versus $96.55 \pm 3.45\%$ in control group). Furthermore, the degree of donor nTreg cells expansion was much more marked when absolute numbers of donor cells recovered from two groups were compared

(Fig. 2c). Activated donor nTreg cells also migrated to the tumour ($0.5821 \pm 0.3011\%$), but only one-third of tumour-infiltrated donor nTreg cells still expressed Foxp3 expression ($33.93 \pm 10.49\%$ in MB49 versus $84.83 \pm 0.08\%$ in dLN).

MB49-induced nTreg-cell expansion seen in Fig. 2(b) can be either antigen driven or not or a combination of both because some tumour cell lines can expand nTreg cells in an antigen-independent fashion.⁹ As MB49 variants lacking expression of HY are not available, the relative contribution of antigen non-specific expansion cannot be determined. To overcome this limitation, we used the alternative strategy of transferring polyclonal nTreg cells from Thy-1.1 WT B6 mice to MB49-bearing B6 Thy-1.2 mice (Fig. 2e). If MB49 could induce nTreg cells to proliferate non-specifically, the expansion of B6 nTreg cells should be seen. Instead, we found no expansion of B6 nTreg cells in response to MB49 in all three tissues tested (Fig. 2e), based on measurement of both frequency (Fig. 2e) and absolute cell number (Fig. 2f). These results do not support the hypothesis that MB49 tumour can non-specifically expand nTreg cells.

Generation and characterization of HY-expressing B16 cell line

Antigen-specific and non-specific nTreg-cell expansion by tumours could be directly compared using antigen-positive and antigen-negative variants of the same tumour cell line. As an HY-negative, mutant MB49 cell line is not available and a knockout of the HY *Dby* gene from MB49 cells is technically challenging, an HY-expressing variant of an HY-negative tumour cell line was produced. The widely used melanoma cell line B16²⁰ was transfected with the HY *Dby* gene and a stable HY-expressing clone was obtained (B16/HY). Although *Dby* expression by B16/HY was lower than MB49, it was significantly higher than B16 ($P < 0.05$) (Fig. 3a). More importantly, *Dby* expression by B16/HY remained following tumour development (Fig. 3b), suggesting that the expression of transgene is stable *in vivo*. Finally, adoptively transferred, CFSE-labelled Marilyn conventional CD4 T cells proliferated in dLN in response to B16/HY (Fig. 3e). They were also detectable in B16/HY tumours. In sharp contrast, the activation of donor cells was not evident in B16-bearing mice (Fig. 3d), or in tumour-free controls (Fig. 3c), as expected. These *in vivo* T-cell recognition assays conclusively demonstrated that transduced *Dby* genes in B16/HY were functional.

Marilyn nTreg cells undergoing antigen-specific expansion, but not those expanded non-specifically, migrate to tumours

Next, we sorted CD4⁺ V β 6⁺ CD25⁺ GFP⁺ cells from (Foxp3/GFP knockin $\times$ Marilyn) F₁ females, and adoptively

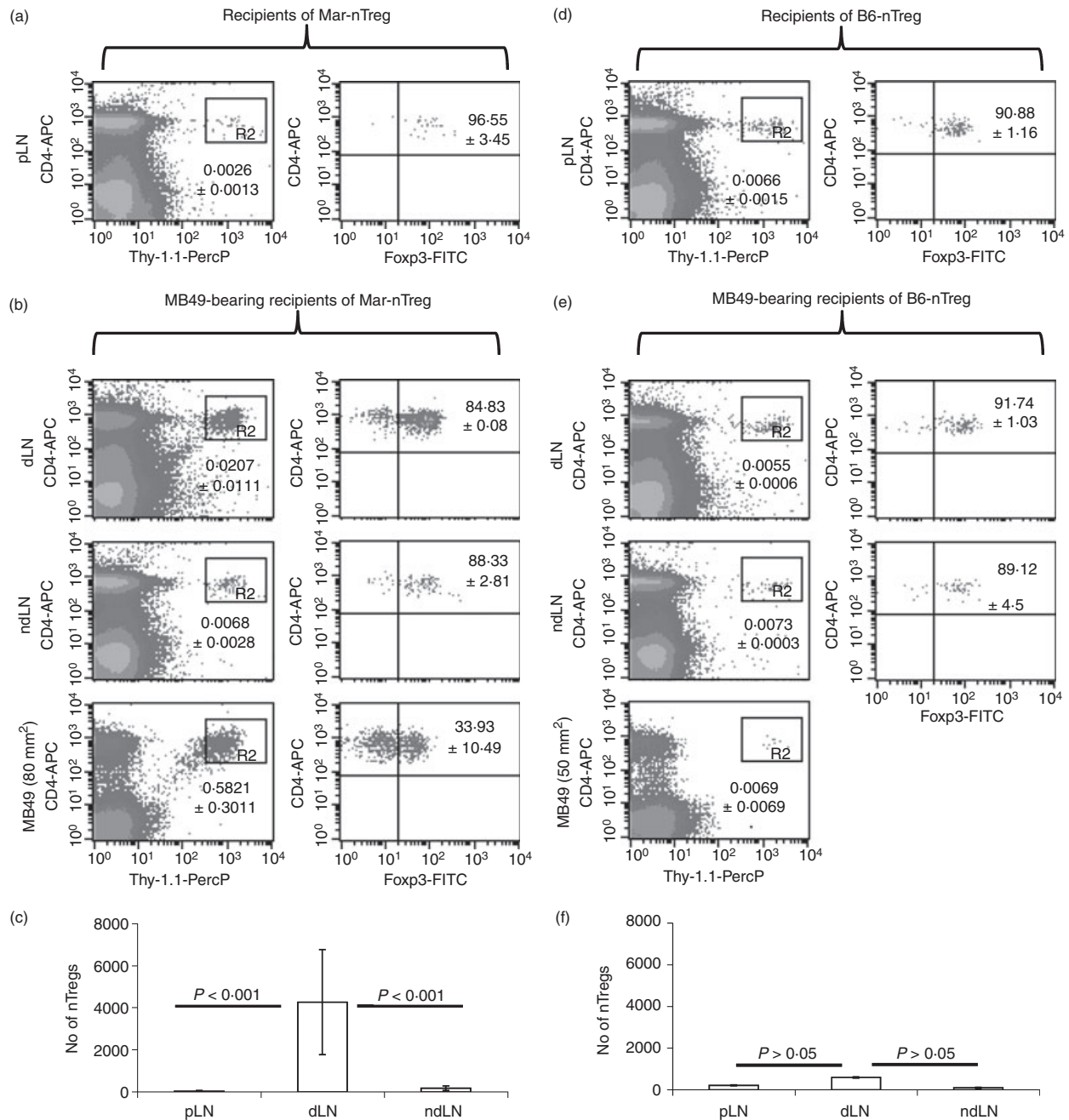


Figure 2. MB49 expands Marilyn but not B6 natural regulatory T (nTreg) cells *in vivo*. Four groups of Thy-1.2 B6 females (three mice/group) were transferred with 10^5 Thy-1.1 Marilyn nTreg (a, b) or Thy-1.1 B6 nTreg cells (c, d), of which two groups of mice were given MB49 cells the next day (b, e) but the other two groups were not (a, d). 11 days after MB49 inoculation, analysis was performed by staining the cells with anti- $\text{V}\beta 6^{\text{PE}}$ or anti-TCR- β^{PE} , anti-Thy-1.1 $^{\text{PerCP}}$ and anti-CD4 $^{\text{APC}}$, before intracellular staining with anti-Foxp3 $^{\text{FITC}}$. 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 (pLN), draining (dLN) and non-draining (ndLN) lymph nodes] were calculated by total cell number $\times$ % of gated lymphocytes (R1) $\times$ % of donor cells (R2) (c, f). One representative experiment of four is shown. A total of 2 million events was acquired for each sample. For the MB49 samples, tumour size is also indicated.

transferred these green nTreg cells (Thy-1.1 $^+$) into three groups of Thy-1.2 females, of which two groups were injected s.c. with either B16 or B16/HY cells next day. The expansion and Foxp3 expression by donor Treg cells were analysed 11 days after tumour inoculation.

We made the following observations regarding the ability of B16 and B16/HY tumours to expand donor Treg cells *in vivo*. First, for expansion of Marilyn nTreg $^{\text{GFP}}$ cells, B16/HY tumour was more efficient than B16 tumour, as 3.8-fold more donor cells accumulated in dLN of B16/HY

(Fig. 4i) than in dLN of B16 (Fig. 4c) ($0.0381 \pm 0.0092\%$ versus $0.0101 \pm 0.0005\%$, $P < 0.05$). Furthermore, the absolute donor Treg-cell numbers recovered from B16/HY-bearing mice was significantly higher than those from B16-bearing mice (data not shown). Second, Marilyn nTreg^{GFP} cells were present in B16/HY tumours ($0.2585 \pm 0.066\%$, Fig. 4m) but were completely absent in B16 tumours ($0.000 \pm 0.000\%$, Fig. 4g), indicating that migration of nTreg cells to the tumour is strictly antigen-dependent. Third, B16 tumour appeared to be able to expand Marilyn nTreg cells non-specifically, as the frequency of donor Treg cells detected in dLN of B16-bearing mice was higher than those in pLN of tumour-free control mice ($0.0101 \pm 0.0005\%$, Fig. 4c versus 0.0013 ± 0.000 , Fig. 4a). Finally, Foxp3 expression by nTreg cells *in vivo* appears to be unstable, following antigen recognition, expanded Treg cells can lose Foxp3. In addition, it seems that loss of Foxp3 by tumour-infiltrated Treg cells is greater ($77.68 \pm 0.02\%$, Fig. 4n) than that by those in dLN ($91.37 \pm 0.08\%$, Fig. 4j).

B16/HY tumours appear to be less potent than MB49 in recruiting donor Marilyn Treg cells in the tumour mass (Fig. 2b versus Fig. 4m). This raises concerns of whether comparing donor Treg-cell recruitment between B16 and B16/HY tumour tissues is feasible, given the few cells detectable (Fig. 4g versus Fig. 4m). To increase sensitivity, we used Rag2^{-/-} B6 females as recipients of Marilyn Treg cells. To make a proper comparison, we also modified the FACS acquisition strategy by collecting the same number of events within a live lymphocyte gate, allowing a more precise measurement of the frequencies of donor Treg cells between the samples.

HY-specific green Treg cells were transferred to two groups of Rag2^{-/-} B6 females, which were inoculated with

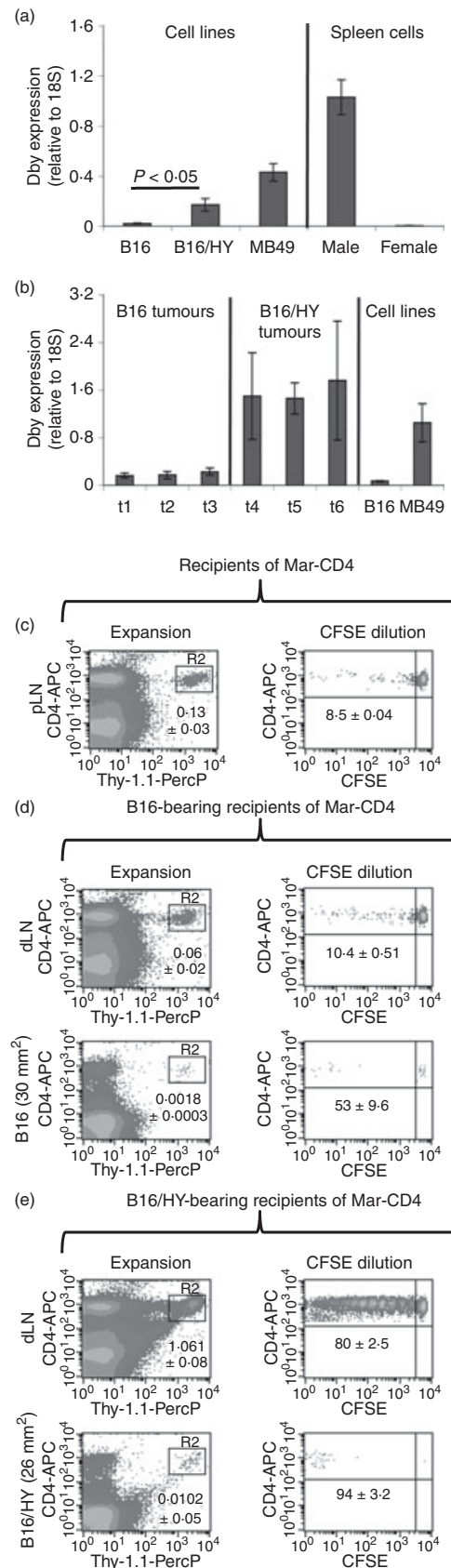


Figure 3. Characterization of HY-expressing B16 cell line (B16/HY). (a) B16, B16/HY and MB49 cells were compared for *Dby* expression by reverse transcription–polymerase chain reaction (RT-PCR) with male or female B6 spleen cells were used as internal controls. One representative experiment of three is shown. (b) Two groups of B6 females (three mice/group) were inoculated subcutaneously (s.c.) with 5×10^5 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. (c–e) Three groups of Thy-1.2 B6 females (three mice/group) were adoptively transferred with 3×10^6 CFSE-labelled Thy-1.1 Marilyn CD4 T cells. Next day the mice in two groups (d, e) were inoculated s.c. with 5×10^5 B16 or B16/HY cells, respectively. Thirteen days after tumour inoculation, analysis was conducted 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 CD4 (R2). The CFSE dilution by donor cells is presented as CFSE versus CD4. One representative experiment of two is shown. A total of 2 million events was acquired for each sample. For the B16 and B16/HY samples, tumour size is also indicated.

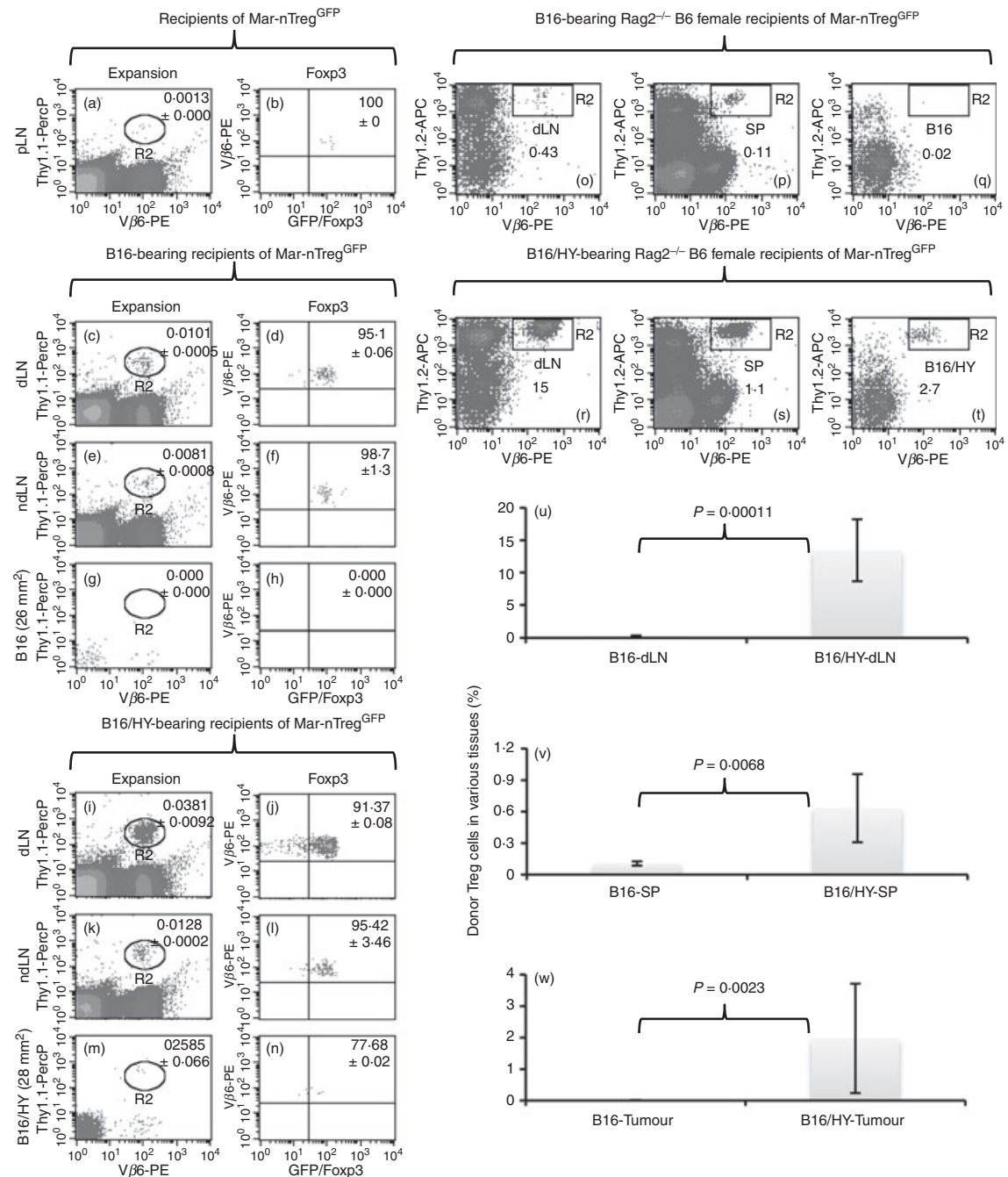


Figure 4. Comparison of the relative contribution by non-specific and antigen-specific natural regulatory T (nTreg) cell expansion. (a–n) Three groups of Thy-1.2 B6 females (three mice/group) were adoptively transferred with 10^5 Thy-1.1 Marilyn nTreg cells co-expressing Foxp3/GFP (Mar-nTreg^{GFP}) by intravenous injection, of which two groups of mice were subcutaneously (s.c.) inoculated with B16 cells (c–h) or B16/HY cells (i–n) (5×10^5) next day. Eleven days after tumour inoculation, analysis was performed by staining of 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 (R2) and their Foxp3 expression was presented as Vβ6 versus GFP. One representative experiment of two is shown. A total of 2 million events was acquired for each sample. For the B16 and B16/HY samples, tumour size is also indicated. (o–t) Two groups of Rag2^{-/-} B6 females (four mice/group) were transferred with 2×10^4 Thy-1.2 Marilyn green Treg cells and were also inoculated s.c. with either 1×10^5 B16 (o–q) or B16/HY cells (r–t) on the same day. After 12 days, analysis was performed by staining the draining lymph nodes (dLN; o, r), spleen (p, s) and B16 or B16/HY tumour tissues (q, t) with anti-Vβ6^{PE}, anti-CD4^{PerCP}, and anti-Thy-1.2^{APC}. Donor Treg cells were identified by co-expression of Thy-1.2 and Vβ6 (R2). Fluorescence-activated cell sorter dot plots of one representative mouse of each group are shown. An equal number of events in the live lymphocyte gate were acquired for the paired samples (2×10^4 for dLN, 5×10^4 for spleen and 5×10^3 for B16 and B16/HY). One representative experiment of two is shown. (u–w) Quantitative comparison of donor Treg frequency in dLN (u), spleen (v) and tumour mass (w) between two groups.

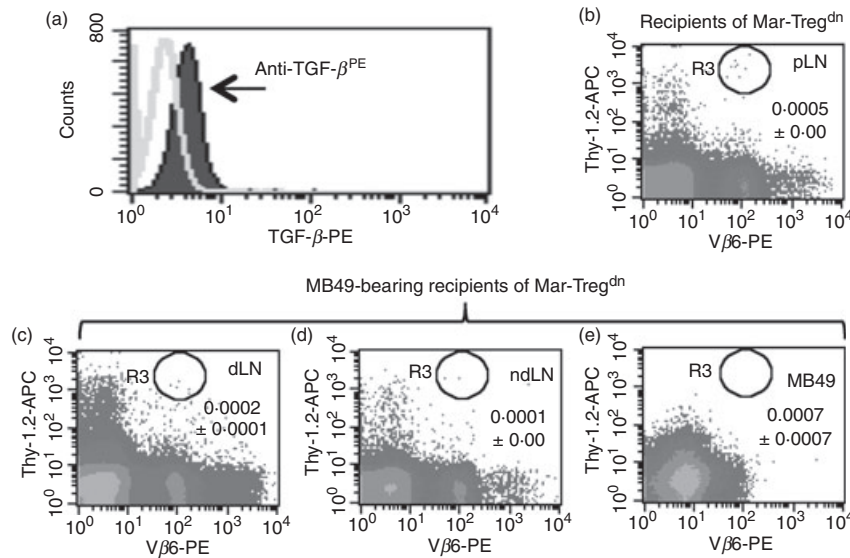


Figure 5. Expansion of Marilyn natural regulatory T (nTreg) cells by MB49 is dependent on transforming growth factor- β (TGF- β). (a) MB49 cells were cultured with 10% fetal calf serum RPMI-1640 in the presence of 1 μ g/ml brefeldin A for 4 hr. After fixing and permeabilizing, the cells were stained for anti-TGF- β -PE (filled) or isotype control (open). (b–e) Two groups of Thy-1.1 B6 females (three mice/group) were transferred with 10^5 Thy-1.2 Marilyn nTreg cells co-expressing dnTGF β RII (Mar-nTreg^{dn}), of which one group of mice received MB49 cells next day (c–e). Eleven days after tumour inoculation, analysis was conducted by staining the cells with anti-V β 6^{PE}, anti-CD4^{PerCP} and anti-Thy-1.2^{APC}, before intracellular staining with anti-Foxp3^{FTTC}. The donor cells were identified by co-expression of Thy-1.2 and V β 6 (R3). A total of 2 million events was acquired for each sample.

either B16 or B16/HY cells on the same day. Twelve days later, FACS analysis was performed by comparing the frequency of donor Treg cells in dLN, spleen and tumour tissues of individual mice between two groups. Unlike the low recovery of donor Treg cells in dLN of B16/HY-bearing WT B6 females (0.04%, Fig. 4i), a much higher proportion was present in dLN of B16/HY-bearing Rag2^{-/-} B6 females (15%, Fig. 4r). A significant fraction of activated donor Treg cells also accumulated in both spleen (1.1%, Fig. 4s) and B16/HY tumours (2.7%, Fig. 4t). In B16-bearing Rag2^{-/-} B6 females, however, the frequency of donor Treg cells was profoundly decreased in both dLN (0.43%, Fig. 4o, 97% reduction) and spleen (0.11%, Fig. 4p, 90% reduction) because of the lack of HY antigens. Consequently, donor Treg cells were barely detectable in B16 mass (0.02%, Fig. 4q, 99% reduction). These results were confirmed by quantitative analysis showing that the frequency of donor Marilyn Treg cells in the presence of HY was significantly higher than that in the absence of HY (Fig. 4u–w), which was also validated by counting absolute donor cell numbers (not shown).

Taken together, these data demonstrate that (i) both antigen-specific and non-specific proliferation of Marilyn nTreg cells contribute to expansion by HY-expressing B16 tumours, (ii) the presence of antigen not only amplifies the expansion in dLN but also is required for expanded Treg cells to migrate into tumour, and (iii) a fraction of Treg cells can lose Foxp3 following *in vivo* activation.

The involvement of TGF- β in the expansion of Marilyn nTreg cells by MB49 tumours

The MB49 cell line constitutively expresses TGF- β , revealed by intracellular staining (Fig. 5a). We hypothesized that this cytokine may play an essential role in tumour-induced expansion of Marilyn nTreg cells. To explore this possibility, Thy-1.2⁺ Marilyn nTreg cells co-expressing the dominant negative TGF- β receptor II (Mar-nTreg^{dn} cells) were transferred to two groups of Thy-1.1 B6 females, of which one group was inoculated with MB49 cells next day. Twelve days after cell transfer, the mice were analysed for the presence of donor cells between these two groups. There was no difference in terms of the proportion of donor cells in pLN of tumour-free control (0.0005 $\pm$ 0.000%, Fig. 5b) and dLN of MB49-bearing mice (0.0002 $\pm$ 0.0001%, Fig. 5c). Donor cells were barely detectable in ndLN (Fig. 5d) and MB49 (Fig. 5e). The defect in TGF- β signal transduction on nTreg cells abrogated their expansion in response to MB49, indicating that nTreg expansion by MB49 appears to be TGF- β dependent.

Characterization of Marilyn Treg cells co-expressing dnTGF β RII

It is unclear whether the data shown in Fig. 5 may result from a developmental defect of nTreg cells expressing the

dnTGF β RII, which may prevent appropriate activation in response to antigen.

To determine the functionality of dnTGF β RII Marilyn Treg cells *in vivo*, we performed co-transfer experiments using Rag2^{-/-} B6 females as recipients. Unlike polyclonal CD4 populations from WT B6 mice, conventional and Treg cells from Marilyn mice failed to repopulate the lymphopenic host, presumably as a result of the lack of cognate HY antigens (manuscript under preparation, C. Addey and J.-G. Chai). In addition, we have previously shown that the efficiency of positive selection of transgenic thymocytes on a Rag-sufficient background is very high, resulting in > 96% CD4 and > 99% CD8 T cells expressing V β 6.¹⁶

To this end HY-specific conventional CD4 T cells alone (CD4⁺ V β 6⁺ CD25⁻ CD44^{low} sorted from Thy-1.1⁺ Rag2^{-/-} Marilyn females), HY-specific Treg cells alone (CD4⁺ V β 6⁺ CD25⁺ sorted from Thy-1.2⁺ Rag2^{+/-} Marilyn females), HY-specific and dnTGF β RII-expressing Treg cells alone (CD4⁺ V β 6⁺ CD25⁺ sorted from Thy-1.2⁺ dnTGF β RII⁺ Rag2^{+/-} Marilyn females), or a mixture of an equal number of conventional and each Treg-cell population were adoptively transferred into five groups of Rag2^{-/-} B6 females which were given MB49 inoculation on the same day. Analysis was conducted 12 days later.

In the absence of donor Treg cells, Marilyn CD4 T cells underwent a robust expansion in response to MB49 tumours with a significant accumulation of donor cells (V β 6⁺ CD4⁺ Thy-1.1⁺) in dLN (50%), spleen (10%) and MB49 mass (16%, Fig. 6a–c). However, this HY-derived systemic activation of donor Marilyn responders was inhibited by co-transferred Marilyn Treg cells with a range of 30–50% suppression in all three tissues tested (Fig. 5d–f). Interestingly, dnTGF β RII-expressing Marilyn Treg cells showed a partial suppression in dLN (20% suppression, Fig. 5g), but they failed to inhibit the accumulation of donor responders in spleen and MB49 tissue (Fig. 6h–i). For the suppression of accumulation of donor responder cells in dLN, both types of Marilyn Treg cells were effective regarding the analysis of both frequency (Fig. 6j) and absolute cell numbers (Fig. 6k), although overall Treg cells without dnTGF β RII are more potent inhibitors than their dnTGF β RII-expressing counterparts. In the presence of donor responder cells, the frequency of Marilyn Treg cells in dLN was significantly higher than that of dnTGF β RII-positive Treg cells (Fig. 6l). Moreover, in the absence of co-transferred donor responder cells, MB49-induced expansion of Marilyn Treg cells (20%) was significantly greater than that of dnTGF β RII-expressing Marilyn Treg cells (4%), Fig. 6m–o).

We also conducted a standard *in vitro* co-culture to determine whether dnTGF β RII-expressing Marilyn Treg cells are functional. Although Marilyn dnTGF β RII⁺ CD4⁺ CD25⁺ cells were apparently anergic, they were also clearly less suppressive, as even at a 10 : 1 ratio of Treg cells to

responder cells, only partial inhibition was achieved (Fig. 6p). In contrast, Marilyn CD4⁺ CD25⁺ cells can readily induce a complete suppression even at 1 : 1 ratio, as shown previously.^{16,23}

CD11c⁺ cells in dLN of MB49 tumours display major histocompatibility complex–peptide complexes

Having established that MB49-induced expansion of Marilyn nTreg cells is antigen-dependent, we wish to determine which antigen-presenting cell subset in dLN is involved in this process by identifying antigen-presenting cells displaying major histocompatibility complex–HY peptide complexes. Three groups of tumour-bearing mice were prepared by s.c. inoculation of MB49 cells on day – 21 (G1), day – 14 (G2) and day – 7 (G3). On day 0, the average tumour sizes in these groups were 90 (G1), 70 (G2) and 30 mm² (G3). A group of naive B6 mice (G4) was used as tumour-free controls. On day 0, dendritic cells (DC), macrophages and B cells isolated from dLN of three groups of MB49-bearing B6 females (G1, G2 and G3) or pLN of naive B6 females (G4), were compared for their ability to prime resting Marilyn CD4 T cells (Fig. 7). CD11c⁺ cells from G1, G2 and G3 induced proliferation whereas those from G4 did not (top panel of Fig. 7), demonstrating that MB49-derived HY antigens were expressed by DC in dLN as early as 7 days after MB49 inoculation, reached the peak expression level at day 14, and sustained this until at least day 21. Responses to CD11b⁺ cells were lower, peaking at day 7 and declining thereafter (middle of Fig. 7), whereas B cells did not stimulate a response (bottom of Fig. 7). When Marilyn CD4 T cells were replaced by WT CD4 T cells in the above assays, no proliferation was induced (not shown), indicating that the responses observed were HY-specific.

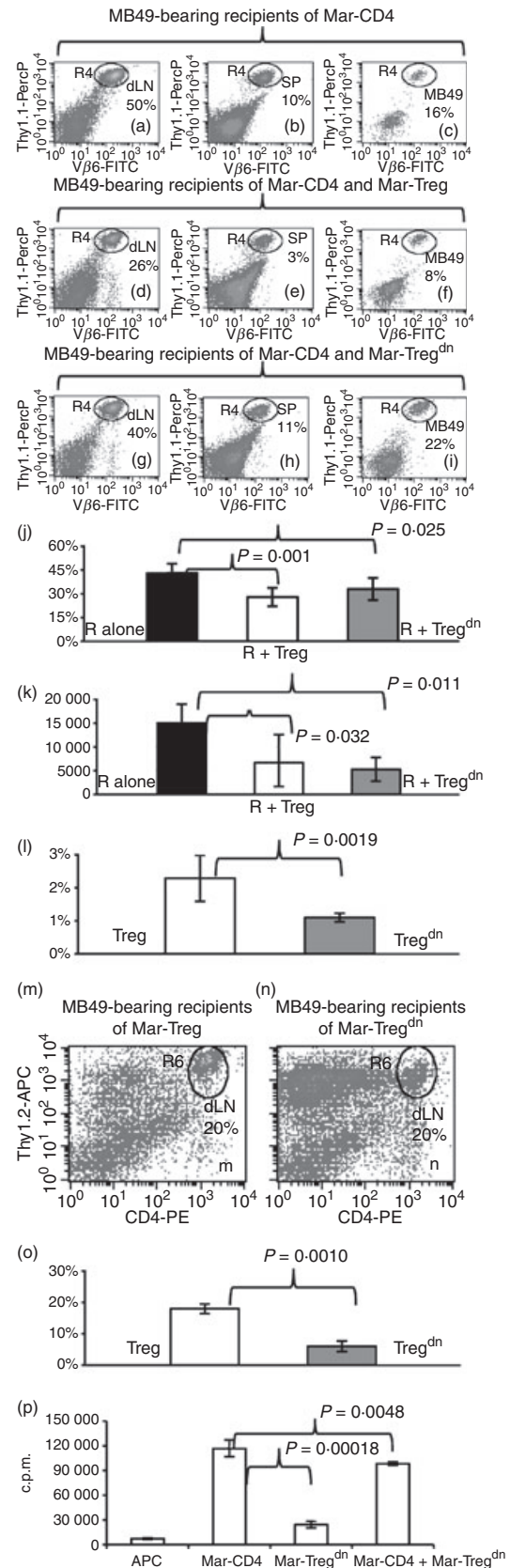
Discussion

Our study establishes an *in vivo* model by which the fate of tumour-antigen-specific nTreg cells in response to a growing tumour can be followed. There are several unique features in this model. First, the surrogate tumour antigen, HY, can be expressed either endogenously (MB49) or exogenously (B16/HY). Importantly, gene transfer does not necessarily result in an over-expression, indeed the B16/HY line expresses a low level of HY, which is stable both *in vitro* (Fig. 3a) and *in vivo* (Fig. 3b). Second, unlike other studies in which the detection of transferred nTreg cells in tumour-bearing mice relies on a viral vaccination,^{10,11} we were able to show the expansion of antigen-specific nTreg cells by tumour without any boost. Third, unlike other investigations in which the detection of nTreg cells is limited to spleen,^{10,11} we were able to detect the progeny of antigen-specific nTreg cells in both tumour tissues and tumour-dLN. Finally, the application of this

model can be extended, for example, MB49 and B16/HY can be injected either s.c. or i.v.²⁴ In addition, a comparison of nTreg behaviour in response to normal (male skin grafts) and tumour tissues can be made.

The first observation of the current study is that the ability of inducing non-specific nTreg-cell expansion is dependent on the tumour cell lines used. This conclusion was based on the results from two sets of adoptive transfer experiments. When B16 and MB49 tumours were compared for their capacities for inducing non-specific nTreg expansion, we found that adoptively transferred polyclonal nTreg cells underwent expansion in dLN of B16-bearing mice (data not shown) but they failed to do so in MB49-bearing mice (Fig. 2d–f), indicating that B16 but not MB49 tumours are able to expand nTreg cells non-specifically. In the second adoptive experiment, we found that the expansion of HY-specific nTreg cells could be induced by both HY-negative B16 and HY-positive B16 tumours *in vivo* (Fig. 4), indicating that B16 is capable of expanding nTreg cells non-specifically. Because MB49 cells constitutively express HY and an HY-negative

Figure 6. Comparison of Marilyn regulatory T (Treg) cells with or without dnTGF β RII *in vivo* and *in vitro*. Five groups of Rag2^{-/-} B6 females (four mice/group) were transferred with 2×10^4 Thy-1.1 Marilyn CD4 T cells alone (a–c), 2×10^4 Thy-1.2 Marilyn Treg cells alone (m), 2×10^4 Thy-1.2 Marilyn Treg cells co-expressing dnTGF β RII alone (n), or Thy-1.1 Marilyn CD4 mixed with either Thy-1.2 Marilyn nTreg cells (d to f) or Thy-1.2 Marilyn Treg cells co-expressing dnTGF β RII (g–i). The mice in all groups were also inoculated subcutaneously with 1×10^5 MB49 cells on the same day. Twelve days later, analysis was performed by staining dLN, spleen and MB49 tissues with anti-V β 6^{FITC}, anti-CD4^{PE}, anti-Thy-1.1^{PerCP} and anti-Thy-1.2^{APC}. The donor responder and donor Treg cells were identified by co-expression of Thy-1.1 and V β 6 (R4, a–i) and Thy-1.2 and CD4 (R6, m–n), respectively. The representation of donor responder cells in dLN of three groups [responders alone (R alone), responders plus Marilyn Treg cells (R+Treg), and responders plus dnTGF β RII-expressing Marilyn Treg cells (R+Treg^{dn})] was displayed as frequency (j) or absolute cell numbers (k). (l) Frequency of donor Treg cells in draining lymph nodes (dLN) of two co-transfer groups [responders plus Marilyn Treg cells (Treg), and responders plus dnTGF β RII-expressing Marilyn Treg cells (Treg^{dn})]. The representation of donor Treg cells in dLN of two groups [Marilyn Treg cells alone (Treg), and dnTGF β RII-expressing Marilyn Treg cells alone (Treg^{dn})] is shown in (m, n), (o) shows the comparison of the frequency of donor Treg cells in dLN of these two groups. One representative experiment of two is shown. A total of 0.5 million events was acquired for each sample. (p) *In vitro* suppression assay. Marilyn CD4⁺ CD25⁻ T cells alone (1×10^4 /well), Marilyn CD4⁺ CD25⁺ dnTGF β RII⁺ cells alone (1×10^5 /well), or both populations were incubated with 50 nM HY/Dby peptide in the presence of antigen-presenting cells (5×10^4 /well, T-cell-depleted B6 female spleen cells) in round-bottomed 96-well plates for 3 days. Thymidine was added to each well for the last 12 hr. One representative experiment of two is shown.



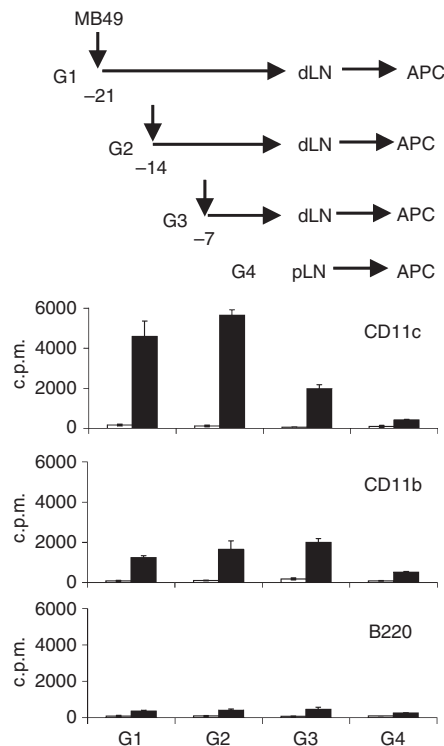


Figure 7. CD11c⁺ cells in draining lymph nodes (dLN) of MB49 tumour display HY antigens. Three groups of B6 females (two mice/group) were inoculated with MB49 cells at day -21 (G1), day -14 (G2) or day -7 (G3) whereas mice in G4 were not. At day 0, pooled dLN cells in G1, G2 and G3 and pooled peripheral lymph node (pLN) cells in G4 were digested before magnetic antibody cell sorting positive selection. Purified CD11c⁺, CD11b⁺ and B220⁺ cells were irradiated before culturing at 3×10^4 /well without (white bars) or with (black bars) purified Marilyn CD4 T cells (1.7×10^5 /well) in 96-well round-bottomed plates for 3 days.

MB49 cell line is not available, MB49-induced antigen-specific nTreg-cell expansion in the presence or absence of HY cannot be assessed. Although we have not used HY-knockout MB49 cells, it is highly unlikely that MB49 is able to induce non-specific nTreg-cell expansion, as MB49 cannot expand polyclonal nTreg cells *in vivo* (Fig. 2d–f). The conclusion that B16 but not MB49 can expand Treg cells non-specifically helps to explain why B16-bearing mice display a significant increase of percentage of nTreg cells in dLN⁹ whereas MB49-bearing mice do not (data not shown). However, the weaker expansion of Treg cells in WT female B6 recipients does not represent the most sensitive model system to examine antigen non-specific Treg-cell expansion by tumours. To make a conclusive demonstration, tumour-bearing Rag2^{-/-} recipients of Treg cells should be used in future studies. Whether the capacity of inducing antigen-non-specific nTreg-cell expansion is restricted to spontaneous tumour cell lines remains to be determined. In addition, B16 cells are less immunogenic than MB49 cells. Whether there is

a correlation between immunogenicity and capacity of inducing non-specific nTreg-cell expansion also needs to be explored further.

Interestingly, there was a significant reduction of Foxp3 expression by a fraction of donor Treg cells in response to HY antigens from MB49 (Fig. 2b) and B16/HY tumours (Fig. 4i–n). Loss of Foxp3 was more marked in tumour-infiltrating donor Treg cells than in dLN. In addition, we show that development of Foxp3-negative donor cells (called ex-Treg cells) represents differentiation from green Treg cells and is not the result of the expansion of contaminating conventional CD4 T cells. Moreover, Treg cells are converted to Th1-like responder cells after losing Foxp3 (manuscript under preparation, C. Addey and J.-G. Chai).

The second observation is that although both antigen-specific and non-specific expansion contribute to the overall pool of adoptively transferred nTreg cells with defined antigen specificity, nTreg cells expanded through these two pathways display distinct tissue distribution. Although several pairs of antigen-negative and antigen-positive tumour cell lines are available, direct comparison of the contributions from antigen-specific and non-specific expansion has not been examined. Consequently, there is very limited information regarding the behaviour of nTreg cells expanded by these two pathways. Our current investigation provides a detailed comparison showing that the expansion of nTreg cells with defined antigen specificity in the presence of antigen is more potent than that in the absence of antigen. It is not surprising, as the expansion of Marilyn nTreg cells by B16/HY results from a combination of two pathways. Importantly, we have shown for the first time that nTreg cells undergoing antigen-specific expansion can migrate to tumours where nTreg cells undergoing non-specific expansion cannot (Fig. 4). Hence, unlike expansion, migration of nTreg cells into tumours is strictly dependent on the presence of antigen.

The third observation is that TGF- β produced by MB49 cells (Fig. 5a) is a key factor promoting expansion of Marilyn nTreg cells (Fig. 5c–e). This conclusion is based on the results showing that the presence of intact TGF- β signalling on Marilyn Treg cells appears to be essential for their expansion in response to MB49 tumours (Fig. 6). A similar strategy has been used by Zitvogel and colleagues to dissect the role of TGF- β in the induction of expansion of polyclonal nTreg cells by B16 tumours *in vivo*,⁹ but their study was restricted to endogenous polyclonal nTreg cells. In this respect, our study offers an example showing that TGF- β is required for the expansion of tumour-antigen-specific, monoclonal nTreg cells (Fig. 6). A caveat to this conclusion is the potential for a developmental defect of nTreg cells expressing the dnTGF β RII which may prevent appropriate activation in response to antigen. To address this concern,

we compared dnTGF β RII-negative and dnTGF β RII-positive Marilyn Treg cells both *in vivo* and *in vitro*. *In vitro*, dnTGF β RII-expressing Marilyn Treg cells are clearly anergic and have weak suppressive activity (Fig. 6p). Moreover, *in vivo*, dnTGF β RII-positive Marilyn Treg cells can inhibit anti-HY T-cell responses (Fig. 6g–i), although they are less potent compared with Marilyn Treg cells (Fig. 6d–f). Finally, dnTGF β RII-positive Treg cells expand less efficiently than dnTGF β RII-negative Treg cells in response to HY antigens from MB49 tumours regardless of the presence or absence of co-transferred conventional Marilyn CD4 T cells (Fig. 6l–o).

Additional *in vivo* TGF- β neutralization experiments are needed to confirm the requirement for TGF- β signalling for *in vivo* expansion of nTreg cells. Although the MB49 cell line can make TGF- β (Fig. 5a), we have not examined whether growing MB49 tumours would produce TGF- β . In this regard, it has been reported that B16 tumours induce a subset of DC in dLN to produce active TGF- β , which then expands polyclonal nTreg cells.⁹ In addition, tumour-specific Treg cells are also able to induce tumour resident immature DC to produce TGF- β .²⁷ Apart from TGF- β , indoleamine 2,3-dioxygenase is another molecule that can directly contribute to Treg-cell expansion.^{25,26}

The final observation is that CD11c⁺ cells in dLN display major histocompatibility complex/HY peptide complexes (Fig. 7) and therefore are likely to be the major APC subset involving in antigen-derived expansion of Marilyn nTreg cells. Although tumour antigens persist in the dLN of tumour-bearing mice,¹² it has not been investigated which population of APC are involved in antigen presentation. In this aspect, our study provides the first direct evidence for the role of CD11c⁺ cells. Although the MB49 cell line does not express H2A^b *in vitro*, it has been suggested that freshly isolated MB49 tumour cells do.²⁰ This raises the possibility of direct antigen presentation by tumour cells to CD4 T cells. We have not investigated whether freshly isolated MB49 tumour cells would be able to stimulate Marilyn CD4 T cells in the presence of interleukin-2 *in vitro*.

In summary, using this HY system, which is composed of HY-expressing tumour cell lines and HY-specific nTreg cells, we have revealed novel aspects of the dynamic behaviour of tumour-antigen-specific nTreg cells for the first time. We demonstrate that the induction of non-specific nTreg-cell expansion does not represent a common rule for all tumour lines. By making a side-by-side comparison between antigen-specific and non-specific nTreg-cell expansion, we provide an example where the relative contribution by two pathways can be evaluated. Moreover, for the first time, we uncover distinct nTreg-cell migration patterns depending on whether expansion is antigen dependent or independent. These findings explore mechanisms responsible for nTreg-cell expansion by

tumours and have implications for understanding and manipulating tumour immune evasion.

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Disclosures

The authors declare that they gave no competing financial interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. The impact of transferred HY-specific nTreg cells on MB49 tumour progress.

Figure S2. The impact of transferred HY-specific nTreg cells on B16/HY tumour progress.

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