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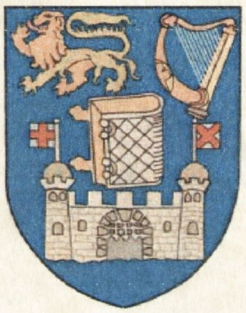
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Manipulating regulatory responses in antitumour immunity

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MSc Immunology and Infectious diseases



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Supervisor: Prof Kingston Mills

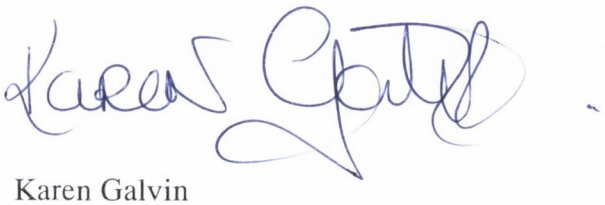
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Karen Galvin

ABSTRACT

The immune system has evolved tolerogenic and regulatory mechanism to control responses to self-antigens and to ensure that the inflammatory immune response to pathogens does not cause excessive tissue damage during infection. Whilst regulatory T cells (Treg) play a pivotal role in maintaining immune tolerance, tumour cells have the capacity to exploit the regulatory mechanisms of the immune system to evade anti-tumour effector immune responses. Immune subversion by tumours, especially their ability to induce or activate Treg cells, is a major barrier in the development of effective immunotherapeutics and vaccines against cancer. Therefore, in order to develop a successful therapeutic vaccine against cancer, immune tolerance must be overcome to allow the generation of anti-tumour $CD4^+$ Th1 cells and $CD8^+$ cytotoxic T lymphocyte (CTL) responses.

This study aimed to manipulate tumour-derived immunosuppressive molecules, such as TGF-β, in an attempt to break immune tolerance and improve the efficacy of cancer immunotherapies. The results revealed that tumour cells secrete high concentrations of TGF-β, a cytokine that can convert naive T-cells into $Foxp3^+$ Treg cells. Transiently silencing TGF-β using small interfering RNA (siRNA) in tumour cells inhibited TGF-β production *in vitro* and reduced tumour growth *in vivo*. Furthermore, inhibiting local TGF-β at the site of the tumour in combination with therapeutic administration of an antigen-pulsed and TLR-activated dendritic cell (DC) vaccine significantly attenuated B16 tumour growth *in vivo*. Protection induced with the vaccine was associated with a significant reduction in tumour infiltrating $FoxP3^+$ Treg cells and $IL\text{-}10^+$ Treg cells and enhancement of tumour infiltrating $CD4^+$ and $CD8^+$ T cells.

Infection with the helminth parasite *Fasciola hepatica* has been shown to promote the induction of Treg cells via TGF-β production. This study found that infection of mice with *F. hepatica* significantly enhanced tumour growth in both lung and subcutaneous tumour models. Exacerbation of tumour growth was associated with a dramatic enrichment of tumour infiltrating $FoxP3^+$ Treg cells. Furthermore, administration of *F. hepatica* excretory secretory products significantly enhanced tumour growth *in vivo,* through TGF-β-dependent mechanisms. This suggests parasite-induced TGF-β and Treg cells can exert bystander suppression of anti-tumour effector T cell responses that control tumour growth *in vivo*.

Retinoic acid (RA) has also been shown to promote conversion of naïve T cell into Treg cells. This study found that a RA receptor inhibitor (RARi) blocked conversion of $CD25^-Foxp3^-$ cells into $CD25^+Foxp3+$ Treg cell by both TGF-β and RA *in vitro*. In addition, RARi significantly suppressed TGF-β and IL-10 and enhanced IL-12 production by DC in response to killed B16 tumour cells or TLR agonists. Furthermore, RARi significantly augmented the efficacy of an antigen-pulsed and TLR-activated DC vaccine, significantly attenuating tumour growth and enhancing survival *in vivo*. This protective effect was associated with a substantial increase in tumour infiltrating Th1 and CTL cells and a corresponding reduction in tumour infiltrating $Foxp3^+$ and $IL\text{-}10^+$ Treg cells. In conclusion, the findings of this study demonstrate that that TGF-β and RA are important targets for the development of effective anti-tumour immunotherapeutics and for improving the efficacy of cancer vaccines.

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LIST OF ABBREVIATIONS

Ag	Antigen
Alum	Aluminium
APC	Antigen presenting cell
APC	Allophycocyan
ATP	Adenosine triphosphate
ATRA	All-trans retinoic acid
A-Treg	Adaptive Treg cells
bp	Base pair
BCG	Bacillus Calmette Guerin
BMDC	Bone marrow-derived dendritic cells
BMPs	Bone morphogenetic proteins
BSA	Bovine serum albumin
CIA	Collagen induced arthritis
CD	Crohn's disease
CD40L	CD40 ligand
CFA	Complete Freunds adjuvant
CpG	Cytosine-phosphate-guanine
CpG-ODN	Cytosine-phosphate-guanine synthetic oligonucleotide motif
CTL	Cytotoxic T lymphocyte
CTLA-4	CTL antigen-4
CXCL	CXC chemokine ligand
DC	Dendritic cell
DLN	Draining lymph node
DNA	Deoxyribonucleic acid

DPT	Diphtheria-pertusis-tetanus
dPBS	Dulbeccos PBS
ds	Double stranded
DT	Diphtheria-tetanus
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
ES	Excretory secretory
ERK	Extracellular signal related kinases
EtOH	Ethanol
FACS	Fluorescence activated cellular sorting
F. Hepatica	*Fasciola hepatica*
FCS	Foetal calf serum
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
FoxP3	Forkhead winged box transcription factor
GALT	Gut associated lymphoid tissue
GATA-3	GATA binding protein 3
GDFs	Growth differentiation factors
GITR	Glucocorticoid-induced TNF-receptor-related protein
GMCSF	Granulocyte-macrophage colony-stimulating factor
GFP	Green fluorescent protein
HBSS	Hanks balanced salt solution
HDACs	Histone deacetylases
HIV	Human immunodeficiency virus

HLA	Human leukocyte antigen
HSP	Heat shock protein
HRP	Horseradish peroxidase
HPV	Human pappiloma virus
IBD	Inflammatory bowel disease
ICOS	Inducible costimulator
iDC	Immature DC
IDO	Indolamine-2, 3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IκB	Inhibitor of NFκB
i.p.	Intraperitoneal
IPEX	Immune dysregulation polyendocrinopathy enteropthy X-linked syndrome
IKK	IκB kinase
IL	Interleukin
IL-R	Interleukin receptor
IRAK	IL-1R-associated kinase
IRF	Interferon regulatory factor
iTreg	Induced Treg
JAK	Janus activated kinase
Jak2	Janus kinase 2
JNK	Jun n-terminal kinase
LAP	Latency associated peptide
Lp	Lamina propria

LPS	Lipopolysaccharide
LRR	Leucine rich repeat
LTA	Lipoteichoic acids
MAPK	Mitogen-activated protein kinase
mDC	Mature DC
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MKK	MAP kinase kinase
MKKK	MAP kinase kinase kinase
MDDC	Monocyte derived dendritic cell
mLN	Mesenteric lymph node
MPL	Monophosphoryl lipid A
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MS	Multiple sclerosis
NFκB	Nuclear factor κB
ng	Nanogram
NK	Natural killer
NLR	NOD-like receptor
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
nTreg	Natural Treg
PAMPs	Pathogen-associated molecular patterns
PAP	Prostatic acid phosphatase
PBS	Phosphate-buffered saline

pDC	Plasmacytoid DC
PE	Phycoerythrin
pg	picogram
PGE_2	Prostaglandin E_2
PI3K	Phosphoinositide 3-kinase
PKA/C	Protein kinase A/C
PLP	Proteolipid protein
PMA	Phorbol mysteric acid
PP	Peyer's patches
PRR	Pattern recognition receptor
PT	Pertussis toxin
RA	Retinoic acid
RA	Rheumatoid arthiritis
RADHs	Retinoid dehydrogenases
RALDHs	Retinaldehyde dehydrogenases
RANTES	Regulated on activation normal T cell expressed
RARE	Retinoic acid response element
RARs	RA receptors
ROR-γt	Retinoic acid receptor-related orphan receptor-γt
RISC	RNA-induced silencing complex
RLHs	RIG-1 like helicases
RNAi	RNA interference
RPMI	Roswell Park Memorial Institute
RXRs	Retinoid X receptors
siRNA	Small interfering RNA

SV	Sendai virus
SOCS	Suppressor of cytokine signalling
STAT	Signal transducer and activator of transcription
s.c.	subcutaneous
TCR	T cell receptor
TGF-β	Transforming growth factor-β
Th	T helper cell
TIL	Tumour infiltrating lymphocytes
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Treg	T regulatory cell
Tr1	Type 1 regulatory T cell
TRAF	TNFR-associated factor

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Chapter 1

Introduction

1.1 Overview of the immune system

The main function of the immune system is to protect the host against invasion by foreign pathogens. The host immune response is a tightly controlled reaction to foreign substances, ranging from pathogens to macromolecules, such as proteins and polysaccharides. The mammalian immune system consists of innate and adaptive arms, functioning together to discriminate self from non-self and protect the host against foreign bodies and cancer. The innate immune system provides the first line of defense, responding rapidly to invading pathogens, while also influencing the nature of the ensuing adaptive immune response. The adaptive immune system acts at a later phase of infection and is responsible for immunological specificity and memory.

1.1.1 The innate immune response

The innate immune system detects the presence and nature of foreign pathogens, providing the initial response of the body to eliminate microbes and prevent infection. The innate immune response consists of two main defense mechanisms: constitutive and inducible. The constitutive mechanisms include the barrier functions of the body such as the skin and epithelial of the respiratory, gastrointestinal and reproductive tracts. When these barriers are breached, the inducible defense mechanisms are activated immediately upon recognition of foreign microbes. The effector mechanisms of the inducible response are largely mediated by phagocytic cells including macrophages, neutrophils and dendritic cells (DCs) [1]. Phagocytic cells either kill invading pathogens following

phagocytosis, or they produce chemokines and cytokines, which attract cells of the adaptive immune system to the site of infection. Natural killer (NK) cells also function as part of the innate immune system, killing virally infected cells or tumour cells [2]. NK cells can kill infected cells by apoptosis, which is mediated by death receptor signalling, or by lysing infected cells through the release of granzyme or perforin granules. Collectively, the inducible defense mechanisms of the innate immune system play a vital role in combating the initial stages of infection and control pathogen proliferation, while directing the nature of the adaptive immune response through the production of cytokines and chemokines.

1.1.2 Innate immune recognition

The innate immune system senses danger signals via pathogen recognition receptors (PRRs), germline encoded non-clonal receptors, that are expressed by a variety of innate cells [3]. PRRs can be expressed on the cell surface, intracellular compartments and they can also be secreted into the blood stream and tissue fluids [4]. The fundamental function of PRRs is to sense danger via the recognition of conserved molecular structures associated with microbial pathogens, termed pathogen associated molecular pathogens (PAMPs) and mediate innate immune responses to pathogens and tumour antigens [1].

PRRs can be divided into several families, including intracellular Nod-like receptors, RIG-1 like helicases (RLHs) as well as extracellular receptors, including scavenger receptors and C-type lectins receptors [5]. However, the toll-like

receptors (TLRs) are the most studied family of PRRs and activate innate immunity via the recognition of PAMPs such as: lipopolysaccharide (LPS) from Gram-negative bacteria, lipoteichoic acids (LTA) from Gram-positive bacteria, cytosine-guanine dinucleotide (CpG) motifs, lipoproteins and double stranded (ds) RNA. TLRs are located extracellularly on the cytoplasmic membrane (TLR 1, 2, 4, 5 and 6) or intracellularly in the lysosome/endosome membranes (TLR 3, 7, 8, and 9) [6]. Activation of TLRs by surface or intracellular TLR agonists results in the induction of a number of downstream signalling pathways, including nuclear factor κB (NFkB), interferon regulatory factor (IRF) pathways and mitogen-activated protein kinase (MAPK) p38 pathways [7]. Depending on the specific TLR activated, a number of signalling cascades leads to the expression of numerous genes that primarily function in inflammation. This pro-inflammatory response, however, is not mutually exclusive as TLRs can also induce regulatory responses, such as IL-10 [8].

1.1.3 Dendritic cells

Dendritic cells (DC) are potent antigen presenting cells (APC) that have the ability of bridging innate and adaptive immune responses by activating and stimulating naïve T cells of the adaptive immune system [9]. DC are derived from haematopoietic stem cells in the bone marrow and are composed of distinct subpopulations classified on the basis of surface antigen expression, such as, CD8α, B220 and CD103. Distinct subtypes of DC have different functions and can activate distinct T cell subtypes or can induce immune tolerance by inducing T

regulatory (Treg) cells or unresponsive effector T cells [4]. It has been proposed that $CD8\alpha^+$ DC promote T helper (Th)1 responses, $CD8\alpha^-$ DC promote Th2 responses, while $CD103^+$ DC of the gut induce regulatory Treg responses [10-12].

DC recognise foreign pathogens directly and indirectly by PAMPs and inflammatory mediators respectively, playing important roles in immunity to both infection and tumours. DC exist in two functional states; immature DC (iDC) and mature DC (mDC) [13]. iDC are located in the epithelia of the skin, respiratory and gastrointestinal systems where they patrol the portals of entry for foreign microbes. iDCs are highly phagocytic and extremely efficient at antigen processing, however, they express very low levels of major histocompatability complex (MHC) class II and co-stimulatory molecules and are therefore poor APC [9]. MHC molecules sample and present the MHC-peptide complex without discriminating between 'self' and 'foreign' peptides. Thus APC convert protein antigens to peptide-MHC complexes, which they display on their cell surface for recognition by T cells [14].

1.1.4 DC Maturation

DC are specialized to capture proteins from foreign antigens and process them into antigenic peptides, which are then presented to naïve T cells on MHC class I and class II molecules [9]. After antigen uptake in the peripheral tissue, DC undergo several morphological, phenotypical and functional changes, a process known as maturation. During maturation DC shut off their antigen capture and processing ability, and increase their expression of CCR7 which, enhances their motility and

controls DC migration to the lymphoid organs [15]. Once in the lymph nodes, DC secrete chemokines to recruit T cells, B cells, NK cells, macrophages and other DC subsets. Mature DC express high levels of MHC I and MHC II in order to efficiently present Ag to naïve T cells; co-stimulatory molecules (CD40, CD80, CD86) are also upregulated, providing the essential second signal for reinforcing the primary antigen specific signals in DC and T cell receptor (TCR) interactions [16]. The nature of the local cytokine milieu from which the DC was primed largely alters the DC response and the ability of DC to prime naïve $CD4^+$ and $CD8^+$ T cells which, subsequently influences T cell polarisation [17].

1.2 The Adaptive Immune System

Foreign pathogens that overcome the innate immune response are met by the second line of defense, the adaptive immune system. Adaptive immunity is mediated via humoral and cell-mediated immune responses, with both systems functioning to protect against various pathogens, such as allergens and self-antigens. Cell-mediated responses are generated by T cells, APC and effector cells, while humoral immune responses are mediated by antigen-specific B cells that secrete antibodies also known as immunoglobulins (Ig). The main effector cells of the adaptive immune system, T and B cells, proliferate clonally in response to foreign antigens, responding more powerfully to repeated exposure of the same microbe, providing specificity and long lived memory for enhanced recall responses [18].

1.2.1 T cell differentiation

Naïve T cells develop in the thymus before entering the periphery as a multipotential precursor, which upon activation with a defined antigen recognition system undergoes proliferation and differentiation via a distinct developmental pathway to attain specialized properties and effector functions. Differentiation of naive T cells is determined by three signals; signal 1 is delivered to the T cell through activation of the TCR, which recognises specific peptide antigens by engagement of MHC peptides by APC [19]. $CD4^+$ T cells recognise MHC class II peptides processed from antigens in the endosome and lysosome, while $CD8^+$ T cells recognise MHC class I peptides generated in the cytosol [20]. Signal 2 is provided by engagement of T cells and DC co-stimulatory surface molecules, such as CD28, CTLA4, CD80 and CD86 [21]. In the absence of this second signal, T cells become anergic, failing to induce an Ag-specific immune response, which can lead to immune tolerance [20, 22]. Signal 3 is generated by the production of cytokines from the primed APC and delivered to the T cells, influencing the direction of T effector cell responses, which can be subdivided into functional subpopulations termed cytotoxic T lymphocytes (CTLs), $CD4^+$ Th and Treg cells [22].

1.2.2 Th1 and Th2 cell lineages

Mosman and colleagues were the first to describe distinct lineages of $CD4^+$ T cell clones based on their function and the pattern of cytokines they express [23]. These cell lineages were classically recognised as Th1 and Th2 effector cells. Th1 cells

function to promote cell-mediated immunity to intracellular pathogens, such as bacteria and viruses, and tumour cells. The Th1 lineage is stereotypically defined on the basis of interferon-γ (IFN-γ) production, while also secreting TNF-α and TNF-β [23]. Conversely, Th2 cells operate in coordinating humoral immunity against parasites and extracellular bacteria, conventionally secreting the cytokines IL-4, IL-5, IL-10 and IL-13 [24-25]. Cytokines produced by mature effector cells of both lineages (Th1/Th2) can influence their own development through positive and negative feedback, acting on both naïve T cells and cells of the innate immune system [26].

1.2.3 Th1 and Th2 differentiation

The differentiation of naïve Th cells into Th1 cells is initiated through coordinate signals from the TCR and activation of signal transducer and activator of transcription (STAT)-1 associated cytokine receptors. Naïve T cells can be activated by IFN-γ produced by pathogen-stimulated cells, such as NK cells or by mature Th1 cells. In addition, IL-12 produced by APCs can also instruct the development of Th1 cells, therefore Th1 differentiation is largely dependent on the cytokine environment which can be TCR dependant or independent. Type I and II interferons, through their respective receptors, can both activate STAT-I as can IL-12, IL-18 and IL-27 [27]. The transcription factor T-bet is upregulated downstream of STAT-I signalling [28]. T-bet subsequently upregulates IL-12Rβ2 and enables IL-12 signalling through STAT4, which further potentiates the expression of IFN-γ and full commitment of the Th1 lineage [29].

Th2 commitment is also initiated by TCR signalling, which acts in conjunction with IL-4 receptor signalling to promote downstream activation of STAT6, which in turn upregulates expression of GATA-3 [30]. Recent reports suggest that basophils are an early source of IL-4, which promotes Th2 differentiation [31]. GATA-3 can further potentiate its own expression by autoactivation, which may subsequently drive the expression of the Th2 cytokines, IL-4, IL-5 and IL-13 [32]. Until recently it was believed that Th1 and Th2 responses were cross regulated, with IFN-γ and IL-12 favouring the activation of the Th1 pathway, while inhibiting the Th2 pathway and vice versa. However recent reports have suggested that Th1 and Th2 cells are not mutually exclusive and that plasticity amongst these committed cell lineages exist. It was shown that fully differentiated Th2 cells can also be reprogrammed into GATA-3^{+}T-bet^{+} cells which produce IFN-γ and IL-4 in response to antigen-specific TCR stimulation and inflammatory stimuli [33].

1.2.4 Th17 cells and their differentiation

A subset of IL-17 producing T cells (Th17) distinct from the Th1 and Th2 cells have recently been described and have been shown to play an important role in host defense against pathogen infections, such as bacterial and fungal infections [34]. IL-17 induces many inflammatory processes and stimulates fibroblasts, endothelial cells, macrophages, and epithelial cells to produce multiple proinflammatory mediators, including IL-1, IL-6, TNF-α, Nos2, and metalloproteases, while also inducing chemokines that enhance neutrophil recruitment [35]. In addition to their

protective role in host defense, Th17 cells also play a central role in various autoimmune and inflammatory disorders, including rheumatoid arthritis (RA), multiple sclerosis (MS), inflammatory bowel disease (IBD) and psoriasis [36-37].

Th17 cells secrete IL-17A, IL-17F, IL-21 and IL-22, however, most of the downstream pathological functions of these cells are mediated via IL-17A and IL-17F [38]. Work by the Weaver, Kuchroo, and Stockinger labatories have demonstrated that transforming growth factor-β (TGF-β) in the presence of IL-6 induces Th17 lineage commitment [39-41]. IL-17 production by committed Th17 cells is amplified by IL-1β or IL-1α in synergy with IL-23 [42]. The initial discovery that TGF-β was involved in promoting Th17 differentiation was unexpected, as TGF-β was primarily recognised as an immunosuppressive molecule associated with the development and function of induced Treg cells. However, recent reports show that TGF-β can block T-bet and STAT-6 signalling, maintaining commitment to the Th17 lineage and preventing conversion/plasticity of the Th17 subset into a Th1/Th2 phenotype [43-44].

While IL-23 is critical for the amplification and survival of Th17 cells, it is not necessary for the differentiation of this T cell subset [39-41]. Thus IL-23 augments IL-17 production from the memory pool of CD4 T cells, but not from naïve T cells, as naïve $CD4^+$ T cells do not express the IL23R [39]. Interestingly, γδ T cells express the IL-23R along with the transcription factor RORγT, producing IL-17, IL-21 and IL-22 in response to IL-1β and IL-23 [45]. This study demonstrated a

novel innate source of IL-17 whereby T cells secrete IL-17 without engagement of the TCR.

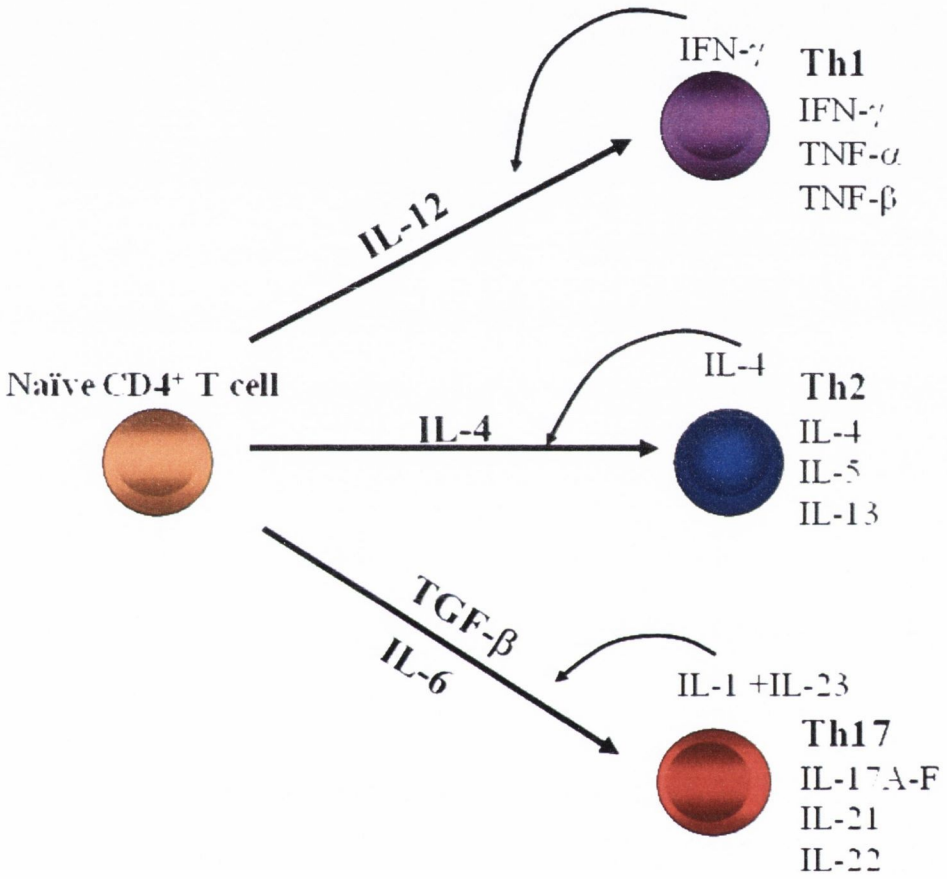


Figure 1.1 Overview of CD4 T helper cell differentiation

1.3 Regulatory T cells

While effector T cell responses are instrumental in protecting the host and mounting specific immune responses to foreign pathogens, uncontrolled or persistent generation of these pathways could prove detrimental for the host, resulting in inflammatory disorders such as allergy or autoimmune disease. Stringent mechanisms are in place to delete self-reactive T and B cell clones during their development in the thymus, however, there is clear evidence that self-reactive lymphocytes are present in the periphery of healthy individuals [46]. To avoid excessive and possibly damaging immune responses, post-thymic host suppressor mechanisms have evolved to dampen over active immune responses, and to prevent immune responses directed towards self-antigens, or commensal flora, thus avoiding autoimmunity.

In 1995 a study by Sakaguchi and colleagues showed that depletion of $CD4^+$ T cells expressing the alpha chain of the IL-2 receptor (CD25) from normal adult mice resulted in the development of a myriad of T cell mediated autoimmune diseases. This affect was ameliorated when $CD4^+CD25^+$ T cells were transferred into the immunocompromised recipient mice [47]. This landmark study resulted in the re-emergence of suppressor cells as Treg cells ($CD4^+$ $CD25^+$ and subsequently $FoxP3^+$ cells), which had been previously discredited due to an inability to identify specific markers [48]. Thus it is now broadly accepted that Treg cells are indispensable for maintaining self-tolerance, preventing immune responses to self-antigens and maintaining immune homeostasis [49].

It was originally believed that Treg cells solely developed and differentiated in the thymus [50] however, it is now known that Treg cells can also develop in the periphery from non-Treg cell progenitors, in a process known as peripheral conversion [51]. Several types of Treg cells have been described, with the identification of two main subsets; natural Treg (nTreg) cells and inducible Treg (iTreg) cells. nTreg cells develop in the thymus as a functionally mature cell population through a tightly regulated homeostatic process [47]. However, iTreg cells originate from uncommitted peripheral naïve or central memory Th cells following antigen stimulation, under the influence of anti-inflammatory molecules generated during pathological conditions, such as infection and transformed malignant progression [52]. Understanding the differentiation, function and balance of nTreg cells and iTreg cells is a major challenge in the field of immune tolerance, as there is a delicate balance between Treg cells dominating the immune responses and thus suppressing host responses to foreign pathogens or cancerous cells, whilst an inadequate Treg response could result in destruction of self-antigens and ultimately lead to autoimmunity.

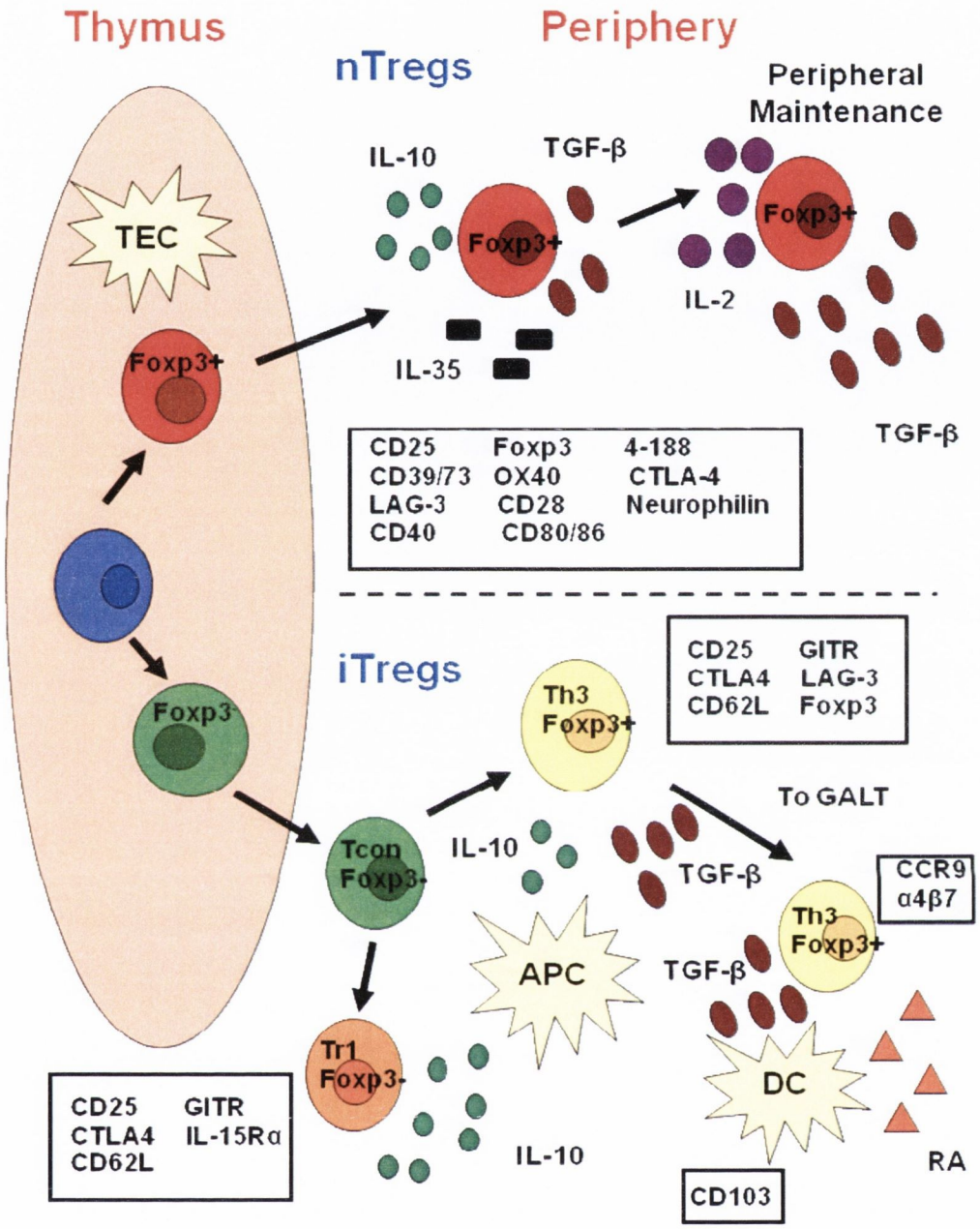


Figure 1.2 Thymic and peripheral generation of FoxP3^{+} Tregs

1.3.1 Natural Treg cells

nTreg cells are continually produced in the thymus as functionally mature T cells, requiring high avidity interactions with cognate self–MHC interactions [53]. They constitute 5-10% of T cells in mice and can be detected in the periphery three days after birth [54]. nTreg cells can be identified by the constitutive self-surface expression of CD25, however, as this marker can become transiently upregulated upon T cell activation it is not unique to Treg cells. Other markers and cell surface proteins have also been identified in the search for an exclusive Treg marker, such as cytotoxic T-lymphocyte antigen 4 (CTLA4) [55], the tumor necrosis factor-receptor family members GITR (glucocorticoid-induced TNF-receptor-related protein), OX40, CD39 and CD73 [56], CD38, CD62L, CD103 [57]. Nevertheless none of these markers are specifically restricted to naturally occurring Treg cells, as they can also be expressed on activated effector T cells [58].

In 2003, it was demonstrated that forkhead winged box transcription factor 3 boxP3 (FoxP3) a transcriptional repressor is specifically required for the development and function of Treg cells [59-61]. $FoxP3^+$ Treg cells are a distinct T cell lineage that the autoreactive TCR repertoire imprints with regulatory functions [62]. Mutations in FoxP3 result in the lymphoprolierative and multiorgan autoimmunity disease in mice, known as Scurfy [63]. The clinical symptoms in scurfy mice are very similar to a fatal autoimmune disorder in humans called immune dysregulation polyendocrinopathy enteropthy X-linked syndrome (IPEX), which also results from mutation in FoxP3 [64]. $FoxP3^+$ Treg cells are widely referred to as the 'master

regulator' of lymphocyte responses, with a proficient capacity to block active immune responses, such as inflammation and tissue destruction via suppressing numerous cell types, including $CD4^{+}$T cells, $CD8^{+}$ CTL activity and APC maturation and function [65]. However, FoxP3 is not an ideal marker as it too can be induced transiently in human naïve T cells upon activation [66] and due to its nuclear expression it cannot be readily used to purify Treg cells.

1.3.2 General Features of nTreg cells

Treg cells are normally anergic, failing to proliferate or to produce IL-2 in response to conventional T cell stimuli. This anergy can be broken by antigenic stimulation of the TCR as well as costimulation; engagement of CD28 is an absolute necessity for the generation of nTreg cells [67]. A two step model has been proposed for the differentiation of nTreg cells, an initial TCR signal of increased strength is required to upregulate CD25, allowing competent utilization of IL-2 signalling, which induces FoxP3 expression in a STAT5-dependent manner [68]. Treg cells express the $\alpha\beta$TCR which is required to confer antigen-specificity [69]. Once the suppressive activity of Treg cells is induced by antigenic stimulation, Treg cells can then go on to suppress in a non-specific fashion [70]. Irrespective of the fact that nTregs express the high affinity IL-2 receptor CD25 they cannot produce IL-2 themselves, due to chromatin inaccessibility of the IL-2 locus [71]. Accordingly, nTregs rely on paracrine IL-2 for survival and growth, while CD28 also promotes this survival through enhancing the production of IL-2 by conventional T cells, while maintaining CD25 expression [72].

1.3.3 Mechanisms of Suppression

The multifaceted function of Treg cells in regulating lymphocyte responses is remarkable given the numerous extrinsic perturbations that affect the host immune response. Treg cells have the capacity to inhibit the activation and expansion of naïve T cells as well as effector T cell function [73]. They can elicit inhibitory effects on the cytotoxic functions of $CD8^+$ T cells and NK cells, while being proficient inhibitors of the maturation and function of DC [74]. Treg cells constitutively express two negative regulators of TCR signalling, CTLA4 and CD5, which function to suppress T cell activation at the level of the TCR, in a cell-cell contact dependent manner [50]. Regulation and suppression of APC function is mediated via blocking or downregulating CD80/CD86 expression, which consequently limits the capacity of DC to stimulate naïve T cells through CD28 [75]. Activation of Treg cells also results in the upregulation of granzyme B, which can kill target cells by direct cytolysis [76].

Treg cells also mediate suppression via the secretion of suppressive cytokines, such as IL-10, TGF-β and IL-35 [77]. In contrast to the cell contact-dependent mechanisms, these cytokines suppress the local environmental milieu in a non-specific bystander fashion [78]. The role of IL-2, a cytokine involved in the growth and differentiation of T cells, in Treg cell mediated suppression has yet to be definitively resolved. Some reports suggest that Treg cells compete with conventional cells for IL-2, consuming high levels of paracrine IL-2, leading to the

deprivation of this growth factor which ultimately results in the incompetent proliferation and eventual apoptosis of conventional T cells [79]. Other reports have suggested that Treg cells inhibit the induction of IL-2 in the responder FoxP3$^-$ cells, which consequently suppresses target cells, [80-81]. Given the numerous methods of suppression utilized by Treg cells, it is highly likely that the exact mechanism by which Treg cells suppress effector immune responses is determined by a variety of contributing factors including, the target cell type, the anatomical location and the local environmental conditions.

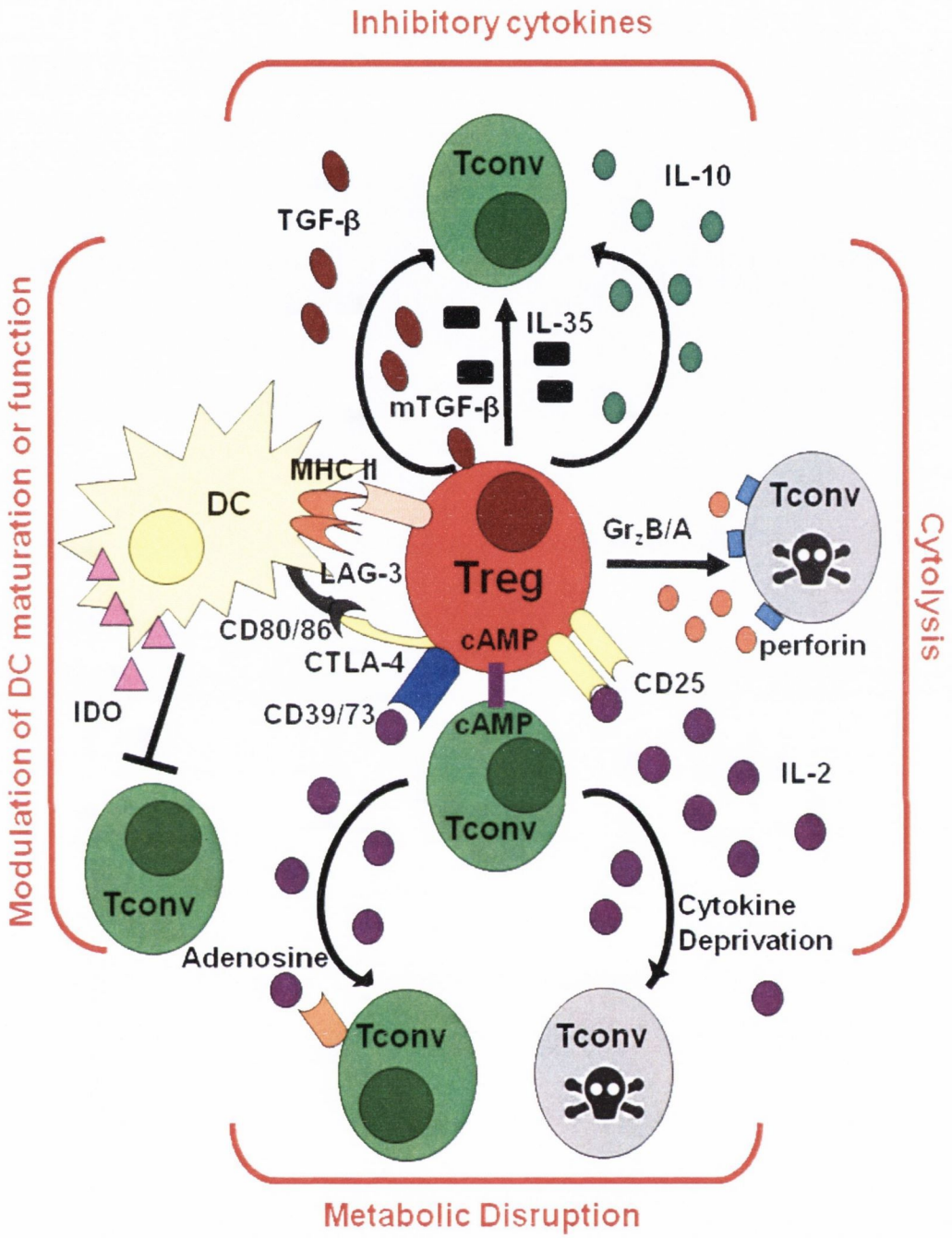


Figure 1.3 Mechanisms of Treg suppression

Adapted from: [76]

1.3.4 Inducible Treg cells

In contrast to nTreg cells, which are thymic derived, iTreg cells develop in the periphery from uncommitted naive $CD4^+$ T cells or central memory Th cells under a variety of conditions of sub-optimal antigenic stimulation [82]. Cytokine production rather than surface markers is used to classify and distinguish iTregs, with the characterization of two main populations: type 1 regulatory T cells (Tr1), which are induced by IL-10 [83] and T helper 3 cells (Th3), which are induced by TGF-β [84]. Weiner and colleagues first identified Th3 cells because of their role in oral tolerance, producing large amounts of TGF-β with varying amounts of IL-10 and IL-4. Tr1 cells are very similar to Th3 cells in that their suppression is also mediated by the same cytokine that is responsible for their induction, producing high levels of IL-10 and TGF-β. These cells were characterized as Treg cells through their role in preventing autoimmune colitis [85].

1.3.5 Tr1 cells

While the bona fide trademark of Tr1 cells is the high production of IL-10, which mediates suppression, low to moderate levels of TGF-β and IL-5 can also be expressed by Tr1 cells depending on the experimental conditions [86]. The suppressor activity of these Tr1 cells is cell-cell contact independent and is mediated primarily through the actions of the anti-inflammatory cytokines IL-10 and TGF-β. The essential role of IL-10 in Tr1 cells was validated by the addition of a neutralizing IL-10 antibody, which reversed the suppressive effects of Tr1 cells [83]. Tr1 cells were first shown to be antigen-specific in a murine model of

Bordatella pertusis infection; Tr1 cell clones shown specific for the bacterial antigens suppress proliferation and IFN-γ production by Th1 cells [86]. Like nTregs, these Tr1 cells have a low proliferative capacity, however they can expand in the presence of IL-2 and IL-15, and express high levels of these receptors subsequent to activation [87]. The generation of Tr1 cells is distinct from nTregs, and unlike nTregs these cells do not express FoxP3, while they exhibit variable expression of CD25 and other surface markers described for nTreg cells [84]. It is believed that the induction of Tr1 cells is essentially mediated by IL-10 producing APCs under conditions of heightened immunological settings, such as immature DCs, plasmacytoid DCs and tolerogenic DCs producing IL-10 in response to pathogens, tumor antigens and allergens [88].

1.3.6 Peripherally converted Treg cells

A large body of evidence has shown that $CD4^+$ T cells can be converted into $FoxP3^+$ Treg cells (iTreg cells) by TGF-β in response to self or non-self antigens in the periphery [89-91]. This 'conversion' can also be observed *in vitro* with naïve $CD4^+CD25^-FoxP3^-$ T cells inducing CD25 and FoxP3 expression in the presence of TGF-β and IL-2 [51]. iTreg cells can be generated from uncommitted naïve or central memory T cells *in vitro* and *in vivo* in mice and humans in the presence of TGF-β [92].

While natural and induced Treg cells are similar in phenotype and function, their induction and development involves two distinct pathways. nTreg cells develop in

the thymus in response to self-antigens that require high avidity interactions, while iTreg cells require weaker suboptimal TCR stimulation presented by DC in the periphery in response to environmental antigens [93]. Initial speculations existed, as to whether these peripherally converted Treg cells are a progeny of nTreg cells, where the CD25 receptor has become downregulated. However, it was then reported that TGF-β-dependent Treg cells could be generated in the periphery of $CD28^{-/-}$ mice, which completely lack nTregs [94]. The strong requirement for CD28 costimulation in nTreg cell development is not required for iTreg cells [95]. Whilst CD28 costimulation is not a prerequisite in the induction of FoxP3 expression it facilitates conversion, a process that is solely related to its capacity to enhance the endogenous production of IL-2, confirming that peripherally converted Treg cells develop from conventional $CD4^+$ T cells in a pathway that is distinct from nTreg cells.

TGF-β is a key cytokine involved in the generation of uncommitted naïve or central memory $CD4^+$ T cells into $FoxP3^+$ Treg cells in mouse and in humans [96]. The requirement for TGF-β in the induction of $FoxP3^+$ Treg cells is seen shortly after TCR stimulation, an effect that decreases over time, and at 72 hrs the ability of TGF-β to convert naïve cells $CD4^+$ $CD25^-$ cells into suppressor cells is lost [97]. In contrast to nTreg cells, TGF-β-induced $FoxP3^+$ expression in iTreg cells is transient and rapidly decreases in the absence of IL-2, as IL-2 works with TGF-β in converting $CD4^+CD25^-$ cells into $CD4^+CD25^+FoxP3^+$ Treg cells [98]. Other γ-chain cytokines, such as IL-15 or IL-7, are not capable of inducing Foxp3 expression in

FoxP3$^-$ T cells in IL2$^{-/-}$ mice [99]. Therefore, the role of IL-2 and TGF-β in the induction and expansion of peripherally converted Treg cells in mice is unique and non-redundant. While, TGF-β and IL-2 are not required for the generation of nTregs in the thymus, as they can develop in TGF-β and IL-2 deficient mice [97], it is required for the function and maintenance of Foxp3 expression in both subsets of Treg cells in the periphery [100]. Conventional T cells when stimulated with antigen produce IL-2, which in turn promotes the induction of TGF-β required to sustain Foxp3 expression [101].

Peripherally converted Treg (iTreg) cells are functionally similar to nTreg cells in that they are anergic, suppressive and capable of inhibiting inflammatory diseases *in vivo* [102]. Lafaille and colleagues demonstrated that mice that lack nTreg cells but have peripherally neoconverted iTreg cells are capable of mediating oral tolerance, as demonstrated by a reduced systemic immune response to antigen previously administered via the oral route [90]. It was then shown that mice incapable of inducing Treg cells in the periphery were incapable of mediating immune tolerance, suggesting that iTreg cell are critical regulators of induced immune tolerance [103].

Until recently, it was not possible to discriminate iTreg from nTreg cells in lymphoid tissue. However, recent studies by Shevach and colleagues have shown that Helios, an Ikaros family transcription factor, is a specific marker that is exclusive to thymic-derived Treg cell populations. The expression of helios is not

observed in peripherally induced Treg cells generated *in vitro* or *in vivo* in mouse or humans, with estimations that 30% of Treg cells in the periphery are generated extrathymically [70]. Accordingly, the expression of helios is a powerful marker to differentiate thymic-derived Treg cells, from peripherally-induced Treg cells and will prove very beneficial in determining the ratio of nTreg cells to iTreg cells in disease models.

1.4 Retinoic Acid

Retinoic acid (RA) is the metabolically active derivative of vitamin A, which functions primarily as a regulator of gene expression. It is an important morphogen that regulates a wide variety of biological processes, such as apoptosis, vision, reproduction and immune homeostasis [104]. A deficiency in vitamin A compromises many aspects of innate and adaptive immune responses, with vitamin A deficient individuals showing defects in immune control of bacterial, viral and protozoan infections [105].

Vitamin A is a fat soluble essential nutrient that cannot be synthesized by the human body, but must be absorbed by the intestine from animal food sources and/or derived from β-carotene, which is found in plants [106]. Subsequent to absorption vitamin A circulates in the peripheral blood as retinol and retinyl esters, which are primarily stored in the liver [107] . These precursors are enzymatically catalysed to RA in a two step process. Once inside a cell retinol is oxidized in a reversible reaction by widely expressed retinoid dehydrogenases (RADHs) to all-trans retinaldehyde (retinal) [108]. Retinal is then irreversibly metabolized into RA in a tightly controlled process by retinaldehyde dehydrogenases (RALDHs) [106]. Multiple isoforms of RA exist, however, all trans retinoic acid (ATRA) is the most predominant isoform in most tissues [109]. The synthesis of dietary vitamin A into ATRA is predominantly mediated by gut associated lymphoid tissue (GALT) DCs and macrophages [110].

ATRA or its isomer 9-cis RA exclusively binds to a family of nuclear hormone receptors termed RA receptors (RARs) and retinoid X receptors (RXR) via heterodimers that act as ligand dependent transcriptional regulators via a retinoic acid response element (RARE) within the promoter of genes [111]. The RARs interact with many different coactivators and corepressor proteins to regulate transcription, with ATRA being the major endogenous agonist for the RARs [112]. The RAR family consists of three subtypes RARα, RARβ and RARγ, which all bind ATRA with high affinity but slightly different binding domains. However, RARα is the predominant signalling receptor involved in immune regulation and homeostasis [104].

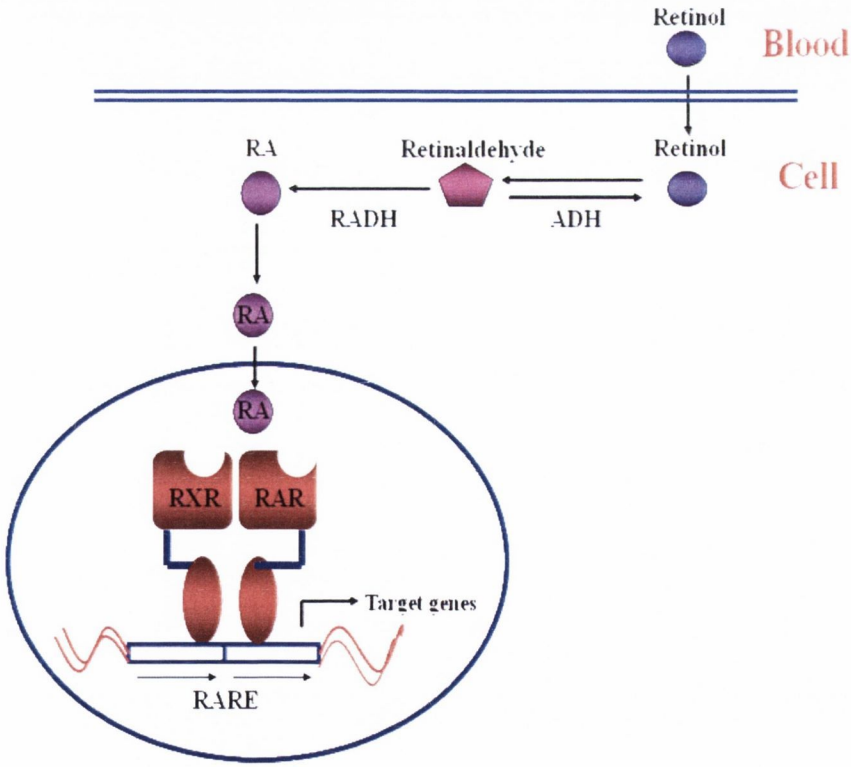


Fig 1.4 Metabolism of vitamin A into retinoic acid

1.4.1 Role of RA as an immune regulator

It is well established that TGF-β is a potent regulator of anti-inflammatory and pro-inflammatory $CD4^+$ T cell responses; contributing to the reciprocal regulation of Treg and Th17 cell development [113]. Several key studies have shown that RA synthesized from GALT DCs and macrophages can synergize with TGF-β to enhance the conversion of naïve $FoxP3^-$ T cells into a unique $FoxP3^+$ Treg cell subset, termed retinoid-induced $FoxP3^+$ T cells [91, 114-117]. Additionally, RA suppresses *de nova* differentiation of naïve $CD4^+$ T cells to Th17 cells in response to TGF-β and IL-6 [39-41]. A recent study also showed that the RA-RARα axis is also required to efficiently activate naïve T cells *in vivo* and subsequently modulate both regulatory and inflammatory arms of the immune response to foreign pathogens. Belkaid and Colleagues showed that vitamin A deficient mice have impaired Th1 and Th17 cell responses to *Toxoplasma gondii* (T. *gondi*), however, short term treatment with RA restored Th1 and Th17 responses and regained host protective immunity to *toxoplasmosis*, enhancing parasite clearance to that of control infected animals [118].

1.4.2 RA iTreg cells

Retinoid- iTreg cells are preferentially induced at mucosal sites, such as mesenteric lymph nodes (mLN) and in the lamina propria (Lp) [117, 119-120]. The enhanced capacity of gastrointestinal tract and associated lymphoid tissue (GALT) DCs to mediate peripheral conversion of $FoxP3^+$ T cells when compared with splenic DCs or DC in the periphery is likely associated with the ability of mucosal DC to synthesize RA [117]. One of the functions of DC is to home lymphocytes to the site of infection, where the foreign antigen is initially encountered [9]. In this regard, RA production by GALT DC directs gut tropism, by upregulating CCR9 and $\alpha_4\beta_7$ on conventional T cells and Treg cells [117].

Recent studies have shown that the intestinal tract is not the only immune privileged site to produce bona fide RA-producing DC that promote iTreg cells. The skin and its draining cutaneous lymph nodes also contain DC that constitutively produce RA and promote *de nova* generation of $FoxP3^+$ T cells in the absence of gut tropic properties such as CCR9 [121]. RA production from DC in the skin and intestinal tract likely promote iTreg cells to dampen down inflammatory responses to innocuous antigens and promote immune tolerance. However, it is interesting to note that RA promotes immune tolerance outside of the mucosal environments.

RA also controls the differentiation of TGF-β-dependent Treg cells in the absence of APC, indicating that RA can also directly control the transcription of naïve $CD4^+$

T cells in the presence of TGF-β [122]. Furthermore, RA promotes TGF-β-dependent FoxP3$^+$ T cell differentiation in CD8$^+$ T cells, suggesting that the regulation by RA is not restricted to the CD4$^+$ T cell lineage [116]. While the modulating effects of RA in immune regulation is largely mediated via the RA-RARα signalling axis [104, 122], the exact molecular mechanisms mediated by RARα in the enhancement of TGF-β-induced Treg cells has been the topic of much debate. Initial reports suggested that instead of enhancing TGF-β signalling directly, RA counteracts the inhibitory effects of memory effector T cells, CD4$^+$CD44^{hi} cells, influencing TGF-β-induced FoxP3 cells through an indirect manner, either by inhibiting the production of inflammatory cytokines (IFN-γ, IL-21, and IL-4), or potently suppressing the IL-6Rα [104]. However, subsequent studies have refuted the central hypothesis put forward by Hill et al., (2008) and proposed that RA can mediate its effects via several mechanisms, including acting directly on naïve CD4$^+$ T cells to enhance TGF-β-induced FoxP3 expression and indirectly via inhibiting the negative regulatory effects of CD4$^+$CD44^{hi} T cells on peripheral conversion [122].

1.4.3 RA maintains Treg stability and function

The *in vitro* suppressive capacity of RA-iTreg cells has been assessed relative to that of nTreg cells and peripherally converted A-Treg cells. Noelle and colleagues reported that not only are these RA iTreg cells potent suppressors of effector T cells *in vitro*, but RA-iTreg cells have the ability to suppress at ratios of Treg:T effector cell that freshly harvested nTreg and A-Treg cells could not suppress [120]. Recent controversial reports have suggested that some FoxP3$^+$ T cells are heterogeneous and can be reprogrammed to lose FoxP3 expression and induce effector T cell functions under inflammatory conditions [123-124]. RA-iTreg cells demonstrated less plasticity *in vivo*, with a lower propensity for reversion to FoxP3$^-$ cells. A-Treg cells and RA-iTreg cells were generated from Ly5.2$^+$OTII$^+$FoxP3/GFP reporter mice and transferred into Ly5.1 mice immunized with CFA or PBS control. After 5 days, up to 40 % of the A-Treg cells lost FoxP3 expression in CFA and PBS treated mice, however, RA-iTreg cells demonstrated more commitment to the Treg lineage with only a 9 % loss in FoxP3 expression [120]. Collectively, these reports show that RA-iTreg cells are functionally competent suppressor cells that are phenotypically more stable, even in the face of overt inflammatory immune responses.

When nTreg cells are stimulated *in vitro* with IL-6 a minority of FoxP3$^+$ cells from reporter mice lose commitment to the FoxP3 lineage and become Th17 cells [125-127]. However, nTreg cells pretreated with ATRA are more refractory to Th17 cell conversion in the presence of IL-6 when compared with untreated Treg cells [128].

Furthermore, when nTreg cells pretreated with ATRA were adoptively transferred into mice with established collagen induced arthritis (CIA), the progression of disease was attenuated and the severity of arthritis was ameliorated [128]. These observations suggest that RA maintains the stability and function of both iTreg and nTreg cell populations.

Understanding the mechanisms involved in peripheral conversion of naïve $CD4^+$ T cells to iTreg cells is imperative in the field of immune tolerance. Furthermore, manipulation of this pathway could prove to be powerful tool in breaking immune tolerance, leading to potential advances in the tumour immunotherapy field. The general consensus is that peripheral conversion contributes to immune tolerance and suppresses antitumour immune responses, therefore antagonising the RA-RARα axis could potentially block or limit the induction of iTreg cells in the periphery and provide immense benefits as a cellular therapy against tumour growth and development.

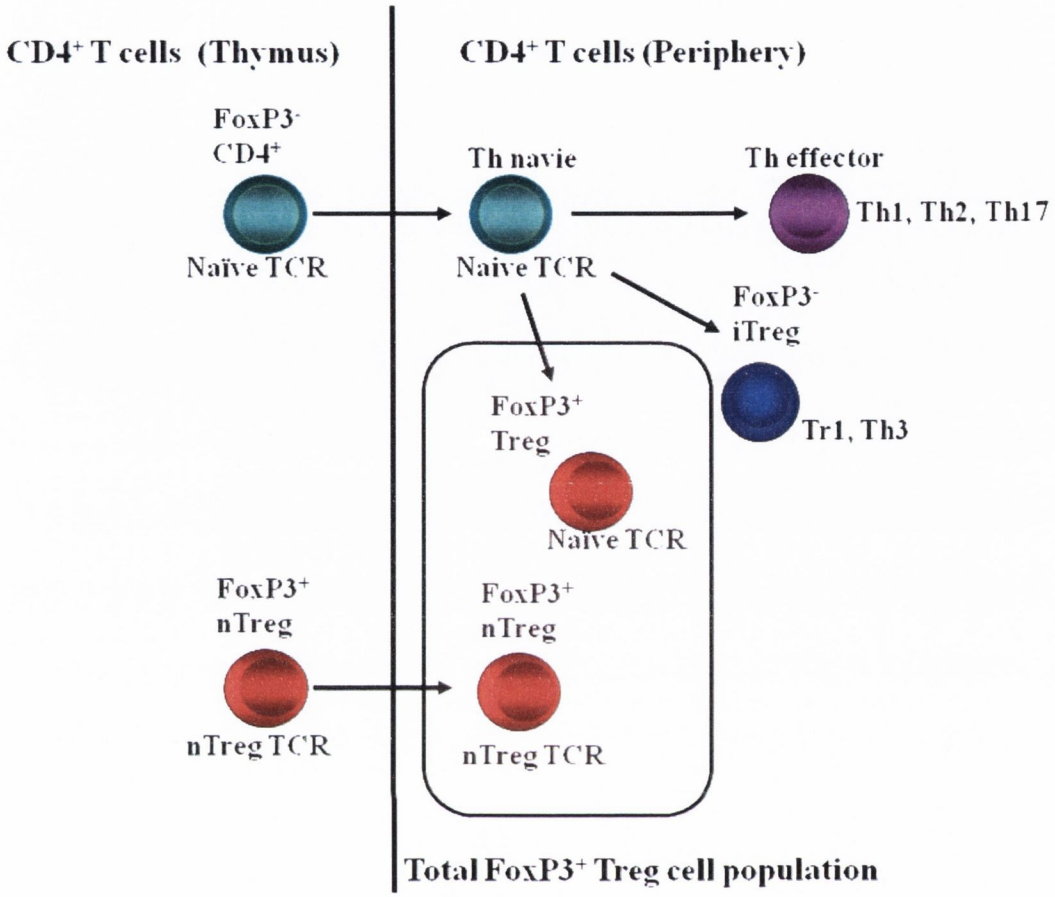


Figure 1.5 Development of nTregs and iTregs

From:[76]

1.5 Immunity to tumours and immune surveillance

"Cancer" is the Latin word for "crab", the ancients used the word to describe malignancy, due to the crab like invasion of cancer into healthy tissue and the migration of cancer cells into distant sites from the primary tumour.

Cancer is a generic term used to classify over a hundred diseases of uncontrolled division of genetically altered cells, followed by the invasion into surrounding tissues and spread to distal sites via the blood or lymphatic systems. To date, cancer is the leading cause of death worldwide and accounted for 7.6 million deaths in 2008. It has been estimated that deaths from cancer worldwide are projected to rise to over 11 million in 2030 [129]. There are several stages in the development of malignancies and carcinogenesis: initially hyperplasia takes place, the proliferation of cancerous cells within an organ or tissue, leading to neoplastic growth whereby the cells have developed into an abnormal mass, this is followed by local invasion and finally distant metastasis, where the cancer can spread to secondary organs [130].

For decades, primary cancer treatments include chemotherapy and radiation to eradicate the bulk of the tumour mass. Whilst these therapies have proven to be of considerable benefit with some cases of complete tumour elimination, the prevalence of relapsing tumours is a huge problem that results from the occurrence of drug resistant mechanisms in a percentage of tumours. Therefore, there is a

crucial need to establish additional therapies to overcome tumour growth and resistantance.

Tumour cell development and survival is dependent on multiple acquired and inherited genetic aberrations of normal cells in the body, this is a major challenge for the immune system as it recognises these tumour antigens as 'self antigens'. A promising strategy to break immune tolerance is active immunotherapy, which is designed to manipulate the immune system into generating a potent antitumour immune response. Active immunotherapy has the potential to be a safer and more effective approach to treating malignancies than current cytotoxic drugs, which have adverse side effects on the host. Pioneering observations made by the German bacteriologist Paul Ehrlich in 1909 lead to the discovery that the immune system can fundamentally control and protect the host against the development of cancer. This lead to the concept of tumour immune surveillance, put forward some 50 years later by Burnet and Thomas, which showed that the immune system recognises and destroys precancerous and cancerous cells that express tumour specific antigens on their surface [131]. However, tumour cells have evolved to evade immune surveillance and subsequent destruction by the immune system, while also inducing a local state of immune tolerance at the site of the tumour. Immunosuppressive mechanisms utilized by the tumour include the secretion of immunosuppressive molecules, the differentiation, recruitment and activation of Treg cells to the site of the tumour, and modifications in antigen expression by the tumour cells, such as downregulating MHC class I on the surface of the tumour cells [132]. Due to the

continuous emergence of tumour variants, which is in part due to the selective pressure from the immune response and the constant immune editing of these cells, new variants are occasionally able to evade immune surveillance resulting in tumour growth and progression of cancer [133].

1.5.1 Immune subversion by Treg cells in the tumour environment

Treg cells are an essential component of a healthy immune system, playing a pivotal role in maintaining self-tolerance and preventing the development of autoimmunity. Paradoxically, tumour cells have adapted ways to exploit the self-regulating mechanism of the immune system, utilising the suppressive functions of Treg cells to subvert host antitumour immunity. For this reason, the role of Treg cells in the development and growth of tumour cells is a key area of research in tumour immunology. A major aim in tumour immunotherapeutics is to develop novel approaches which target Treg cells and break immune tolerance to the growing tumour.

In the majority of studies increased numbers of Treg cells have been found in peripheral blood, lymph node and spleen of patients with cancer when compared with healthy controls [134-136]. Moreover, the tumour itself contains with significant numbers of infiltrating Treg cells [137-139], an accumulation of which is associated with poor prognosis [140]. With increasing evidence that Treg cells play an important role in the immune evasion mechanisms induced by the tumour, a number of studies in mice have reported that the removal of $CD4^+CD25^+$ cells prior

to tumour challenge greatly enhances immune surveillance and anti-tumour immunity, with subsequent rejection in a wide range of tumour models [141]. Additionally, depletion of nTreg cells *in vivo* enhances the efficacy of therapeutic cancer vaccines and restores tumour immune surveillance [142]. Intratumoral Treg cells are highly activated and undergo extensive proliferation, with enhanced expression of CD25, CTLA4 and GITR [143-145]. Recruitment of Treg cells has been seen in the early hyperplasia stages in pancreatic carcinoma model and lung adenocarcinoma, while studies in a fibrosarcoma model have shown recruitment of $CD4^+CD25^+Foxp3^+$ cells even before tumour transformation [146-148]. Thus elucidating the mechanisms by which tumour cells recruit and expand Treg cells in tumour development could provide an attractive therapeutic target for the treatment of tumours.

1.5.2 Recruitment and expansion of nTreg cells at the site of the tumour

Tumour cells exploit the immune system in a variety of ways to potently abrogate anti-tumour immune responses. One mechanism utilised by tumour cells to manipulate the immune response is to mimic a chronic inflammatory environment, where the tumour cells secrete various homing chemokines, one of which is CCL22, to recruit Treg cells [140]. CCR4 engagement of CCL22 actively recruits nTreg cells to the site of the tumour and surrounding lymph nodes, where they potently suppress antitumour immune responses [149]. Furthermore, the production of immune suppressive molecules in the growing tumour can stimulate local immature myeloid DCs to expand in tumours and draining lymph nodes (DLN) and

to produce TGF-β more actively than mature DCs [150]. TGF-β produced by DC can drive the proliferation of $CD4^+CD25^+Foxp3^+$ Treg cells in a tumour-derived TGF-β dependent fashion, an interaction which is MHC class II restricted and was not observed in naive mice [151]. While the origin of Treg suppression in tumour immunity remains elusive, it is likely that both nTreg cells and iTreg cells contribute to the suppression of tumour immunity and maintaining immunological self-tolerance to tumour antigens.

1.5.3 iTreg cells at the site of the tumour

Many types of tumour cells secrete high levels of TGF-β [152], which plays a key role in the peripheral conversion of naïve T cells to iTreg cells. TGF-β can act either directly or indirectly contributing to the generation of extrathymic Treg cells. The direct mechanism involves the conversion of $CD4^+$ T cells to Treg cells by tumour-derived TGF-β [143], whilst the indirect mechanism is mediated by immature myeloid DC that produce TGF-β [151]. Recruitment of nTreg cells by trafficking or the direct expansion of nTregs by DCs is not dependant on TGF-β, however, neutralisation of TGF-β can reduce Treg cells locally and within the tumour environment [153]. This suggests that $CD4^+CD25^+Foxp3^+$ Treg cells found in the tumour mass are peripherally converted by tumour-derived TGF-β.

In a further attempt to elucidate the contribution of iTreg cells to the total Treg population in a tumour environment, Valzasina and colleagues reported that thymectomized (tx) mice depleted of CD25 demonstrated that the percentage of

Treg cells in the spleen and lymph node of tumour bearing mice were respectively two-fold and five-fold greater than the total number of $CD4^+$ Treg cells in tumour free mice 20 days post TX-CD25 depletion, whilst also showing a reduction in $CD4^+CD25^-$ cell numbers [143]. This confirmed that the generation of *de nova* Treg cells contributes to the overall pool of regulatory cells in the tumour environment, occurring independently of nTregs and is greatly enhanced under immunosuppressive conditions. While there is a clear rationale for developing immunotherapies that dampen the immunosuppressive effects of Treg cells in malignancies, understanding the mechanisms involved in peripheral conversion of Treg cells is imperative to manipulate this pathway. This should facilitate attempts to enhance the efficacy of cancer vaccines.

1.5.4 TGF-β

The TGF-β superfamily represents a diverse set of growth factors which have been classified into two main groups; TGF-β/Activin and bone morphogenetic proteins (BMPs) and growth differentiation factors (GDFs) [154]. There are three homologous TGF-β isoforms in mammals, TGF-β1, TGF-β2 and TGF-β3, however, TGF-β1 is the predominant isoform expressed in the immune system [155]. TGF-β is produced in an biologically inactive form, bound to a latency associated peptide (LAP) to form the latent TGF-β complex, which is secreted by cells and can be activated by numerous methods, including enzymatic cleavage by furins and other convertases [156].

TGF-β evolved to regulate epithelial, neural tissue, the immune system and wound repair [156]. The primary function of TGF-β in the immune system is to limit the development of immunopathology to self or innocuous antigens while ensuring that the immune system generates a robust response to foreign pathogens. While virtually all human cell types are responsive to the immunosuppressive signalling of TGF-β, it exerts the most potent effects on T lymphocytes, mediated via the regulation of lymphocyte proliferation, differentiation, and survival [157]. TGF-β is for the most part a beneficial component of a healthy immune system, however, when this signalling pathway malfunctions, there can be serious consequences including tumorigenesis or excessive immunopathology.

1.5.5 TGF-β in the tumour microenvironment

Basal TGF-β is constantly released to maintain homeostasis in normal healthy tissue, however, under conditions of stress or injury, blood platelets and numerous stromal components release abundant TGF-β to prevent inflammation and tissue damage [156]. These conditions are mimicked in the tumour microenvironment, with most tumour cells producing TGF-β. TGF-β has a dichotomous role in anti-tumour immunity. At the initial stages of tumour growth, the primary function of TGF-β is to halt pre-malignant growth by inducing cell cycle arrest and apoptosis [158]. However, at later stages, cancer cells can become insensitive to TGFβ receptor signalling or the downstream SMAD proteins [159], allowing the tumour cells and their surrounding tumour associated T cells, NK cells, macrophages, DCs, epithelial cells and stromal cells to over express TGF-β in a paracrine manner

[160]. Subsequently TGF-β can inhibit the host's antitumour immune response while also contributing to the immunosuppressive microenvironment of the growing tumour. It is well established that tumour cells have evolved the capacity to manipulate and dysregulate the TGF-β signalling pathway with studies in humans showing that gastric, pancreatic and colon cancer can selectively subvert the TGF-β signalling pathway by inactivating core components, such as SMAD transcription factors, effectively shutting down TGF-β signalling and eliminating tumor suppression [156]. However, melanoma, gliomas, prostate and breast cancer cells can exploit the TGF-β signalling pathway by blocking the tumour suppressive arm, while utilising the regulatory functions of the TGF-β pathway for tumour growth and survival [161].

1.5.6 Evasion of antitumour immune responses by tumour-derived TGF-β

Misuse of the TGF-β signalling pathway can benefit tumour growth and suppress antitumour T cell responses at multiple levels. TGF-β signalling can inhibit the transcription of IL-2 [94], a cytokine involved in the growth and maintenance of T cells. Inhibition of IL-2 has a detrimental effect on the proliferation of immature T cells, while also inhibiting the activation and function of antitumour CTL [162]. TGF-β also potently regulates $CD4^+$ T cell differentiation, typically inhibiting effector T cell functions by controlling the transcription factors that regulate the expression of these Th effector subsets [163]. TGF-β targets multiple signalling pathways that regulate Th1 responses. It inhibits T-bet expression [29], and STAT4 expression [157], providing a negative feedback loop that will block the induction

and interfere with the stability of anti-tumour Th1 cell responses. TGF-β also inhibits GATA-3 expression, which controls Th2 cell differentiation, while promoting FoxP3 and retinoic acid receptor-related orphan receptor-γt (ROR-γt), directing Treg and Th17 differentiation respectively [164]. In addition, TGF-β also potently inhibits anti-tumour $CD8^+$ cytolytic functions, by directly suppressing the expression of the pore forming protein perforin, caspase-activating secreted factors granzyme A and B and pro-apoptotic cytokines FAS Ligand [165]. Similar to the Th1 signalling pathway, TGF-β attenuates the expression of IFN-γ from $CD8^+$ memory T cells by antagonising T-bet expression [166]. Studies have shown that the adoptive transfer of autologous $CD8^+$ T cells insensitive to TGF-β signalling can attenuate tumour growth by infiltrating into tumours, secreting inflammatory cytokines, such as IFN-γ and successfully killing tumour cells [167-168]. In addition, reports by Gorelik and colleagues have also demonstrated that blocking TGF-β signalling in CD4 and CD8 T cells can eradicate tumours in mice [153]. TGF-β is also expressed by Treg cells, playing a major role in the expansion and conversion of Treg cells in the periphery. Treg cells can suppress activation, proliferation, differentiation and effector function of $CD4^+$ Th cells, CD8 CTL, B cells, NK cells, and DCs [169].

In addition to the antitumour effects of TGF-β on tumour infiltrating lymphocytes (TIL), there is growing evidence to link TGF-β to the promotion of distal metastasis, with recent studies demonstrating a prominent role for TGF-β in bone metastases, a common site for dissemination in both breast and prostate cancer

[161]. Consistent with this hypothesis, TGF-β expression has been shown to be stronger in the local lymph nodes when compared with the primary tumour site in colorectal and breast cancer [170]. Several studies have demonstrated that systemic inhibition of TGF-β signalling using a variety of methods, including pan-TGF-β neutralising antibodies, small molecule inhibitors and TGF-β receptor antagonists, reduced the number and size of metastasis formation in breast, lung and bone cancer models [161, 171].

1.5.7 The Role of $CD8^+$ T cells in antitumour immunity

The majority of antitumour immune therapies under development to date have focused primarily on expanding the function of $CD8^+$ CTLs as mediators of antitumour immunity. $CD8^+$ cells recognise intracellular antigens that are presented as peptides by MHC class I, which is ubiquitously expressed by normal and cancerous tissues [172]. $CD8^+$ T cells can be naturally occurring tumour-specific T cells, and have been found in melanomas and other tumour types [173]. Naïve $CD8^+$ T cells are ineffective at killing tumours, but after the initial encounter with tumour antigen, naïve $CD8^+$ T cells become activated, proliferating and differentiating into effector CTLs [174]. Effector $CD8^+$ T cells employ various mechanisms to kill tumour cells that express MHC class I; they secrete IFN-γ and TNF-α, which orchestrates a cascade of events ultimately killing tumour cells both locally and systemically [175]. They can also recruit and activate other effector cells, such as macrophages, which kill tumour cells by phagocytosis. In addition, $CD8^+$ T cells can kill tumour cells through FAS-mediated cell death, which

involves activation of apoptosis by the caspase cascade [176]. However, the cytotoxic activity of $CD8^+$ T cells is largely mediated by the release of proteins, such as perforin and granzyme B, which are present in large quantities in the cytoplasmic granules of effector $CD8^+$ T cells, and directly lyse and kill the tumour cells [177].

However, tumour cells have evolved many strategies to escape immune surveillance, including the downregulation of MHC class I on their surface, a process known as immunoediting. In addition, many tumour cells have developed strategies to overcome the host protective effects of CTL, such as biasing the immune response towards a regulatory or Th2 response, which leads to ineffective CTL activation and prevents tumour cells being killed by CTLs [178]. Intratumoural Treg cells can inhibit naïve $CD8^+$ T cell priming, while $CD8^+$ T cells primed in the presence of Treg cells can be rendered anergic [77]. Furthermore, Treg cells have also been shown to suppress the differentiation and function of primed $CD8^+$ T cells [179]. In the lymph node (LN) CTLs can kill target cells, detach and migrate to the next cell in as little as 15 mins to less than 1 hour [180]. However flourescent studies have shown that it can take up to 6 hours for one CTL to kill one tumour cell [180], suggesting that other antitumour cells are recruited into the site of the tumour to aid CTLs in the rejection of tumors.

1.5.8 The role of CD4 Th cells in tumour immunity

The role of $CD4^+$ Th cells in antitumour immunity has received far less attention than $CD8^+$ T cells, as most tumour cells do not express MHC class II on their surface [172]. However, studies have shown that $CD4^+$ T cells can efficiently induce tumour clearance, without the direct expression of MHC II on the tumour cell, suggesting that $CD4^+$ can indirectly recognise tumour associated antigens (TAA) [181]. This suggests a crucial role for tumour infiltrating APCs in mediating cross presentation of TAAs, resulting in $CD4^+$ T cell activation and a primed anti-tumour effector Th response, while recruiting other effector cells of the innate and adaptive response.

The original hypothesis was that $CD4^+$ T cells functioned to provide cytokines and added stimuli for the priming of $CD8^+$ CTLs in a tumour environment [182]. However, recent studies demonstrated that naïve $CD4^+$ T cells transferred into a lymphopenic host caused the regression of established melanomas without the aid of $CD8^+$ T cells, an effect that was associated with the naïve $CD4^+$ T cells differentiating into cytotoxic T cells expressing IFN-γ, granzyme B and perforin [183]. Furthermore, blocking CTLA4 on the transferred naïve $CD4^+$ T cells resulted in a greater expansion of effector T cells, a reduction in Treg cells and a more efficient antitumour immune response, which was associated with greater tumour regression [183].

A study by Koebel et al provided unequivocal evidence that the adaptive immune system has the ability to inhibit the expansion of transformed cells in a state of quiescence [184]. The model involved injection of a low dose MCA carcinogen on day 0, which results in spontaneous growth of tumours over a long period in a proportion of mice. In mice that did not develop tumours, when the Th1 effector arm of $CD4^+$ T cells was neutralized using anti-IL-12 or anti-IFN-γ antibodies, equilibrium was lost and spontaneous cancer developed. Up to 60% of mice developed progressively growing sarcomas at the MCA (3-methylcholanthrene) injection site when depleted of $CD4^+$/$CD8^+$ T cells and anti-IFN-γ or anti-IL12p40, in contrast to the untreated control mice where none developed tumours. This study highlighted the importance of an efficient adaptive immune response, with emphasis on the protective role of Th1 effector cells, in antitumour immunity.

The importance of $CD4^+$ T cells in tumour clearance was further substantiated when it was demonstrated that tumour specific $CD4^+$ T cells were more effective than tumour specific $CD8^+$ T cells at eliminating a variety of tumours in mice, even when the tumour cells expressed MHC I and not MHC II [181]. While the majority of antitumour therapies continue to focus on CD8 CTLs, clinical trials have shown that the transfer of mixed populations of $CD4^+$ and $CD8^+$ T cells were more effective at eliminating the tumour compared with transfer of $CD8^+$ T cells alone [185]. These findings emphasize the importance of activating tumour-specific $CD4^+$ and $CD8^+$ T cells in establishing optimal antitumour immunity.

1.6 DC Immunotherapeutic approaches to cancer

Successful immunotherapeutic approaches to cancer must break immunological self-tolerance and stimulate tumour-specific immune responses. A major obstacle in antitumour immunotherapeutics is that breaking self-tolerance can also be detrimental to the host, where the immune response becomes self-reactive. Thus achieving the fine balance between tolerance and self-reactivity is one of the fundamental challenges in cancer immunotherapeutics.

Since DC are crucial in orchestrating immune responses, numerous DC vaccines are in development for the treatment of cancer, with the aim to amplify tumour specific Th1 and CTL responses. While mature DC should effectively prime naïve T cell to induce an antigen-specific CTL response to tumour antigens, several reports have shown that DC function in cancer patients is significantly impaired in terms of tumour antigen presentation and T cell activation [186] [187-188]. Tumour cells secrete immunosuppressive molecules such as, IL-10, TGF-β, VEGF [189] soluble Fas ligand, and indolamine-2, 3-dioxygenase (IDO)[190], which all contribute to modulating iDC into becoming tolerogenic DCs with regulatory and suppressive functions, including the secretion of IL-10 and TGF-β [151].

A successful DC vaccine primes autologous DCs with tumour antigens *ex-vivo* in the absence of the immunosuppressive tumour microenvironment and adoptively transfers these manipulated DC *in vivo,* as a promising strategy for cancer immunotherapy [149]. To date many methods have been developed to successfully

load TAAs onto DCs, including pulsing with heat shocked and irradiated tumour cells [191], and pulsing with synthetic peptides derived from the known antigens [192]. Tumour lysates have also been used to load antigens onto DCs [193]. Additionally, heat shock proteins (HSPs) purified from the tumour can be used to load DC with tumour peptides and induce maturation of DC for induction of protective immunity against tumours in mice [194]. However, clinical studies have shown that the use of whole tumour cells for the loading of DCs provides a broader range of TAAs for the DC and correlates with a favourable antitumour immune response in comparison with peptides or gene transfection methods, where knowledge of the antigen is essential [195-196].

Immunotherapeutic approaches that involve DC vaccines pulsed with tumour antigens have been used to treat a variety of malignancies in pre-clinical and clinical studies, with the first human clinical trial of a DC vaccine carried out in patients with B cell lymphoma over a decade ago [197]. The safety profile of DC vaccines is very good and whilst this immunotherapeutic approach has generated encouraging antitumour immune responses, the efficacy in the clinic to date has been limited. In April 2010, Dendreon Corporation received the first and only FDA approval for the licensing of cell-based tumour vaccines, Sipuleucel-T. Sipuleucel-T is a patient specific autologous cellular immunotherapy designed to stimulate specific antitumour immune responses in patients with prostate cancer. This vaccine consists of APC, including DCs, incubated with a fusion protein, or recombinant prostatic acid phosphatase (PAP), a tissue antigen expressed by

prostate cancer cells, with rGM-CSF, which activates APCs (PAP-GM-CSF) [198]. The patient's immune system is primed and boosted with activated PAP-specific APCs, which are capable of recognizing and killing PAP-expressing cancer cells, while also expanding antigen-specific memory T cell responses. While this vaccine demonstrates promise, it's improvement on survival time is limited, enhancing the survival of prostate cancer patients by 4.2 months [198].

A recent Phase I/II clinical trial has also demonstrated the benefits of utilizing immune modulatory approaches to improve the efficacy of cell-based vaccines manipulated *ex-vivo* to generate efficient antitumour immune responses. This autologous cell vaccine also used GM-CSF to enhance immune activation while simultaneously inhibiting TGF-β2 gene expression to limit the inhibitory effects of TGF-β2 on DC activation and effector T cell responses. Results from this clinical trial, TGF-β2 Antisense rhGMCSF (TAG) demonstrated prolonged disease stability and complete response in combination with immune activation, without toxic effects, in patients with advanced cancer [199]. Thus, the pioneering work by Dendreon has highlighted the benefit of *ex vivo* manipulation of APC away from the immunosuppressive tumour environment, whilst also validating the potential and high safety profile of DC as promising cancer therapies.

1.6.1 DC vaccination and TLR adjuvants

Adjuvants are compounds that enhance the quality, strength and durability of specific immune responses to foreign antigens, but have minimal toxicity or lasting effects on their own [200]. An association between infection and cancer was first demonstrated in the 1890s by William Coley, when he administered *Streptococcal* organisms into an inoperable tumour in order to shrink the growing tumour. It was Coleys work with bacterial components, "Coley's toxins" that paved the way for utilizing synthetic PAMPs to enhance antitumour immunity [201]. Research focusing on the activation of innate immune responses, via highly conserved PRRs on APCs, has led to the appreciation that proficient DC activation and maturation is essential to prime protective antitumour adaptive immune responses and overcome immunological self-tolerance. While dying tumour cells may adversely affect DC function [202], activating with a TLR ligand can enhance antigen uptake and presentation by DCs [9] and thus may be a potential strategy for priming antitumour immune responses. Consequently TLR agonists are widely used as vaccine adjuvants to improve the immunogenicity of tumour vaccines and elicit a proinflammatory Th1 response.

Of the TLR agonist family, CpG has shown the most promise as a vaccine adjuvant for protein, peptide, DC and autologous tumour cell vaccines [203]. The TLR9 agonist cytosine-phosphate-guanine (CpG) induces potent Th1 type responses [204], whilst TLR9 responses are severely suppressed in DC isolated from human tumours [205]. CpG has been studied as a monotherapy and as an adjuvant for a

cancer vaccine in phase I and II clinical trials. In addition to the potent inflammatory immune responses elicited by TLRs, PAMPs also induce regulatory responses as a strategy of feedback control to dampen down over active immune responses during infection [149]. A study by Jarnicki and colleagues showed that CpG-activated DC can promote both Th1 and Tr1 cells and this was related to the ability of CpG to promote IL-10 as well as IL-12 production by DC. However, CpG induced IL-10 by DC can be inhibited by blocking the p38 MAPK signalling pathway, which is induced downstream of TLR9 activation. Furthermore, addition of a P38 inhibitor (P38i) enhanced the efficacy of a CpG-activated DC vaccine against B16 tumours in mice [191]. Another method of inhibiting TLR-induced Treg cells and enhancing tumour reactive CTL responses involved administration of a DC-based vaccine with IL-12, CpG and anti-CD3 and anti-CD28 [206]. The ability to selectively block the immunosuppressive response induced by TLR agonists can further potentiate the Th1 response and improve the efficiency of TLR agonists as adjuvants for DC tumour vaccines.

1.6.2 Licensed TLR adjuvants for cancer immunotherapy

In the late 1920s, it was demonstrated that aluminium (alum) based compounds could enhance the immunogenicity of a diptheria-toxoid vaccine via activating cellular sensors. Since then alum has become the most commonly used adjuvant used in infectious disease vaccines. It has been approved by the FDA and licensed for several human vaccines, including diphtheria-tetanus (DT), diphtheria-pertusis-tetanus (DPT), streptococcus pneumonia and meningococcal [200]. To date, a

considerable number of compounds have been evaluated as adjuvants for cancer immunotherapeutics, however, monophosphoryl lipid A (MPL) was the first and only TLR ligand licensed by the FDA as part of a prophylactic vaccine against cervical cancer. MPL is a derivative of LPS (a TLR-4 agonist) from *salmonella Minnesota*, which stimulates a potent T cell and antibody responses [200]. Fendrix® and Cervarix® vaccines, contain a combination of alum and the TLR agonist MPL and are approved vaccines that protect against the human pappiloma virus (HPV) which causes cervical cancer [207]. The approval of these TLR-containing prophylactic vaccines has increased the credibility of TLR ligands as safe, well tolerated, and efficient adjuvant molecules.

Numerous adjuvants have been developed and exploited to enhance the potency of cancer vaccines and have also been used as direct therapeutics. These include Bacillus Calmette Guerin (BCG), a mycobacterium bovis derivative and the synthetic TLR agonists, Imiquimod. Imiquimod is a TLR7 agonist that is used as a topical treatment for various skin diseases, such as keratosis and basal cell carcinoma. Imiquimod activates both mDC and pDC to stimulate innate and adaptive immune responses [208-209]. BCG interactes with TLR2 and TLR4 and is the most effective agent for the treatment of bladder cancer. Moreover, BCG was shown to increase IL-12 production in the bladder cancer T24 cell line [210]. Until the recent approval of provenge, BCG and Imiquimod were the only cancer therapy to be approved by the FDA in over 30 years.

1.6.3 Immunotherapeutic approaches by inhibiting Treg cells

The high frequency of Treg cells in patients with tumours is a major barrier to the success of many tumour immunotherapies [140]. Targeted inhibition of Treg cells showed encouraging results in animal models, which subsequently progressed to clinical trials. This lead to the development of ONTAK, a recombinant fusion protein composed of IL-2 and diphtheria toxin. ONTAK binds $CD25^+$ cells (including Treg cells) to enter the cell via endocytosis to subsequently induce death via the diphtheria toxin portion [211]. Given that CD25 is also upregulated on activated T cells, the targeted inhibition of the CD25 receptor can also eliminate activated effector T cells, including antitumour T cells, and therefore compromise host immunity. For these reasons, ONTAK has had limited clinical success to date [212]

Another approach to inhibiting Treg cell function involves blocking negative regulators of T cell activation, such as CTLA4. CTLA4 which is expressed on $CD4^+$ and $CD8^+$ T cells subsequent to activation negatively regulates T cell responses through interaction with CD80 and CD86 on a DC. Furthermore CTLA4 is constitutively expressed on Treg cells, inhibiting T cell functions, with confirmed functions in antitumour immune suppression [55, 213]. Therefore inhibiting CTLA4 should break immunosuppression during tumour growth and promote expansion of CTL responses. Anti-CTLA4 therapy augments antitumour immune responses [214-215]. Clinical trials in cancer patients with various tumour types, such as melanomas, bladder cancer and prostate cancer, have demonstrated that

anti-CTLA4 treatment enhances antitumour immune responses. However, the beneficial response to treatment with anti-CTLA4 was associated with adverse side effects, such as dermatitis, colitis and hepatitis [216-217]. Thus, while successful in promoting antitumour immunity, attenuation of Treg function has serious consequences resulting in autoimmunity, limiting its success as a future immunotherapy. While significant discoveries have been found in the field of cancer immunotherapy, the success of next generation therapies is dependent on overcoming immune tolerance to the growing tumour while simultaneously preventing self-reactivity to the detriment of the host.

AIMS

The overall objectives of this study were to investigate the effects of manipulating the frequency of Treg cells *in vivo* and determine how this affects tumour growth *in vivo*.

The specific aims were as follows:

- To determine the effect of transiently inhibiting TGF-β mRNA in tumour cells *in vitro*.
- To determine the effect of transiently silencing TGF-β gene expression on tumour growth *in vivo*.
- To investigate whether silencing TGF-β gene expression can enhance the efficacy of a TLR-activated DC vaccine.
- To determine if silencing TGF-β can affect the frequency of tumour infiltrating $FoxP3^+$ Treg cells.
- To investigate the effect of TGF-β-induced by a parasitic infection, *Fasciola hepatica*, on tumour growth *in vivo*.
- To determine if administration of *Fasciola hepatica* secretory products can alter tumour growth *in vivo*.
- To investigate if *Fasciola hepatica* infection or it's secretory products can alter the frequency of tumour infiltrating Treg cells.
- To determine if blocking RA can suppress TGF-β-mediated conversion of $CD4^+CD25^+$ $FoxP3^-$ T cells into $CD4^+CD25^+FoxP3^+$ T cells.
- To investigate if blocking RA can affect DC activation *in vitro*.
- To determine if blocking RA can enhance the efficacy of a TLR-activated DC vaccine against tumour growth *in vivo*.
- To determine if blocking RA in a TLR-activated DC vaccine can alter the frequency of tumour infiltrating Treg cells *in vivo*.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Ammonium chloride lysis solution

0.77 g Ammonium chloride (NH_4Cl)

Dissolved in 100 ml dH_2O and filter sterilised.

2.1.2 Cell culture medium

Roswell Park Memorial Institute (RMPI)-1640 medium (BioSera), supplemented with 10% heat inactivated (56°C for 30 min) foetal calf serum (FCS), 100 mM L-glutamine (Gibco), 100 U/ml penicillin/100 μg/ml streptomycin (Gibco). Complete RPMI (cRPMI) was used to culture murine bone marrow DC (BMDC) and $CD4^+$ T cells in vitro.

2.1.3 EDTA (25mM)

0.93g of EDTA

Dissolved in 50mls Baxters water

2.1.4 ELISA developing solution

1 x 10 mg OPD tablet

25 mls Phosphate citrate buffer

7 μl 1M H_2O_2

2.1.5 ELISA stopping solution ($1MH_2SO_4$)

26.74 mls 18 M H_2SO_4

473.26 mls dH_2O

2.1.6 ELISA washing buffer

0.5L of 1X PBS

9.5L distilled water

5ml of Tween 20

Made up to 10 L with dH_20

2.1.7 FACS buffer

2% FCS

0.1% Sodium azide

Made up in 1X PBS

2.1.8 FACS blocking buffer

50% FCS

50% FACS Buffer

2.1.9 MACS buffer

0.2% FCS

5ml 25mM EDTA

Made up in 500ml 1X PBS

2.1.10 MACS rinsing buffer

0.5 mM EDTA in PBS (filter sterilised)

2.1.11 MACS running buffer

0.5 mM EDTA / 0.5% FCS in PBS (filter sterilised)

2.1.12 Phosphate buffered saline (PBS)

800g Sodium chloride (NaCl, 1.4M)

92g Sodium hydrogen phosphate (Na_2HPO_4, 0.08M)

20g Potassium dihydrogen phosphate (KH_2PO_4, 0.01M)

20g Potassium chloride (KCL, 0.03M)

Dissolved in 5L of dH_2O, pH 7

2.1.13 Phosphate citrate buffer

10.19g Anhydrous citric acid

36.69g di-Sodium hydrogen orthophosphate dodecahydrate ($Na_2HPO_4.12H_2O$)

Make up in 1L with dH_2O, pH 5.0.

2.2 Methods

2.2.1 Animals

Specific pathogen free BALB/c (H-2^d) mice and C57/BL6 (H-2^d) mice were purchased from Harlan UK Ltd (Bicester, Olac, UK) and were maintained according to the regulations and guidelines of the European Union and the Irish Department of Health. All experiments were conducted under university ethical approval and under license from the Department of Health. All mice were 6-8 weeks old at the initiation of experiments. Five to fifteen mice were used per experimental group.

2.2.2 Subcutaneous tumour model

The B16F10 murine melanoma and CT26 colon carcinoma cell lines were purchased from ATCC (American Type Culture Collection, Manassas, VA). Cells were maintained in culture in DMEM (B16F10) or RPMI (CT26) supplemented with 10% heat inactivated FCS (Biosera), 2mM L-Glutamine, 100 μg/ml streptomycin (Life Technologies, Gaithersburg, MD) and 100 U/ml penicillin at 37°C, 5% CO_2. Cells were passaged approximately every three to four days. CT26 and B16F10 tumour cells were adherent and were removed using 0.5% trypsin. B16F10 were used for the tumour induction in C57/BL6 and CT26 cells for tumour induction in BALB/c mice. For tumour induction (day 0), tumour cells were washed twice in PBS, viable cells counted and resuspended at 2 x 10^6 /ml in endotoxin free sterile PBS. 100 μl of the cell suspension was injected into the shaved flank of a mouse using a 27 gauge

needle. Tumour volume was measured every 2-3 days from day 7 following initial challenge. Animals were sacrificed before tumour diameter reached $16mm^3$.

2.2.3 Tumour measurements

Tumour size was determined by measuring tumour length and breadth with digital callipers. Tumour volume was determined using the following formula: $(D1)^2 \times D2 \times \pi/6$ where D1 is the smaller of the two diameters.

2.2.4 Establishment of experimental lung metastasis model

CT26 or B16F10 tumour cells were resuspended at 3×10^6/ml in endotoxin free sterile PBS. Mice were placed in a cage under an infrared lamp for approximately 5 minutes to dilate the blood vessels in the tail prior to intravenous (i.v.) injection (300 µl of cell suspension). Mice were sacrificed 14 days post challenge for tumour enumeration and immunological readouts.

2.2.5 Enumeration of tumour numbers in lung tissues

The lungs were removed, the tumours on the large lung lobe were counted under a binocular microscope.

2.2.6 siRNA oligos

siRNA oligo specific for TGF-β were purchased from AMBION, (Austin, Texas) siRNA ID 187280 with the sequence CCAAGGAGACGGAAUACAGtt. Non-specific control siRNA (RISC-free) was purchased from Pharmacon, (Lafayette, CO).

2.2.7 siRNA transfection

CT26 cells were cultured at $2x10^4$ / ml in RPMI-1640 medium with 10 % FCS overnight and transfected with siRNA (100 nM) using oligofectamine (Invitrogen) according to manufacturer's protocol. Supernatants were removed on indicated days to determine TGF-β concentrations by ELISA.

2.2.8 TGF-β siRNA in combination with the DC vaccine

BMDC were derived as described in section 2.2.17. DC were loaded at a 1:1 ratio with heat-shocked (43°C for 1 hr), γ-irradiated (200G) B16F10 tumor cells. After 2 hours cells were stimulated with CpG 5 μg/ml (CpG-oligodeoxynucleotide (ODN) 1668; 5'-tccatgacgttccgatgct-3'; Sigma-Genosys) and incubated for 18 hours. Mice were treated with up to 3 injections of treated DC (5×10^5), one week apart starting on day 3 s.c. into the tumour site. For TGF-β siRNA treatments on day 2, 3, 4, 9, 10, 11, 16, 17 and 18, 5μg of siRNA was complexed with oligofectamine in optimem and administered s.c. in a volume of 100 μl.

2.2.9 Infection with *Fasciola hepatica*

10 viable metacaceria of *F. hepatica* Gordon Graham strain (Gerald Coles, Department of Veterinary Clinical Sciences, University of Bristol) were selected using a light microscope and entangled in cotton wool. C57BL6 mice were then infected by oral inoculation by placing the cotton wool ball at the back of the mouth using a forceps.

2.2.10 Preparation of ES from *F. hepatica*

Adult flukes were collected from infected bovine livers at the Kildare abattoir, washed in several changes of PBS containing 100μg/ml penicillin/streptomycin (PBS/PS) and transported to the lab. Live flukes were incubated overnight in (PBS/PS) in cell culture incubator at 37° C/ 5%$C0_2$. Supernatent fluid was harvested, cleared by centrifugation at 3000 rpm 30 min, sterilized by filtration through 0.22 μM filter attached to sterile filter unit, aliquoted and stored at – 80^0C.

2.2.11 Restimulation with LFH Cytokine detection

Single cell suspensions were prepared by homogenising spleens (from infected mice) through a 70 μm cell strainer. Cells were centrifuged at 1200 rpm for 5 mins and red blood cells were lysed using 0.87% ammonium chloride solution. Cells were resuspended at 2 x 10^6 /ml and 100 μl of the cell suspension was added to wells of a 96 well U bottom plate. 100 μl of LFH antigen, medium (negative control) or the T cell mitogen PMA (5 ng/ml) and anti-CD3 (500

ng/ml) (positive control) was added to the triplicate wells. Cells were incubated for 72 hrs at 37°C, 5% CO_2 and then supernatants were removed and tested for cytokines by enzyme linked immunosorbent assays (ELISA).

2.2.12 Treatment with RARi at the site of the tumour

C57BL/6 mice were injected s.c. into the right flank with 2 x 10^5 B16F10 tumour cells on day 0. Mice were also injected s.c. into the right flank with either PBS, DMSO or RARα antagonist (5 uM) on days -3, -1, +1, +3, +5 and +7 in a volume of 100 μl.

2.2.13 Determine ALDHact

ALDHact in DC was estimated using ALDEFLUOR® staining kit (STEMCELL Technologies, Vancouver, British Columbia, Canada) according to the manufacturer's protocol. Briefly, BMDC were derived as described in section 2.2.17, cells were suspended at 1 x 10^6 cells/ml in ALDEFLUOR assay buffer containing 5 μl of activated ALDEFLUOR substrate (150 nM) with or without 5 μl of the DEAB stock solution (1.5 mM). Samples were incubated for 30-60 mins at 37 ^{o} C. Cells were then centrifuged at 1200 rpm for 5 mins and the samples were resuspended in 0.5 ml of cold ALDEFLUOR assay buffer and analysed by flow cytometry, gating on the FL1 positive population of cells versus side scatter.

2.2.14 CpG-activated DC pulsed with tumour antigen and pretreated with RARi and/or p38i (analysed *in vitro*)

In studies to determine the effect of RARi and or p38i on killed tumour cell and CpG-induced cytokine production, BMDC ($1x10^6$) were cultured in 24-well tissue culture plates (Cellstar). BMDC were pretreated with 1 μM p38i for 1 hour and/or with 5 μM of RARi for 15 minutes, DC were then loaded at a 1:1 ratio with heat-shocked (43°C for 1 hr), γ-irradiated (200G) B16F10 tumor cells. After 2 hours cells were stimulated with CpG 5 μg/ml and incubated for 18 hours. Supernatants were removed and concentrations of IL-10, IL-17, IL-6, TNF-α, IFN-γ, IL-12p40/70 and TGF-β were determined by ELISA.

2.2.15 CpG-activated DC pulsed with tumour antigen and pretreated with RARi and/or p38i (administered *in vivo*)

BMDC were pretreated with 1 μM of SB203580 (p38i; Enzo life sciences) for 1 hour and/or 5 μM of Ro41-5253 (RARα-specific antagonist; Biomol) for 15 minutes. DC were then loaded at a 1:1 ratio with heat-shocked (43°C for 1 hr), γ-irradiated (200G) B16F10 tumour cells. After 2 hours, cells were stimulated with CpG 5 μg/ml and incubated for 18 hours. Mice were treated with up to 3 injections of treated DC (5×10^5), one week apart starting on day 3 s.c. into the tumour site. For neutralising TGF-β studies, mice were also injected every second day from day +1 with 100 μg/mouse of anti-TGF-β antibody (clone 1D11, Bioceros BV) or isotype control until day + 19.

2.2.16 Assessment of cell viability using trypan blue exclusion

Cell counts were performed by diluting cells (usually 1/20 dilution) with trypan blue (Sigma). A 10µl volume of the cell suspension was then loaded onto a disposable hemocytometer (Hycor Biomedical, UK). The number of viable cells (white) was counted using a light microscope. The number of cells per ml was calculated using the following formula:

Number of cells/ml=cell number x 10^4 x dilution factor.

2.2.17 Generation of murine BMDC

BMDC were generated from C57BL/6 mice, using a method similar to that described by Lutz et al (Lutz et al., 1999). Mice were euthanized and their femurs and tibiae dissected from the surrounding muscle tissues. The bone marrow was flushed out with cRPMI using a 27G needle attached to a 20 ml syringe barrel containing cRPMI. The cell aggregates were dissociated using a 19G needle attached to a 20ml syringe containing cRPMI. The cell suspension was pelleted by centrifugation at 1200 rpm for 5 min at 4°C and cells were resuspended in 2 ml of ammonium chloride lysis solution (section 2.1) that was heated to 37°C for 2 minutes, to lyse red blood cells. The cells were washed in fresh cRPMI and pelleted by centrifugation at 1200 rpm for 5 minutes, resuspended in 10 ml of cRPMI and counted (section 2.2.2). Immature BMDC were cultured at 0.5 x 10^6/ml in cRPMI supplemented with 40 ng/ml of GM-CSF in the form of supernatant from a GM-CSF expressing, J558 cell line.

After 3 days of culture, 30 ml of fresh cRPMI containing 40 ng/ml of GM-CSF was added to each culture flask.

On day 6, the flasks were gently removed from the incubator and cell culture supernatant was carefully removed and discarded, eliminating contaminating cells such as granulocytes, from the culture. 15 ml of sterile PBS, heated to 37^0C, was added to each culture flask. Flasks were then gently agitated and the PBS suspension was transferred into 50 ml tubes containing 10 ml of fresh cRPMI. 15 ml of sterile EDTA (0.02%; Sigma), heated to 37^0C, was added to each culture flask and then incubated at 37^0C for 10 minutes. Meanwhile, the cells removed in the PBS step were pelleted by centrifugation at 1200 rpm for 5 minutes at 4^0C and resuspended in cRPMI. After 10 minutes of incubation, cells in EDTA solution were removed by continuous pipetting and EDTA cell suspension was added to fresh cRPMI. Cells were pelleted by centrifugation at 1200 rpm for 5 mins at 4^0C and resuspended in 10 mls of fresh cRPMI. Cells removed by the PBS and EDTA steps were pooled and counted. Cells were re-cultured at 0.5 x 10^6/ml in cRPMI supplemented with 40 ng/ml of GM-CSF. On day 8, 30 ml of cRPMI containing 40 ng/ml GM-CSF was added to each flask. On day 10 of culture the loosely adherent cells were harvested by gentle pipetting of the culture medium. BMDC were counted and cell viability was assayed (section 2.2.2). Cells were plated at 1 x 10^6 cells per/ml in tissue culture plates. Prior to use BMDC were rested over night.

2.2.18 $CD4^+CD25^-$ T cell purification

Isolated spleens were homogenised and passed through a 70 μM cell strainer (Nunc) to obtain single cell suspensions. Spleen cells were centrifuged at 1200 rpm for 5 mins and red blood cells were lysed by resuspending cells in 1 ml of ammonium chloride solution for 2 min. Cells suspensions were counted and washed in 20 ml of MACS buffer and pelleted by centrifugation at 1200 rpm for 10 minutes at room temperature. Spleen cells were resuspended in 800 μl of MACS buffer and 200 μl of $CD4^+$ magnetic beads (Mitenyl Biotech) was added to the cell suspensions as follows:

10^7 cells: 80 μl of MACS buffer

10^7 cells: 20 μl of $CD4^+$ magnetic beads

Cell suspensions and beads were incubated for 15 minutes at 4°C, after which they were washed in 20 ml of MACS buffer and pelleted by centrifugation at 1200 rpm for 10 minutes at room temperature. Pelleted cells were resuspended in 1 ml of MACS buffer and were placed in the AutoMacs (Mitenyl Biotech) uptake port. The possel 1 programme was selected and purified $CD4^+$ T cells were collected from the positive selection port. Purified $CD4^+$ T cells were washed in cRPMI and pelleted by centrifugation at 1200 rpm for 5 minutes, at room temperature.

$CD4^+$ T cells were counted and stained with CD25 PE-conjugated antibody (Section 2.2). For MACS PE-conjugated antibodies, 10^7 cells were resuspended in 10 µl PE conjugate. Cells were incubated for 10 minutes in the dark at 4^o C. Cells were washed twice in 1-2 ml of buffer to remove unbound primary antibody and pelleted by centrifugation at 1200 rpm for 5 minutes. Cells were then resuspended in 20 µl of Anti-PE Micro-beads per 10^7 cells and incubated for 15 minutes at 4^o C. Cells were then washed twice by adding 1-2 ml of buffer and pelleted by centrifugation at 1200 rpm for 5 minutes. Pelleted cells were resuspended in 1 ml of MACS buffer and were placed in the AutoMacs (Miltenyi Biotech) uptake port. The possel 1 programme was selected purified $CD4^+CD25^-$ T cells were collected from the positive selection port. The purified $CD4^+CD25^-$ T cells were washed in cRPMI and pelleted by centrifugation at 1200 rpm for 5 minutes, at room temperature.

2.2.19 *In vitro* activation and conversion assays.

$CD4^+CD25^-$ T cells were isolated from spleens of C57BL/6 mice. $CD4^+$ T cells were isolation with the $CD4^+$ T cell isolation kit (Militenyi Biotech), then subsequently incubated with PE-conjugated antibody to CD25 (from eBioscience) and then with anti-PE beads (Militenyi Biotech) to isolate naïve $CD4^+CD25^-$ T cells by an autoMACS separator (Militenyi Biotech). $CD4^+CD25^-$ T cells were activated with plate-bound anti-CD3 at a concentration of 1 µg/ml, anti-CD28 at a concentration of 2 µg/ml and recombinant mouse IL-2 (rIL-2) at 5 ng/ml, in the presence or absence of rTGF-

β (5 ng/ml or 10 ng/ml), all-trans-retinoic acid (ATRA; Sigma-Aldrich at10 nM or 100 nM) and Ro41-5253 (retinoic acid receptor α (RARα)-specific antagonist; Biomol; 5 μM) for 3 days and further cultured in the presence of rIL-2 and rTGF-β for an additional 3-4 days. On days 3, 6, and 7 cells were surface stained with CD4-Pecy5, CD25-PeCy7 and intracellularly stained with FoxP3-FITC after fixation and permeabilisation with a specific FoxP3 kit in accordance with the manufacture's protocol (eBioscience).

2.2.20 Standard ELISA

The concentrations of IFN-γ, IL-10, IL-17 were measured by ELISA using matched pairs of antibodies described in Table 2.1. Cytokine specific capture antibodies (50 μl/well) were added to high binding certified 96-well ELISA plates (Greiner Bio-one) and incubated overnight at 4°C. The plates were washed 4-5 times with wash buffer (PBS/0.05% Tween 20) and then incubated with 200 μl/well of blocking buffer at RT for 2 hours to prevent non-specific binding. Plates were then washed 5 times in wash buffer. Plates were incubated overnight at 4°C with 50 μl/well of the test supernatant and 50 μl/well of the corresponding serially diluted cytokine standard in assay diluent. The plates were then washed and incubated with 50 μl/well of the appropriate biotinylated anti-mouse antibody at RT for 2 hours and then washed. The plates were incubated for 20 mins in the dark at RT with 50 μl/well of HRP conjugated StreptAvidin (Pharmingen) and then washed 6 times in wash buffer. Finally the plates were developed with 100 μl/well of OPD dissolved in phosphate citrate

buffer with 1M H_2O_2. The reaction was stopped by adding 50 µl/well of 1M H_2SO_4 Absorbance was measured at 492 nm using a microtitre plate reader (Molecular Devices, VERSAmax tunable microplate reader) with Softmax Pro 3.0 software. Cytokine concentrations for test samples were determined with reference to a standard curve prepared from recombinant cytokines of known concentration.

2.2.21 Detection of TGF-β by ELISA

The concentrations of murine TGF-β were determined by specific immunoassay using a multispecies TGF-β1 duoset (R&D systems). 96-well microtitre plates were coated overnight at 4°C with 50 µl/well of coating antibody (4 µg/ml) diluted in PBS. Wash plates and then block with 200 µl/well of PBS/1% BSA for 2 hours at RT. Plates were then washed three times with PBS/ 0.05% Tween. Samples were activated by acid treating with 1N HCl to adjust to pH 3.0 and incubated for 15 minutes at RT. The samples were then neutralised with 1N NaOH/0.5M Hepes and the samples were added directly to the plates. Alternatively cells were heat activated at 80 ^{o} C for 10 min. The TGF-β standard was serially diluted in 1% BSA and ranged from 0-1000 pg/ml. Plates were incubated overnight at 4°C. Plates were washed and 50 µl of biotinylated chicken anti-TGF-β1 (200 ng/ml) was added to each well. Plates were incubated for 2 hrs at RT. Plates were washed and 50 µl/well of streptavidin-HRP (1:200 dilution in PBS /1% BSA) was added for 30 minutes at RT in the dark. Plates were washed and developed by adding 100 µl/well of OPD

dissolved in phosphate citrate buffer with 1M H_2O_2, once developed the reaction was stopped by adding 50 µl/well of 1M H_2SO_4. Plates were read at 492 nm. TGF-β concentrations in the samples were calculated with reference to a standard curve generated using recombinant TGF-β of a known concentration.

Table 2.1 Origin of antibodies and recombinant cytokines used in murine ELISA

Cytokine	Supplier
IL-10	R&D
TGF-β	R&D
IL-17	R&D
IL-12p70	R&D
IL-6	R&D
TNF-α	R&D
IFN-γ	BD Pharmingen
IL-12p40	BD Pharmingen

2.2.22 FACS analysis

Spleen or lymph node cell suspensions were prepared by passing the organs through a 70 μm cell strainer. Cells were washed in cRPMI and centrifuged at 1200 rpm for 5 mins. Red blood cells were then lysed using ammonium chloride solution. Cells were resuspended and counted. Lung and tumour samples were first washed in RPMI to remove excess blot clots and were then chopped up finely with a scalpel blade. The tissue was then transferred into a sterile tube with 1ml of Hanks balanced salts solution (HBSS, Sigma) per tissue sample with 100 μl of a 10 mg/ml solution of collagenase D (Sigma) dissolved in HBSS. The tube was covered with parafilm and incubated for 30 mins (lungs) or 1 hour (tumour) with rocking at 37°C. Cells suspensions were then passed through a 70 μm cell strainer and red blood cells were lysed. Cells were washed and counted.

2.2.23 Flow cytometry analysis for cell surface markers

Cell suspensions were washed once with FACS buffer and blocked for 5 mins in FACS blocking solution. The cells were then washed twice in FACS buffer. Cells were then transferred to FACS tubes in 100 μl of FACS buffer or 96 well U bottom plates and incubated for 30 minutes with the appropriate PE, FITC or Tri-labelled FACS antibodies (listed in Table 2.2). Cells were washed three times and resuspended in 300 μl of FACS buffer and immunoflourescence analysis was performed using a FACScalibur™ flow cytometer (Becton-Dickson, San Jose, CA) with CELLQuest™.

2.2.24 Detection of intracellular cytokines by FACS analysis

Cell suspensions from spleen, lymph node, lung and subcutaneous tumour were stained directly ex-vivo or stimulated with PMA (10 ng/ml), ionomycin (1 μg/ml) for 6 hr, then Brefeldin A (10 μg/ml) was added for a minimum of 2 hrs at 37°C / 5% CO2. Brefeldin A is a fungal metabolite, which disrupts the structure and function of the golgi apparatus and ER membrane transport and thus prevents the secretion of cytokines from cells. Cells were centrifuged for 5 mins at 1200 rpm and supernatant removed. Cells were then washed in FACS buffer. Non-specific binding was prevented by incubating cells with FACS buffer supplemented with 50% FCS for at least 5 mins. Cells were washed twice with FACS buffer, resuspended with the antibody specific for the cell surface marker (1/50 dilution) in 100 μl of FACS buffer and incubated at 4°C for 30 mins in the dark. Cells were washed twice with FACS buffer and then fixed with 50 μl of fixation medium A (Fix & Perm cell permeabilization kit, Caltag Laboratories) for 15 mins at RT. After washing twice, cells were incubated with 50 μl of the permeabilization medium B (Fix & Perm cell permeabilization kit, Caltag Laboratories) and with 5 μl of the antibody specific for the appropriate cytokine and incubated at room temperature for 15 mins. Cells were then washed three times in FACS buffer and resuspended in 200 μl FACS buffer and analysed immediately using CELLQuest™ software on FACScalibur™ flow cytometer (Becton-Dickson, San Jose, CA). 20,000 live cells were analysed per sample. Gates were set using forward scatter versus the cell subset marker such as CD4 or CD8, similar to method used for surface

antigen expression analysis described in the previous section. Cytokine production from the cells within the appropriate region was analysed by dot plot and quadrants were set using fluorescent minus one (FMO) control.

Table 2.2 FACS Antibodies for surface markers

Surface Marker	Fluorochrome	Source
CD3	PeCy5	eBioscience
CD3	A780	eBioscience
CD3	FITC	eBioscience
CD4	PeCy5	eBioscience
CD4	FITC	eBioscience
CD4	PeCy5	eBioscience
CD8	PeCy7	eBioscience
CD8	PeCy5	eBioscience
CD25	APC	eBioscience
CD25	FITC	eBioscience
CD25	PE	eBioscience

Table 2.3 Intracellular FACs Antibodies

Intracellular Antibody	Flourochrome	Source
FoxP3	FITC	eBioscience

FoxP3	PE	eBioscience
FoxP3	APC	EBioscience
IL-10	PE	eBioscience
IL-17	PE	eBioscience
IFN-γ	APC	eBioscience
TGF-β	PE	R&D

Table 2.4 Recombinant cytokines

Cytokine	**Supplier**	**Concentration used in vitro**
rTGF-β	R&D	5-10 ng/ml
rIL-2	R&D	10 U/ml = 5 ng/ml

Table 2.5 Antibodies used in neutralisation studies.

Antibody	Clone	Supplier	Concentration in vivo
Anti-TGF-β	1D11	Bioceros BV	100 μg/mouse

2.2.25 Statistical analysis

Statistical Analysis was performed using the computer based mathematical package GraphPad Prism. Student's T test was used to compare the mean values between two groups. Statistical differences in mean values between more than two experimental groups were determined by Analysis of Variance (ANOVA). P values less than 0.05 were considered significant.

Chapter 3

Effect of transiently silencing TGF-β on antitumour immunity

3.1 INTRODUCTION

Pioneering observations made by the German bacteriologist Paul Ehrlich in 1909 led to the discovery that the immune system can control tumour growth and protect the host against the development of cancer. Indeed in recent years the concept of continual immune surveillance controlling the development of new neoplasms has gained support. Likewise a role for the immune system in killing tumour cells is now widely accepted. Such immunological control of tumours exerts selective pressure on the development of malignancies that results in several mechanisms by which tumour cells seek to avoid detection by and responses of the immune system. One immune evasion mechanism commonly utilized by tumour cells is the induction of a profoundly immunosuppressed environment both locally in the tumour microenvironment and also, to a certain extent, systemically with increased immunosuppressive factors and cells commonly detected in peripheral blood and unrelated organs in tumour patients. Suppressive mechanisms utilized by the tumour include downregulated expression of immune recognition molecules, such as tumour associated antigens, secretion of immunosuppressive molecules, such as TGF-β, and substantial recruitment, activation and/or *de nova* generation of Treg cells within the tumour microenvironment [189].

Active immunotherapy is a promising strategy to enhance anti-tumour effector responses by manipulating the immune system into generating potent CTL and Th1 responses against the tumour. DC are key antigen presenting cells thought to initiate most, if not all, primary immune responses and are recognised to play a

crucial role in orchestrating adaptive immunity. Due to this key role in controlling and shaping immune responses, a number of cancer vaccines are in development, aimed at exploiting DC. One approach utilises autologous DC which are pulsed *in vitro*, away from the immunosuppressive environment of the growing tumour, with tumour antigens and immunomodulatory molecules, such as TLR agonists, prior to their adoptive transfer *in vivo*. DC vaccines have been shown to induce anti-tumour effector T-cell responses *in vivo* and as such are a promising strategy to elicit pro-inflammatory Th1 and CTL responses in cancer patients [149]. However, despite encouraging results in animal models and early clinical trials, DC based immunotherapies have demonstrated limited success in the clinic to date. This lack of widespread efficacy is attributed, at least in part, to the immunosuppressive nature of the growing tumour.

In order to enhance the efficacy of DC based therapies it will be necessary to develop strategies to overcome the immunosuppressive microenvironment created by the growing tumour. One promising target, which plays a key role in the development and maintenance of tumour immunosuppression is TGF-β. The suppressive effects of tumour derived-TGF-β on immune responses, including those against tumours, is well characterized. TGF-β-mediated immunosuppressive mechanisms include potent inhibition of activation and function of anti-tumour $CD8^+$ CTL and IFN-γ producing $CD4^+$ T cells [163, 165]. Furthermore, TGF-β directly enhances the peripheral conversion of naïve $CD4^+$ T cells into $FoxP3^+$ Treg

cells, which in turn suppress the activation, proliferation, differentiation and effector function of $CD4^+$ Th cells, $CD8^+$ CTL, B cells, NK cells, and DCs [169].

The permanent removal of TGF-β would be deleterious to the host due to its key role in maintaining immune homeostasis and preventing autoimmune diseases. However, strategies that give a transient and local effect, preventing TGF-β-mediated suppression within the tumour for long enough to allow DC vaccines to initiate, expand and potentiate sufficient Th1 and CTL responses should allow tumour killing whilst preventing auto-immune or inflammatory damage to unrelated organs. RNA interference (RNAi) by small interfering RNA (siRNA) is a novel approach for post-translational gene silencing of messenger RNA (mRNA), accumulating in cells for up to 4 hours before a plateau effect [218]. This method provides a short-term effect to target genes with high selectivity and specificity by directing the RNA-induced silencing complex (RISC) to mediate site-specific cleavage, and hence destruction of the targeted mRNA, having shown a greater efficacy for knocking down gene expression than anti-sense technologies [219]. To date, siRNA technology has already been applied in a number of clinical trials, targeting genes in human immunodeficiency virus (HIV), respiratory disease and cancer [220]. This technology could also be of use in the regulation of immune cell functions by manipulating DC gene expression to improve the potency of future therapies [218]. This study examined the hypothesis that combining siRNA technology to knock down tumour derived TGF-β, with simultaneous treatment with an adoptively transferred DC vaccine would circumvent immune tolerance and

allow the induction of significant antitumour immune responses by the transferred DC, thus improving the overall efficacy of such treatments and resulting in a reduction in tumour growth.

3.2.1 TGF-β induces the peripheral conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+FoxP3^+$ T cells

The majority of nTreg cells found in lymphoid organs have been generated in the thymus [221]. However, conventional $CD4^+$ T cells can be induced to express FoxP3 in the periphery under a variety of suboptimal TCR stimulations by agonist peptides [222]. In addition, a growing body of literature has demonstrated that naïve $CD4^+$ T cells can be converted to $FoxP3^+$ Treg cells when activated in the presence of IL-2 and TGF-β [51, 159]. These iTreg cells are functionally similar to nTreg cells in that they are anergic, suppressive and capable of inhibiting inflammatory diseases *in vivo* [51, 102]. This study examined the role of TGF-β in the peripheral conversion of naive $CD4^+$ T cells into $CD4^+CD25^+FoxP3^+$ T cells. $CD4^+CD25^-$ T cells were purified from spleens of C57BL/6 mice and stimulated with αCD3, αCD28 and recombinant (r) IL-2, in the presence or absence of various concentrations of rTGF-β. After 3 days of stimulation, the frequency of $CD4^+CD25^+FoxP3^+$ T cells was quantified by flow cytometry. The results demonstrate that stimulation with IL-2 alone did not induce significant peripheral conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+FoxP3^+$ T cells (Fig 3.1). However, $CD4^+CD25^-$ T cells treated with TGF-β and IL-2 were induced to convert to $CD4^+CD25^+FoxP3^+$ T cells in a dose-dependent manner (Fig 3.1). These findings demonstrate that peripheral conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+FoxP3^+$ T cells can be directly mediated by TGF-β.

3.2.2 B16 tumour cells secrete TGF-β both *in vitro* and *ex-vivo*

Having shown a direct role for TGF-β in the peripheral conversion of conventional $CD4^+CD25^-$ T cells into $CD4^+CD25^+FoxP3^+$ T cells, experiments were carried out to determine if tumour cells secrete a high quantity of TGF-β relative to non tumour cells. B16F10 melanoma tumour cells, CT26 colon carcinoma cells, bone marrow derived DC (BMDC) and $CD4^+$ T cells were cultured in FCS-free medium for 24 hours, supernatants were removed and the concentration of active TGF-β protein was quantified by ELISA. The results demonstrate that cultured B16 tumour cells and CT26 tumour cells both secrete a significantly greater amount of TGF-β when compared with BMDC or $CD4^+$ T cells purified from C57BL/6 mice (Fig. 3.2, *** $p < 0.001$).

In order to examine the production of TGF-β during tumour growth *in vivo*, C57BL6 mice or Balb/c mice were injected s.c. with 2×10^5 B16 tumour cells or CT26 tumour cells respectively, after 18 days the tumours were removed, homogenised and cultured *ex vivo* for 24 hours in FCS-free media. The results demonstrate that the growing B16 and CT26 tumour mass, which also contains infiltrating leukocytes, produces substantial amounts of TGF-β relative to the cultured tumour cell lines (Fig. 3.2). Furthermore, the data demonstrates that the CT26 carcinoma cells produce a significantly greater concentration of TGF-β when compared with the B16 melanoma tumour cells both *in vitro* and *ex-vivo*. Collectively, this data suggests that B16 and CT26 tumour cells produce vast quantities of TGF-β in comparison to non transformed cells, this tumour derived-

TGF-β is likely to contribute to immunosuppression in the growing tumour microenvironment.

3.2.3 Production of tumour-derived TGF-β promotes tumour growth *in vivo*

Having demonstrated that both the B16 and CT26 tumour cell lines produce TGF-β, with CT26 producing significantly greater quantities of the protein, work was carried out in conjunction with Dr. Conroy in the lab to investigate the effect of disrupting TGF-β gene expression in the CT26 colon carcinoma cell line. CT26 tumour cells were transfected with TGF-β siRNA. The expression of TGF-β was significantly reduced 96 hours post transfection with TGF-β siRNA when compared with untransfected cells (Fig 3.3). However, CT26 cells that were transfected with control siRNA or treated with the oligofectamine alone also demonstrated a reduction in the concentration of TGF-β, though not as significant as that observed with TGF-β siRNA. This down-regulation of TGF-β continued up to 144 hours post transfection.

The effect of reduced TGF-β gene expression in CT26 cells on their tumour growth *in vivo* was assessed by inoculating mice s.c. with siRNA transfected CT26 cells. Balb/c mice were injected with 2 x 10^5 tumour cells on day 0 (4 days after transfection) and tumours were measured every 3 days. The results indicate that the CT26 cells transfected with the TGF-β siRNA significantly reduced tumour growth when compared with tumour cells transfected with a control siRNA or

untransfected cells (Fig 3.4). Mice inoculated with CT26 cells that had been transfected with a control siRNA also demonstrated a modest but non-significant attenuation of tumour growth (Fig 3.4). These data indicate that tumour derived TGF-β assists tumour growth *in vivo*.

3.2.4 Therapeutic administration of a DC vaccine with TGF-β siRNA attenuated tumour growth in mice

Studies carried out in the lab by Dr. Conroy showed that prophylactic but not therapeutic administration of TGF-β siRNA injected intra-tumourally reduces tumour growth *in vivo* (Conroy et al., unpublished). An alternative approach was deisgned to derive a therapeutic effect from blocking TGF-β signalling, this involved combining the administration of TGF-β siRNA with a cell-based vaccine. Since DC have a unique capacity to prime immune responses, they are an attractive candidate for tumour immunotherapy. Current DC vaccines utilise DCs primed with tumour antigens *ex-vivo* in the absence of the immunosuppressive tumour microenvironment, which are then adoptively transferred *in vivo*. In April 2010, the FDA approved the first and only autologous DC immunotherapy, Sipuleucel-T (PROVENGE; Dendreon), for the treatment of asymptomatic or minimally symptomatic metastatic prostate cancer [198]. The licensing of Sipuleucel-T is a landmark event for cancer immunotherapy as prior to this vaccine the efficacy of DC based therapies has had limited success when translating into the clinic. A fundamental reason for the poor efficacy of DC in the tumour vaccine may in part be attributed to the immunosuppressive microenvironment created by the growing

tumour [223]. Having established that B16 tumour cells secrete TGF-β which aids tumour growth, experiments were designed to investigate the hypothesis that TGF-β siRNA could enhance the efficacy of a DC based vaccine in the treatment of B16 melanoma tumours by inhibiting TGF-β mediated immunosuppression at the site of the tumour. Mice were s.c. inoculated with the poorly immunogenic B16F10 melanoma tumour cells. DCs that had been pulsed with heat-shocked and irradiated (hs/irr) B16 tumour cells and stimulated with CpG were injected intra-tumourally on days 3 and 10. Mice were simultaneously injected intra-tumourally with TGF-β siRNA, or control siRNA (Oligofectamine or RISC-free siRNA), on days 2, 3, 4, 9, 10 and 11. Tumour volumes were measured every 2-3 days from day 14.

Therapeutic administration of the DC vaccine alone did not have a protective anti-tumour effect (Fig 3.5). However, when the DC based vaccine was combined with administration of the TGF-β siRNA a significant attenuation of tumour growth was observed, when compared with the DC vaccine alone or the DC vaccine with control siRNA (RISC-free siRNA) (Fig 3.5, $p<0.001$). This data demonstrates that reducing TGF-β gene expression at the site of tumour enhanced the efficacy of the DC vaccine in the treatment of B16 melanoma, presumably by reversing the suppressive affect of TGF-β in the tumour microenvironment.

3.2.5 Therapeutic vaccination is associated with a higher frequency of tumour infiltrating $CD4^+$ and $CD8^+$ T cells and a dramatically reduction in tumour infiltrating $CD4^+IL10^+$ and $CD8^+IL10^+$ T cells

Having demonstrated that a DC vaccine when used in combination with TGF-β gene silencing attenuated tumour growth *in vivo*, experiments were designed to determine if this treatment approach altered the phenotype of tumour infiltrating Treg cells. Tumours were excised 15 days post tumour inoculation, in mice treated with a DC vaccine with or without TGF-β siRNA, and tumour infiltrating T cells were examined *ex-vivo* for expression of CD4, CD8, CD25, IL-10 and FoxP3 by staining with surface and intracellular antibody and analysed by FACS. Tumours from untreated mice contained very low frequencies of tumour infiltrating $CD4^+$ and $CD8^+$ T cells, whereas mice treated with the DC vaccine had a significant increase in tumour infiltrating $CD4^+$ T cells and a dramatic increase $CD8^+$ T cell infiltration (Fig. 3.6, $p<0.05$). Administration of TGF-β siRNA did not further enhance the frequency of tumour infiltrating $CD4^+$ or $CD8^+$ T cells.

A very high frequency of tumour infiltrating $CD4^+$IL-10^+ T cells was observed in untreated tumour bearing mice, however, the frequency of tumour infiltrating $CD4^+$IL-10^+ was significantly reduced in mice given the DC vaccine, DC vaccine plus control siRNA or DC vaccine plus TGF-β siRNA (Fig. 3.7A. $p<0.01$). Similarly, the absolute numbers of tumour infiltrating $CD4^+$IL-10^+ cells were also considerably reduced in tumours from mice treated with a DC vaccine alone or in the presence of TGF-β siRNA or control siRNA (Fig. 3.7B). In addition, the

frequency of $CD4^+CD25^+IL\text{-}10^+$ T cells infiltrating the tumour were also significantly reduced following administration of the DC vaccine alone or in combination with TGF-β siRNA (Fig 3.8A $p<0.001$). This reduction in $CD4^+CD25^+IL\text{-}10^+$ T cell infiltration was also reflected in the absolute numbers of tumour infiltrating nTreg cells (Fig 3.8B). Furthermore, similar results were observed for $CD8^+IL\text{-}10^+$ tumour infiltrating T cells, with mice in all treatment groups showing a dramatic reduction in the percent (Fig 3.9A $p<0.01$), and absolute numbers of $CD8^+IL\text{-}10^+$ cells (Fig 3.9B) within the tumour mass when compared with tumours from the untreated mice. This data suggests that the therapeutic administration of the DC vaccine was associated with a significant increase in the percentage of $CD4^+$ and $CD8^+$ T cell infiltration, with a significant reduction in the percentage of $CD4^+IL\text{-}10^+$, $CD4^+CD25^+IL\text{-}10^+$ and $CD8^+IL\text{-}10^+$ T cell infiltration. While therapeutic administration of TGF-β siRNA with the DC vaccine attenuated tumour growth, it did not alter the percentage of infiltrating $CD4^+IL\text{-}10^+$, $CD4^+CD25^+IL\text{-}10^+$ and $CD8^+IL\text{-}10^+$ T cells when compared with treatment with a DC vaccine alone or in combination with control siRNA. This could possibly indicate that recruitment or expansion of Treg cells secreting IL-10, such as Tr1 cells, within the tumour environment is not TGF-β-dependent but is significantly suppressed by adoptive DC transfer.

3.2.6 Therapeutic treatment is associated with a significant reduction in tumour infiltrating $CD4^+CD25^+FoxP3^+$ T cells

Interestingly, untreated tumour-bearing mice showed a very high frequency of tumour infiltrating nTreg cells, with up 52% of infiltrating $CD4^+$ T cells staining positive for $CD25^+FoxP3^+$. In contrast, the frequency nTreg cells infiltrating the tumour was significantly reduced in mice treated with the DC vaccine alone or in the presence of TGF-β siRNA or control siRNA (Fig 3.10A $p<0.001$). The mice treated with TGF-β siRNA showed the lowest mean percentage of $CD4^+CD25^+FoxP3^+$ infiltrating the tumour. Similarly, DC vaccination was associated with a considerable reduction in the absolute numbers of tumour infiltrating $CD4^+CD25^+FoxP3^+$ T cells when compared with untreated mice (Fig 3.10B). This data suggests that the significant reduction in nTreg cell infiltration is primarily due to treatment with the DC based vaccine, however, a modest but not significant reduction in tumour infiltrating $CD4^+CD25^+FoxP3^+$ T cells was observed when mice were treated with TGF-β siRNA.

3.3 DISCUSSION

It is well documented that the microenvironment of a growing tumour induces a state of local immune tolerance that potently inhibits anti-tumour immunity. Such local immune suppression is mediated by several immune evasion strategies, which include secretion or activation of immunosuppressive molecules such as TGF-β and IL-10, and the trafficking, activation and/or *de novo* induction of Treg cells within the tumour environment [140, 189, 191, 224]. This study demonstrated that targeted removal of TGF-β enhanced the efficacy of a DC vaccine in a poorly immunogenic B16 melanoma model, an effect that was associated with a significant reduction of tumour infiltrating Treg cells.

The present study showed that TGF-β and IL-2 can convert $CD4^+CD25^-$ T cells into $CD4^+CD25^+FoxP3^+$ T cells. Such observations are consistent with published reports demonstrating that TGF-β is the key cytokine involved in peripheral conversion of naïve $CD4^+$ T cells into $FoxP3^+$ Treg cells [51, 84, 159]. Treg cells potently impair anti-tumour immune responses *in vivo*, with many reports demonstrating that depletion of $CD4^+CD25^+$ cells dramatically enhances tumour immune surveillance and greatly improves the therapeutic efficacy of cancer vaccines, which subsequently allowed rejection of several experimental tumour models [141-142].

The present study demonstrated that B16 melanoma tumour cells and CT26 carcinoma cells secrete significantly greater concentrations of TGF-β when

compared with non-transformed cells. This finding is consistent with other reports demonstrating that many tumour cells over express TGF-β [225-226]. Transformed cells can become insensitive to TGF-β receptor signalling or the downstream SMAD proteins [159], allowing tumour cells to over express TGF-β in a paracrine manner [160]. This is a particular challenge for the immune system as numerous malignancies such as melanomas, gliomas, prostate and breast cancer cells block the tumour suppressive arm of TGF-β, while utilizing the regulatory functions of the TGF-β pathway to suppress anti-tumour immune responses, and promote peripheral conversion of naïve $CD4^+$ T cells into iTreg cells [227]. Furthermore, TGF-β-secreting T cells, NK cells, macrophages and DCs are found at tumour sites, where it is thought that they too function to suppress effector T cell responses and induce *de nova* generation of Treg cells, thus promoting immune tolerance and maintaining tumour growth [160, 228] . Studies have shown that the adoptive transfer of autologous $CD8^+$ T cells insensitive to TGF-β signalling can attenuate tumour growth by infiltrating into tumours, secreting inflammatory cytokines, such as IFN-γ and successfully killing tumour cells [167-168]. Therefore, because of it's effects on both tumour cells and immune cells TGF-β contributes significantly to the survival and growth of tumours making it an attractive target for tumour therapies.

Overcoming local immune tolerance and immune suppression is one of the fundamental challenges for active immunotherapies against cancer, therefore reducing local TGF-β provides a potential strategy for enhancing anti-tumor

immunity. Whilst the permanent removal of TGF-β would be harmful to the host, due to its key role in maintaining immune homeostasis and preventing autoimmune diseases, strategies that transiently silence TGF-β gene expression, using innovative techniques such as siRNA technology, may be a safer approach than those that completely block TGF-β. The present study revealed that transfection of CT26 tumour cells with TGF-β siRNA successfully reduced the expression of endogenous TGF-β for up to 6 days post transfection. Furthermore, the current study found that modified CT26 cells, transiently expressing TGF-β siRNA, markedly decreased the ability of the tumour cells to grow *in vivo*. This highlights the influence of tumour-derived TGF-β in promoting tumour growth in vivo. These results are consistent with those of Moore et al who reported that transfection of a highly metastatic breast cancer cell line, MDA-MB-435 cells, with TGF-β siRNA resulted in a significant decrease in the number of lung metastasis when compared with unaltered MDA-MB-435 cells [229]. Furthermore, the targeted inhibition of TGF-β_2 using anti-sense technology is now in phase III clinical trials in patients with high-grade gliomas [230], having demonstrated that the antisense therapy significantly enhanced survival time with no serious toxicities in a phase I/II study [231]. In order to maximize the efficacy of such therapies it is likely to be necessary to develop strategies to enhance anti-tumour effector responses by manipulating the immune system into generating potent CTL and Th1 responses against the tumour.

The recent FDA approval of Provenge, a DC based vaccine for the treatment of prostate cancer, has reinforced the potential of active immunotherapies for the

treatment of cancer [198]. However, despite encouraging results, DC based immunotherapies have demonstrated limited success in the clinic to date. This may be due in part to the immunosuppressive nature of the growing tumour as a result of significant levels of tumour-derived TGF-β and concomitantly tumour-induced Treg cells. In an attempt to overcome the immunosuppressive nature of the tumour, the present study tested a combination therapy consisting of the therapeutic silencing of TGF-β in the region of the tumour, with the administration of a DC vaccine, consisting of CpG-activated DC pulsed with hs/irr tumour cells, for the ability to generate antitumour immunity and reduce tumour growth. The results of this study showed that CpG-activated DC primed with hs/irr tumour cells had no significant effects on tumour growth. However, silencing TGF-β at the site of the tumour significantly enhanced the efficacy of this DC-based therapy in the poorly immunogenic B16F10 melanoma model. These results indicate that the transient silencing of TGF-β gene expression at the site of the tumour is a powerful strategy to overcome local immune tolerance induced by the growing tumour. The potential of a combined therapeutic approach is currently under investigation in a phase I clinical trial, targeting the removal of tumour derived TGF-β_2 using anti-sense technology, while simultaneously treating the patient with an autologous tumour cell-based vaccine [199]. This trial has demonstrated promising results, successfully inducing immunological responses to the tumour and improving survival time, while being well tolerated with no grade 3 or 4 toxicities.

Whilst DC therapy alone did not attenuate tumour growth, it was associated with a significant increase in the numbers of tumour infiltrating $CD4^+$ and $CD8^+$ T cells, an effect that predominantly correlates with a good prognosis in the clinic [232]. Interestingly, the enhancement of $CD4^+$ T cells was inversely correlated with the percentages of $CD4^+CD25^+FoxP3^+$ T cells in the tumour. All treatment groups demonstrated significant reductions in the frequency of tumour infiltrating $Foxp3^+$ Treg cells when compared with untreated mice, however, mice that were simultaneously treated with TGF-β siRNA consistently displayed the lowest percentages of Treg cells. The present findings are in agreement with a report by Fujita et al who showed reduced numbers of Treg cells in the DLN of tumour bearing mice when local TGF-β expression was inhibited [160]. Furthermore, the present study demonstrated that therapeutic treatment with the DC vaccine was associated with a significant reduction in frequency of IL-10-producing $CD4^+$ and $CD8^+$ T cells. While the suppressive capabilities of Tr1 cells have been amply demonstrated, $CD8^+$ T cells have traditionally been found to be protective in antitumour immunity, through their CTL activity and IFN-γ expression [175]. However, it is now broadly accepted that $CD8^+$ T cells expressing IL-10 are recruited to the site of the tumour and actively suppress anti-tumour immune responses [189].

The key finding of the present study is the demonstration of a promising siRNA application that transiently reduces local TGF-β, which circumvents the immunosuppressive nature of the tumour environment, and subsequently enhances

the efficacy of a DC vaccine in a poorly immunogenic melanoma model. While reducing TGF-β at the site of the tumour did not significantly inhibit the ratio of tumour infiltrating Treg cells when compared with the DC vaccine alone, it is possible that inhibition of TGF-β may be attenuating the immunosuppressive nature of the tumour microenvironment, and as a result allows the development of anti-tumour effector T cell responses, thereby reducing tumour growth. It is well established that DC purified from tumour-bearing mice have defective function, including abnormal maturation and migration [233-234], whilst tumour-derived TGF-β compromises the capacity of tumour infiltrating DC to present antigen [235-236]. Therefore, an alternative interpretation of the findings in the present study is that reducing tumour-derived TGF-β may enhance the efficacy of adoptively transferred DC vaccines, whilst simultaneously alleviating the suppressive effects of TGF-β on DC differentiation, maturation and trafficking to the secondary lymph organs where they prime anti-tumour effector T cell responses.

In summary, this study highlights the promising potential of combining a DC vaccine approach with silencing TGF-β gene expression. As monotherapies both approaches have shown promise in treating tumours however this study provides dramatic evidence of a synergistic effect of such a combination therapy which results in significantly decreased tumour growth by reducing infiltration of nTreg cells, Tr1 cells and $CD8^+IL10^+$ Treg cells and simultaneously recruiting or expanding $CD4^+$ and $CD8^+$ effector T cells. Given the fact that both approaches have been well tolerated in the clinic this combined approach is likely to be

similarly well tolerated and if translated into the clinic may function as a highly effective anti-cancer immunotherapy.

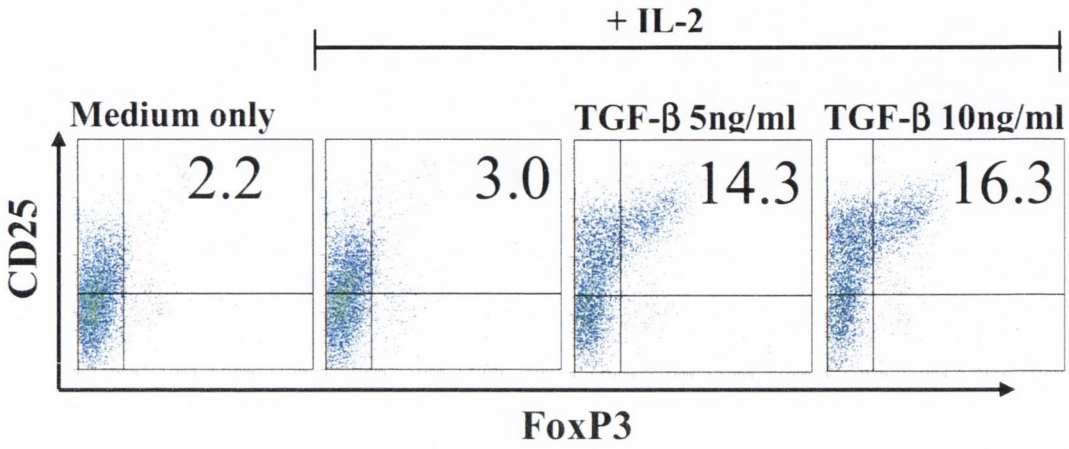


Figure 3.1 TGF-β induces peripheral conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+FoxP3^+$ T cells. $CD4^+CD25^-$ T cells isolated from spleen cells were stimulated with platebound αCD3 (1 μg/ml), soluble αCD28 (2 μg/ml), rIL-2 (5 ng/ml), with or without rTGF-β (5 ng/ml and 10 ng/ml). The frequency of $CD4^+CD25^+FoxP3^+$ T cells was determined on day 3 by surface labelling with αCD4, αCD25 and intracellular labelling with FoxP3. This experiment is a representative of at least six experiments.

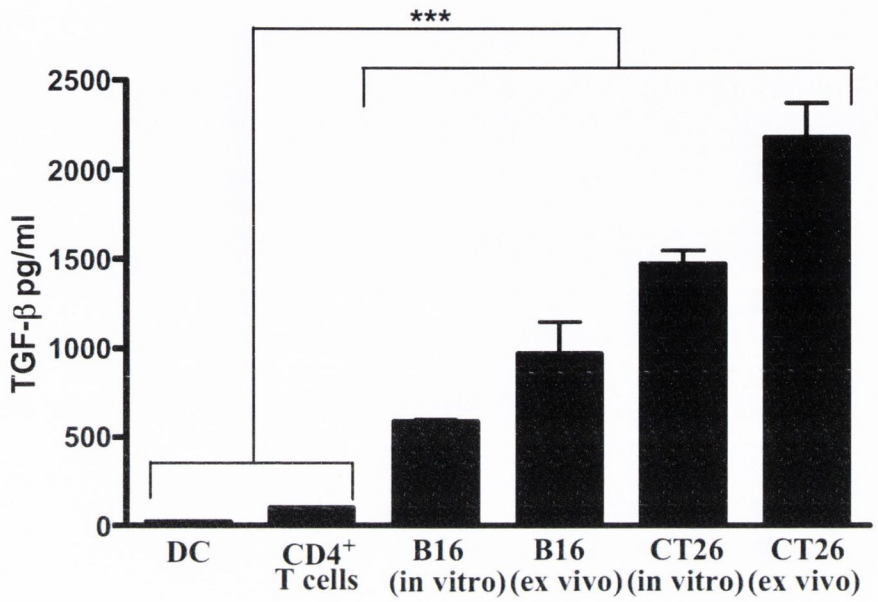


Figure 3.2 TGF-β is produced by both B16 and CT26 tumour cell lines. BMDC, $CD4^+$ T cells, B16 melanoma tumour cells and CT26 colon carcinoma cells were cultured in FCS free medium and the concentration of TGF-β in supernatants was quantified by ELISA (*in vitro*). C57BL6 mice were injected s.c. with 2 x 10^5 B16 cells. Balb/c mice were injected s.c. with 2 x 10^5 CT26 cells. Tumours were removed on day 18 and homogenised and the concentration of TGF-β in tumour homogenate was quantified by ELISA (*ex-vivo*). Results are means (+/- SD) of triplicate assays. (***, $p < 0.001$ non transformed cells versus B16 or CT26 tumour cells).

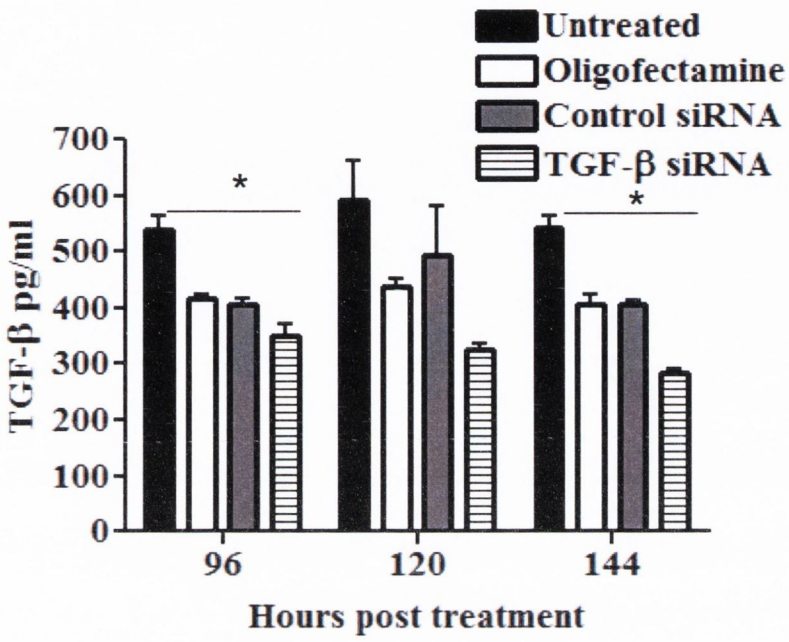


Figure 3.3 TGF-β siRNA disrupts the expression of the TGF-β gene in the CT26 colon carcinoma cell line. CT26 tumour cells were cultured in RPMI medium at 2 x 10^4/ml overnight and transfected with oligofectamine according to manufacturer's protocol (siRNA at a final concentration of 100 nM). Supernatants were removed after 96 hours, 120 hours and 144 hours post transfection and the concentration of TGF-β was determined by ELISA.

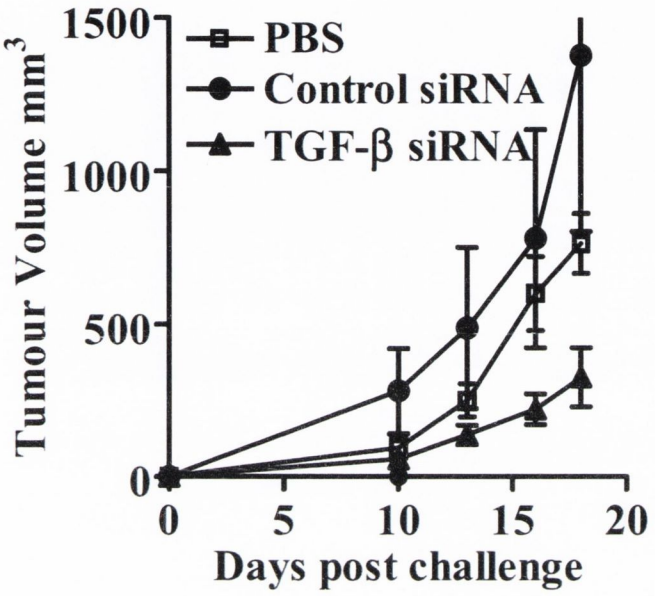


Figure 3.4 Transfection with TGF-β siRNA attenuates tumour growth in vivo. CT26 tumour cells were transfected with TGF-β siRNA or control siRNA at a final concentration of 100 nM. Balb/c mice were injected with 2 x 10^5 CT26 tumour cells that were transfected with TGF-β siRNA or siRNA control. Tumour growth was monitored and recorded every 2-3 days for 18 days. The data represents the mean tumour volume from 6 mice per group. This experiment was carried out by Dr. Helen Conroy.

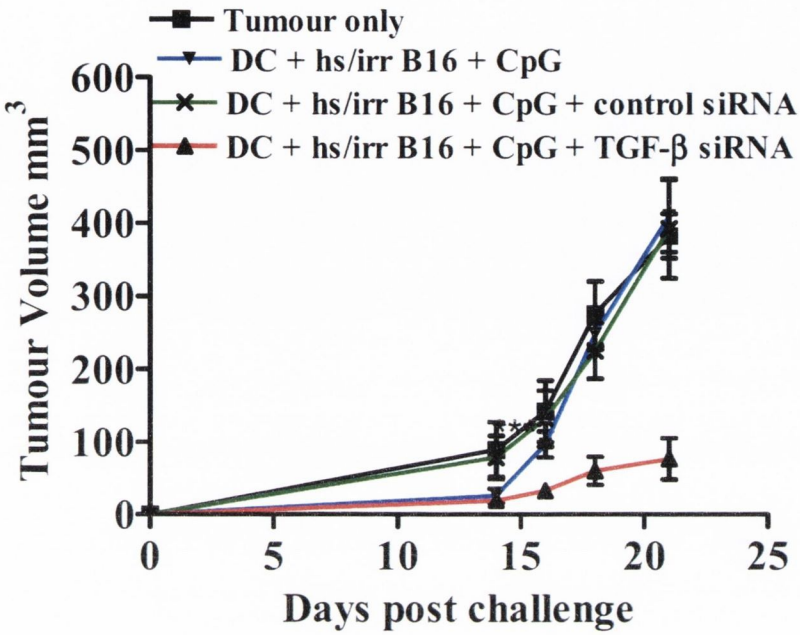


Figure 3.5 Therapeutic administration of a DC vaccine with TGF-β siRNA attenuated tumour growth in mice. C57BL/6 mice were injected s.c. with 2 x 10^5 B16 melanoma tumour cells. Mice were injected s.c. in the region of the tumour 3, 10 and 17 days later with 1–5 x 10^5 DC pulsed for 24 h with heat-shocked and irradiated tumour cells (ratio 1:1). siRNA for TGF-β or siRNA controls (RISC-free siRNA or Oligofectamine alone) were injected on days 2, 3, 4, 8, 9 10, 11, 16, 17, and 18. Tumour growth was monitored over 21 days. Results are mean tumour volume for 6 mice per group (***,$p < 0.001$ Medium control versus TGF-β siRNA treatment group). This experiment is a representative from five experiments.

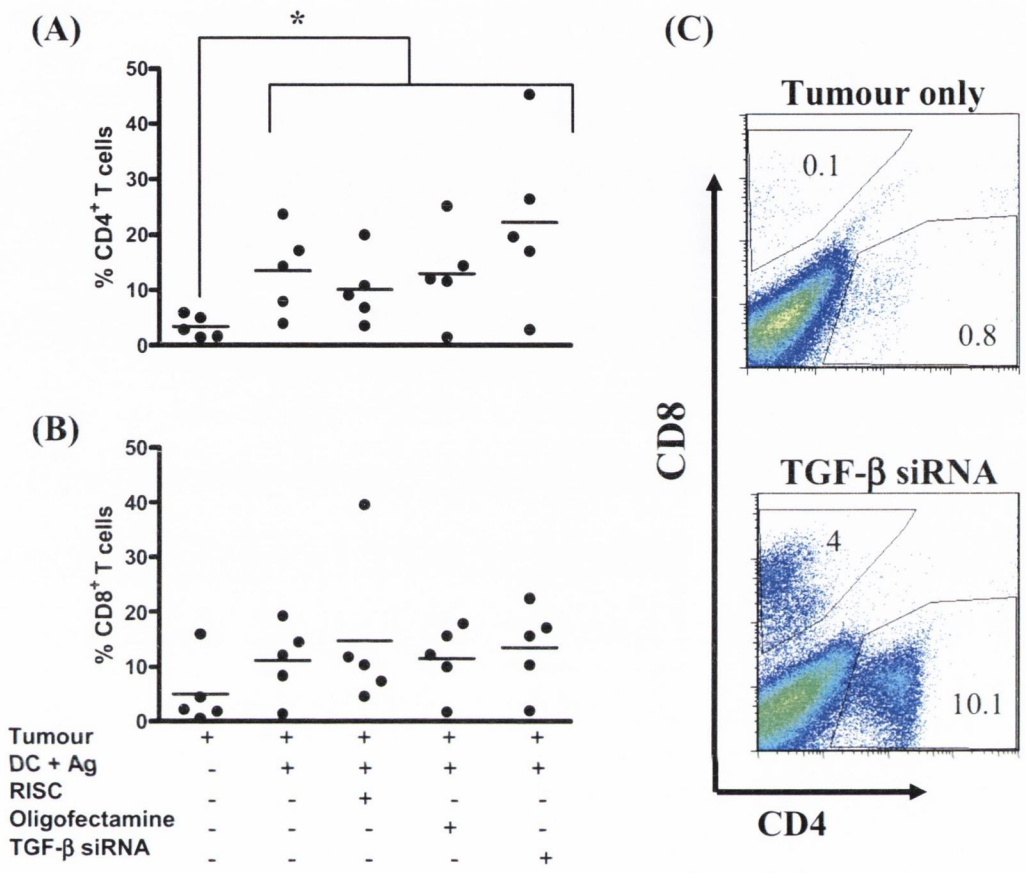


Figure 3.6 Treatment with a DC vaccine and TGF-β siRNA promotes $CD4^+$ and $CD8^+$ T cell recruitment to the site of B16 subcutaneous tumours. Mice were s.c. challenged with B16 tumour and treated as described in Fig 3.5. On day 21 tumours were excised and tumour infiltrating lymphocytes were labelled directly *ex-vivo* with antibodies specific for CD4, CD8 and analysed by flow cytometry. (A) The percent of $CD4^+$ T cells from the total lymphocyte population (Untreated tumour versus treatment groups. *$p<0.05$ by unpaired t test), (B) The percent of $CD8^+$ T cells from the total lymphocyte population and (C) A representative dot plot from the tumour only and TGF-β siRNA treated groups showing dot plots of CD4 versus CD8 (C). The percentage of cells in each quadrant are gated on FMO controls.

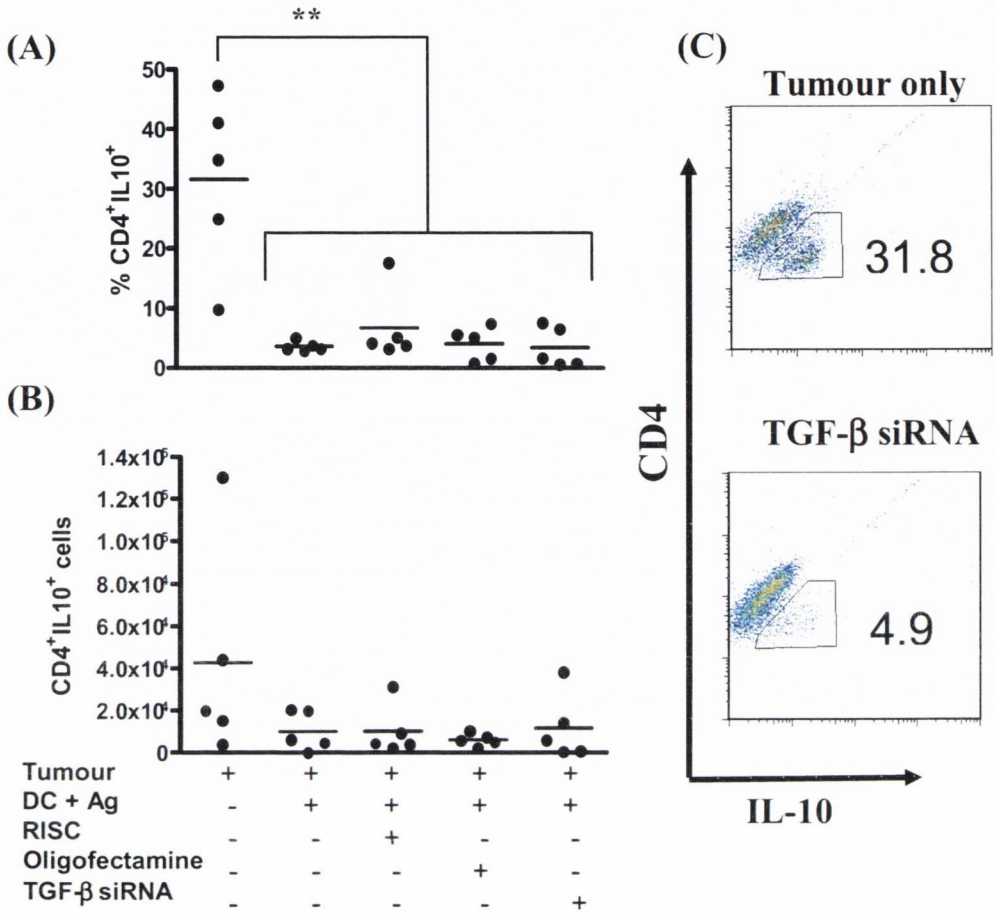


Figure 3.7 Therapeutic administration of a DC vaccine was associated with a reduction in tumour infiltrating $CD4^+$ $IL\text{-}10^+$ T cells. Mice were s.c. challenged with B16 tumours and treated as described in Figure 3.5. Tumour infiltrating lymphocytes were surface and intracellularly labelled directly *ex-vivo* with antibodies specific for CD4 and IL-10 and analysed by flow cytometry. (A) The percent of tumour infiltrating $CD4^+IL\text{-}10^+$ cells (untreated tumour versus treatment groups. $^{**}p<0.01$ by unpaired t test). (B) The absolute numbers of $CD4^+IL\text{-}10^+$ cells and (C) A representative dot plot from the tumour only and TGF-β siRNA treated groups, the percentage of cells in each quadrant are gated on FMO controls.

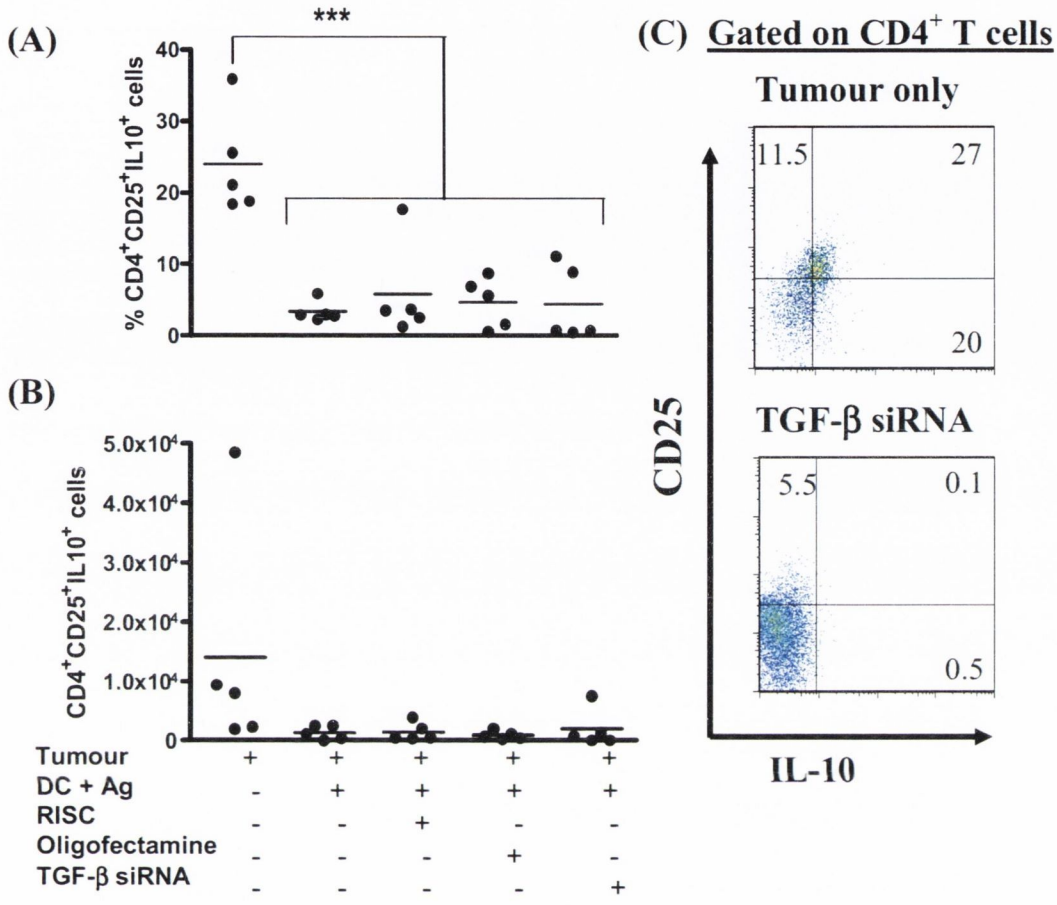


Figure 3.8 Reduced $CD4^+CD25^+IL\text{-}10^+$ cells infiltration into the subcutaneous B16 tumours when treated with a DC vaccine. Mice were s.c. challenged with B16 tumour and treated as described in Figure 3.5. Tumour infiltrating lymphocytes were labelled directly *ex-vivo* with antibodies specific for surface CD4, CD8, CD25 and intracellularly stained for IL-10. (A) The percent of of $CD4^+CD25^+IL\text{-}10^+$ cells (Untreated tumour versus treatment groups. $^{**}p<0.001$, by unpaired t test). (B) The absolute numbers of $CD4^+CD25^+IL\text{-}10^+$ cells and (C) A representative dot plot from the tumour only and TGFβ siRNA treated groups, the percentage of cells in each quadrant are gated on FMO controls.

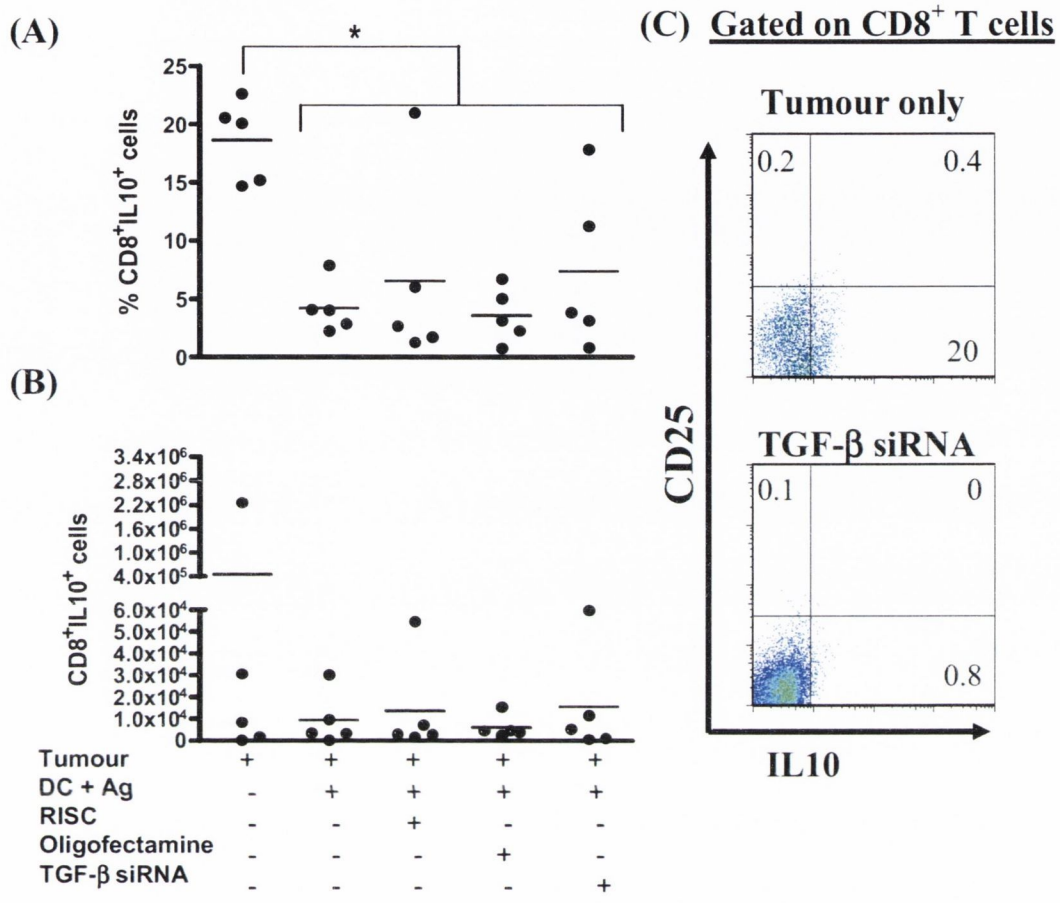


Figure 3.9 A reduction in IL-10 secreting $CD8^+$ T cells infiltrating the subcutaneous B16 tumour when treated with a DC vaccine. Mice were s.c. challenged with B16 tumour and treated as described in Figure 3.5. Tumour infiltrating lymphocytes were labelled directly *ex-vivo* with antibodies specific for surface CD8, CD25 and intracellularly stained for IL-10. (A) The percent of tumour infiltrating $CD8^+IL\text{-}10^+$ (Untreated tumour versus treatment groups. *$p<0.05$, by unpaired t test). (B) Absolute numbers of $CD8^+IL\text{-}10^+$ cells and (C) A representative dot plot from the tumour only and TGFβ siRNA treated groups, the percentage of cells in each quadrant are gated on FMO controls.

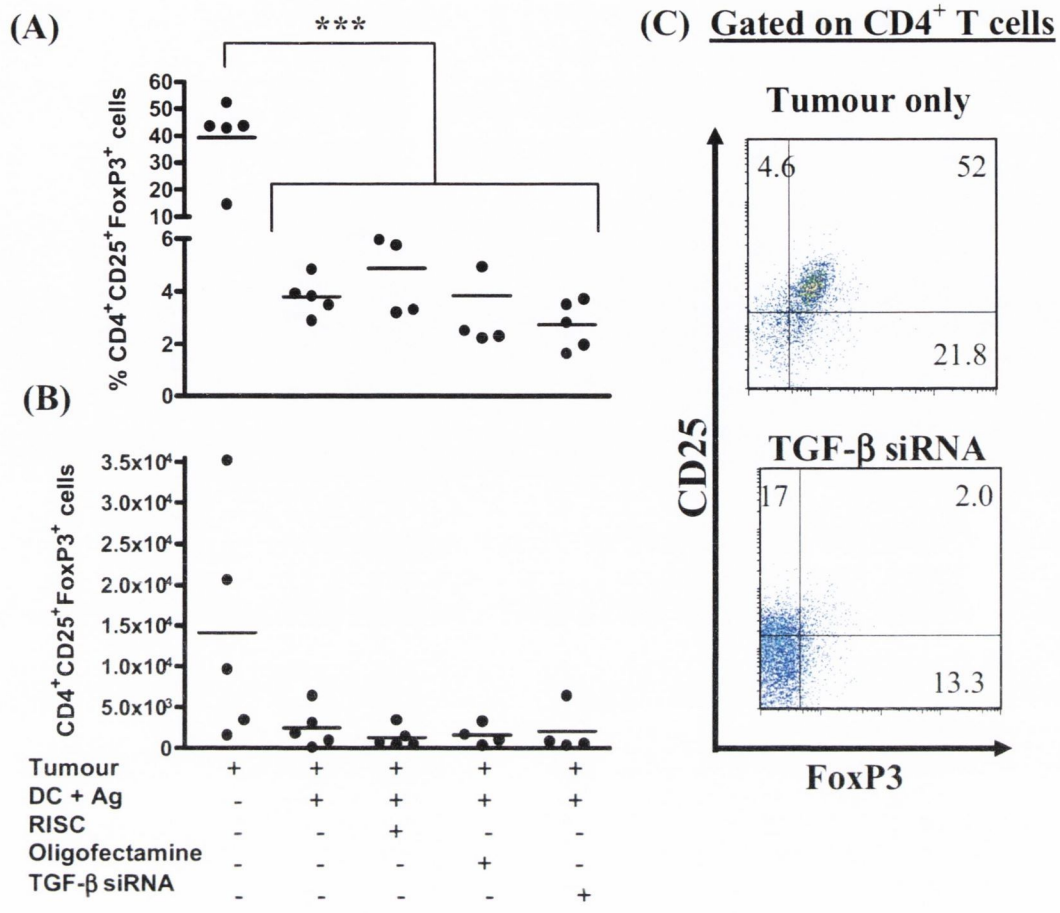


Figure 3.10 Therapeutic administration of a DC vaccine was associated with a significant reduction in tumour infiltrating $CD4^+CD25^+FoxP3^+$ T cells. Mice were s.c. challenged with B16 tumour and treated as described in Figure 3.5. Tumour infiltrating lymphocytes were labelled directly *ex-vivo* with antibodies specific for surface CD4, CD25 and intracellularly stained with FoxP3. (A) The percent of $CD4^+CD25^+FoxP3^+$ T cells infiltrating the tumour (Untreated tumour versus treatment groups ***$p<0.001$, by unpaired T test). (B) The absolute numbers of tumour infiltrating $CD4^+CD25^+FoxP3^+$ T cells and (C) A representative dot plot from the tumour only and TGFβ siRNA treated groups, the percentage of cells in each quadrant are gated on FMO controls.

Chapter 4

Effect of TGF-β-inducing pathogen on antitumour immunity

4.1 INTRODUCTION

Treg cells are an essential component of a healthy immune system, playing a crucial role in maintaining self-tolerance and maintaining immune homeostasis via their capacity to suppress over-active or undesired immune responses [49]. When the immune system is challenged with a foreign pathogen, Treg cells ordinarily control the balance of immune responses to limit harmful immune-mediated pathology, whilst allowing the host to generate protective immune responses against the foreign pathogen [237]. However, Treg cells are often induced by parasitic infections, including *Fasciola hepatic* (*F. hepatic*) [238], *Heligmosomoides polygyrus* (*H. Polygyrus*) [239], *Litomosoides sigmodontis* [240] and *leishmaniasis* [241], whereby they can subvert host immunity, inducing a state of immune tolerance to the parasite, resulting in long lasting parasitic infections. Studies in murine models of parasitic infections have confirmed the capacity of Treg cells to subvert immune responses against parasitic infections. Depletion of Treg cells *in vivo* led to a protective immune response and expulsion of the parasite, *Litomosoides sigmodontis* [242]. Recent reports have also revealed that helminth parasites, such as *H. polygyrus*, can secrete TGF-β, a key cytokine involved in promoting *de nova* generation of $FoxP3^+$ Treg cells in the periphery, as a mechanism by which helminths directly induce $FoxP3^+$ Treg cells to down-regulate host effector responses [243].

F. hepatica is a parasitic trematode that infects mammals causing liver fluke disease. The mammalian infection is initiated by the consumption of plant material

that harbours encysted metacaceria. The metacaceria excysts in the duodenum and migrates through the intestinal wall, crosses the peritoneal cavity before entering the liver. The juvenile fluke spends between 8-12 weeks feeding on parenchymal cells of the liver, which causes extensive haemorrhaging and perforation [244]. Due to the long periods within a vertebrate organism, *F. hepatica* has evolved an array of immune evasion strategies to subvert the host immune response against the parasite and induce an active state of immune tolerance. The infected host initially mounts a Th2 dominated immune response against the parasite [245-246], however, this Th2 response is ineffective at clearing the parasite, and within 12 weeks, a chronic infection develops characterized by reduced levels of IL-4 and enhanced IL-10 and TGF-β production by PBMC [247] Interestingly, experimental neutralisation of TGF-β and IL-10 resulted in enhanced parasite specific IL-4 and IFN-γ production, suggesting that the parasite may induce these regulatory cytokines as a strategy to suppress protective immune responses [247]. It has also been reported that infection with *F. hepatica* induces generalized systemic immune suppression, ablating the host immune response to the parasite, but also to other pathogens. Mice co-infected with *F. hepatica* and *B. pertusis* had suppressed Th1 responses against *B. pertussis* and delayed bacterial clearance from the lung of infected mice [248]. A more recent study by Walsh et al., revealed that infection with *F. hepatica* resulted in an influx of immune cells into the peritoneum, including DC, macrophages and $CD4^+$ T cells expressing significant amounts of TGF-β and IL-10 [238]. Importantly, this study also demonstrated that *F. hepatica* infection is associated with expansion or recruitment of $FoxP3^+$ Treg cells and

induction of adaptive Ag-specific Treg cells, which suppress host immune responses to the parasite and bystander Ags, indicating that an active state of immune suppression is maintained by the parasite [238]. Biasing the immune response toward Treg cells could be a natural mechanism of the immune system to control collateral damage and limit immune pathology or alternatively it may be a strategy utilized by the parasite to circumvent the protective immune response directed at the parasite. Interestingly, observations by McSorley et al., have revealed that live filarial parasites promote murine FoxP3$^+$ Treg cells *in vivo*, an effect that was not observed with killed parasites [249]. These findings support the hypothesis that parasites can exploit the host's regulatory immune response as a means of subverting protective immunity. It is highly likely that secretion of such immunomodulatory mediators by parasites is one of the key mechanisms employed by the parasite to down-regulate host immune responses, with several studies showing that helminth parasites secrete biologically active excretory secretory (ES) products that directly modulate host immune responses [250].

Experimental models of infection have proved to be an invaluable tool in mechanistic studies of helminth–induced immune responses *in vivo*, with several studies reporting that helminth parasites induce a state of active immune tolerance in the host, which in part was attributed to the recruitment, activation and expansion of Treg cells. Since Treg cells also subvert immune responses to tumours, it is possible that infection with a parasite may have a negative effect in immunity to tumours. Thus, this study examined how infection with a helminth parasite, *F.*

hepatica, may influence antitumour immunity and alter tumour development. Finally this study examined the mechanistic ability of ES products from *F. hepatica* to modulate immune responses *in vitro* and subsequently alter antitumour immunity and tumour development *in vivo*.

4.2 F. hepatica infection exacerbates B16 tumour growth in mice

F. hepatica is a helminth parasite that induces a potent Treg cell response in mice and can suppress immune responses to self-antigen via TGF-β-dependent mechanisms [238]. In order to investigate the effect of altering the ratio of effector to regulatory T cells, following parasitic infection, on tumour development, an experimental model of B16 melanoma was established in the presence and absence of *F. hepatica* infection. C57BL6 mice were injected s.c. with B16 melanoma tumour cells on day 0, one group of mice were also orally infected with *F. hepatica* on day 0, while one group of tumour bearing mice were left uninfected. Tumour volumes were measured from day 7. The results revealed that infection with *F. hepatica* enhanced tumour growth with palpable tumour detected after 10 days; untreated control mice did not develop palpable tumours until day 15 (Fig 4.1). Furthermore, the rate of tumour growth was significantly exacerbated in mice infected with *F. hepatica* (Fig. 4.1, $p<0.001$). These findings indicate that infection with *F. hepatica* results in earlier tumour progression and significant exacerbation of B16 s.c. tumour growth in mice.

4.2.1 *F. hepatica* infection induces *F. hepatica*-specific IL-10 production in the spleen

In order to confirm that tumour bearing mice were successfully infected with *F. hepatica,* and that infection generated a Treg type response, experiments were designed to determine if mice orally challenged with *F.hepatica* developed IL-10 secreting memory T cell responses to the parasite. Spleens from control tumour

bearing mice and tumour bearing mice infected with *F. hepatica* were removed 18 days post challenge, and spleen cells were re-stimulated with liver fluke homogenate (LFH). After 72 hours the concentration of IL-10 in the supernatant of spleen cells was determined by ELISA. Spleen cells from mice infected with *F. hepatica* produced high concentrations of IL-10 following re-stimulation with LFH (Fig 4.2). However, spleen cells from the control tumour bearing group did not generate a T cell response to the parasitic antigen but did respond to polyclonal activation with anti-CD3 and PMA (Fig 4.2). These findings suggest that the strong induction of IL-10 to LFH in the infected mice is indicative of an IL-10 secreting memory T cell response, indicating that the mice were successfully infected with the *F. hepatica* parasite and that induces IL-10 secreting cells *ex-vivo*.

4.2.2 Enhanced $CD4^+FoxP3^+$ T cells in the B16 tumour mass of mice infected with *F. hepatica*

Having established that infection with the *F. hepatica* parasite was associated with exacerbation of tumour growth, experiments were designed to determine if this was associated with an enhanced frequency of tumour infiltrating Treg cells. Mice were injected with B16 melanoma tumour cells and infected with *F. hepatica*. Tumours were removed and TIL were stained with surface CD4 and intracellular FoxP3. There were no significant differences observed in the percent of tumour infiltrating $CD4^+$ T cells, $CD4^+CD25^+$ T cells and $CD4^+IL\text{-}10^+$ T cells, between the infected and control tumour bearing mice (Fig 4.3A). However, exacerbation of tumour growth by *F. hepatica* infection was associated with a substantial increase in the

percentage of tumour infiltrating $CD4^+$ T cells expressing FoxP3 (Fig 4.3A). Infection with *F. hepatica* was not associated with any significant differences in the percent of $CD4^+CD25^+IL\text{-}10^+$ T cells or $FoxP3^+IL\text{-}10^+$ T cells, however, the percent of nTreg cells, as defined by $CD4^+CD25^+FoxP3^+$, infiltrating the tumour was dramatically enhanced in mice infected with *F. hepatica* (Fig 4.3B). These results indicate that infection with *F. hepatica* is associated with a dramatic increase in the percentage of tumour infiltrating $CD4^+FoxP3^+$ T cells and $CD4^+CD25^+FoxP3^+$ T cells, and this may be attributed in part to the exacerbation of tumour growth following parasitic infection.

4.2.3 Enhanced B16 tumour burden in the lungs of mice infected with *F. hepatica*

Having established that infection with *F. hepatica* exacerbated tumour growth in the experimental s.c. melanoma model, experiments were designed to confirm these findings using a lung tumour model. C57BL/6 mice were injected i.v. with B16 melanoma tumour cells, with one group of mice simultaneously infected by oral challenge with metacaceria from *F. hepatica*. After 14 days, lungs were removed, fixed and tumour nodules on the large lung lobe were counted. Mice that were infected with *F. hepatica* displayed a significant increase in the number of lung tumour metastasis when compared with the tumour only control group (Fig 4.4, $p<0.05$). This study is consistent with previous findings demonstrating that infection with the helminth parasite, *F. hepatica*, exacerbated tumour growth. It also confirms that the parasitic infection systemically renders the host immune

response into an active state of tolerance, as demonstrated by exacerbation of tumour growth in both lung and s.c. tumour models.

4.2.4 Enhanced $CD4^+$ and $CD8^+$ T cell infiltration in the lungs of B16 tumour bearing mice infected with *F. hepatica*

Having shown that infection with *F. hepatica* exacerbated tumour burden in the lung melanoma model, subsequent studies examined the effect of the parasitic infection on infiltrating $CD4^+$ and $CD8^+$ T cells in the lungs of tumour bearing mice. Mice were challenged with B16 melanoma only or B16 melanoma and *F. hepatica* infection. Lungs were removed 14 days post challenge and the percentages of infiltrating $CD4^+$ and $CD8^+$ lymphocytes were determined by flow cytometry. The results demonstrate a significant increase in $CD4^+$ and $CD8^+$ T cell infiltration into the lungs of tumour bearing mice infected with *F. hepatica* (Fig 4.5, $p<0.01$).

4.2.5 Enhanced CT26 tumour burden in the lungs of mice infected with *F. hepatica*

In order to determine if the exacerbating effects of *F. hepatica* on tumour growth is specific to the melanoma tumour models, experiments were designed to examine the effect of *F. hepatica* on a CT26 colon carcinoma model using a different strain of mice. BALB/c mice were injected i.v. with CT26 colon carcinoma tumour cells, with one group of mice simultaneously infected with the *F. hepatica*. After 14 days, lungs from each group were removed, fixed and tumour nodules on the large lung lobe were counted. A significant enhancement in CT26 tumour burden was evident

in the lungs of *F. hepatica* infected mice when compared with the tumour only control group (Fig 4.6, $p<0.01$). Together with the previous results, these findings confirm that *F. hepatica* significantly exacerbated tumour growth, an effect that is not unique to one experimental tumour model, site of tumour growth or strain of mice.

4.2.6 $CD4^+$ and $CD8^+$ T cell populations in the lungs of CT26 tumour bearing mice infected with *F. hepatica*

Having demonstrated an increase in CT26 tumour burden in the lungs of mice infected with *F. hepatica*, the next experiment was designed to determine the effect of infection with *F. hepatica* on infiltrating Treg cells in the lungs of tumour bearing mice. Lungs were removed 14 days post tumour challenge from infected and non infected mice and the mononuclear cells were stained for CD4, CD8, CD25, IL-10 and FoxP3. In contrast to the B16 lung tumour model, there was not a significant difference observed in the percent of $CD4^+$ T cells infiltrating into the lungs of CT26 tumour bearing with or without *F. hepatica* infection. However, a marginal but non-significant increase was observed in $CD4^+$ T cells expressing IL-10 in the lungs of infected mice (Fig 4.7). In contrast to the s.c. tumour model (Fig 4.3), a reduction in the percentage of $CD4^+FoxP3^+$ T cells was observed in the lungs of tumour bearing mice infected with *F. hepatica*. Similarly, infection with *F. hepatica* was associated with a slight increase in $CD8^+$ T cell expressing IL-10, while a reduction in $CD8^+$ T cells expressing FoxP3 was also evident (Fig 4.7 B). These results indicate that while infection with *F. hepatica* exacerbated tumour

burden in the lungs of *F. hepatica* infected mice, this was not associated with increased percentages of nTreg cells in the lung.

4.2.7 The ES products of *F. hepatica* modulates a DC response to promote TGF-β and IL-10 production

Previous studies demonstrated a major role for *F. hepatica* in suppressing antitumour immunity, the next experiments were designed to determine if the ES products secreted by the parasite would also have immunosuppressive effects on antitumour immune responses. The first experiment examined the ability of ES to modulate cytokine production by BMDC. DC from C57BL6 mice were incubated with ES (1, 5, 10, 20, 30 and 40 μl/ml) and the concentration of TGF-β was quantified 24 hours later. The data demonstrates that incubation of DC with ES significantly enhanced the production of TGF-β (Fig 4.8, $p<0.05$, $p<0.001$)). The increase in ES-induced TGF-β was dose dependent and could be observed with as little as 1 μl/ml of ES (Fig 4.8). Furthermore, the production of IL-10 from DC was significantly enhanced in a dose dependent manner in response to ES (Fig 4.8, $p<0.05$, $p<0.01$), however, this effect was only observed with higher concentrations of ES (< 20 μl/ml). In contrast to the enhancing effect of ES on active TGF-β and IL-10, ES attenuated the production of IL-12p40, TNF-α and IL-6 from DC in a dose dependent manner (Fig 4.9, $p<0.05$, $p<0.01$, $p<0.001$). This data suggests that ES products from the *F. Hepatica* parasite significantly modulates the innate immune response, suppressing pro-inflammatory cytokine production, while driving the production of immunosuppressive cytokines.

4.2.8 ES exacerbates B16 tumour growth in mice

Having established that ES can modulate the innate immune response, suppressing the induction of inflammatory cytokines, while reciprocally enhancing TGF-β and IL-10 production from DC *in vitro*, experiments were designed to determine if the immunosuppressive properties of ES could hinder effective antitumour adaptive immune responses and subsequently exacerbate tumour growth *in vivo*. C57BL6 mice were injected s.c. with B16 melanoma tumour cells and injected into the region of the tumour with 20 μl of ES or PBS as a control on day 1 and every second day thereafter. Similar to the *F. hepatica* infection model, palpable tumours were detected earlier in mice that received ES, and these mice demonstrated significant exacerbation of tumour growth when compared with the tumour mice treated with PBS (Fig 4.10, $p<0.001$). Collectively, this data confirms that both infection with *F. hepatica* parasite and administration of the products secreted by the parasite has the ability to exacerbate tumour growth *in vivo*.

The effect of ES on TILs was determined by surface and intracellular staining. Interestingly, there was a significant enhancement in both the frequency and absolute numbers of tumour-infiltrating $CD4^+$ T cells expressing TGF-β in ES-treated mice when compared with the tumour only control group (Fig. 4.11, $p<0.05$). In contrast to the effect of *F. hepatica* infection on tumour immunity, the percentage of $CD4^+CD25^+FoxP3^+$ T cells recruited into the tumour was significantly reduced in response to ES treatment (Fig 4.11), however, the absolute numbers of tumour infiltrating $CD4^+CD25^+FoxP3^+$ cells indicate that there was no

differences between tumour bearing mice injected with ES or a PBS control (Fig 4.11). Since the analysis is based on T cells that infiltrate the tumour, it was important to determine both the percent and absolute numbers of TILs as the proportion of tumour cells to lymphocytes is a lot greater in larger tumour volumes and therefore the percent of TILs is relatively lower. Collectively, these findings indicate that similar to infection with *F. hepatica*, ES significantly exacerbated tumour growth. However, unlike *F. hepatica* infection, exacerbation of tumour growth by ES was not associated with the recruitment of tumour infiltrating nTreg cells but could be in part mediated by enhancing or recruiting tumour infiltrating $CD4^+TGF\text{-}\beta^+$ T cells.

4.2.9 Exacerbation of tumour growth by ES is mediated through TGF-β

Having shown that ES significantly exacerbated tumour growth, an effect which was associated with a significant enhancement in the recruitment of tumour infiltrating $CD4^+TGF\text{-}\beta^+$ cells, the next question to be addressed was the role of TGF-β in ES-mediated enhancement of tumour growth. C57BL6 mice were challenged s.c. with B16 melanoma tumour cells on day 0 and ES (20 μl) or PBS as a control (20 μl) were s.c. injected in the presence or absence of a neutralizing TGF-β antibody (100 μg per mouse) near the site of the tumour every second day from day 1. Consistent with previous findings, mice that received ES demonstrated an earlier development of palpable tumours and tumour growth was significantly exacerbated (Fig 4.12B, $p<0.001$). Inhibiting TGF-β at the site of the tumour with a neutralizing TGF-β antibody was associated with a significant reduction in tumour

growth when compared with the control mice treated with PBS (Fig 4.12B, $p<0.05$, $p<0.01$). This experiment was consistent with the data presented in chapter 3 where silencing TGF-β gene expression using siRNA significantly attenuated tumour growth and improved the efficacy of a cell-based therapy (Fig 3.5). Interestingly, anti-TGF-β treatment completely blocked the exacerbating effects of ES on tumour growth (Fig 4.12B, $p<0.001$). Furthermore, the ES-treated mice that received anti-TGF-β demonstrated a significant attenuation of tumour growth when compared to the tumour only control group (Fig 4.12B, $p<0.05$, $p<0.01$). Moreover, it was evident from the individual tumour plots that the mice that received both ES and anti-TGF-β exhibited the smallest tumour volumes, with only two out of six mice developing measurable tumours compared with all other treatment and control groups, where at least 5 out of 6 mice developed palpable tumours (Fig 4.12A).

4.2.10 Neutralizing TGF-β inhibits ES induced $CD4^+TGF\text{-}\beta^+$ T cells within the B16 tumour mass

Having confirmed that the mechanism by which ES exacerbated tumour growth was mediated via TGF-β, experiments were carried out to establish the effects of ES and anti-TGF-β on T cell responses within the tumour. Mice were challenged s.c. with B16 tumour cells and treated with PBS, ES and/or anti-TGF-β. After 14 days, the phenotype of TILs for each group was determined by surface and intracellular staining. Consistent with previous findings, the results demonstrate that exacerbation of tumour growth in response to ES was associated with an increase in the percentage and absolute numbers of tumour infiltrating $CD4^+$TGF-

β^+ T cells (Fig 4.13). A modest reduction in the percent of tumour infiltrating $CD4^+TGF\text{-}\beta^+$ T cells was observed when ES-treated mice were treated with anti-TGF-β (Fig 4.13). However, a clearer trend was evident in the absolute numbers of tumour infiltrating $CD4^+TGF\text{-}\beta^+$ T cells; treatment with anti-TGF-β completely inhibited the enhancement of tumor infiltrating $CD4^+TGF\text{-}\beta^+$ T cells in response to ES (4.13).

In support of the data presented in chapter 3, these experiments demonstrated that blocking TGF-β at the site of the tumour reduced the frequency of tumour infiltrating nTreg cells (Fig 3.10). Moreover, the percent of $CD4^+CD25^+FoxP3^+$ cells recruited into the tumour was attenuated in tumour-bearing mice treated with ES and/or α-TGF-β (Fig 4.14). Consistent with previous experiments, the reduction in the percent of tumour infiltrating nTreg cells observed in the ES-treated mice was not reflected in the absolute numbers of tumour infiltrating nTreg cells (Fig 4.14). However, a comparison of mice with untreated tumours to tumour bearing mice treated with ES and/or αTGF-β revealed a consistent reduction in the absolute numbers of tumour infiltrating $CD4^+CD25^+FoxP3^+$ T cells (Fig 4.14).

Assessment of the percent and absolute numbers of tumour infiltrating $CD4^+IL\text{-}10^+$ T cells did not reveal any significant difference between tumour bearing mice that received ES, PBS, and/or anti-TGF-β (Fig 4.14). However, ES-treated mice that received anti-TGF-β demonstrated an increase in the percent of $CD4^+IL\text{-}10^+$ cells when compared with mice that received PBS, ES or αTGF-β. In addition, a modest

reduction in the absolute numbers of $CD4^+IL\text{-}10^+$ was observed when mice treated with ES or a PBS control, were compared with mice that received anti-αTGF-β (Fig 4.14).

The frequency of tumour infiltrating $CD8^+$ T cells expressing IFN-γ was reduced in the tumours of mice that received ES (Fig 4.15). Moreover, treatment with anti-TGF-β blocked the suppressive effect of ES on the percent of tumour infiltrating $CD8^+IFN\text{-}\gamma^+$ T cells (Fig 4.15). However, this pattern was not reflected in the absolute numbers of tumour infiltrating $CD8^+IFN\text{-}\gamma^+$ T cells. Similar to the trend observed for tumour infiltrating $CD4^+IL\text{-}10^+$ T cells, tumour infiltrating $CD8^+IL\text{-}10^+$ T cells did not reveal any significant difference between control tumour mice and tumour bearing mice that received ES and/or anti-TGF-β (Fig 4.15).

Collectively, this study reveals that ES exacerbated tumour growth through a mechanism involving TGF-β. Neutralizing TGF-β at the site of the tumour completely reversed the effects of ES, while also attenuating tumour growth below that of the tumour only control. The cytokine profile within the B16 tumour mass demonstrated enhancement of tumour infiltrating $CD4^+TGF\text{-}\beta^+$ T cells in the ES-treated mice and this was completely inhibited in mice that received anti-TGF-β. This data suggests that exacerbation by ES is mediated by TGF-β and neutralizing this cytokine blocks the effects of ES and also neutralized endogenous TGF-β, which attenuated tumour growth below the volumes observed in control tumor bearing mice.

4.3 DISCUSSION

Chronic infections with parasites, viruses and bacteria contribute to ~18% of cancers worldwide [251]. While only a small portion of such cases are attributed to helminth infections, overall the influence of such persistent infections remains a huge difficulty in the developing world, where the number of people exposed or challenged with parasitic infections is extremely high [252]. The influence of helminth infection on the development and growth of malignancies is a complicated process consisting of numerous different mechanisms, however, the significant new finding of this study is that infection with the helminth parasite, *F. hepatica*, significantly exacerbated tumour growth *in vivo*, an effect that was associated with a dramatic enrichment of tumour infiltrating $FoxP3^+$ Treg cells in the s.c. B16 model. Additionally, administration of *F. hepatica* ES products alone were capable of promoting tumour growth *in vivo* through a TGF-β-dependent mechanism.

Several reports have suggested significant positive associations between the occurrence of helminth infections and cancer, with bladder carcinomas being geographically associated with *S. haematobium* infection [253]. However, given the long latency periods of both diseases it is challenging to confirm a definitive effect of infection on development or progression of cancer, as numerous other factors associated with each condition can play a role in altering the immune response. However, animal studies have provided evidence of strong correlations between helminth infections and the development of cancer. For example monkeys and opossums infected with *S. haematobium* develop transitional cell carcinomas [254].

Interestingly, the present study demonstrated that *F. hepatica*-mediated exacerbation of tumour growth in the s.c. B16 melanoma model was associated with a dramatic enhancement of $FoxP3^+$ Treg cells, in some cases showing over 50% of tumour infiltrating $CD4^+$ T cells co-expressed CD25 and FoxP3. While there is limited data on the immunological effects of a parasitic infection in experimental tumour models, several studies have reported parasitic infections significantly amplify Treg cell responses. $FoxP3^+$ Treg cells contribute to immune suppression during infection with the malaria parasite, allowing the parasite to escape from host immunity [255]. Furthermore FoxP3 expression was induced at a high rate in naïve $FoxP3^-$ ovalbumin-specific T cells in mice infected with *H. polygyrus* [256]. It has also been demonstrated that helminth parasites can employ indirect mechanisms to convert naïve $CD4^+$ T cells into $FoxP3^+$ Treg cells, with schistosome egg antigens from *S. mansoni* acting on DCs to promote the induction of $FoxP3^+$ Treg cells [257].

The present study also showed that infection with *F. hepatica* significantly enhanced B16 tumour metastases in the lung, indicating that infection with *F. hepatica* promoted systemic immune tolerance, as demonstrated by exacerbation of tumour growth in both lung and s.c. tumour models. Furthermore, the present study demonstrated that exacerbation of tumour growth *in vivo* was not specific to one tumour type, as a significant enhancement of CT26 tumour metastases was observed in the lungs of *F. hepatica* infected mice. This data is consistent with a report by Walsh et al., that *F. hepatica* induces immunosuppression *in vivo* and this

can compromise host immune responses to the parasite and to autoantigens [238]. In contrast to the s.c. tumour model, enrichment of FoxP3 expressing T cells was not observed in the lungs of tumour bearing mice infected with *F. hepatica*, however, the expression of the IL-10 suppressive cytokine was enhanced from both $CD4^+$ and $CD8^+$ T cell populations in the lungs of infected mice. The reason for these differences are unclear, however, a possible explanation may be that enrichment of nTreg cells or iTreg cells can be influenced by the tissue and environment where the immune response is generated. Thus the immune response in the lung may be more conducive to the induction of iTreg cells, such as Tr1 cells, and peripheral sites more suited to the recuitment/expansion of nTreg cells. This is supported by observations that DCs in the lung readily produce IL-10 to mediate tolerance [258], which would preferentially support the induction of Tr1 type Treg cells.

Helminth parasites secrete biologically active ES products that can directly modulate host immune responses [259]. DC are key APCs that play a crucial role in orchestrating adaptive immunity and as such are a prime target cell for multiple immune modulating signals delivered by the parasite in order to subvert the development of protective immune responses. A significant finding of the present study was that ES products from *F. hepatica* induced TGF-β and IL-10 production by DC, a phenotype associated with the induction of Th3 cells and Tr1 cells, respectively. Consistent with this finding, it was recently shown that one of the most abundant proteins released by the adult hookworm, a tissue inhibitor of

metalloproteases (Ac-TMP-1), enhanced the production of TGF-β and IL-10 by DC [260]. Indeed the present study also demonstrated that the *F. hepatica* ES significantly inhibited IL-12p40, IL-6 and TNF-α production by DC, possibly suggesting that inflammatory responses by DC were significantly constrained by ES-induced TGF-β and IL-10. These findings are consistent with reports from Hamilton et al., that showed that *F. hepatica* tegumental antigen (Teg) significantly suppressed IL-12p70, TNF-α and IL-6 production by LPS-activated DC [259]. Collectively, this study reveals a mechanism by which live *F. hepatica* parasites secrete biologically active products to exert suppressive effects and evade host immune responses to the parasite.

Furthermore, the present study demonstrated that F. *hepatica* ES products significantly exacerbated B16 tumour growth *in vivo*, an effect that was associated with a significant enhancement of tumour infiltrating $CD4^{+}TGF\text{-}\beta^{+}$ T cells. A subversive role for TGF-β in tumour growth is well established and consistent with the findings established in Chapter 3. Interestingly, ES-mediated exacerbation of tumour growth was not associated with enrichment of tumor infiltrating $FoxP3^{+}$ Treg cells. Moreover, the present study established that ES-mediated exacerbation of tumour growth was completely reversed by neutralization of TGF-β *in vivo*, formally demonstrating that ES exacerbated tumour growth via TGF-β-dependent mechanisms. Similarly, it has recently been reported by Maizel and colleagues that *H. polygyrus* ES products mediate their effects via the TGF-β pathway, however, in

contrast to the present study, they have reported that this TGF-β induces *de nova* FoxP3$^+$ Treg cells [256].

As expected, neutralising TGF-β at the site of the tumour reduced the ES-mediated enhancement of TGF-β, however, the percentage of tumour infiltrating CD4$^+$IL-10$^+$ Tr1cells was modestly increased under these conditions. A possible explanation for this observation may be that when endogenous TGF-β is inhibited, the host's intrinsic immune response is compromised and therefore it enhances CD4$^+$IL-10$^+$ T cells to dampen down overactive immune responses to the *F. hepatica* ES products. Furthermore, mice treated with both anti-TGF-β and ES had the smallest mean tumour volumes when compared with all other experimental groups, suggesting that neutralising TGF-β also reduced endogenous TGF-β and/or tumour derived TGF-β and attenuated tumour growth *in vivo*, highlighting the suppressive role of TGF-β in subverting antitumour immunity, and supporting the findings in chapter 3.

This study also demonstrated that ES had effects on antitumour effector T cells, reducing the percentage of tumour infiltrating CD8$^+$IFN-γ$^+$ T-cells; an effect that was reversed by neutralising TGF-β. It is well established that CD8$^+$ CTLs have a protective role in antitumour immunity, able to mediate direct killing of tumour cells and to activate other immune cells via the secretion of IFN-γ [175]. In addition, ES enhanced tumour infiltrating CD8$^+$IL-10$^+$ T cells, which have been shown to have regulatory functions, actively suppressing antitumour immune

responses [189]. Furthermore, neutralising TGF-β in the ES-treated mice modestly enhanced tumour infiltrating $CD8^+IL\text{-}10^+$ T cells, which may also reflect the intrinsic imbalance in host immune responses. Collectively, ES reduced the frequency of proinflammatory $CD8^+$ IFN-γ^+ T cells within the tumour mass, while promoting the expression of tumour infiltrating $CD8^+$ T cells expressing IL-10, highlighting the capacity of ES to skew immunological responses to that of a regulatory phenotype.

Collectively the present study has established several novel findings on the immunomodulatory effects of *F. hepatica* and *F. hepatica* ES products on tumour growth and development. Firstly, the present study established that infection with *F. hepatica* significantly exacerbated tumour growth *in vivo*. Likewise, exacerbation of tumour growth was also mediated by the ES products of *F. hepatica*, suggesting that the parasite has evolved mechanisms to induce systemic immune tolerance to down-regulate protective immune responses. Secondly, this study demonstrated that exacerbation of tumour growth was associated with enhancement of tumour infiltrating Treg cells. Exacerbation of tumour growth in response to *F. hepatica* was associated with a dramatic enrichment of nTreg cells, while ES-mediated exacerbation of tumour growth was associated with a significant enhancement of tumour infiltrating Th3 type Treg cells. The differential immune-mediated responses elicited by the parasite and its secreted products can be rationally interpreted. The dominant nTreg response observed within the tumour mass of *F. hepatica* infected mice may reflect the intrinsic immune response

elicited by the host to systemically limit harmful immune-mediated pathology against the parasite. However, ES enhanced tumour growth via TGF-β-dependent mechanisms, indicating that the parasite secretes biologically active products to suppress and subvert immunity to the parasite, which have a systemic effect on immunity. Thus, this study provides direct experimental evidence that parasite induced regulatory cells and their immunosuppressive cytokines can systemically suppress the immune response and allow growth of tumours to be unhindered by effector immune responses. It is estimated that that up to 17 million people are infected with *F. hepatica* worldwide and while this disease is recognized as a serious public health problem by the WHO, these novel findings raise more worrying concerns for *F. hepatica* infected individuals and the impact this induced systemic immune suppression may have on tumour growth and development.

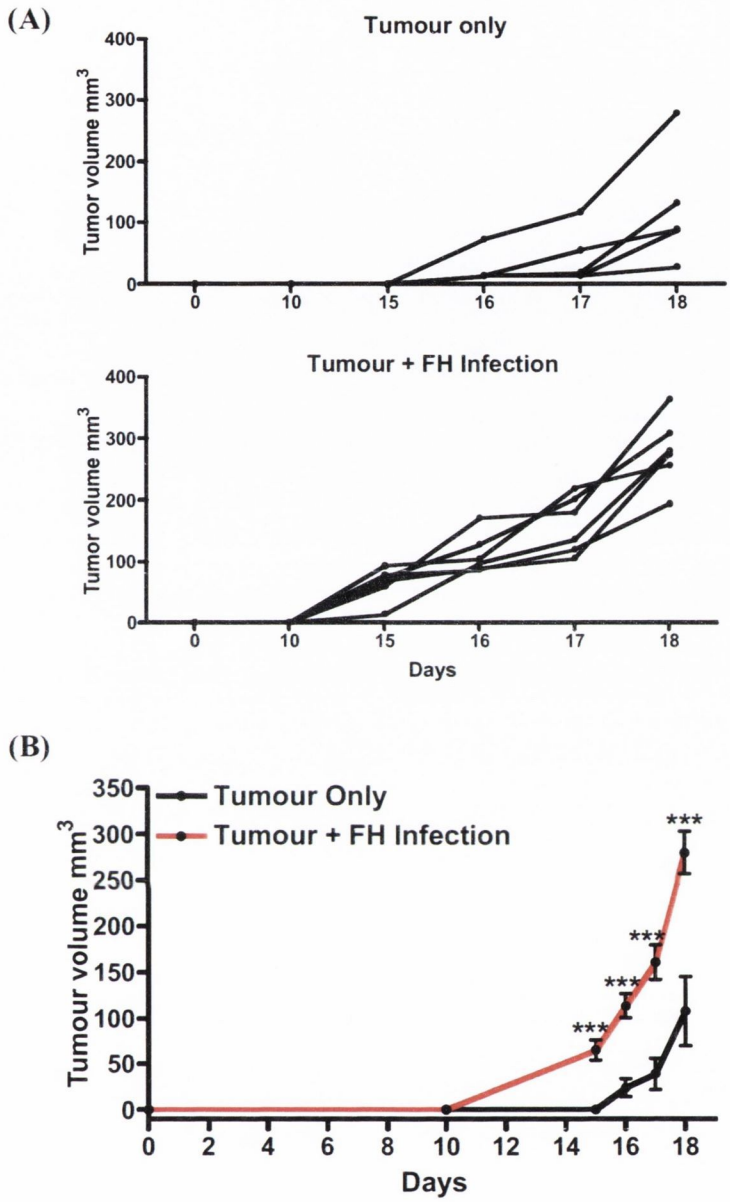


Figure 4.1 *F. hepatica* infection exacerbates B16 tumour growth in mice. C57BL/6 mice were injected s.c. with 2 x 10^5 B16 melanoma tumour cells on day 0. One group of mice were infected with *F. hepatica* by oral administration of 10 viable metacaceria per mouse on day 0, and one group were left untreated. Tumour volumes were measured from day 10. (A) Tumour growth rates for individual mice (n=6). (B) Mean tumour volume per group +/- SEM for 6 mice: ***p < 0.001 by two way ANOVA. This experiment is a representative from five experiments.

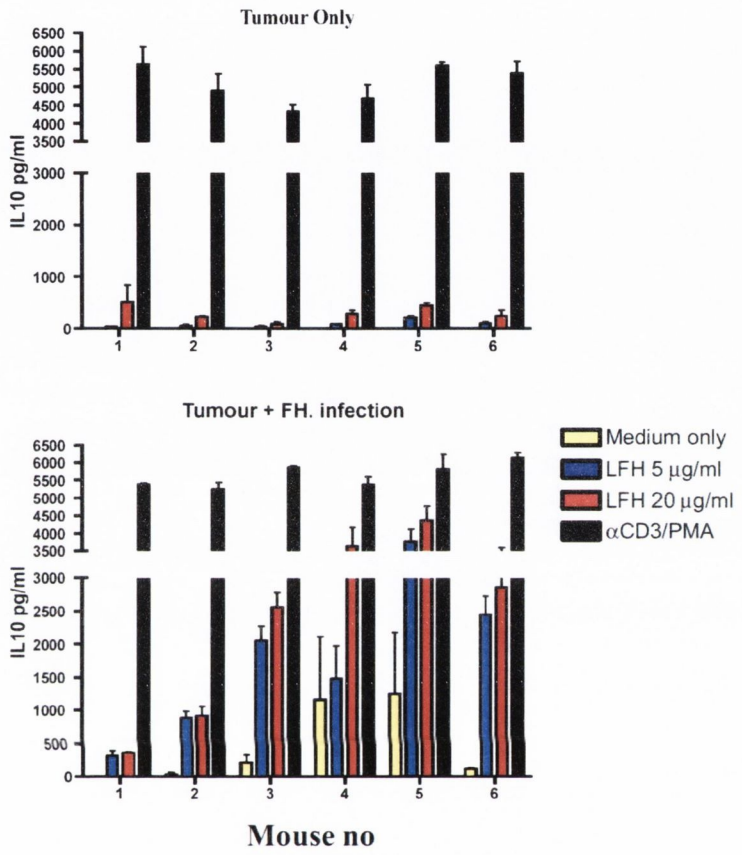


Figure 4.2 *F. hepatica*-specific IL-10 production by spleen cells from tumour bearing mice with or without *F. hepatica* infection. C57BL/6 mice were challenged with B16 and infected with F. *hepatica* as described in legend Fig 4.1. Spleen cells were recovered after 18 days and restimulated *in vitro* with liver fluke homogenate (5 µg/ml and 20 µg/ml), medium only or PMA and anti-CD3 stimulated cells. After 3 days the concentration of IL-10 in supernatents was quantified by ELISA. Results are shown for 6 individual mice on samples quantified in triplicate assays. This experiment is a representative from three independent experiments.

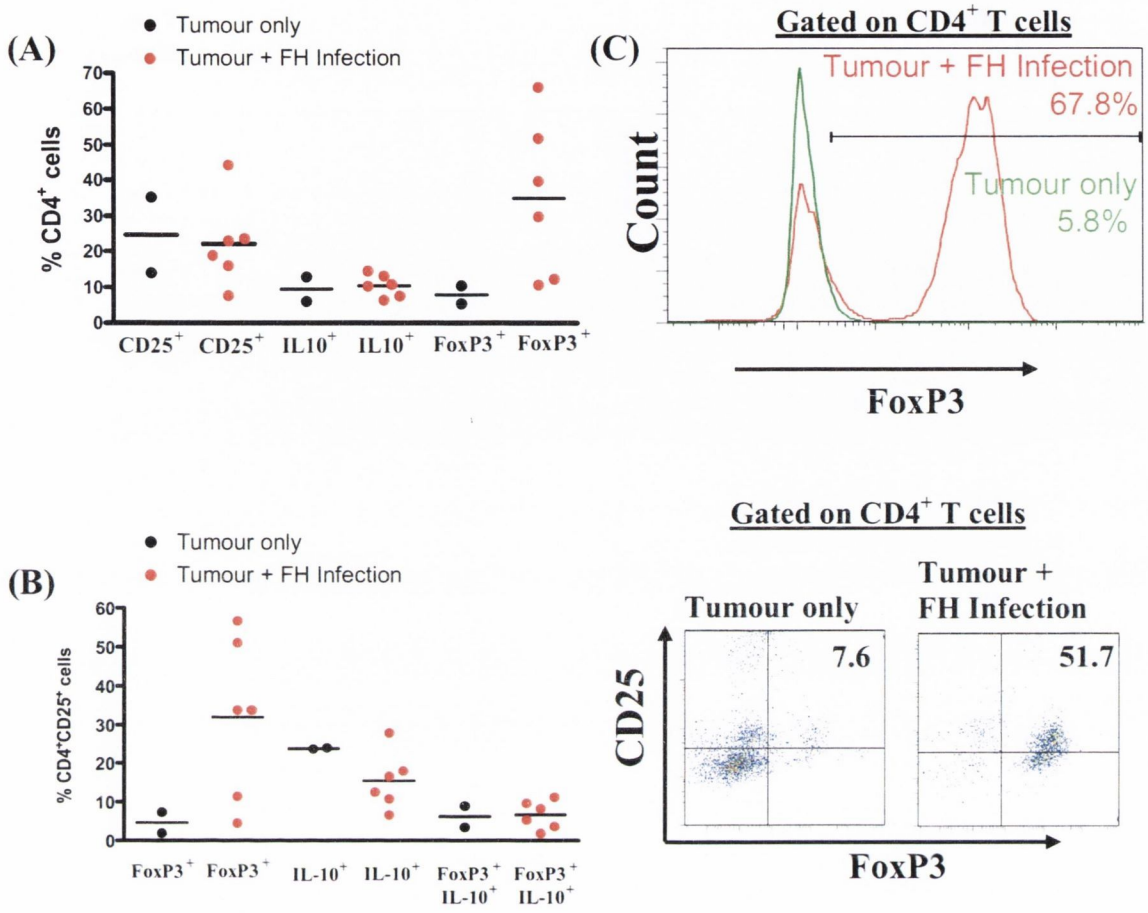


Figure 4.3 *F. hepatica* significantly enhances tumour infiltrating $CD4^+CD25^+FoxP3^+$ T cells. C57BL/6 mice were challenged s.c. with B16 tumour cells. One group of mice were infected with *F. hepatica*, and one group were left untreated. Subcutaneous tumours were excised 18 days after tumour challenge. Tumour infiltrating cells were stimulated with PMA (50 ng/ml), ionomycin (500 ng/ml) and Brefeldin A (5 µg/ml) for 6 hours. The tumours from un-infected mice were pooled into two samples due to small tumour volumes and a lower yield of cells. (A) Frequency of tumour infiltrating $CD4^+$ T cells staining for CD25, IL-10 and FoxP3. (B) Representative flow cytometry demonstrating $CD4^+$ T cells from tumour only and *F. hepatica* infected tumour-bearing mice, gating on $CD25^+$ and $FoxP3^+$ cells. (C) Representative mean fluorescence intensity measuring total FoxP3 from both tumour only and tumour and infected mice.

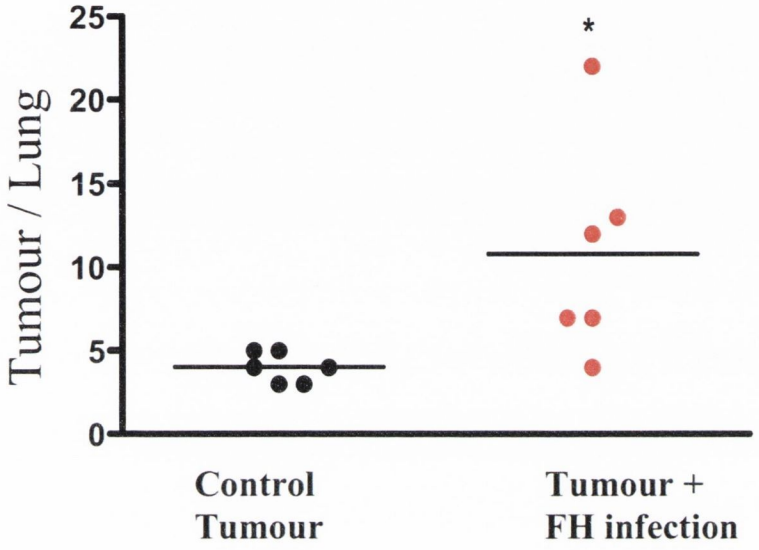


Figure 4.4 Enhanced B16 tumour burden in lungs of mice infected with *F. hepatica*. C57BL6 mice were injected i.v. with $3x10^5$ B16 melanoma cells on day 0. One group of mice were infected with *F. hepatica* by oral administration of 10 viable metacaceria per mouse on day 0, and one group were left untreated. Lungs from each group were removed on day 14, fixed and tumour nodules on the large lung lobe were counted. (*$p<0.05$ by unpaired T test).

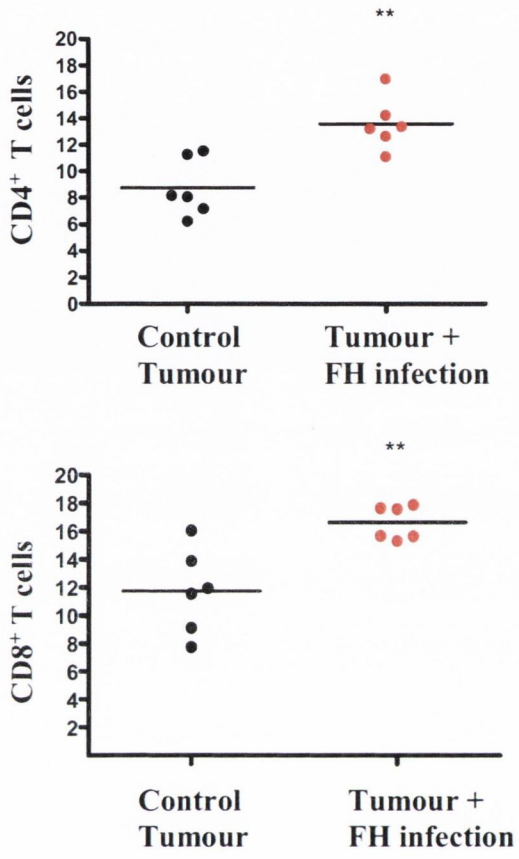


Figure 4.5 Enhanced $CD4^+$ and $CD8^+$ T cells in lungs of B16 tumour bearing mice challenged with *F. hepatica* infection. C57BL6 mice were injected i.v. with $3x10^5$ B16 melanoma cells on day 0. One group of mice were infected with *F. hepatica* by oral administration of 10 viable metacaceria per mouse on day 0, and one group were left untreated. Lungs from each group were removed on day 14 and the frequency of $CD4^+$ and $CD8^+$ T cells were determined by flow cytometry. (*$p < 0.01$ by unpaired T test).

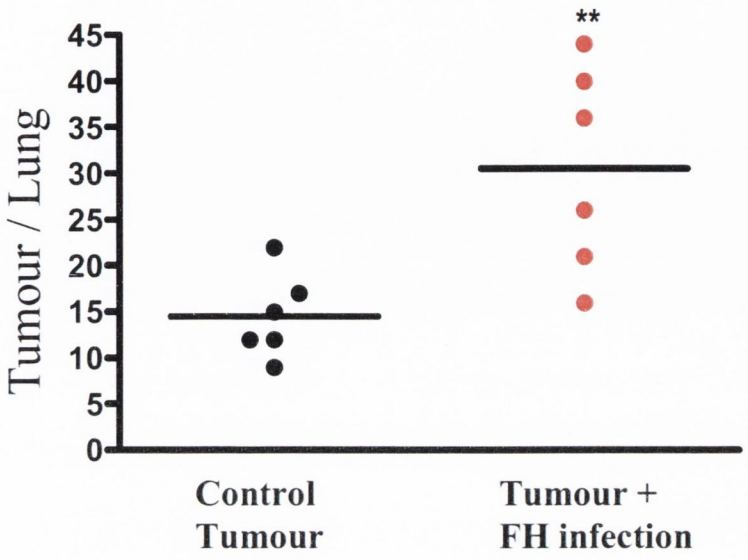


Figure 4.6 Enhanced CT26 tumour burden in lungs of mice infected with *F. hepatica*. BALB/c mice were injected i.v. with $3x10^5$ CT26 colon carcinoma cells on day 0. One group of mice were infected with *F. hepatica* by oral administration of 10 viable metacaceria per mouse on day 0 and one group were not infected. Lungs from each group were removed on day 14, fixed and tumour nodules on the large lung lobe were counted (**$p < 0.01$ by unpaired T test).

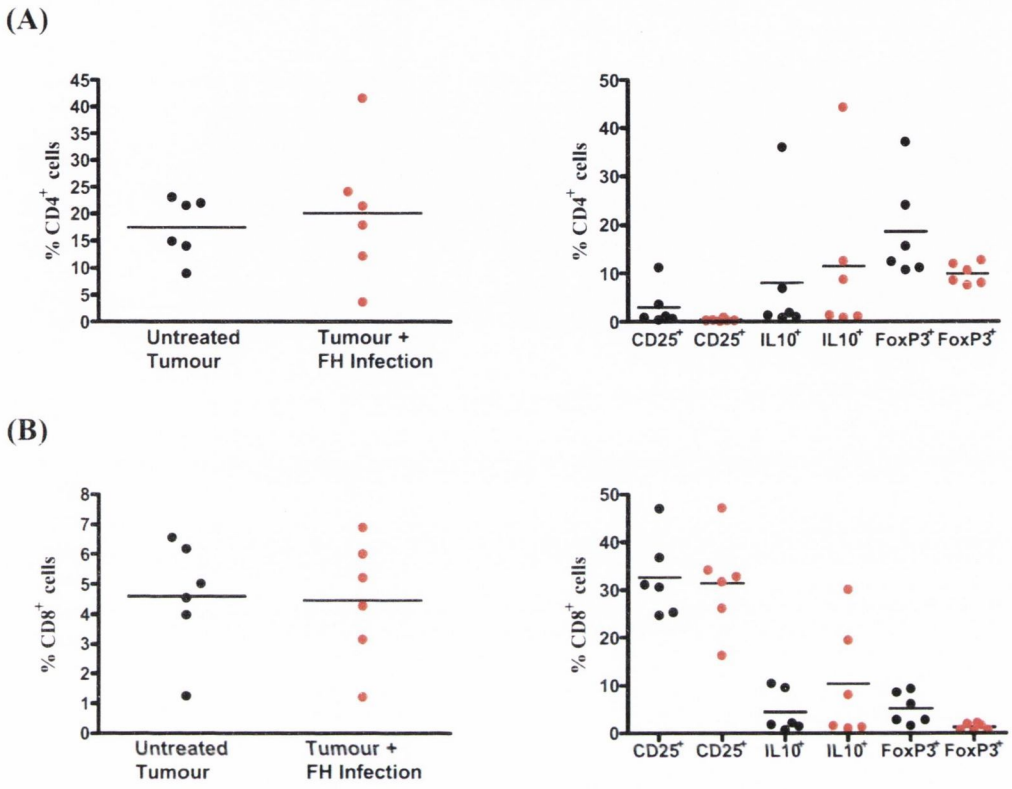


Figure 4.7 Influence of *F. hepatica* infection on infiltrating $CD4^+$ and $CD8^+$ T cell populations into the lung of CT26 tumour bearing mice. BALB/C mice were injected i.v. with $3x10^5$ CT26 colon carcinoma cells on day 0. One group of mice were infected with *F. hepatica* by oral administration of 10 viable metacaceria per mouse on day 0, and one group were left untreated. Lungs from each group were removed on day 14 and collagenase digested. Cells infiltrating the lung were stimulated with PMA (50 ng/ml), ionomycin (500 ng/ml) and Brefeldin A (5 µg/ml) for 6 hours. The Frequency of tumour infiltrating $CD4^+$ T cells (A) and $CD8^+$ T cells (B) expressing CD25, IL-10 and FoxP3 were determined by flow cytometry.

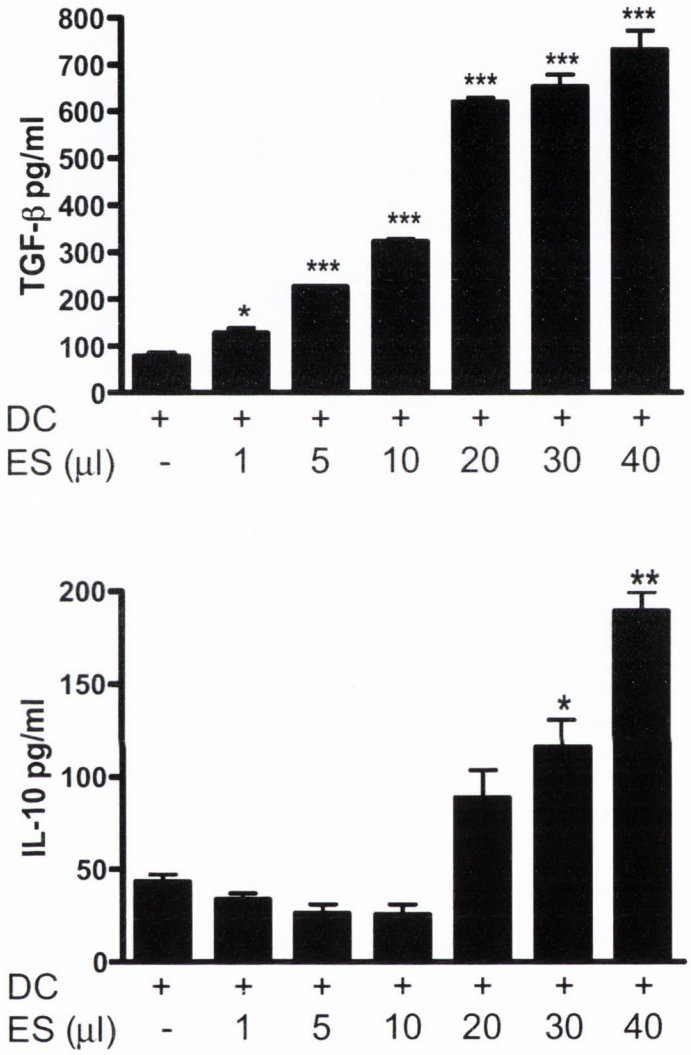


Figure 4.8 ES products from *F. hepatica* induces TGF-β and IL-10 from DC. BMDC (1 x 10^6 cells/ml) were stimulated with *F. hepatica* ES products (1, 5, 10, 20, 30 or 40 µl). Supernatents were removed after 24 hours and the concentration of TGF-β was quantified by ELISA. This experiment is a representative of three independent experiments. Results are means (+/- SD) of triplicate assays; *$p<0.05$, **$p<0.01$, ***$p<0.001$ DC only versus DC + ES by unpaired t test.

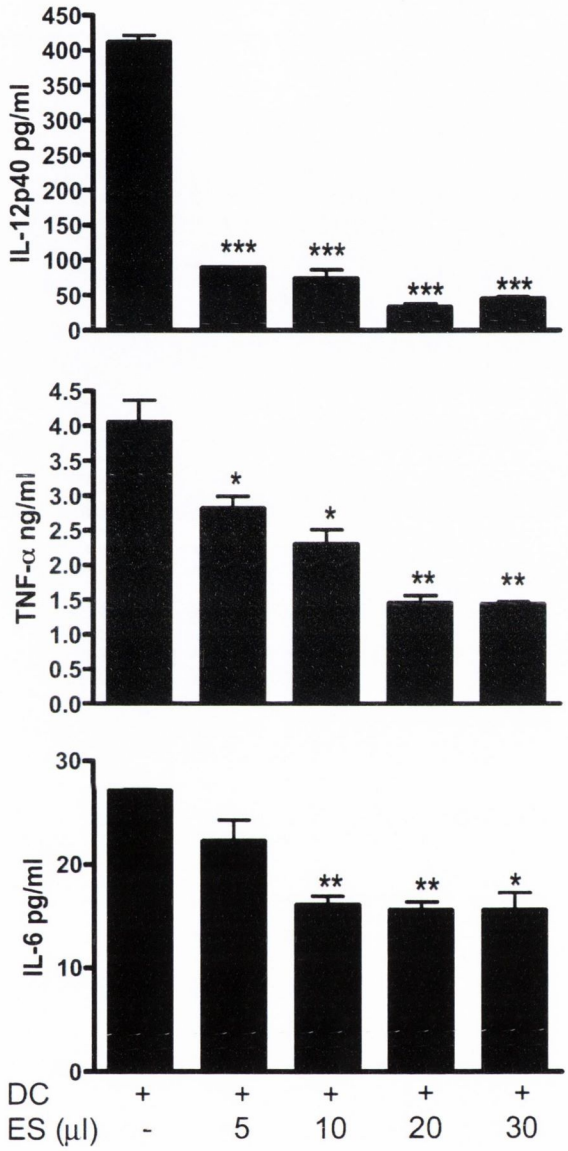


Figure 4.9 ES attenuates the production of the pro-inflammatory cytokines IL-12p40, TNF-α and IL-6 from DC. BMDC (1 x 10^6 cells/ml) were incubates with *F. hepatica* ES products (5, 10, 20 or 30 µl). Supernatents were removed after 24 hours and the concentrations of IL-12p40, TNF-α, IL-6 and IL-10 were quantified by ELISA. This experiment is a representative of three independent experiments. Results are means (+/- SD) of triplicate assays; *$p<0.05$, **$p<0.01$, ***$p<0.001$ DC only versus DC + ES by unpaired t test

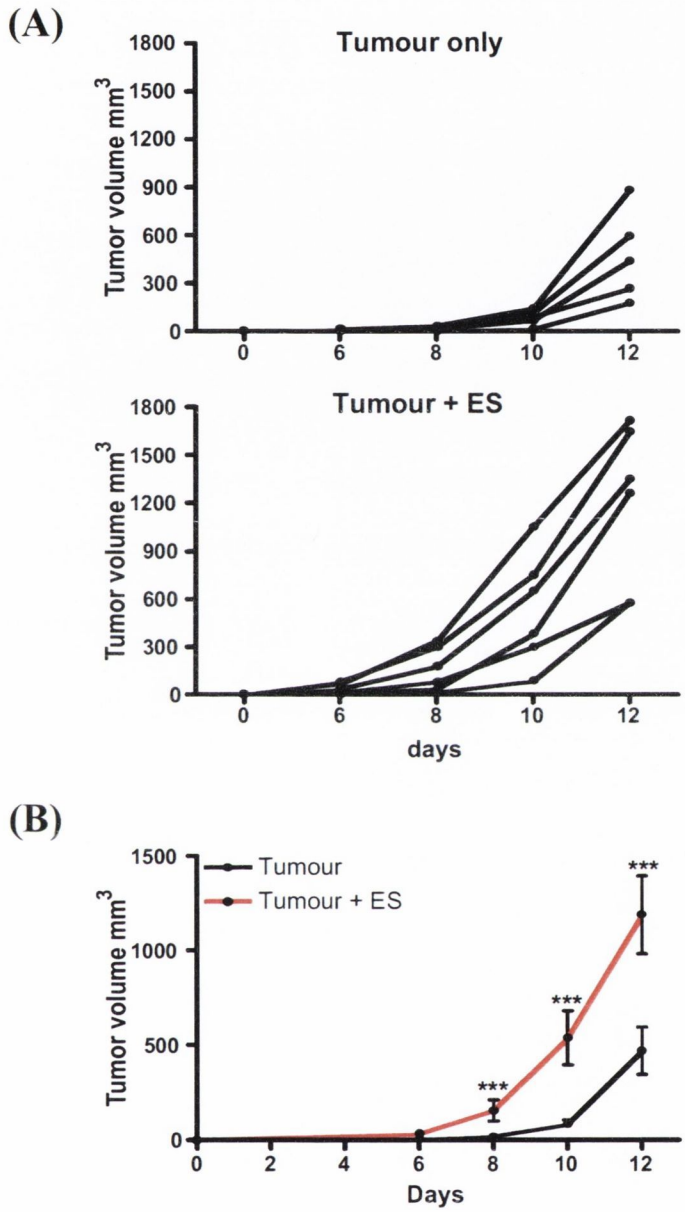


Figure 4.10 ES products from *F. Hepatica* exacerbates tumour growth *in vivo*. C57BL/6 mice were injected s.c. with 2 x 10^5 B16 melanoma tumour cells. ES (20 µl) or PBS (20 µl) was injected s.c. into the site of the tumour from day 1 and every second day thereafter. (A) Tumour growth rates (B) Mean tumour volume per group. This experiment is a representative from 4 experiments. Results are mean score +/- SEM for 6 mice, ***$p < 0.001$ by two way ANOVA.

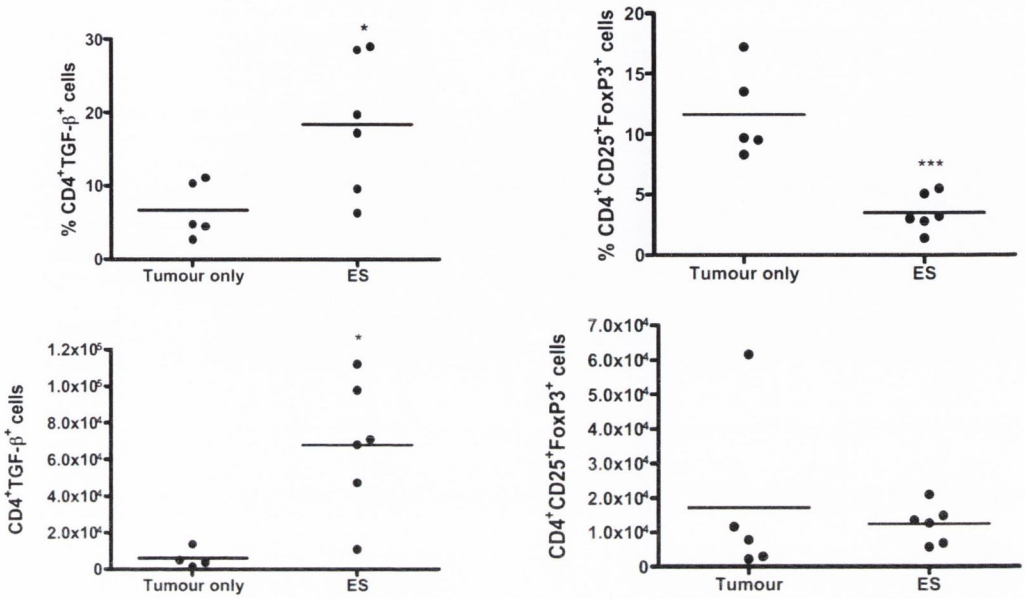


Figure 4.11 ES products from *F. Hepatica* significantly enhances tumour infiltrating CD4$^+$TGF-β$^+$ T cells. C57BL/6 mice were injected s.c. with 2 x 10^5 B16 melanoma tumour cells. ES (20 µl) or PBS (20 µl) was injected s.c. into the site of the tumour from day 1 and every second day thereafter. Tumours were removed 12 days post tumour and infection challenges. The frequency of tumour infiltrating CD4$^+$TGF-β$^+$ T cells and CD4$^+$CD25$^+$FoxP3$^+$ T cells was determined directly *ex-vivo* by flow cytometry.

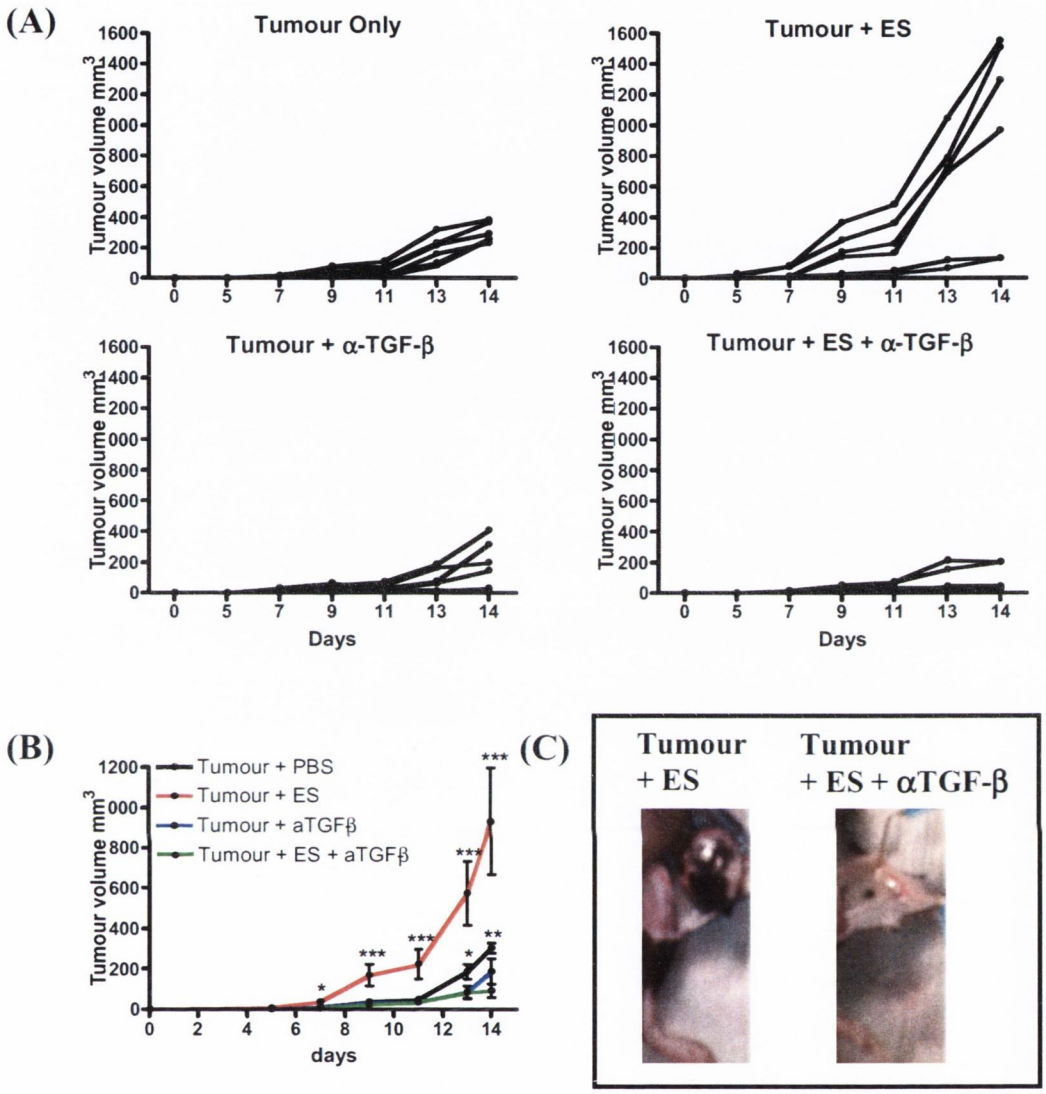


Figure 4.12 Neutralising TGF-β at the site of the tumour completely reverses the effects of ES on tumour growth. Mice were injected s.c. with 2 x 10^5 B16 tumour cells. ES (20 µl) or PBS (20 µl) with and without anti-TGF-β (100 µg) was injected s.c. into the site of the tumour from day 1 and every second day thereafter. (A) Tumour growth rates from each group (B) Mean tumour volume +/- SD for 6 mice: *$p<0.05$, ***$p < 0.001$ by two way anova. (C) A representative picture of tumour size from Tumour + ES group and Tumour + ES and anti-TGF-β group.

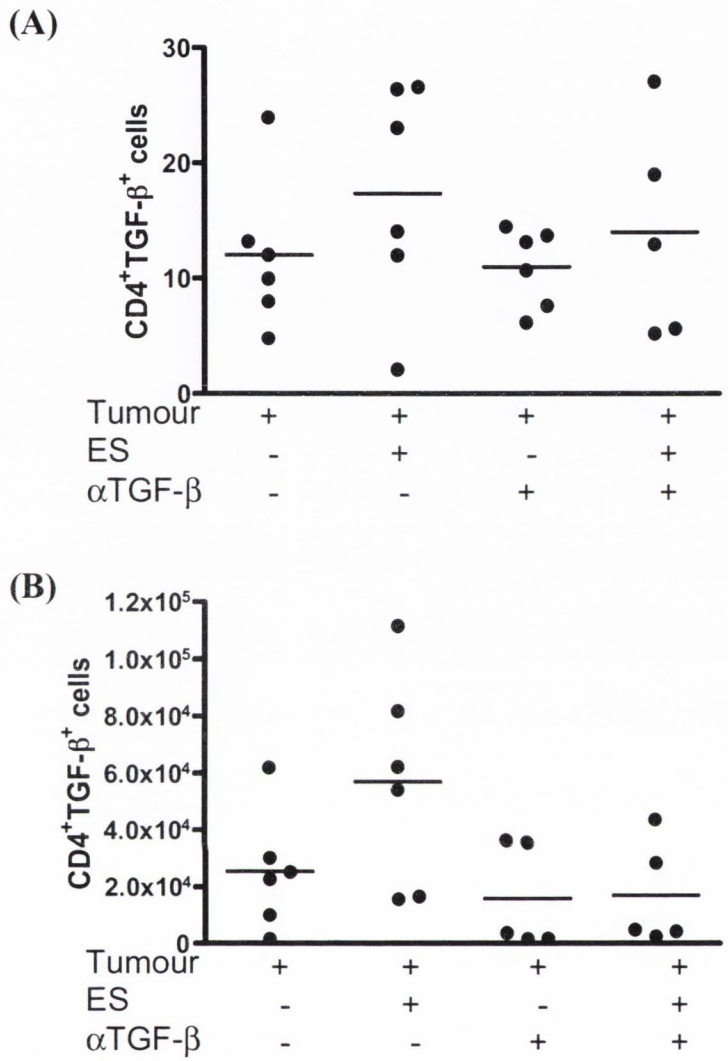


Figure 4.13 Neutralising TGF-β at the site of the tumour inhibits the *F. hepatica* ES-meditated induction of tumour infiltrating CD4$^+$TGF-β$^+$ T cells. Mice were injected s.c. with 2 x 10^5 B16 tumour cells. ES (20 µl) or PBS (20 µl) with and without anti-TGF-β (100 µg) was injected s.c. into the site of the tumour from day 1 and every second day thereafter. Tumours were removed 14 days post tumour challenge. The frequency of tumour infiltrating CD4$^+$TGF-β$^+$ T cells was determined directly *ex-vivo* by flow cytometry. (A) Percent of tumour infiltrating CD4$^+$TGF-β$^+$ T cells, (B) Absolute numbers of tumour infiltrating CD4$^+$TGF-β$^+$ T cells.

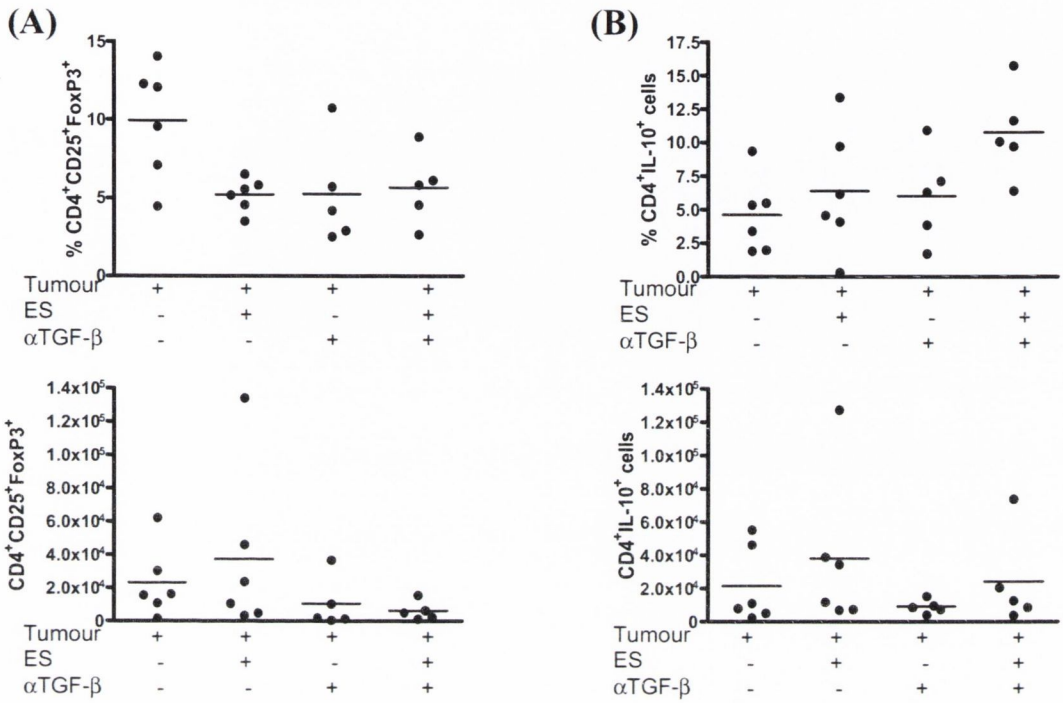


Figure 4.14 The effect of ES and anti-TGF-β on tumour infiltrating nTreg cells and Tr1 cells. Mice were injected s.c. with 2 x 10^5 B16 tumour cells. ES (20 µl) or PBS (20 µl) with and without anti-TGF-β (100 µg) was injected s.c. into the site of the tumour from day 1 and every second day thereafter. Tumours were removed 14 days post tumour and infection challenges. (A) The frequency and absolute numbers of tumour infiltrating $CD4^+CD25^+FoxP3^+$ T cells was determined directly *ex-vivo* by flow cytometry. (B) Percent and absolute number of tumour infiltrating $CD4^+IL\text{-}10^+$ T cells was determined by flow cytometry after 6 hours of stimulation with PMA (50 ng/ml), ionomycin (500 ng/ml) and Brefeldin A (5 µg/ml).

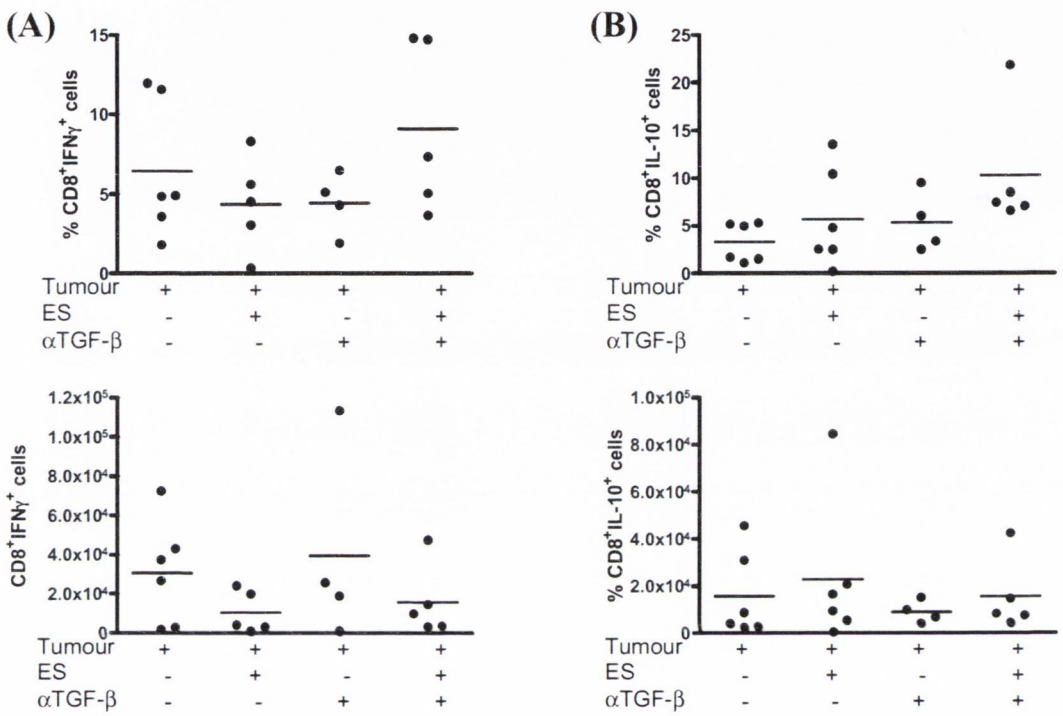


Figure 4.15 The effect of ES and anti-TGF-β on tumour infiltrating $CD8^+$IFN-γ T cells and $CD8^+$IL-10^+ T cells. Mice were injected s.c. with 2 x 10^5 B16 tumour cells. ES (20 µl) or PBS (20 µl) with and without anti-TGF-β (100 µg) was injected s.c. into the site of the tumour from day 1 and every second day thereafter. Tumours were removed 14 days post tumour challenge and stimulated for 6 hours with PMA (50 ng/ml), ionomycin (500 ng/ml) and Brefeldin A (5 µg/ml). The frequency and absolute numbers of tumour infiltrating $CD4^+$IFN-γ^+ T cells (A) and $CD8^+$IL-10^+ T cells (B) was determined by flow cytometry.

Chapter 5

Blocking RA-induced Treg cells as a cancer therapy

5.1 INTRODUCTION

It has long been recognised that dietary constituents and vitamin metabolism can play an influential role in mediating biological processes and immune functions. Indeed in recent years the pleiotropic influence of vitamin A has been an active area of research. Vitamin A has regulatory roles in a wide variety of biological processes, ranging from vision and reproduction to apoptosis and immune homeostasis [104]. The essential role of vitamin A in immunological functions has been amply demonstrated by the fact that supplementation with vitamin A dramatically reduces childhood mortality from lung and gastrointestinal infections in regions of endemic malnutrition [261-262]. Likewise, a deficiency in vitamin A compromises many aspects of innate and adaptive immune responses, with vitamin A deficient individuals showing defects in immune control of bacterial, viral and protozoan infections [105]. Over the last number of years it has become apparent that vitamin A influences multiple immune cell lineages and immunological cell functions [263-264] [265].

Vitamin A is a fat-soluble essential nutrient that cannot be synthesized by the human body, but must be absorbed by the intestine from animal food sources and/or derived from β-carotene, which is found in plants [106]. Subsequent to absorption vitamin A circulates in the peripheral blood as retinol and retinyl esters, which are primarily stored in the liver [107]. These precursors are enzymatically catalysed to RA in a two step process. Once inside a cell retinol is oxidized in a reversible reaction by widely expressed retinoid dehydrogenases (ADHs) to all-trans

retinaldehyde (retinal) [108]. Retinal is then irreversibly metabolized into retinoic acid (RA) in a tightly controlled process by retinaldehyde dehydrogenases (ALDHs) [106]. Multiple isoforms of RA exist, however, all-trans retinoic acid (ATRA) is the most predominant isoform in most tissues [109]. The synthesis of dietary vitamin A into ATRA is mediated at immune privileged sites, by bona fide RA producing DCs, such as gut associated lymphoid tissue (GALT) DCs and macrophages [110].

ATRA or its isomer 9-cis RA exclusively binds to a family of nuclear hormone receptors, termed RA receptors (RARs) and retinoid X receptors (RXR) via heterodimers that act as ligand dependent transcriptional regulators via a retinoic acid response element (RARE) within the promoter of genes [111]. The RARs interact with many different coactivators and corepressor proteins to regulate transcription, with ATRA being the major endogenous agonist for the RARs [266]. The RAR family consists of three subtypes RARα, RARβ and RARγ, which all bind ATRA with high affinity but slightly different binding domains. However, it has been amply demonstrated that RARα is the predominant signalling receptor involved in immune regulation and immune homeostasis [104, 118].

It has recently been discovered that RA, the active derivative of vitamin A, is a key regulator of TGF-β-dependent immune homeostasis, contributing to the reciprocal regulation of Treg and Th17 cell development. Several key studies have shown that RA synthesized by DCs and macrophages can synergize with TGF-β to enhance the

conversion of naïve FoxP3$^-$ T cells into a unique FoxP3$^+$ Treg subset, that are functionally competent suppressor cells termed retinoid-induced FoxP3$^+$ T (RA-iTreg) cells [91, 114] [115-116] [117]. Furthermore, RA-iTreg cells have demonstrated less plasticity *in vivo* than conventional iTreg cells, with a lower propensity for reversion to FoxP3$^-$ cells under conditions that generally affect FoxP3 stability, such as overt inflammation [120]. Conversely, RA is capable of inhibiting *de nova* differentiation of naïve CD4$^+$ T cells into Th17 cells in response to TGF-β and IL-6 [39-41]. Since vitamin A must be absorbed from the diet and metabolized by DC or macrophages, it can be postulated that environmental factors modulate DC responses to mediate pro and anti-inflammatory immunity [12]. Thus, DCs have the capacity to sense and sample microbial Ags and inflammatory responses, migrate toward lymphoid organs and release variable amounts of RA to influence the differentiation of TGF-β-dependent FoxP3$^+$ cells or TGF-β-dependent Th17 cells, providing a self-regulating mechanism for TGF-β-mediated immune homeostasis [116, 265].

The immune system has evolved tolerogenic and regulatory mechanisms to control responses to self-antigens, and these have been exploited by tumours to evade effector immune responses. One of the fundamental challenges in the development of effective immunotherapeutics and vaccines against cancer is the identification of strategies to overcome the suppressive environment created by the tumour. Suppressive mechanisms elicited by the tumour include the secretion of immunosuppressive molecules, such as TGF-β, and the substantial recruitment and

activation of Treg cells locally within the tumour microenvironment but also systemically, with increased Treg cells commonly detected in peripheral blood and unrelated organs in cancer patients [148, 189].

Several studies have demonstrated that FoxP3$^+$ Treg cells can be induced from naïve CD4$^+$ T cells in response to growing tumours, contributing to the overall suppressive environment of the growing tumour [143, 151]. Furthermore, reports have demonstrated significant negative correlations between tumour infiltrating Treg cell numbers and overall patient survival [140, 145]. Removal or depletion of Treg cells in murine tumour models has been shown to dramatically enhance tumour immune surveillance, greatly improving the efficacy of therapeutic cancer vaccines, and in some cases allowing spontaneous tumour rejection [141-142]. However, approaches that persistently suppress Treg cell functions may not be generally applicable for the treatment of human tumours, considering that Treg cells are an essential component of a healthy immune system. While blocking CTLA4, which is constitutively expressed on Treg cells, has shown efficacy against a range of human tumours, harmful side effects were associated with CTLA4 blockade, including the induction of colitis and grade 3 and 4 autoimmune manifestations [216-217].

An alternative strategy and less harmful approach than the systemic inhibition of Treg cells may be to block or limit *de nova* induction of Treg cells at the site of the tumour, whilst simultaneously enhancing effector T cell responses. This combined

approach should facilitate attempts to enhance the efficacy of cancer vaccines, whilst preventing the induction of autoimmune or inflammatory disorders. Much of the published work on active immunotherapy and the data presented in chapter 3 have highlighted the promising potential of using a autologous DC pulsed *in vitro*, away from the immunosuppressive environment of the growing tumour, with tumour antigens and immunomodulatory molecules, such as TLR agonists, prior to being adoptively transferred *in vivo*. Since RA, which is metabolised by DC, has a central role in promoting the generation of TGF-β-dependent iTreg cells, targeting RA metabolism or RARα signalling in the DC could be an attractive therapeutic target to integrate into DC immunotherapies. Therefore, the aim of the present study was to investigate if antagonising the RA-RARα axis could inhibit the induction of $FoxP3^+$ iTreg cells and improve the efficacy of a DC vaccine for the treatment of tumours by reducing the ratio of Treg cells and enhancing Th1 and CTL responses.

5.2.1 RA enhances TGF-β-mediated conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+FoxP3^+$ T cells *in vitro*

A number of studies have demonstrated that conventional $CD4^+$ T cells can be converted into $FoxP3^+$ Treg cells (iTreg cells) by TGF-β in response to self or non-self antigens in the periphery [89-91]. This 'conversion' can also observed *in vitro* with naïve $CD4^+CD25^-FoxP3^-$ T cells converting to $CD4^+CD25^+FoxP3^+$ T cells in the presence of TGF-β and IL-2 [51]. Recently, a number of studies have highlighted a role for RA as a positive regulator of TGF-β-induced conversion of $CD4^+$ T cells into $FoxP3^+$ T cells [91, 116-117]. Initial experiments were designed to assess the ability of RA to enhance TGF-β-mediated peripheral conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+FoxP3^+$ T cells. $CD4^+CD25^-$ T cells were purified from C57BL/6 spleen cells and stimulated with αCD3, αCD28, rIL-2, in the presence or absence of rTGF-β and ATRA. The results demonstrate that after 3 days TGF-β-induced the peripheral conversion of conventional $CD4^+25^-FoxP3^-$ T cells into $CD4^+FoxP3^+$ T cells (Fig 5.1). These converted T cells also co-expressed CD25, a surface marker for T cell activation and Treg cells (Fig 5.1). RA worked in synergy with TGF-β to enhance the peripheral conversion of $CD4^+CD25^+FoxP3^+$ T cells. When TGF-β and IL-2 were added back into the culture on day 3, the TGF-β-mediated conversion of $FoxP3^+$ T cells was lost by days 6 and 7 (Fig 5.1). Critically, the conversion of $CD4^+CD25^-FoxP3^-$ T cells to $CD4^+CD25^+FoxP3^+$ T cells was maintained when cells were initially stimulated with RA (Fig 5.1). In conclusion this data suggests that RA works in synergy with TGF-β to enhance and sustain the peripheral conversion of $CD4^+CD25^+FoxP3^+$ T cells.

5.2.2 TGF-β-mediated conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+FoxP3^+$ T cells is inhibited by RARi

Having shown that RA worked in synergy with TGF-β to positively regulate the expression of FoxP3 in conventional $CD4^+$ T cells, experiments were designed to determine if blocking RA at the α receptor, with an antagonist (RARi), could inhibit the co-operative interaction of TGF-β and RA to induce peripheral Treg cell conversion. $CD4^+CD25^-$ T cells were purified from C57BL/6 spleen cells and stimulated with αCD3, αCD28 and recombinant IL-2, in the presence or absence of rTGF-β, ATRA and RARi. After 3 days the frequency of $CD4^+CD25^+FoxP3^+$ T cells was quantified by flow cytometry. The results demonstrate that TGF-β-mediated peripheral conversion of $CD4^+CD25^-$ into $CD4^+CD25^+FoxP3^+$ T cells was significantly enhanced in the presence of ATRA, while blocking the RARα signalling pathway reduced this synergistic effect (Fig. 5.2). Interestingly, in the absence of exogenous RA, blocking RARα also inhibited TGF-β-mediated peripheral conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+FoxP3^+$T cells (Fig. 5.2). While conversion by RA is exclusively dependent on TGF-β, this data suggests that the effects of TGF-β on peripheral conversion of Treg cells could be in part mediated through RA. Alternatively, the RA-RARα axis in naive $CD4^+$ T may be required for sufficient development of iTreg cells.

5.2.3 Treatment with RARi did not alter tumour growth

The data in Fig 5.2 demonstrated that RARi can directly inhibit TGF-β-mediated peripheral conversion of Treg cells from conventional $CD4^+$ T cells. Given the essential role of Treg cells in the pathogenesis of tumour development, this study investigated the direct effect of administration of RARi in a B16 melanoma tumour model. Mice were injected s.c. with RARi, or a vehicle DMSO control on days –3, –1, +1, +3, +5 and +7 , B16 tumour cells were injected s.c. on day 0 and tumour volumes were measured every second day from day 7. Treatment with RARi did not have any effect on tumour development when compared with the vehicle control (Fig 5.3). These results suggest that while RARi can inhibit TGF-β mediated peripheral conversion of Treg cells, the direct administration of RARi into the region of the tumour does not alter tumour growth.

5.2.4 BMDC express ALDHact

It is broadly accepted that Treg cells can compromise the efficacy of tumour vaccines. Since RA is metabolised by DC, an alternative approach to the direct administration of RARi was to test the ability of RARi to enhance the efficacy of a DC vaccine. While the eventual aim is to test the *in vivo* efficacy of DC pulsed with hs/irr tumour cells, as a source of antigen, and CpG, as a immunomodulator, the initial experiments were set up to test the effect of RARi on activation of DC *in vitro*. Given that much of the published data on RA is based on RA producing $CD103^+$ DC from the gut associated lymphoid tissue (GALT), the next study investigated the possibility that bone marrow derived DC (BMDC) could produce

RA. RA is synthesized via a multi-step pathway where retinol is converted by the ubiquitously expressed alcohol dehydrogenase enzymes into retinal and then converted into RA in a more restricted manner by a family of RALDH enzymes [108]. BMDC were harvested after overnight culture and RALDH activity was determined using the commercially available substrate aldeflour (ALD), which is converted into a fluorescent product by RALDH enzymes, thus allowing the identification of active RALDH-expressing cells by flow cytometry. The results indicate that BMDC produce RA, as demonstrated by functional RALDH enzyme activity (Fig 5.4). Expression of ALDEFLUOR was enhanced by stimulation with the TLR9 ligand CpG or killed B16 tumour cells, suggesting that a higher concentration of RA is produced by the DC in response to these stimuli (Fig 5.4).

5.2.5 Blocking RARα signalling in the DC significantly inhibits CpG and tumour antigen induced TGF-β and IL-10 production

Having confirmed that BMDC produce RA, the next study examined the effect of blocking RARα signalling (RARi) in Ag-pulsed and TLR-activated DC. BMDC from C57BL/6 mice were pretreated with medium or RARi, prior to incubation with hs/irr B16F10 tumour cells for 2 hours before the addition of CpG. After 24 hours the concentrations of TGF-β, IL-10, TNF-α, IL12-p40, IL12-p70 and IL-6 were determined by ELISA. Pretreatment of the DC with RARi significantly inhibited TGF-β-induced with hs/irr B16 tumour cells, and completely inhibited the synergistic effects of CpG and hs/irr B16 tumour cells in driving the production of TGF-β (Fig 5.5 $p<0.001$). In addition, pretreatment with RARi also significantly

inhibited CpG-induced IL-10 by DC (Fig 5.5 $p<0.001$). Killed B16 cells did not induce IL-10 production from DC and partially suppressed the production of CpG-induced IL-10. However, RARi suppressed IL-10 production from CpG-activated DC pulsed with hs/irr B16 cells (Fig 5.5 $p<0.001$). Pretreatment with RARi also inhibited TNF-α production by CpG-activated DC (Fig 5.6 $p< 0.01$) Interestingly, RARi had no effect on CpG-induced IL-12p40, IL-12-p70, or IL-6 production from DC, while RARi significantly enhanced IL-12p70 production from CpG-activated DC pulsed with hs/irr B16 cells (Fig 5.6 $p<0.001$). These findings demonstrate a positive role for RA signalling in TGF-β, TNF-α and IL-10 induced by CpG-activated DC pulsed with hs/irr B16 cells, while having no effect on IL-12p40 and IL-6 and inhibiting IL-12p70 production.

5.2.6 The RARα signalling pathway in a DC does not affect DC maturation

Having established that blocking RARα inhibits the production of immunosuppressive cytokines while enhancing IL-12p70 from CpG-activated DC pulsed with hs/irr B16 cells, the next experiment sought to determine if antagonising RARα signalling also affected DC maturation. BMDC were pretreated with RARi or medium control for 15 minutes, prior to incubation with hs/irr B16 tumour cells for 2 hours before the addition of CpG. After 24 hours DC were washed and surface stained for CD40, MHC class II, CD80 and CD86. Incubation of DC with the RARi alone had little or no effect on CD40, MHC II, CD80 or CD86 expression (Fig 5.7 and 5.8). In addition, hs/irr B16 cells did not alter the expression of these maturation markers on the surface of DC (Fig 5.7 and

5.8). Incubation of DC with CpG-induced maturation, upregulating the expression of CD40, MHC II, CD80 and CD86 (Fig 5.7 and 5.8). The enhancement of MHC class II and CD80 in response to CpG was unaffected by pretreatment with RARi, however, the expression of CD40 and CD86 in response to CpG was slightly reduced by pretreatment with RARi (Fig 5.7 and 5.8). A similar trend was observed when Ag-pulsed DC stimulated with CpG were pretreated with RARi, in that MHC class II and CD80 expresssion was unaffected, but CpG-induced CD40 and CD86 was slightly reduced (Fig 5.7 and 5.8). These results suggest that the ability of CpG to upregulate MHC II and CD80 expression is independent of RARα signalling. However, the enhancement of CpG induced CD40 and CD86 may be partially RARα dependent.

5.2.7 Blocking RARα signalling in a DC inhibits the ability to induce TGF-β production by $CD4^+$ T cells and enhances IFN-γ

Having shown that blocking RARα signalling can modulate the innate immune response by DC, the next experiment examined the capacity of these modulated DCs to direct $CD4^+$ T cell responses *in vitro*. BMDC were pretreated with medium or RARi, prior to incubation with hs/irr B16 tumour cells for 2 hours before the addition of CpG for 24 hours. DC were washed and added to purified $CD4^+$ T cells that were activated with plate-bound αCD3 at a T cell: DC cell ratio of 5:1. After 3 days, TGF-β, IL-10, IL-17 and IFN-γ production was assessed by ELISA. The data demonstrates that $CD4^+$ T cells secrete TGF-β following co-culture with CpG-activated DC, this was further augmented when DC were also stimulated with hs/irr

B16 cells (Fig 5.9). Pretreatment of DC with RARi did not affect the expression of TGF-β from $CD4^+$ T cells following co-culture with CpG-activated DC (Fig 5.9). However, pretreatment of DC with RARi resulted in significant inhibition of TGF-β from $CD4^+$ T cells following co-culture with CpG-activated DC pulsed with hs/irr B16 cells (Fig 5.9 $p<0.001$). In contrast, pretreatment of DC with RARi did not significantly affect the ability of DC to induce IL-10 or IL-17 from $CD4^+$ T cells (Fig 5.9 and 5.10). Furthermore, blocking RARα signalling in antigen-pulsed and CpG-activated DC significantly enhanced the ability of the DC to induce IFN-γ production from $CD4^+$ T cells (Fig 5.10 $p<0.001$). Collectively, these experiments suggest that inhibition of RARα in antigen-pulsed and CpG-activated DC directed $CD4^+$ T cells away from TGF-β regulation and toward a pro-inflammatory Th1 response.

5.2.8 Blocking RA enhanced the therapeutic efficacy of a DC vaccine against B16 melanoma in mice

Having shown that RARi can suppress IL-10 and TGF-β production by DC, while enhancing the induction of IFN-γ from a $CD4^+$T cells, experiments were carried out to investigate if blocking RARα, using a receptor antagonist, could improve the efficacy of a DC-based tumour vaccine using the poorly immunogenic B16F10 melanoma model. Following s.c. tumour challenge, C57BL/6 mice were injected on days 3 and 10 into the region of the tumour with a DC vaccine, comprising of CpG-activated DC pulsed with hs/irr B16F10 tumour cells, in the presence or absence of RARi. Tumour volumes were measured every second day from day 7. The results

demonstrated that treatment with the DC vaccine alone had no significant effect on tumour growth (Fig 5.11). However, when the nuclear receptor RARα is blocked in the DC, the DC vaccine significantly attenuated tumour growth when compared with untreated mice or mice treated with the DC vaccine alone (Fig. 5.11 $p<0.01$, <0.001). In untreated mice or mice treated with the DC vaccine alone, 100% of mice had palpable tumours by day 9. In contrast, 5/15 mice from the RARi-treated DC vaccine group remained tumour free at day 9. Furthermore, by day 14 only 1/15 mice from mice treated with the RARi-treated DC vaccine had a tumour volume that was comparable to the average tumour volume in both the control groups. These findings indicate that RARα is an important therapeutic target in antitumour immunity and blocking RARα is a useful approach for enhancing the efficacy of a DC vaccine for cancer immunotherapy.

5.2.9 Mice treated with the RARi-treated DC vaccine have enhanced antitumour immune responses within the tumour microenvironment

In order to determine if blocking RARα in the DC vaccine attenuated tumour growth by inhibiting the regulatory responses and boosting antitumour immunity, tumours were removed and assessed for TIL and cytokine production by FACS analysis. Attenuation of tumour growth by treatment with the RARi-treated DC vaccine was associated with a substantial increase in the percentage of tumour infiltrating $CD4^+$ T cells when compared with untreated mice or mice treated with DC vaccine only (Fig. 5.12). Intracellular cytokine staining for FoxP3 in TIL demonstrated that the increase in infiltrating $CD4^+$ T cells in mice treated with the

RARi-treated DC vaccine was inversely correlated with a significant reduction in the percentage and absolute numbers of $CD4^+CD25^+FoxP3^+$ T cells infiltrating the tumours when compared with untreated mice or mice treated with DC vaccine only (Fig 5.13 $p<0.01$). In addition, there was a significant reduction in the percentage of $CD4^+IL\text{-}10^+$ Tr1 type Treg cells infiltrating the tumour in the mice treated with the RARi-treated DC vaccine (Fig 5.14 $p<0.05$). Furthermore, the protective effect of treatment with the RARi-treated DC vaccine was also associated with an enhanced frequency of tumour infiltrating $CD4^+$ T cells expressing IFN-γ (Fig 5.15) and $CD8^+$ T cells producing IFN-γ (Fig 5.16). Collectively, this data suggests that blocking RAR-α enhanced the efficacy of a DC vaccine by significantly inhibiting the induction of Treg cells, allowing the infiltration and activation of protective IFN-γ secreting T cells.

5.2.10 Effect of blocking both p38 and RARi on the efficacy of DC vaccine against B16 tumour growth *in vivo*

Activation of TLR ligands result in the induction of a number of downstream signalling pathways, including p38 MAP kinase, NFkB and IRF pathways [7]. Depending on the specific TLR activated, a number of signalling cascades lead to the expression of numerous genes that primarily function in inflammatory immune responses, including the production of type I IFN, chemokines and inflammatory cytokines. However, it is broadly accepted that activation of TLRs can also induce anti-inflammatory cytokines from DC. Reports by Bowie and colleagues demonstrated that blocking the p38 MAPK signalling cascade suppressed LPS-

induced IL-10 production [8]. Consistent with this study, it was shown that a TLR agonist promotes both Th1-producing cells and IL-10 secreting Treg cells through the MAPK p38 signalling pathway [191]. Furthermore, blocking the p38 signalling pathway significantly inhibited CpG-induced IL-10 production by DC and augmented the efficacy of a CpG-activated DC vaccine against B16 tumour growth *in vivo*, and this was associated with enhanced $CD4^{+}IFN\text{-}\gamma^{+}$ T cells and a reduction in tumour infiltrating Treg cells [191].

The next experiment examined the possibility that blocking both the P38 and RARα signalling pathways could improve the efficacy of the CpG-activated DC vaccine against tumour growth in mice. C57BL/6 mice were challenged s.c. with B16F10 tumour cells, mice were injected s.c. into the region of the tumour on days 3 and 10 with antigen-pulsed CpG-activated DC that were pretreated with RARi and/or P38i day. Tumour volumes were measured every 2-3 days from day 7. The results demonstrate that administration of the DC vaccine had little effect on tumour growth, however, the RARi-treated DC vaccine significantly attenuated tumour growth when compared with untreated mice or mice treated with the DC vaccine alone (Fig 5.17 $p<0.01$). Consistent with published data [191], this study demonstrated that blocking the p38 signalling pathway significantly improved the efficacy of the DC vaccine against tumour growth *in vivo* (Fig 5.17 $p<0.01$). In contrast, blocking both p38 and RARα completely inhibited the beneficial effects of DC vaccines pre-treated with either p38i or RARi alone (Fig 5.17). While this data validated the significance of the p38 signalling pathway as an important

therapeutic target for cancer immunotherapy, the discovery that blocking both the p38 and RARα pathways completely inhibited the beneficial effects of single treatments was unexpected.

5.2.11 Effect of blocking both P38 and RARi on cytokine production by DC

Having established that the protective effect of inhibiting RARα or P38 was lost when both pathways were blocked, experiments were set up to investigate the effect of blocking both P38 and RARi on cytokine production by DC. DC from C57BL/6 mice were preincubated with p38i for 1 hour before the addition of RARi for 15 minutes, DCs were then activated with hs/irr B16 tumour cells for 2 hours before stimulating with CpG. After 24 hours the concentrations of TGF-β and IL-10 were determined by ELISA. Consistent with previous experiments (Fig 5.5), pretreatment with RARi significantly inhibited CpG-induced IL-10 production by DC (Fig 5.18 $p<0.01$). Incubation with hs/irr B16 cells reduced the production of CpG-induced IL-10, however, the negative regulatory effect of RARi on IL-10 production from the DC was still significant (Fig 5.18 $p<0.001$). Pretreatment of DC with the P38i significantly inhibited IL-10 production in response to CpG and hs/irr B16 cells in the presence or absence of RARi (Fig 5.18 $p<0.001$). However, inhibition of IL-10 was most significant when both the P38 and RARα pathways were blocked in the DC (Fig 5.18 $p<0.01$). These results highlight the importance of p38 and RA signalling in the production of CpG-induced IL-10 by DC and suggest that the P38 and RARα signalling pathways may both be involved in the production of CpG-induced IL-10 by DC.

Blocking RARα significantly inhibited TGF-β induced with hs/irr B16 tumour cells and/or CpG (Fig 5.5 and 5.18 $p<0.001$). However, blocking P38 did not affect the production of TGF-β from DC stimulated with CpG or CpG and killed tumour cells. In contrast, pretreatment with the P38i partially enhanced TGF-β production from RARi-pretreated CpG-activated DC pulsed with hs/irr B16 cells (Fig 5.18 $p<0.01$). However, a significant reduction in TGF-β was observed in all conditions when DC were pretreated with RARi (Fig 5.18 $p<0.001$). This data suggests that the induction of TGF-β from DC is independent of the P38 signalling pathway, however, the p38i partially suppressed RARi-mediated inhibition of TGF-β, suggesting that RARα signalling may be enhanced when the p38 signalling pathway is blocked.

5.2.12 Effect on $CD4^+$ T cells to produce cytokine following co-culture with DC pretreated with P38i and RARi

Given the unexpected findings observed when both the RARα and p38 signalling pathways were blocked in the DC vaccine *in vivo*, the next study examined the capacity of these modulated DCs to direct $CD4^+$ T cell responses *in vitro*. BMDC were pretreated with the p38i for 1 hour and RARi for 15 minutes, DCs were then incubated with hs/irr B16 cells for 2 hours before the addition of CpG for 24 hours. DC were washed and added to purified $CD4^+$ T cells that were activated with plate-bound αCD3 at a T cell: DC cell ratio of 5:1. After 3 days, the expression of TGF-β and IL-10 was determined by ELISA. The data demonstrates that $CD4^+$ T cells

secrete TGF-β following co-culture with CpG-activated DC, this is further augmented when DC were also co-stimulated with killed tumour cells (Fig 5.19). Pretreatment with RARi or P38i did not affect the expression of TGF-β from $CD4^+$ T cells following co-culture with CpG-activated DC (Fig 5.19). However, pretreatment with RARi or P38i resulted in significant inhibition of TGF-β expression from $CD4^+$ T cells following co-culture with CpG-activated DC pulsed with hs/irr B16 cells (Fig 5.19 $p<0.001$). In contrast, pretreatment with both RARi and P38i at different time points did not affect the expression of TGF-β from $CD4^+$ T cells following co-culture with CpG-activated DC pulsed with hs/irr B16 cells (Fig 5.19).

The results demonstrate that pretreatment with RARi did not significantly affect the induction of IL-10 from $CD4^+$ T cells (Fig 5.19). However, pretreatment with the P38i significantly inhibited the ability of $CD4^+$ T cells to induce IL-10 following co-culture with CpG-activated DC, or DC pulsed with hs/irr B16 cells, or CpG-activated DC pulsed with hs/irr B16 cells (Fig 5.19 $p<0.05$; $p<0.001$). In contrast, pretreatment with RARi and p38i significantly increased the expression of IL-10 from $CD4^+$ T cells following co-culture with CpG-activated DC pulsed with hs/irr B16 cells (Fig 5.19 $p<0.01$). Furthermore pretreatment with RARi and p38i significantly reduced IL-10 expression below the basal level observed for incubation of $CD4^+$T cells with a DC medium control (Fig 5.19). In addition, pretreatment with RARi or P38i significantly enhanced the expression of IFN-γ from $CD4^+$ T cells following co-culture with CpG-activated DC pulsed with hs/irr

B16 (Fig 5.19 $p<0.05$, $p<0.001$). In contrast, pretreatment with both RARi and P38i does not significantly affect the expression of IFN-γ from $CD4^+$ T cells following co-culture with CpG-activated DC pulsed with hs/irr B16 (Fig 5.19). Collectively, the present *in vitro* findings demonstrate that blocking RARα or P38 in CpG-activated DC pulsed with killed tumour cells significantly modulated the capacity of the DC to direct $CD4^+$ T cell responses, reducing TGF-β while promoting IFN-γ. However, when both RARα and P38 pathways are blocked the capacity of these CpG-activated DC pulsed with hs/irr B16 cells to modulate T cell responses is lost. This *in vitro* data correlates with the *in vivo* findings showing that blocking either RARα or P38 alone significantly modulates DC function, however, the significance of this effect is lost when both pathways are simultaneously blocked in the DC.

5.2.13 TGF-β-dependent conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+FoxP3^+$ T cells is enhanced in the presence of ATRA and P38i

Having established that a negative regulatory effect exists between P38 and RA for the induction of TGF-β by DC, the next experiment examined the effect of the p38i and/or the RARα inhibitor on peripheral conversion of $CD4^+CD25^-$ into $CD4^+CD25^+FoxP3^+$ T cells. $CD4^+CD25^-$ T cells were purified from C57BL6 spleen cells and stimulated with αCD3, αCD28, rIL-2, in the presence or absence of rTGF-β, ATRA, RARi and P38i. The results demonstrate that after 3 days TGF-β-induced the peripheral conversion of conventional $CD4^+25^-FoxP3^-$ T cells into $CD4^+CD25^+FoxP3^+$ T cells (Fig 5.20). Pretreatment with the p38i did not affect TGF-β-mediated peripheral conversion (Fig 5.20). In addition, pretreatment with

p38i and RARi did not affect TGF-β-mediated peripheral conversion of $CD4^+CD25^+FoxP3^+$ T cells (Fig 5.20). Consistent with previous experiments, ATRA dramatically enhanced TGF-β-mediated peripheral conversion of $CD4^+25^-FoxP3^-$ T cells into $CD4^+CD25^+FoxP3^+$ T cells (Fig 5.20). Furthermore, blocking RARα signalling suppressed ATRA and TGF-β-mediated peripheral conversion (Fig 5.20). However, blocking the p38 signalling pathway modestly enhanced ATRA and TGF-β-mediated peripheral conversion of $CD4^+25^-FoxP3^-$ T cells into $CD4^+CD25^+FoxP3^+$ T cells, while also demonstrating a marked increase in $CD4^+CD25^-FoxP3^+$ T cells (Fig 5.20). Collectively, this data suggests that the p38i does not affect TGF-β-mediated peripheral conversion. However, blocking p38 slightly enhanced RA and TGF-β-mediated peripheral conversion, suggesting that p38 may negatively regulate the RA-RARα axis in TGF-β-mediated peripheral conversion.

5.2.14 TGF-β-dependent conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+FoxP3^+$ T cells in a DC co-culture

Given that much of the work carried examined the effects of RARi and P38i in DC, the next experiment investigated the effects of RARi and P38i in conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+FoxP3^+$ T cells in DC and T cell culture. $CD4^+CD25^-$ T cells were purified from C57BL6 spleen cells and incubated with BMDC from C57BL6 mice. Cells were stimulated with αCD3, in the presence or absence of rTGF-β, ATRA, RARi and P38i. After 3 days, TGF-β-induced the conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+FoxP3^+$ T cells, however, the

percentage of $CD4^+CD25^+FoxP3^+$ T cells was very low (Fig 5.21). Pre-incubation of cells with p38i alone or in combination with RARi suppressed TGF-β-mediated peripheral conversion of $CD4^+CD25^+FoxP3^+$ T cells (Fig 5.21). However, treatment of cells with ATRA and p38i dramatically enhanced TGF-β-mediated peripheral conversion of $CD4^+25^-FoxP3^-$ T cells into $CD4^+CD25^+FoxP3^+$ T cells (Fig 5.21). This data could suggest that p38 is negatively regulating RARα signalling in the TGF-β pathway.

5.2.15 A single administration of the RARi-treated DC vaccine can attenuate tumour growth *in vivo*

Having established that two treatments with the RARi-treated DC vaccine significantly attenuated tumour growth and augmented antitumour effector T-cell responses, the next experiments sought to determine if a single administration of the vaccine would have protective effects if administered during the early or late stage of tumour growth. Mice were injected with B16F10 melanoma tumour cells on day 0, and a single dose of the DC vaccine alone, or RARi-treated DC vaccine was administered on day 3 or day 10. Treatment of mice on day 3 with a single dose of the RARi-treated DC vaccine resulted in a delayed onset in tumour growth, but the effect of the RARi-treated DC vaccine was no longer evident at 17 days (Fig 5.22). However, when a single administration of the vaccine was injected on day 10, a significant attenuation of tumour growth was observed in mice treated with the RARi-treated DC vaccine (Fig. 5.23 $p<0.01$). Collectively, this data indicates that a single administration of the RARi-treated DC vaccine can

transiently attenuate tumour growth *in vivo*, however, the data suggests that early and repeated administration of at least 2 doses is required to maintain attenuation of tumour growth.

5.2.16 Three doses of a DC vaccine and RARi significantly enhanced the survival of tumour bearing mice

Having established that 2 doses of the RARi-treated DC vaccine confers a level of protection against tumour growth, the next experiment examined if 3 doses could confer a greater level of protection. Mice were s.c. challenged with B16 melanoma tumour cells on day 0, and on days 3, 10 and 17 post tumour challenge the mice were s.c. injected into the region of the tumour with DC vaccine alone, or the RARi-treated DC vaccine. Treatment with 3 doses of the RARi-treated DC vaccine demonstrated a significant attenuation of tumour growth and a significant improvement in survival time when compared with untreated mice or mice treated with the DC vaccine alone, were 100% of mice reached the maximum ethical tumour volume and had to be sacrificed by day 20 (Fig. 5.24 $p<0.01$). While a significant improvement in survival time was observed, all of the mice treated with the RARi-treated DC vaccine developed tumours and had to be sacrificed by day 40. These findings suggest that blocking RARα significantly augments the efficacy of a DC vaccine, attenuating tumour growth and improving the survival time. However, the RARi-treated DC vaccine does not demonstrate tumour regression or complete survival in tumour bearing mice.

5.2.17 Neutralising TGF-β at the site of the tumour does not significantly improve the efficacy of the RARi-treated DC vaccine as a cancer immunotherapy

The findings in chapters 3 established that attenuating TGF-β with siRNA significantly enhanced the efficacy of a DC vaccine against B16 tumour growth *in vivo*. The next experiment examined the possibility that neutralising TGF-β at the site of the tumour in combination with the RARi-treated DC vaccine might be a more successful vaccine approach against tumour growth. C57BL/6 mice were challenged s.c. with B16F10 tumour cells, mice were injected s.c. into the region of the tumour with CpG-activated DC pulsed with hs/irr B16 cells that were pretreated with and without the RARi on days 3, 10 and 17. A neutralizing antibody for TGF-β was also administered s.c. into region of the tumour every second day from day +1. Tumour volumes were measured every second day from day 7. The data demonstrated that administration of the DC vaccine alone did not significantly affect tumour growth (Fig 5.25). However, neutralising TGF-β at the site of the tumour significantly improved the efficacy of the DC vaccine (Fig 5.25 $p<0.01$, $p<0.001$). This data supports the data presented in Fig 3.5 where silencing of TGF-β gene expression using TGF-β siRNA significantly attenuated tumour growth *in vivo,* when compared with untreated mice or mice treated with the DC vaccine alone. Consistent with previous studies, blocking RARα significantly improved the efficacy of the DC vaccine (Fig 5.25 $p<0.01$, $p<0.001$). Furthermore, the combined treatment of neutralizing TGF-β and administering the RARi-treated DC vaccine demonstrated a significant reduction in tumour burden when compared with the

untreated mice or mice treated with the DC vaccine alone (Fig 5.25 $p<0.01$, $p<0.001$). However, neutralizing TGF-β in combination with the RARi-treated DC vaccine did not significantly improve the efficacy of the DC vaccine when compared with mice treated with the RARi-treated DC vaccine, or mice treated with the DC vaccine in combination with anti-TGF-β (Fig 5.25). The individual tumour plots suggest that the RARi-treated DC vaccine is the most effective treatment for delaying the onset and development of tumour growth, when compared with untreated mice or mice treated with the DC vaccine alone or in combination with anti-TGF-β (Fig 5.25). In summary, this data suggests that neutralising TGF-β at site of the tumour does not dramatically improve the efficacy of the RARi-treated DC vaccine as a successful immunotherapy.

5.2.18 The TLR9 agonist CpG induces a Th1 type cells

A central requirement for an effective DC vaccine is optimal maturation of a DC in order to generate robust Ag-specific immune responses. Consistent with the data presented in Fig 5.11 and 5.17, TLR agonists are widely used as adjuvants to improve the immunogenicity of DC-based tumour vaccines, as they can promote DC maturation and induce inflammatory immune responses by DC. The DC vaccine in this study uses CpG as a maturation stimulus. Previous studies showed that CpG-induced large concentrations of TNF-α, IL-12p40, IL-12p70 and IL-6 from a DC (Fig 5.6), and up-regulated the expression of MHC II and the co-stimulatory molecules CD80, CD86 and CD40 on a DC (Fig 5.7 and 5.8). The next experiments were designed to examine the capacity of CpG-activated DC to direct

$CD4^+$ T cell responses *in vitro*. BMDC were stimulated with CpG or medium only for 24 hours, DCs were then washed and added to αCD3-stimulated $CD4^+$ T cells for 72 hours. Cells were stimulated with PMA/Ionomycin and BFA for 6 hours and stained for CD4, CD25, IFN-γ and FoxP3. DCs stimulated with CpG induced dramatically enhanced $CD4^+IFN\text{-}\gamma^+$ T cells when compared with unstimulated DCs (Fig 5.26). In addition, there were no significant differences in the frequency of $CD4^+CD25^+FoxP3^+$ T cells from DC stimulated with the TLR-9 agonist CpG or medium control (Fig 5.26). Collectively, this data demonstrates that CpG is an efficient maturation stimulus, which modulates DC function to enhance a Th1 type immune response but not $FoxP3^+$ Treg cells.

5.2.19 Contribution of CpG to the efficacy of the RARi-treated DC vaccine

Previous experiments have shown that blocking RARα in DCs pulsed with tumour Ag and co-stimulated with CpG significantly attenuated tumour growth by inhibiting the frequency of tumour infiltrating Treg cells and enhancing the frequency of effector cells. The next experiment examined the importance of CpG as an immunopotentiator, and its contribution to the RARi-treated DC vaccine. C57BL/6 mice were inoculated s.c. with B16 tumours, and mice were then treated with DC pulsed with tumour Ag alone, Ag + CpG, Ag + RARi or Ag + CpG + RARi on days 3, 10 and 17. Tumour volumes were measured every 2-3 days from day 6. Consistent with previous findings, treatment with CpG-activated DC pulsed with B16 Ag did not have a significant effect on tumour growth (Fig 5.27). As expected, the efficacy of the Ag-pulsed CpG-activated DC vaccine was

significantly enhanced by blocking RARα signalling in the DC vaccine (Fig 5.27, $p<0.01$). In contrast, in the absence of CpG co-stimulation, RARi did not enhance the efficacy of the DC vaccine, and if anything a modest enhancement in tumour growth was observed when compared with untreated mice (Fig 5.27). Furthermore, mice that received the DC vaccine pulsed with hs/irr B16 cells in the absence of both RARi and CpG stimulation demonstrated a transient non-significant attenuation of tumour growth, which was completely lost by day 18 (Fig 5.27). This transient attenuation however was not consistent across all experiments. Collectively, these findings highlight the crucial role of a TLR stimulus, in this case CpG, which acts as a potent adjuvant to improve the efficacy of the RARi-treated DC vaccine.

5.2.20 Blocking RARα signalling in the DC vaccine negatively regulates suppressor T cells

The data presented in Fig 5.27 indicated that it is a combination of CpG and RARi that significantly enhanced the efficacy of the DC based vaccine. Given the necessity of CpG stimulation in the DC immunotherapy, experiments were carried out to determine the influence of CpG-activation on antitumour immune responses. Mice were s.c. challenged with B16 tumours and treated therapeutically as described in Fig 5.27. Tumours were removed and assessed for TIL and cytokine production by FACS analysis. Consistent with earlier findings, blocking RARα in CpG-activated DC pulsed with hs/irr B16 cells significantly reduced the frequency of tumour infiltrating $CD4^+CD25^+FoxP3^+$ T cells when compared with untreated

mice or mice treated with CpG-activated DC pulsed with hs/irr B16 cells (Fig 5.28 $p<0.05$). In the absence of CpG-activation the DC vaccine in combination with RARi did not attenuate tumour growth (Fig 5.27), however, a significant reduction in tumour infiltrating $CD4^+CD25^+FoxP3^+$ T cells was observed when compared with the untreated mice and mice treated with the DC vaccine alone (Fig 5.28 $p<0.05$, $p<0.01$). In addition, mice treated with Ag-pulsed DC alone or in combination with CpG had a slight but non-significant reduction in tumour infiltrating $CD4^+CD25^+FoxP3^+$ T cells when compared with untreated mice (Fig 5.28).

Treatment with Ag-pulsed CpG-activated DC that were pretreated with RARi significantly inhibited the frequency of tumour infiltrating Tr1 type Treg cells (Fig 5.28 $p<0.001$). Furthermore, treatment with Ag-pulsed DC that were pretreated with RARi was also associated with a significant reduction in tumour infiltrating $CD4^+IL\text{-}10^+$ T cells, when compared with untreated mice or mice treated with the DC vaccine (Fig 5.28 $p<0.001$) In addition, treatment with Ag-pulsed CpG-activated DC without RARi resulted in a significant reduction in tumour infiltrating Tr1 cells when compared with the untreated mice (Fig 5.28 $p<0.001$). This trend is consistent with the data presented in Fig 5.14, demonstrating that tumour infiltrating $CD4^+IL\text{-}10^+$ T cells are reduced in the mice treated with DC vaccine alone, however, the most significant reduction is observed in the mice treated with the RARi-treated DC vaccine (Fig 5.14 $p<0.05$). There were no significant differences observed in the frequency of tumour infiltrating $CD4^+TGF\text{-}\beta^+$ T cells

amongst treated and untreated groups. However, the trend suggests that the inclusion of RARi in the DC vaccine reduced the frequency of tumour infiltrating $CD4^+TGF\text{-}\beta^+$ T cells, Th3 type Treg cells (Fig 5.28).

The frequency of tumour infiltrating $CD4^+IFN\text{-}\gamma^+$ T cells was dramatically increased in mice treated with the CpG-activated DC vaccine with or without RARi (Fig 5.28). Treatment of mice with the RARi-treated DC vaccine without CpG co-stimulation had an enhanced frequency of tumour infiltrating $CD4^+IFN\text{-}\gamma^+$ T cells when compared with untreated mice or mice treated with Ag-pulsed DC (Fig 5.28). However, the frequency of $CD4^+IFN\text{-}\gamma^+$ T cells was further enhanced when Ag pulsed DC were co-stimulated with both CpG and RARi (Fig 5.28). In addition, the percentage of tumour infiltrating $CD8^+IFN\text{-}\gamma^+$ T cells was also dramatically enhanced in mice treated with DC vaccine with or without CpG and/or RARi (Fig 5.28). However, the most dramatic enhancement was observed in mice that were treated with Ag-pulsed CpG-activated DC pretreated with RARi (Fig 5.28). The trend observed for tumour infiltrating $CD4^+IFN\text{-}\gamma^+$ T cells and $CD8^+IFN\text{-}\gamma^+$ T cells was consistent with previous findings where the DC pulsed with tumour Ag, CpG and RARi demonstrated the highest frequency of antitumour effector T cells (Fig 5.15 and 5.16). These results suggest that the reduction in tumour infiltrating nTreg cells, Tr1 type Treg cells and Th3 type Treg cells is a direct effect of blocking RARα in an Ag pulsed DC vaccine.

5.3 DISCUSSION

DC are key APCs that play a crucial role in bridging innate and adaptive immune responses. Indeed, DC are central to directing the fate of naïve T cells, either polarising effector T cell responses through the upregulation of MHC and co-stimulatory molecule expression and production of polarising cytokines and chemokines or inducing immune tolerance. In addition, it has recently been discovered that DC can produce RA, the active derivative of vitamin A, which has been identified as a key regulator of TGF-β-dependent immune homeostasis. RA can synergize with TGF-β to enhance the conversion of naïve $FoxP3^-$ T cells into a unique $FoxP3^+$ Treg subset, whilst reciprocally mediating the generation of Th17 cells in response to TGF-β and IL-6 [39-41, 91, 114-117]. Thus, it has been hypothesized that RA is central to a self-regulating mechanism for maintaining the balance between TGF-β-mediated effector and regulatory responses. The significant new finding of the present study is that blocking RARα signalling in a DC vaccine significantly augments the efficacy of the cell-based therapy against murine tumours, reducing tumour growth and enhancing survival. The reduction in tumour growth following administration of the RAR-treated DC vaccine was associated with a substantial reduction in tumour infiltrating $CD4^+CD25^+FoxP3^+$ T cells and $CD4^+IL10^+$ T cells and a corresponding increase in the percent of tumour infiltrating $CD4^+$ T cells, $CD4^+IFN\text{-}\gamma^+$ T cells and $CD8^+IFN\text{-}\gamma^+$ T cells.

The present study showed that RA enhanced TGF-β-dependent peripheral conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+FoxP3^+$ T cells *in vitro*. This is

consistent with a number of reports showing that RA can synergize with TGF-β to enhance the conversion of naïve FoxP3$^-$ T cells into a unique FoxP3$^+$Treg cell subset, termed retinoid-induced FoxP3$^+$ T cells [91, 114] [115-117]. In addition, the present study found that when additional TGF-β and IL-2 were added to the culture on day 3, TGF-β-mediated conversion of FoxP3$^+$ T cells was reversed by days 6 and 7. In contrast, conversion of CD4$^+$CD25$^-$FoxP3$^-$ T cells into CD4$^+$CD25$^+$FoxP3$^+$ T cells was maintained for 6-7 days when cells were stimulated with RA as well as TGF-β, suggesting that RA stabilised as well as enhanced TGF-β-mediated peripheral conversion of CD4$^+$CD25$^+$FoxP3$^+$ T cells. This finding is consistent with reports that RA-iTreg cells have a lower propensity for reversion to FoxP3$^-$ cells under conditions that generally oppose FoxP3 stability, such as high co-stimulatory environments and certain inflammatory cytokines, including IL-6 and IL-21 [120] [122]. Whilst RA does not influence the selection or proportion of nTreg cells in the thymus or lymphoid organs respectively, it has been shown that nTreg cells pretreated with RA are more refractory to Th17 conversion in the presence of IL-6 when compared with untreated control nTreg cells [128]. These observations suggest that RA maintains the stability and function of both iTreg and nTreg cell lineages.

In agreement with much of the published data, the present study demonstrated that blocking RARα inhibited the synergistic effects of TGF-β and RA on Treg cell conversion [91, 116-117]. Furthermore, the present study demonstrated that in the absence of exogenous RA, antagonising RARα inhibited TGF-β-mediated

peripheral conversion of naïve T cells into $CD4^+CD25^+FoxP3^+$ T cells. One explanation for this observation is that TGF-β-dependent conversion of Treg cells may be in part mediated through RA. Alternatively the RA-RARα axis on naive $CD4^+$ T may be required for sufficient development of iTreg cells. The latter hypothesis is consistent with recent findings by Belkaid and colleagues who showed that antagonism of RAR signalling in naive $CD4^+$ T cells impedes signal transduction events upon engagement of TCR, resulting in $CD4^+$ T cell activation defects and impaired development of regulatory and inflammatory responses [118].

It was originally thought that RA-producing DC were restricted to immune privileged mucosal sites, such as GALT and Lp [117]. However, recent studies have shown that synthesis of RA from stored or dietary retinol occurs in other DC populations in tissues at environmental interfaces, including the skin and lungs and their corresponding draining lymph nodes [121]. However, not all DC populations at environmental interfaces produce RA, as monocyte-derived DCs in the intestine do not produce RA during periods of inflammation [267]. In addition, the present study demonstrated that BMDC can produce RA. Taken together, these findings suggest that RA-producing DCs are not restricted to lineage specific DC. In addition, the present study showed that activation of DC with CpG or hs/irr B16 cell promoted ALDH activity, suggesting that RA expression might be induced by immunogenic stimuli in the local microenvironment to regulate TGF-β-mediated immune responses. These findings are consistent with several reports demonstrating that ALDH activity in DC is increased following stimulation with

multiple factors, including GM-CSF, IL-4, IL-13 or the TLR2 ligand zymosan [121].

The recent FDA approval of Sipuleucel-T, a patient specific autologous cell-based vaccine for the treatment of prostate cancer, has highlighted the promise of active immunotherapy for the treatment of cancer. However, despite encouraging results, DC-based immunotherapeutic approaches have had limited success in the clinic to date. This has been attributed in part to tumour-derived immunosuppressive molecules, including TGF-β, and tumour-induced Treg cells. A central requirement for an effective DC vaccine is proficient activation and maturation of the DC in order to induce robust anti-tumour adaptive immune responses to overcome immunological self-tolerance. A number of studies, including DC vaccine trials in humans, have demonstrated that antigen-pulsed immature DC are very poorly immunogenic with a tendency to induce Treg cells [268], however, with optimal maturation they can induce antigen-specific Th1 responses [269]. Consequently TLR agonists are used as adjuvants to improve the immunogenicity of tumour vaccines and to elicit pro-inflammatory Th1 responses. The TLR-9 agonist, CpG has been widely used as a vaccine adjuvant for protein, peptide, DC and autologous tumour cell vaccines [116, 203]. In agreement with much of the published data, the present study demonstrated that CpG-activated DC can induce potent IFN-γ-secreting T cell responses. Consistent with other reports [191], the present study also found that CpG can simultaneously induce IL-10 as well as IL-12p40/p70 production by DC, however, the present study also uncovered a role for RA

signalling in CpG-induced IL-10 by DC. Furthermore blocking RA signalling, via antagonising RARα, significantly inhibited CpG-induced IL-10 production from DC.

In an attempt to limit tumor-induced immune suppression and break immune tolerance, the present study tested the *in vitro* effects of blocking RARα in a CpG-activated DC pulsed with tumour Ag, as a potential cell-based therapy for treatment of tumours *in vivo*. The results showed that blocking RARα significantly inhibited the production of TGF-β, TNF-α and IL-10, while sparing IL-12p40 and IL-6, and significantly enhanced IL-12p70 from CpG-activated DC pulsed with hs/irr B16 cells. The demonstration that blocking RARα significantly suppressed TGF-β and IL-10 while promoting IL-12p70 production by CpG-activated BMDC pulsed with tumour Ag suggests that the RARα antagonist can modulate BMDC responses to inhibit the induction of adaptive or inducible Treg cells, while also enhancing Th1 immunity and CTL cytotoxicity via augmented IL-12p70 production. Recent studies have revealed that RA can differentially modulate DC subsets under different environmental conditions, for example intestinal DCs activated with IL-15 and RA unexpectedly enhanced the proinflammatory cytokines IL-12p70 and IL-23 in response to dietary antigens [270]. However, additional research carried out by Belkaid and colleagues showed that Lp DC from vitamin A insufficiency (VAI) mice produced a lower concentration of IL-6, whilst other proinflammatory mediators such as TNF-α, IL-12 and IL-23p40 remained intact [265]. These conflicting results indicate that RA signalling differentially regulates pro-

inflammatory and anti-inflammatory immune responses by DC, depending on the specific DC subtype and surrounding environmental conditions.

The present study also found that blocking RARα in CpG-activated DC pulsed with hs/irr B16 cells significantly influenced the capacity of these DC to direct $CD4^+$ T cells responses, inhibiting TGF-β expression while enhancing IFN-γ production from $CD4^+$ T cells. These data suggest that antagonising RARα modulates BMDC away from regulation and towards a pro-inflammatory Th1 response. The demonstration that antagonising RAR signalling in DC can inhibit the generation of Treg cells is consistent with other studies which showed that RAR antagonism inhibits mucosal DC-induced $FoxP3^+$ Treg cells generation *in vitro* [116]. Taken together, these results illustrate a critical role for RA in the production of immunosuppressive cytokines by DC, providing a novel target for improving the efficacy of DC based vaccines.

Consistent with the data presented in chapter 3, the present study showed that therapeutic administration of CpG-activated DC primed with hs/irr B16 tumour cells had no significant effects on tumour growth *in vivo*. However, blocking RARα in CpG-activated DC pulsed with hs/irr B16 cells before therapeutic administration to mice with the poor immunogenic B16F10 melanoma model, significantly reduced tumour growth and improved survival. Attenuation of tumour growth was associated with a substantial increase in the percentage of tumour infiltrating $CD4^+$ T cells, an effect that predominantly correlates with a good

prognosis in the clinic [232]. In addition, therapeutic treatment with the DC vaccine in combination with RARi results in a marked increase in tumour infiltrating $CD4^+IFN\text{-}\gamma^+$ T cells and $CD8^+IFN\text{-}\gamma^+$ T cells. It is well established that $CD8^+$ CTLs and $CD4^+$ Th1 cells have a protective role in antitumour immunity, with the capacity to mediate direct killing of tumour cells and to activate other immune cells via the secretion of IFN-γ [175]. Furthermore, blocking RARα in the DC vaccine resulted in a significant reduction in the frequency of tumour infiltrating $CD4^+CD25^+FoxP3^+$ T cells and $CD4^+IL\text{-}10^+$ T cells. It is now broadly accepted that Treg cells play a major role in the failure of many immunotherapeutic approaches against cancer, inhibiting the protective functions of a wide range of anti-tumour immune responses involving $CD4^+$ T cells, $CD8^+$ T cells and NKT cells [49]. The results from the present study suggest that blocking RARα in a DC vaccine is a promising immunotherapeutic approach to overcome local immune tolerance and attenuate tumour growth by blocking RA-iTreg cells and promoting CTL responses.

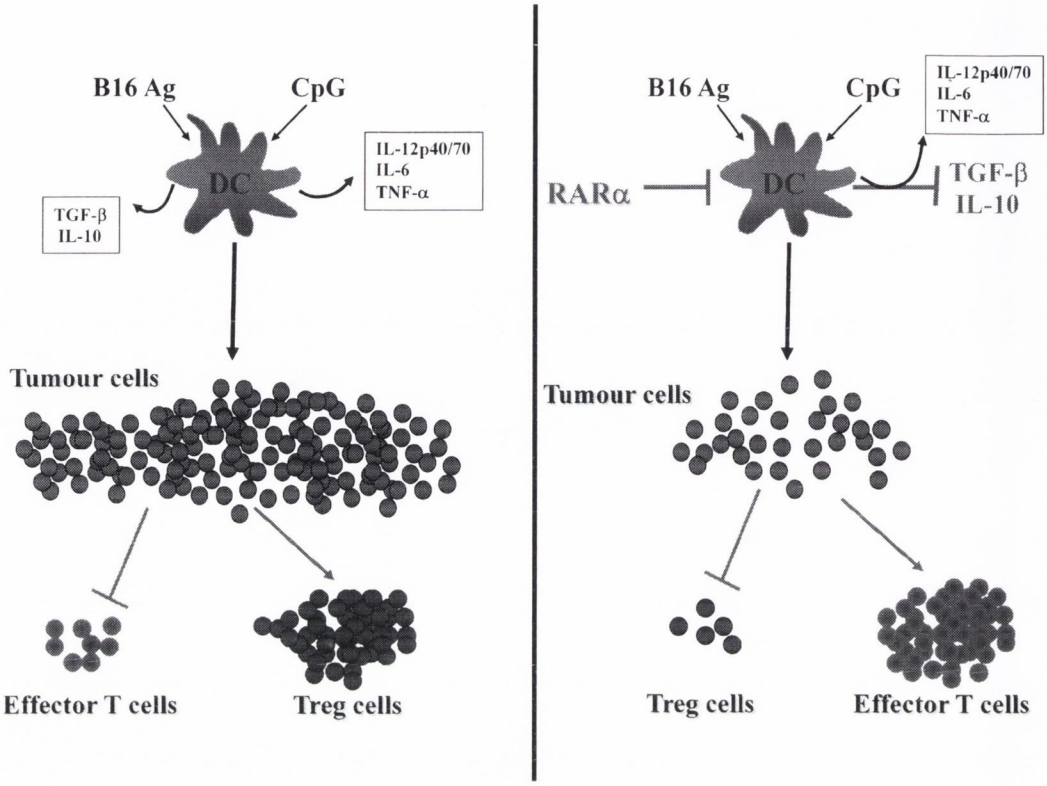


Fig. 5.35 Blocking RARα significantly improved the efficacy of a CpG-activated DC based vaccine against tumour growth *in vivo*

Pretreatment with the RARα antagonist significantly inhibited TGF-β and IL-10, while enhancing IL-12p70 production from CpG-activated DC pulsed with hs/irr B16 tumour cells. Therapeutic treatment of this Ag-pulsed and TLR-activated DC vaccine in combination with RARi significantly attenuated tumour growth and is associated with a significant reduction in Treg cells and a corresponding enhancement of IFN-γ producing $CD4^+$ and $CD8^+$ T cells.

In an attempt to improve the efficacy of the RARi-treated DC vaccine, the present study examined the effects of blocking both P38 and RA signalling in a CpG-activated DC vaccine. Consistent with reports by Jarnicki et al, blocking p38 enhanced the protective efficacy of DC activated with CpG and pulsed with hs/irr B16 cells against tumour growth *in vivo*. However, blocking both p38 and RARα completely inhibited the beneficial effects of either the p38i or RARi on the efficacy of the DC vaccine. This observation was also reflected in the capacity of CpG-activated DC pulsed with tumour Ag to direct $CD4^+$ T cell responses *in vitro*. Pretreatment with RARi or P38i inhibited the production of TGF-β and enhanced IFN-γ from $CD4^+$ T cells following co-culture with CpG-activated DC pulsed with tumour Ag, however, when both pathways were simultaneously blocked this effect was lost.

While inhibition of p38 suppressed TLR-induced IL-10, the findings of the present study demonstrated that p38 alone does not appear to play a role in the production of TGF-β from DC. However, inhibition of p38 partially enhanced TGF-β production from Ag-pulsed CpG-activated DCs that were pretreated with RARi, possibly suggesting that p38 may negatively modulate the effect of RARα on TGF-β-mediated signalling. Furthermore, blocking p38 in the presence of TGF-β and ATRA enhanced the conversion of naïve $CD4^+$ T cells into $CD4^+CD25^+FoxP3^+$ cells. Taken together, these findings suggest that blocking p38 may enhance the activity of RARα to bind to RA and positively regulate the TGF-β signalling pathway. Collectively, the present study suggests that a co-operative interaction

between the RARα and p38 signalling pathways exist, however, the mechanism of activation of p38MAPK/ RARα is unknown and requires extensive future work to draw definitive conclusions.

The present study found that CpG-activation was an essential component of the RARi-treated DC vaccine, playing a key role in immunopotentiation. In the absence of CpG, the RARi-treated DC vaccine was not protective against tumour growth *in vivo*. A plausible explanation for the lack of efficacy may be that CpG was required to induce maturation of the DC and IL-12 production to promote Th1 and CD8 CTL responses. Furthermore, the greatest frequency of tumour infiltrating $CD8^+IFN\text{-}\gamma^+$ T cells was observed when DC pulsed with tumour Ag were co-stimulated with both CpG and RARi. Interestingly, CpG-activation was not required for the DC vaccine to significantly inhibit the frequency of tumour infiltrating $CD4^+CD25^+FoxP3^+$ cells, indicating that blocking RARα directly modulates DC to inhibit the frequency of RA-induced $FoxP3^+$ Treg cells within the tumour. Overall these findings demonstrate that manipulating pro-inflammatory and anti-inflammatory immune responses with RARi is a useful approach for circumventing immune tolerance to drive antitumour immune responses.

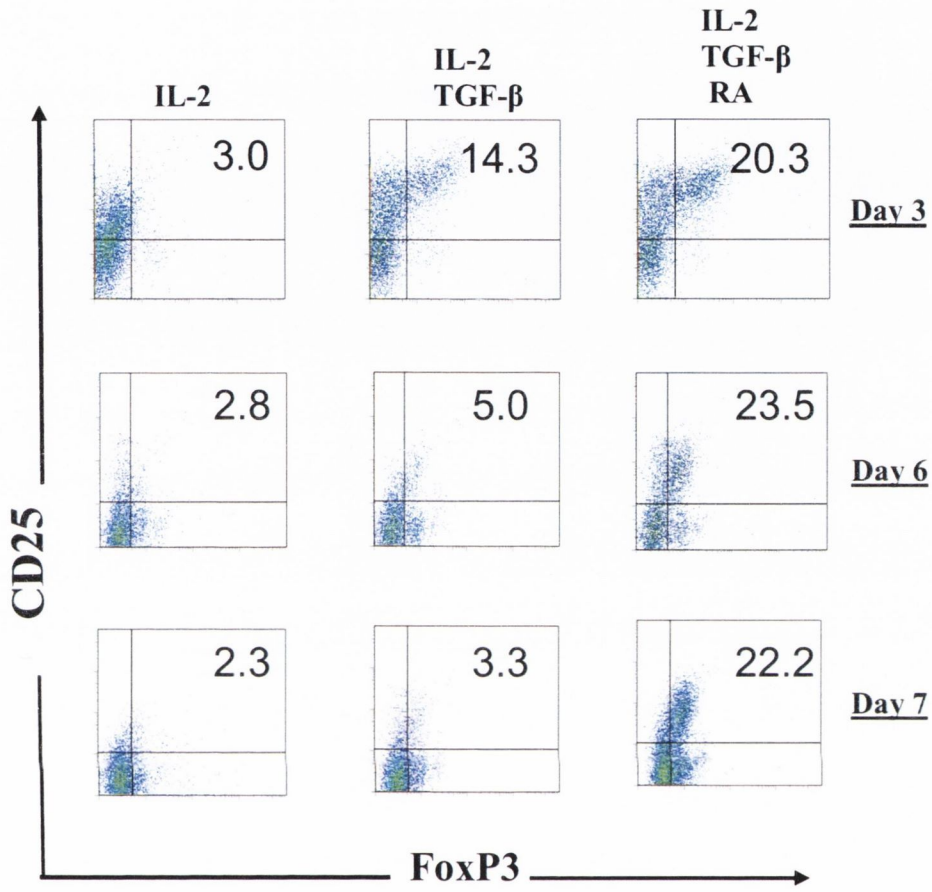


Figure 5.1. TGF-β dependent conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+FoxP3^+$ T cells is enhanced and sustained in the presence of ATRA. $CD4^+CD25^-$ T cells were stimulated with platebound αCD3 (1 μg/ml), soluble αCD28 (5 μg/ml), rIL-2 (5 ng/ml), with or without rTGF-β (10 ng/ml) and ATRA (10 nM). Fresh rIL-2 and rTGF-β were added to the culture again on day 3. The frequency of $CD4^+CD25^+FoxP3^+$ T cells was determined on day 3, 6 and 7 by surface labelling with CD4, CD25 and intracellular labelling with FoxP3.

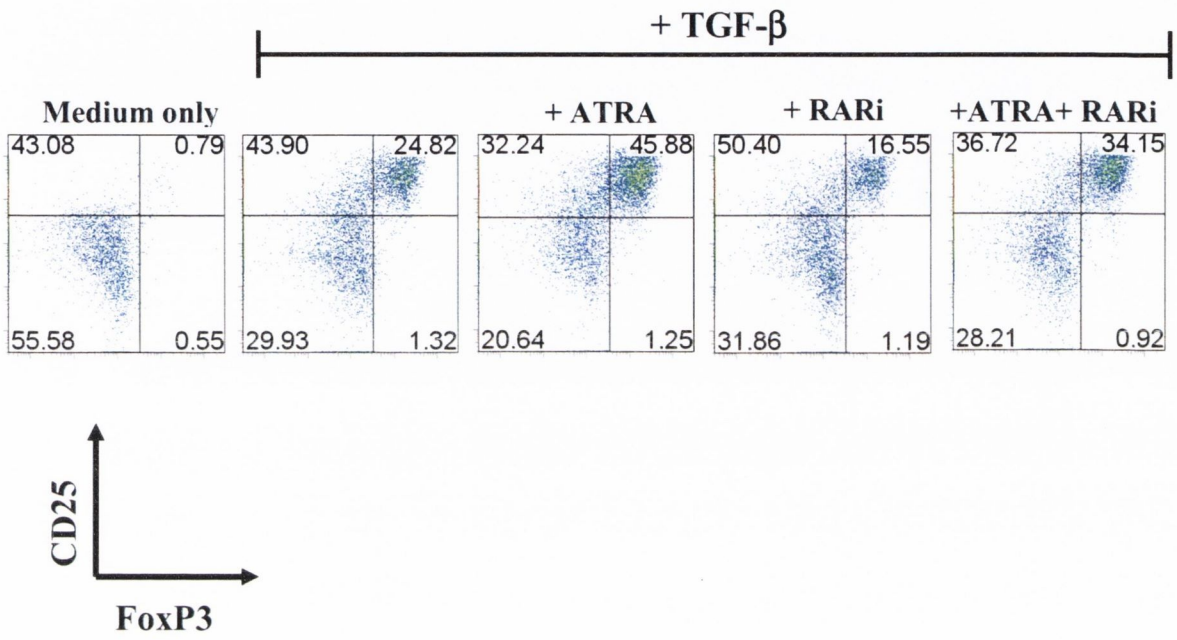


Figure 5.2 TGF-β and ATRA dependent conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+FoxP3^+$ T cells is inhibited by RARi. $CD4^+CD25^-$ T cells isolated from spleen cells were stimulated with platebound αCD3 (1 μg/ml), soluble αCD28 (5 μg/ml), rIL-2 (10 ng/ml), with medium, rTGF-β (5 ng/ml), rTGF-β and ATRA (100 nM), rTGF-β and RARi (5 μM), or rTGF-β, ATRA and RARi. The frequency of $CD4^+CD25^+FoxP3^+$ T cells was determined on day 3 by surface labelling with CD4, CD25 and intracellular labelling with FoxP3. This experiment is a representative of six experiments.

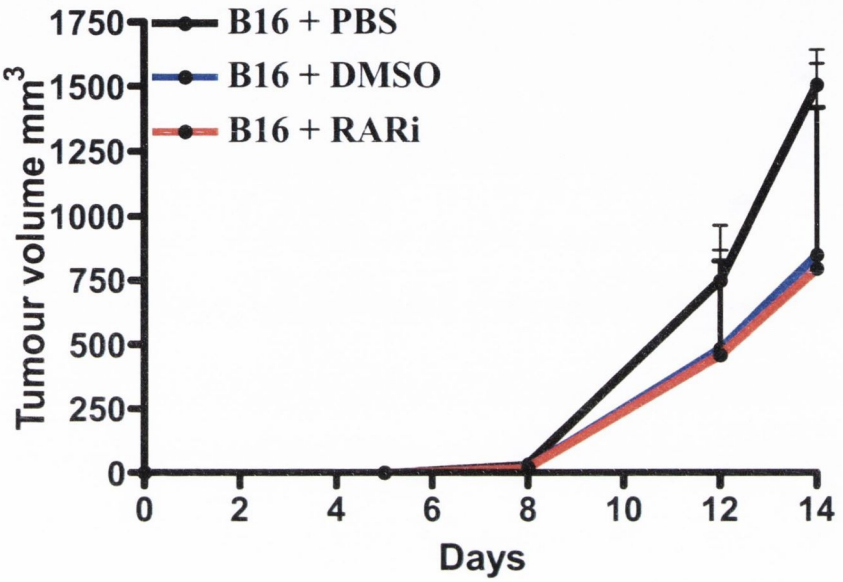


Figure 5.3 Treatment with RARi at the site of the tumour does not alter tumour growth. C57BL/6 mice were injected s.c. into the right flank with 2 x 10^5 B16 melanoma tumour cells on day 0. Mice were also injected s.c. at the same site with either PBS, DMSO or RARi on days –3, –1, +1, +3, +5, and + 7. Tumour volumes were measured from day 5. Results are means of tumour volumes +/- SEM for 6 mice per group. Data are representative of two independent experiments.

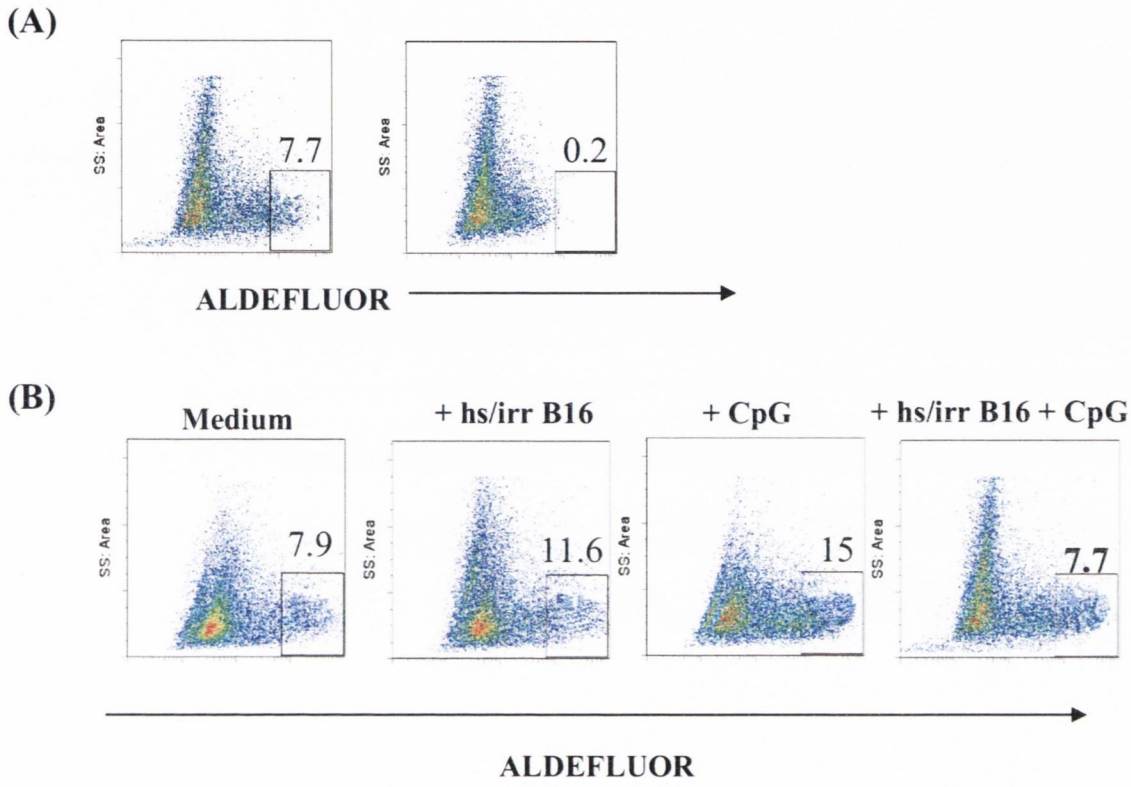


Figure 5.4 DC express ALDHact. BMDC were preincubated with medium only (control) or RARi (5 μM) for 15 minutes and then stimulated with hs/irr B16 tumour cells for 2 hours before the addition of CpG (5 μg/ml), or with CpG alone. After 24 hours cells were incubated with ALDEFLOUR in the presence or absence of the ALDH inhibitor DEAB and analysed by flow cytometry. (A) ALDHFLOUR expression in unstimulated DC or DC in the presence of DEAB inhibitor. (B) ALDEFLOUR expression in DC after 24 hour stimulation with hs/irr B16 tumour cells and CpG. Data are representative of three independent experiments.

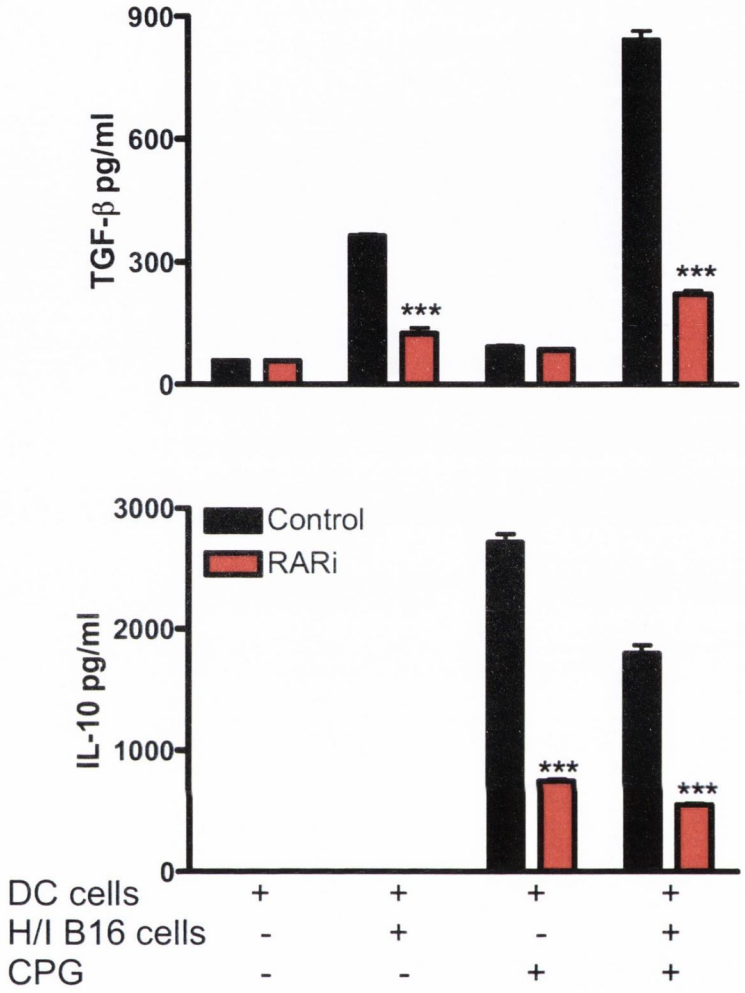


Figure 5.5 RARi significantly inhibited the production of TGF-β and IL-10 from DC stimulated with CpG and killed tumour cells. BMDC were pre-incubated with medium only (control) or RARi (5 μM) for 15 minutes and then stimulated with hs/irr B16 tumour cells for 2 hours before the addition of CpG (5 μg/ml). After 24 hours supernatants were recovered and concentrations of TGF-β and IL-10 were determined by ELISA. Results are means (+/- SD) of triplicate assays. Data is representative of six independent experiments (***p <0.001 medium control versus RARi).

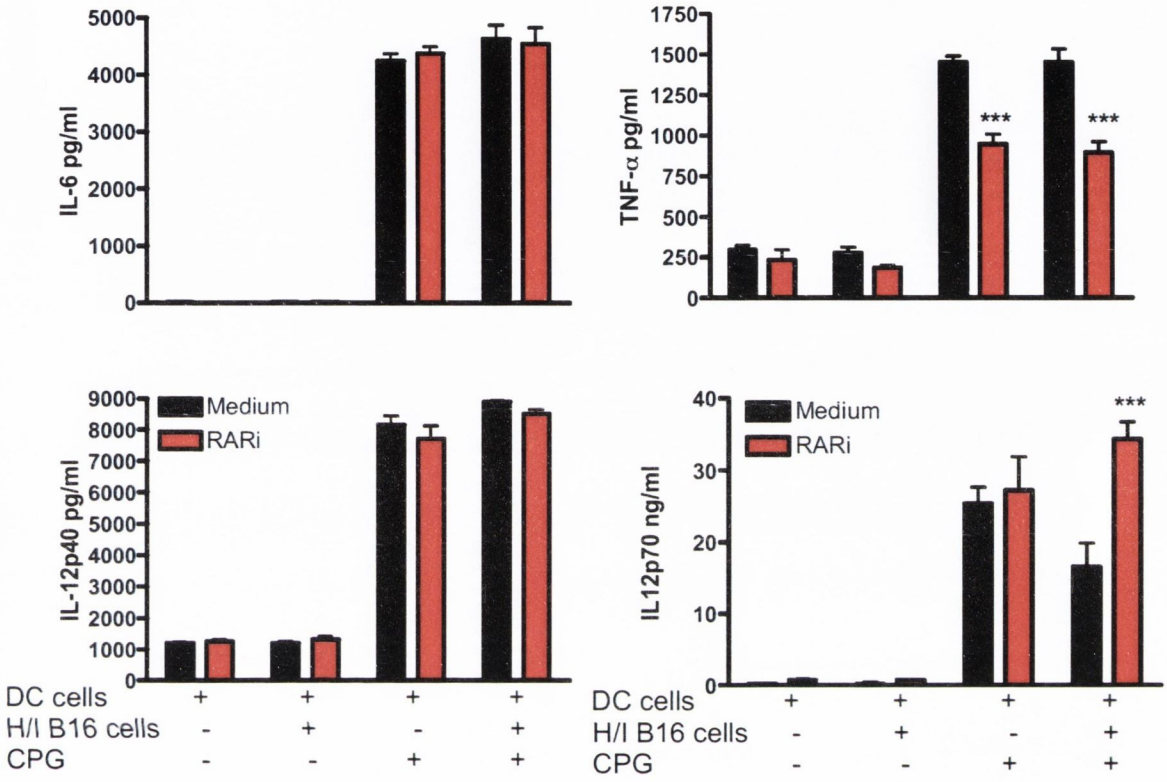


Figure 5.6 RARi did not alter IL-12p40 and IL-6, but enhanced IL-12p70 production from DC stimulated with CpG and killed tumour cells. BMDC were stimulated as described in Fig legend 5.5. After 24 hours supernatants were recovered and concentrations of TNF-α, IL-12p40, IL-12p70 and IL-6 were determined by ELISA. Results are means (+/- SD) of triplicate assays. Data is representative of six independent experiments.

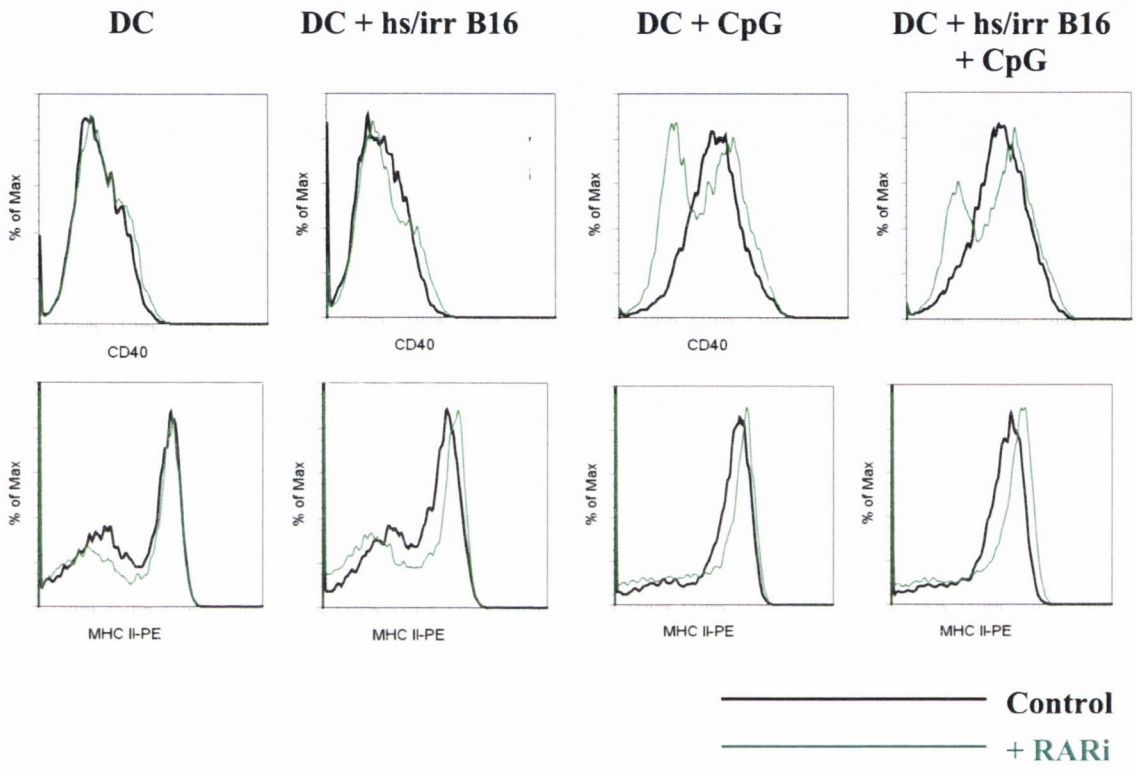


Figure 5.7 The effect of RARi on expression of CD40 and MHC II on DC stimulated with CpG and hs/irr tumour cells. BMDC were preincubated with medium only (control) or RARi (5 μM) for 15 minutes and then stimulated with hs/irr B16 tumour cells for 2 hours before the addition of CpG (5 μg/ml). After 24 hours cells were stained with FITC conjugated αCD40 antibody and PE conjugated MHC II antibody and analysed by flow cytometry. Data is representative of three independent experiments.

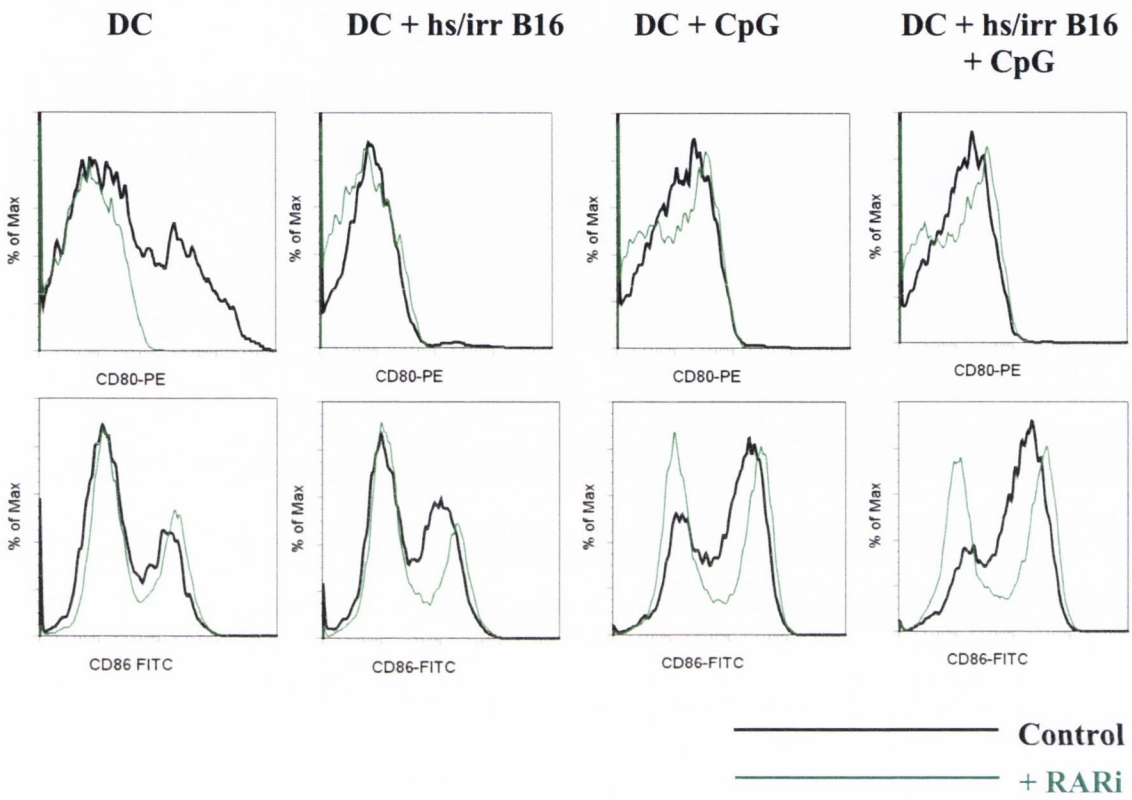


Figure 5.8 The effect of RARi on expression of CD80 and CD86 on DC stimulated with CpG and hs/irrd tumour cells. BMDC were stimulated as described in Fig legend 5.9. After 24 hours cells were stained with FITC conjugated αCD80 antibody and PE conjugated αCD86 antibody and analysed by flow cytometry. Data is representative of three independent experiments.

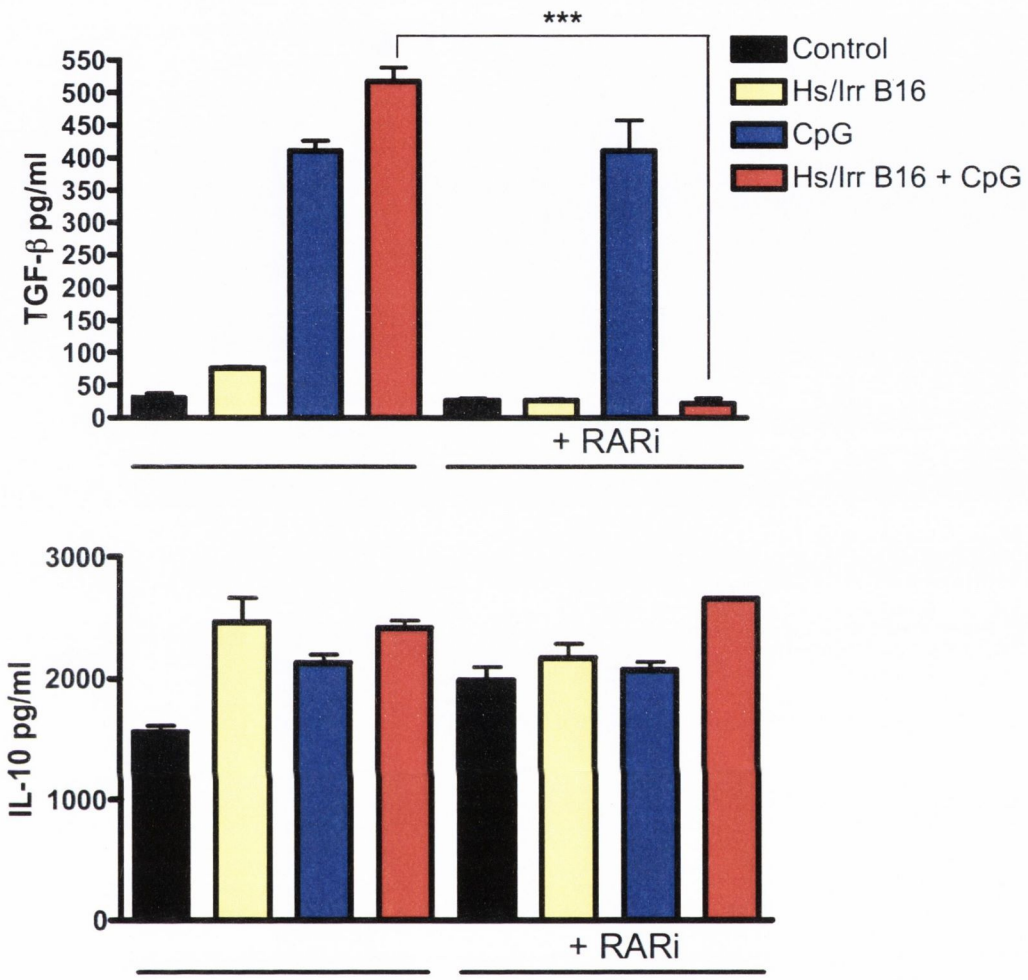


Figure 5.9 RARi inhibited the capacity of DC stimulated with CpG and killed tumour cells to produce TGF-β by $CD4^+$ T cells. BMDC were pre-incubated with medium only or RARi (5 μM) for 15 minutes, then activated with hs/irr B16 tumour cells for 2 hours before stimulating with CpG (5 μg/ml). After 24 hours DC were washed and added to purified $CD4^+$ T cells activated with αCD3 at a T cell: DC ratio of 5:1. After 3 days supernatents were recovered and concentrations of TGF-β and IL-10 were determined in triplicate assays by ELISA.

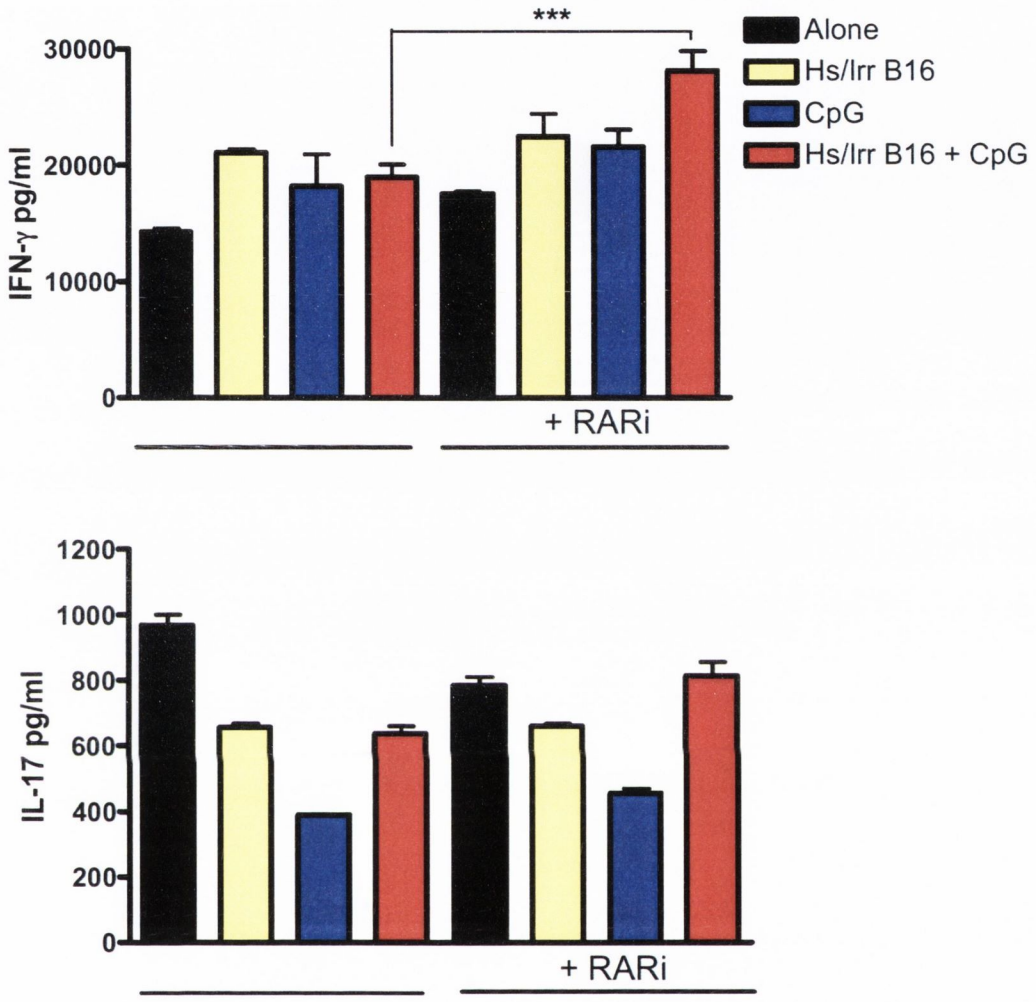


Figure 5.10 RARi enhances the capacity of DC stimulated with CpG and killed tumour cells to produce IFN-γ by $CD4^+$ T cells. BMDC were stimulated as described in Fig legend 5.7. After 24 hours DC were washed and added to allogeneic αCD3 activated $CD4^+$ T cells at a T cell: DC ratio of 5:1. After 3 days supernatents were recovered and concentrations of IFN-γ and IL-17 were determined in triplicate assays by ELISA.

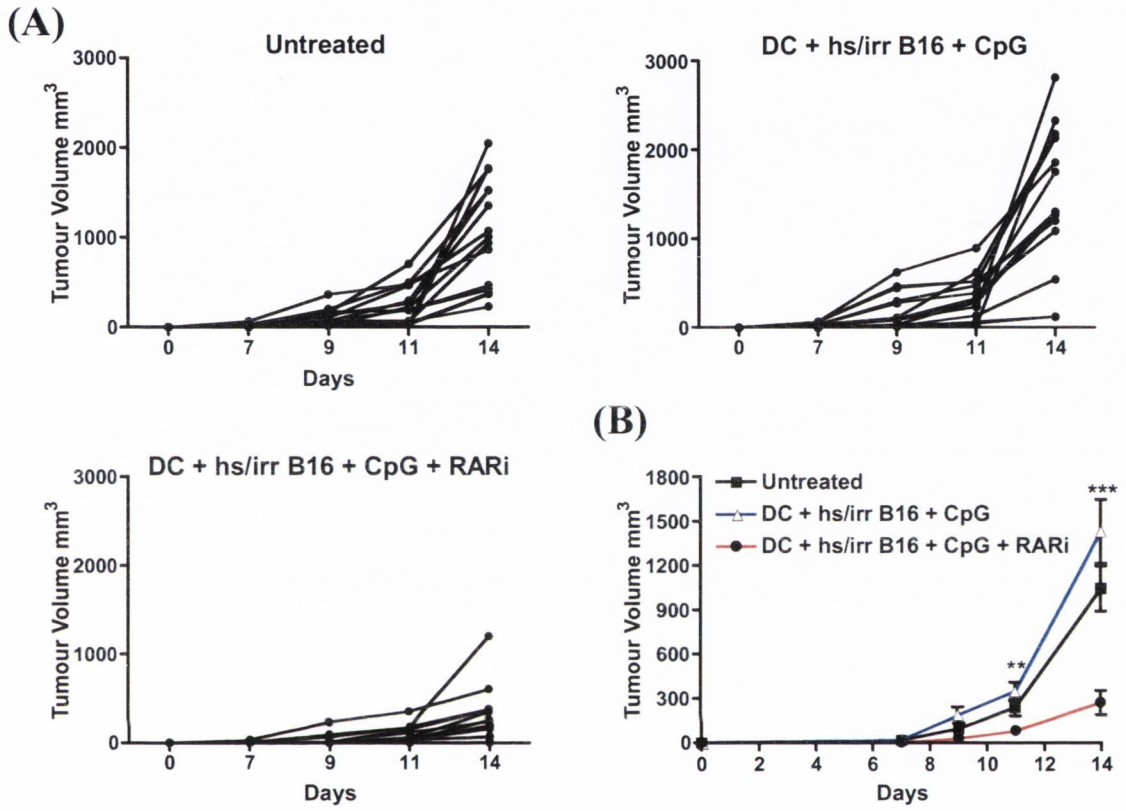


Figure 5.11 Therapeutic administration of a DC vaccine with RARi significantly attenuated tumour growth in mice. C57BL/6 mice were injected s.c. with 2 x 10^5 B16 melanoma tumour cells. Mice were injected s.c. in the region of the tumour 3 and 10 days later with 1–5 x 10^5 DC that were preincubated with medium only (control) or RARi (5 μM) for 15 minutes and then pulsed for 24 hours with hs/irr B16 tumour cells (ratio 1:1) and CpG (5 μg/ml). Tumour volumes were measured every second day from day 7. (A) Represents the tumour volumes for individual mice (n=15), (B) Represents the mean tumour volume +/- SEM for 15 mice per group. This experiment is a representative from 6 experiments. (** $p<0.05$, *** $p<0.001$ by two way ANOVA).

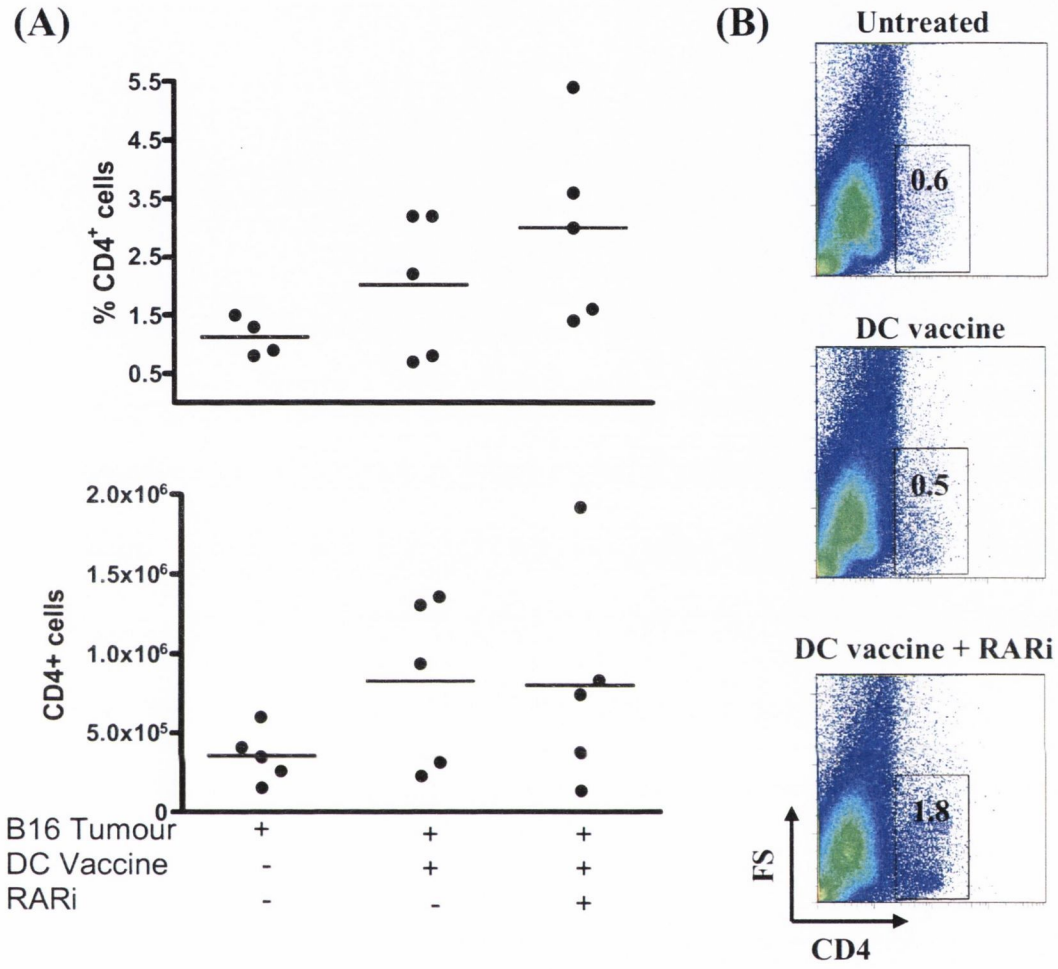


Figure 5.12 Treatment with DC vaccine and RARi promotes the recruitment of tumour infiltrating CD4$^+$ T cells to the site of the B16 tumour. C57BL/6 mice were injected s.c. with 2 x 10^5 B16 melanoma tumour cells. Mice were injected s.c. in the region of the tumour 3 and 10 days later with 1–5 x 10^5 DC that were pretreated with or without RARi for 15 minutes and then pulsed for 24 h with hs/irr B16 tumour cells (ratio 1:1) and CpG. Subcutaneous tumours were removed on day 14 tumour infiltrating CD4$^+$ T cells were labelled directly *ex-vivo* and analysed by flow cytometry. (A) Frequency and absolute numbers of tumour infiltrating CD4$^+$ T cells (B) Representative dot plot from one mouse for each group.

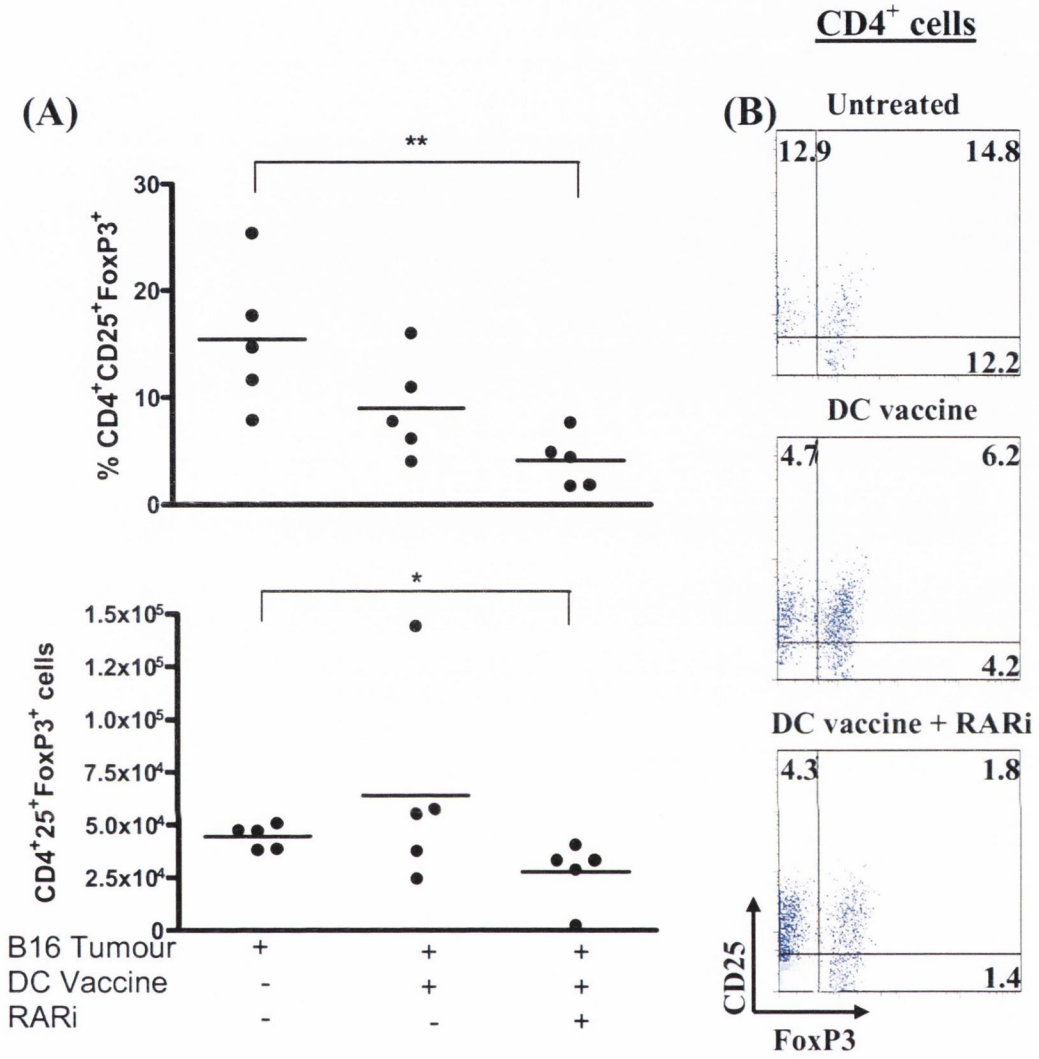


Figure 5.13 Treatment with DC vaccine and RARi is associated with a significant reduction in the tumour infiltrating $CD4^+CD25^+FoxP3^+$ T cells. C57BL/6 mice were treated as described in Fig legend 5.12. Tumours were excised on day 14 and labelled directly ex-vivo with antibodies specific for surface CD4, CD25 and intracellularly stained with FoxP3. (A) The percent and absolute numbers of $CD4^+CD25^+FoxP3^+$ T cells infiltrating the tumour (** $p<0.005$, * $p<0.05$ by unpaired t test) (B) A representative dot plot from each group gated on tumour infiltrating $CD4^+$ T cells.

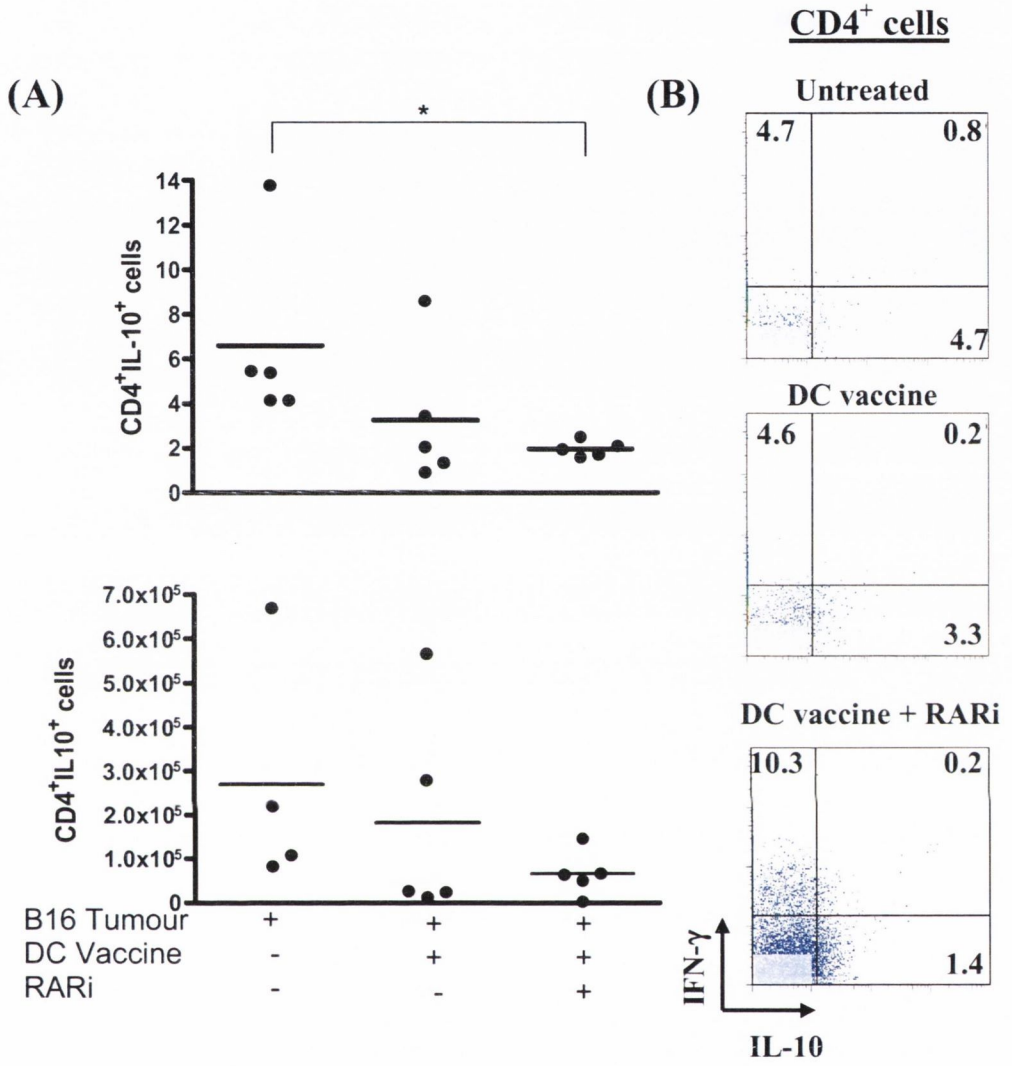


Figure 5.14 Therapeutic treatment with a DC vaccine and RARi is associated with reduction in tumour infiltrating $CD4^+IL\text{-}10^+$ T cells. C57BL/6 mice were treated as described in Fig legend 5.12. Tumours were excised on day 14 and labelled directly ex-vivo with antibodies specific for surface for surface CD4, and intracellularly stained with IL-10. (A) The percent and absolute numbers of $CD4^+IL\text{-}10^+$ T cells infiltrating the tumour (*$p<0.05$ by unpaired t test) (B) A representative dot plot from each group gated on tumour infiltrating $CD4^+$ T cells.

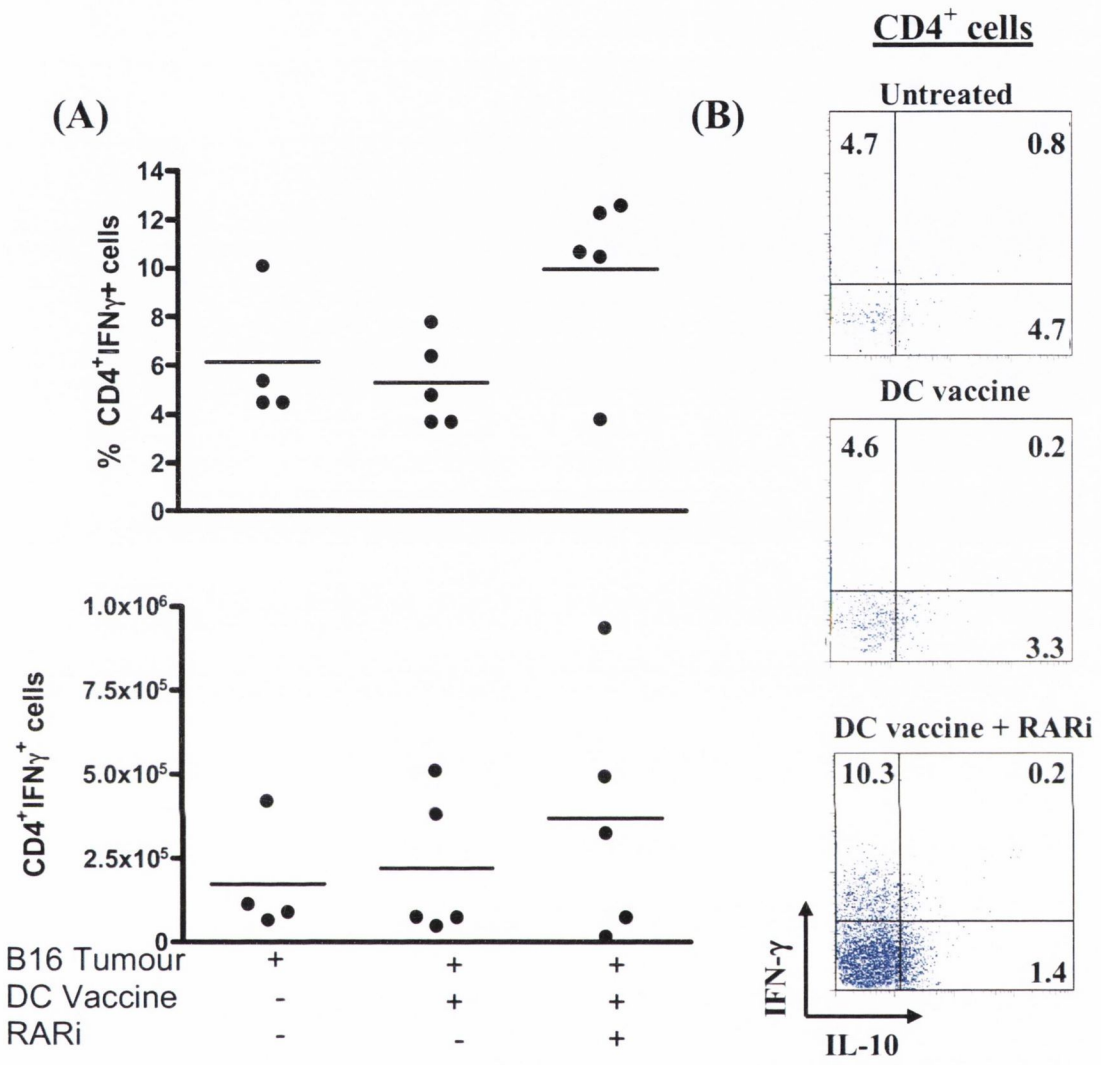


Figure 5.15 Therapeutic treatment with a DC vaccine and RARi is associated with an enhancement in tumour infiltrating $CD4^+$ IFN-γ^+ T cells. C57BL/6 mice were treated as described in Fig legend 5.12. Subcutaneous tumours were removed 14 days post challenge and infiltrating lymphocytes were stimulated with PMA (50 ng/ml), ionomycin (500 ng/ml) and Brefeldin A (5 μg/ml) for 6 hours. (A) The percent and absolute numbers of tumour infiltrating $CD4^+$IFN-γ^+ T cells (B) A representative dot plot from each group, gated on $CD4^+$ T cells showing the frequency of IFN-γ and IL-10.

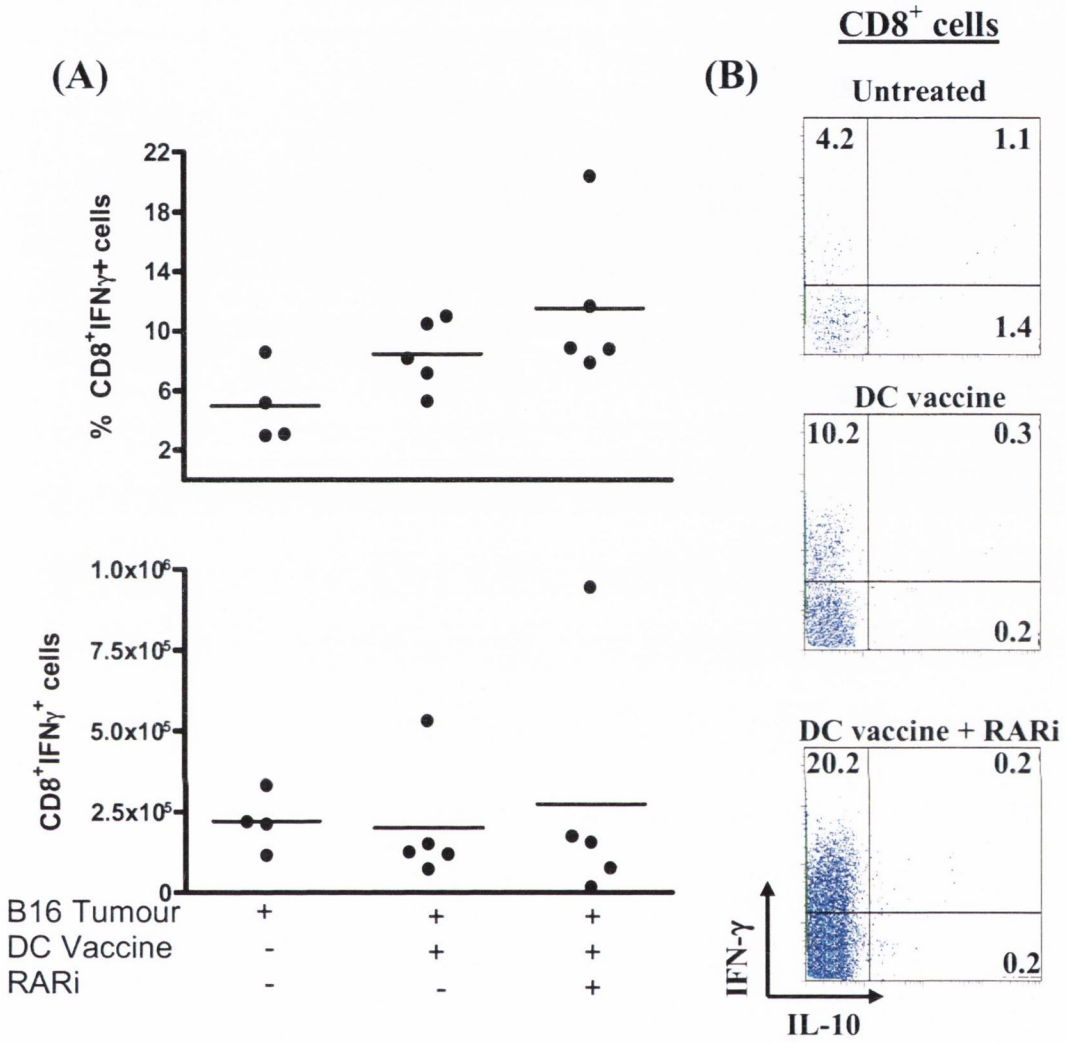


Figure 5.16 Therapeutic treatment with a DC vaccine and RARi is associated with an enhancement in tumour infiltrating CD8$^+$ IFN-γ$^+$ T cells. C57BL/6 mice were treated as described in Fig 5.12. Tumours were removed 14 days post challenge and stimulated with PMA (50 ng/ml), ionomycin (500 ng/ml) and Brefeldin A (5 μg/ml) for 6 hours. (A) The percent and absolute numbers of tumour infiltrating CD8$^+$IFN-γ$^+$ T cells (B) A representative dot plot from each group, gated on CD8$^+$ T cells demonstrating the frequency of IFN-γ and IL-10.

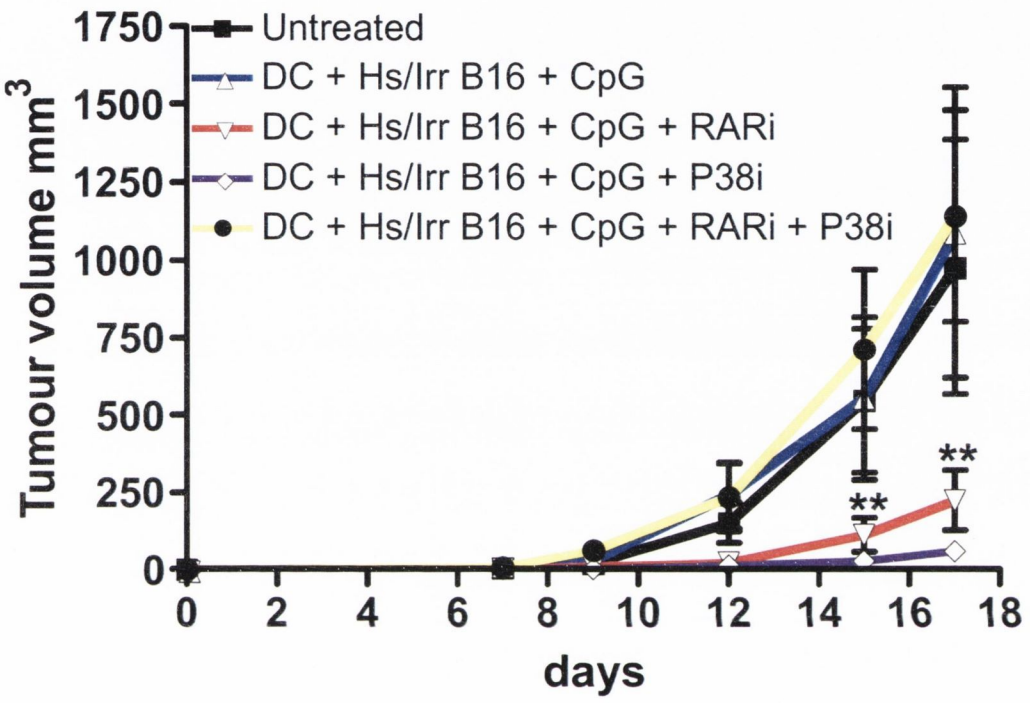


Fig 5.17 Therapeutic administration of a DC vaccine pretreated with RARi or P38i alone but not in combination significantly attenuated tumour growth *in vivo*. Mice were challenged with 2 x 10^5 B16 tumour cells and injected s.c. in the region of the tumour 3 and 10 days later with 1–5 x 10^5 DC that had been pulsed for 24 hr with hs/irr B16 cells (ratio 1:1), and CpG (5 μg/ml), a P38 inhibitor SB203580 (1 μM) or RARi (5 μM) + P38i. Tumour volumes were measured every second day from day 7. (A) Represents the mean tumour volume +/- SEM for 6 mice per group. (** $p<0.05$ by two way ANOVA).

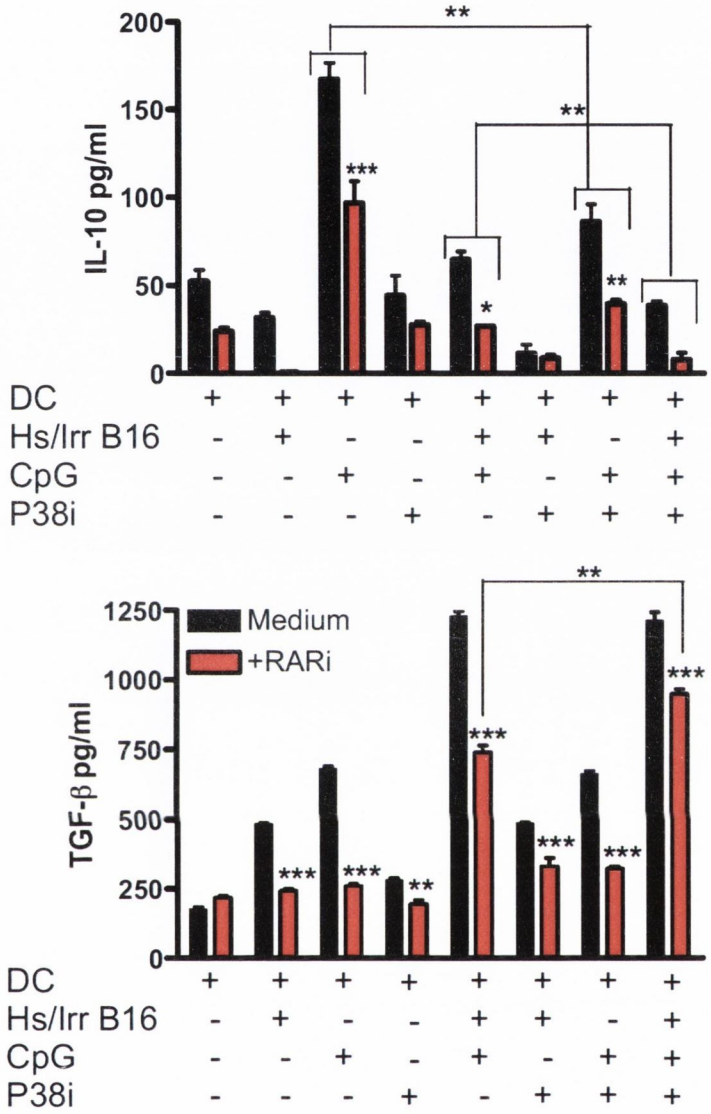


Fig 5.18 Inhibition of P38 suppresses IL-10 production from DC. BMDC were preincubated with medium only (control) or P38i (1 μM) for 1 hour before the addition of RARi (5 μM) for 15 minutes, then activated with hs/irr B16 tumour cells for 2 hours before stimulating with CpG (5 μg/ml). After 24 hours supernatants were recovered and concentrations of IL-10 and TGF-β were determined by ELISA. Results are means (+/- SD) of triplicate assays.

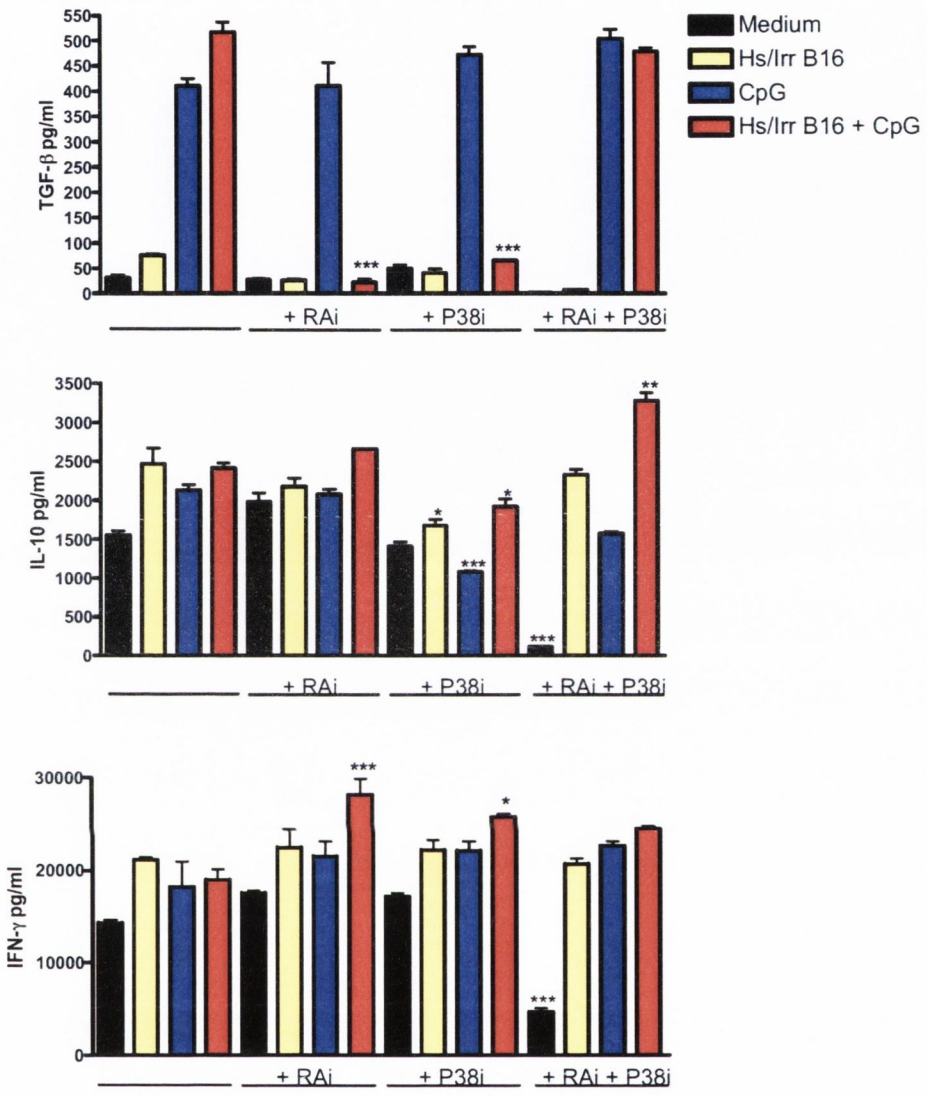


Fig 5.19 RARi or P38i alone completely suppressed the ability of killed tumour cell and CpG-activated DC to induce TGF-β production by allogeneic $CD4^+$ T cells. BMDC were preincubated with medium only or P38i (1 μM) for 1 hour before the addition of RARi (5 μM) for 15 minutes, then activated with hs/irr B16 tumour cells for 2 hours before stimulating with CpG (5 μg/ml). After 24 hours DC were washed and added to allogeneic $CD4^+$ T cells at a T cell: DC ratio of 5:1. After 3 days supernatants were recovered and concentration of TGF-β and IL-10 were determined by ELISA. Results are means (+/- SD) of triplicate assays (*$p<0.05$, **$p<0.01$, ***$p<0.001$ by one way ANOVA).

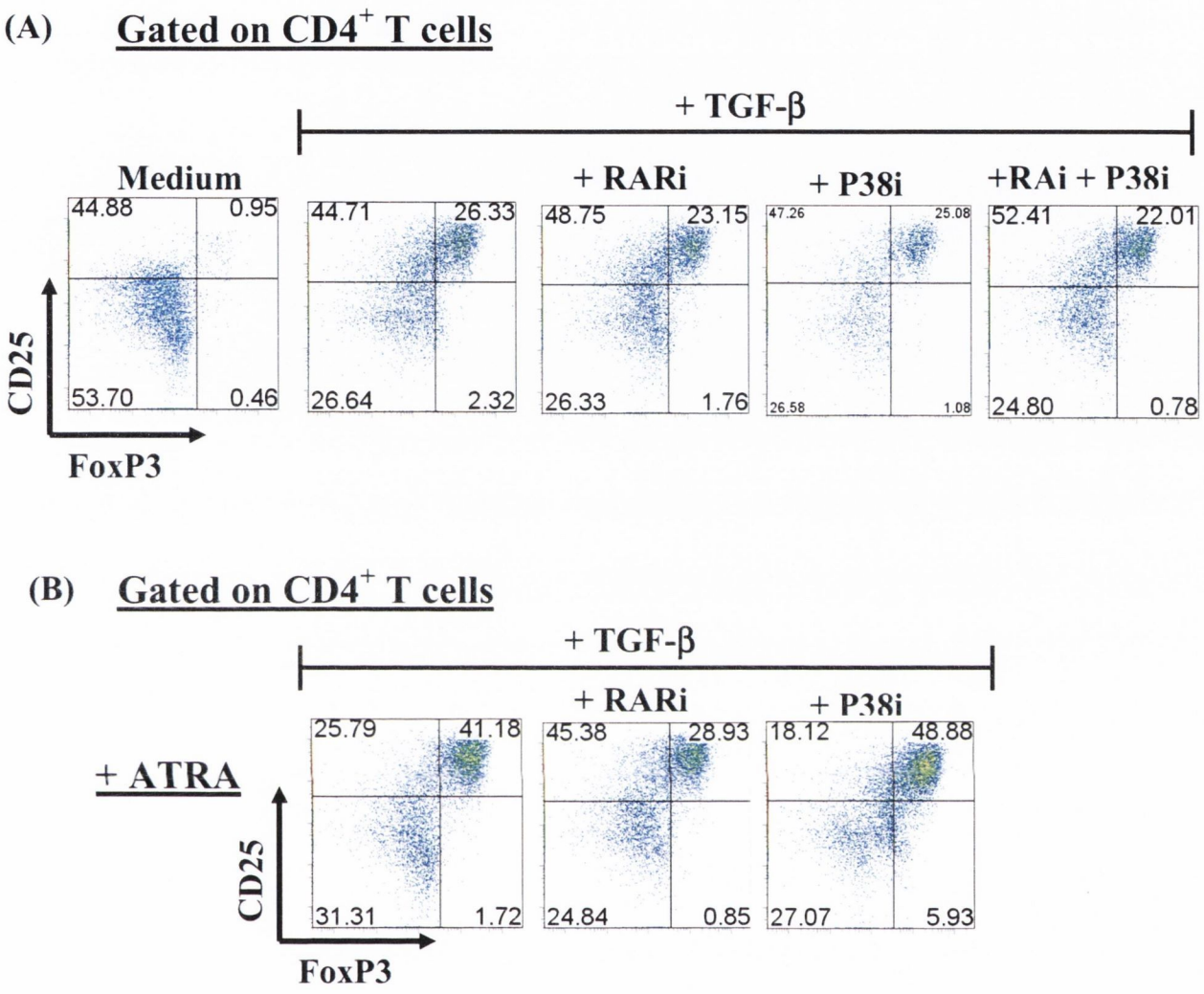


Fig 5.20 TGF-β dependent conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+FoxP3^+$ T cells is enhanced in the presence of ATRA and the P38i. $CD4^+CD25^-$ T cells isolated from spleen cells were stimulated with platebound αCD3, soluble CD28, rIL-2, with or without rTGF-β (5 ng/ml), ATRA (100 nM), P38i (1 μM) and RARi (5 μM). The frequency of $CD4^+CD25^+FoxP3^+$ T cells was determined on day 3 by surface labelling with CD4, CD25 and intracellular labelling with FoxP3. This experiment is a representative of three experiments.

Gated on $CD4^+$ T cells

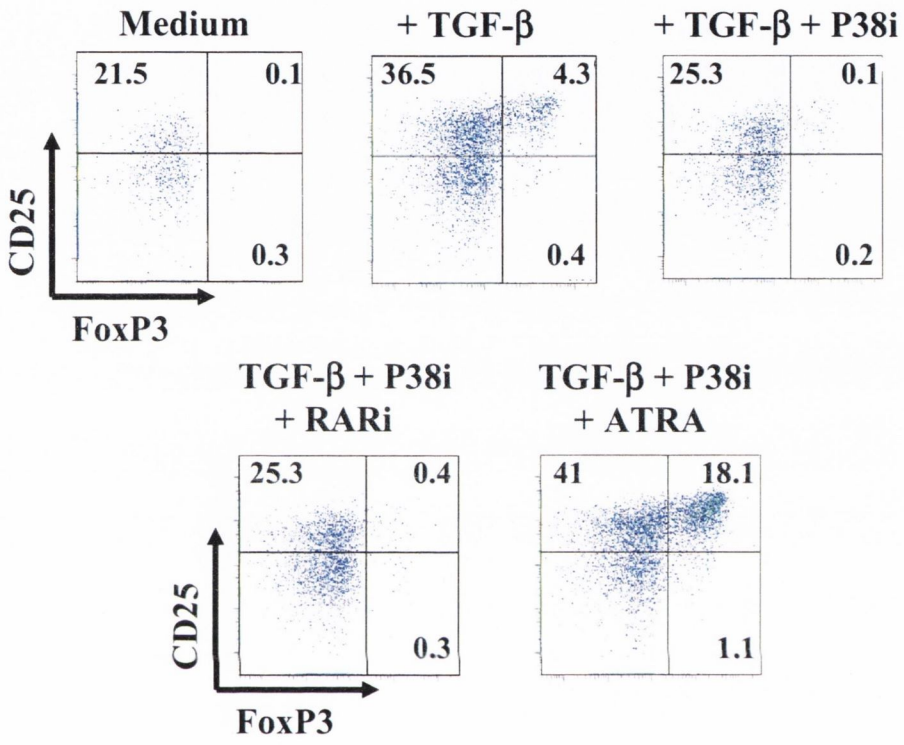


Fig 5.21 Conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+FoxP3^+$ T cells in DC and $CD4^+CD25^-$ co-cultures is enhanced in the presence of ATRA and P38i. $CD4^+CD25^-$ T cells isolated from spleen cells were stimulated with platebound αCD3 (1 μg/ml) and co-cultured with BMDC at a T cell:DC ratio of 5:1. Cells were stimulated with and without rTGF-β (5 ng/ml), ATRA (100 nM), P38i (1 μM) and RARi (5 μM). The frequency of $CD4^+CD25^+FoxP3^+$ T cells was determined on day 3 by surface labelling with CD4, CD25 and intracellular labelling with FoxP3.

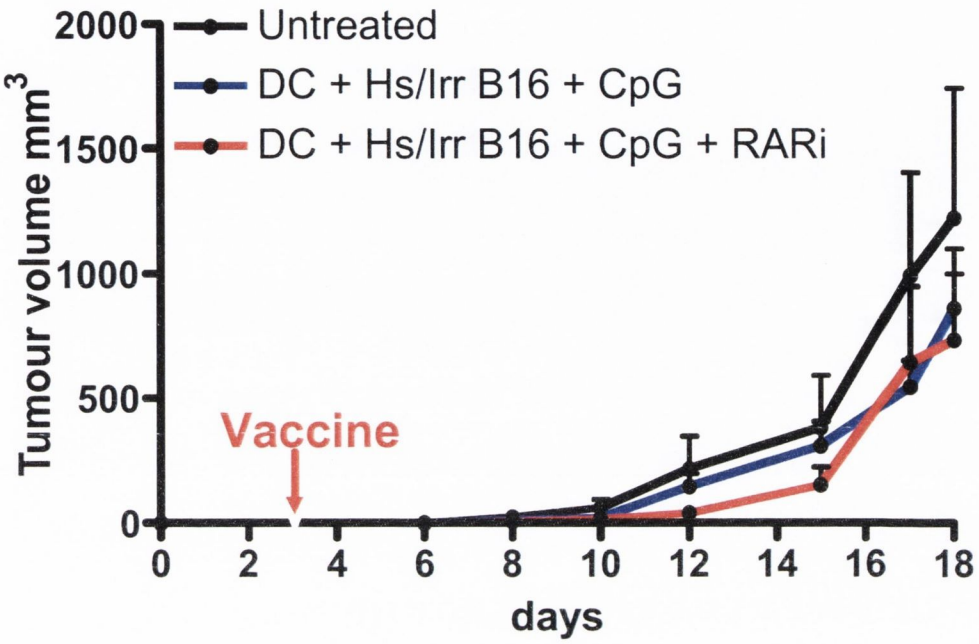


Figure 5.22 A single treatment with a DC vaccine and RARi during the initial stages of tumour development delays tumour growth and moderately attenuates early tumour growth. C57BL/6 mice were injected s.c. with 2 x 10^5 B16 melanoma tumour cells. Mice were injected s.c. in the region of the tumour on day 3 with 1–5 x 10^5 CpG-activated DC that were pretreated with or without RARi for 15 minutes and then pulsed for 24 h with hs/irr B16 tumour cells (ratio 1:1). Tumour volumes were measured every second day from day 6. Results are mean tumour measurements +/- SEM for 9 mice per group.

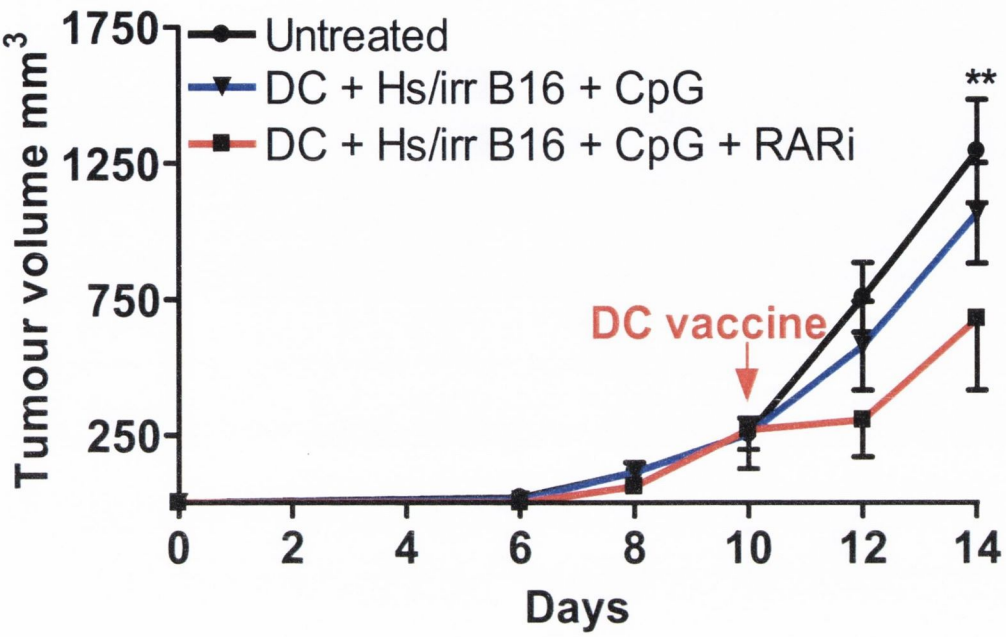


Figure 5.23 A single treatment with a DC vaccine and RARi during the aggressive stages of tumour development moderately attenuates tumour growth. C57BL/6 mice were injected s.c. with 2 x 10^5 B16 melanoma tumour cells. Mice were injected s.c. in the region of the tumour on day 10 with 1–5 x 10^5 CpG-activated DC that were pretreated with or without RARi for 15 minutes and then pulsed for 24 h with hs/irr cells (ratio 1:1). Tumour volumes were measured every second day from day 6. Results are mean tumour measurements +/- SEM for 8 mice per group. ** $p<0.01$ RARi-treated DC vaccine versus untreated by two way ANOVA.

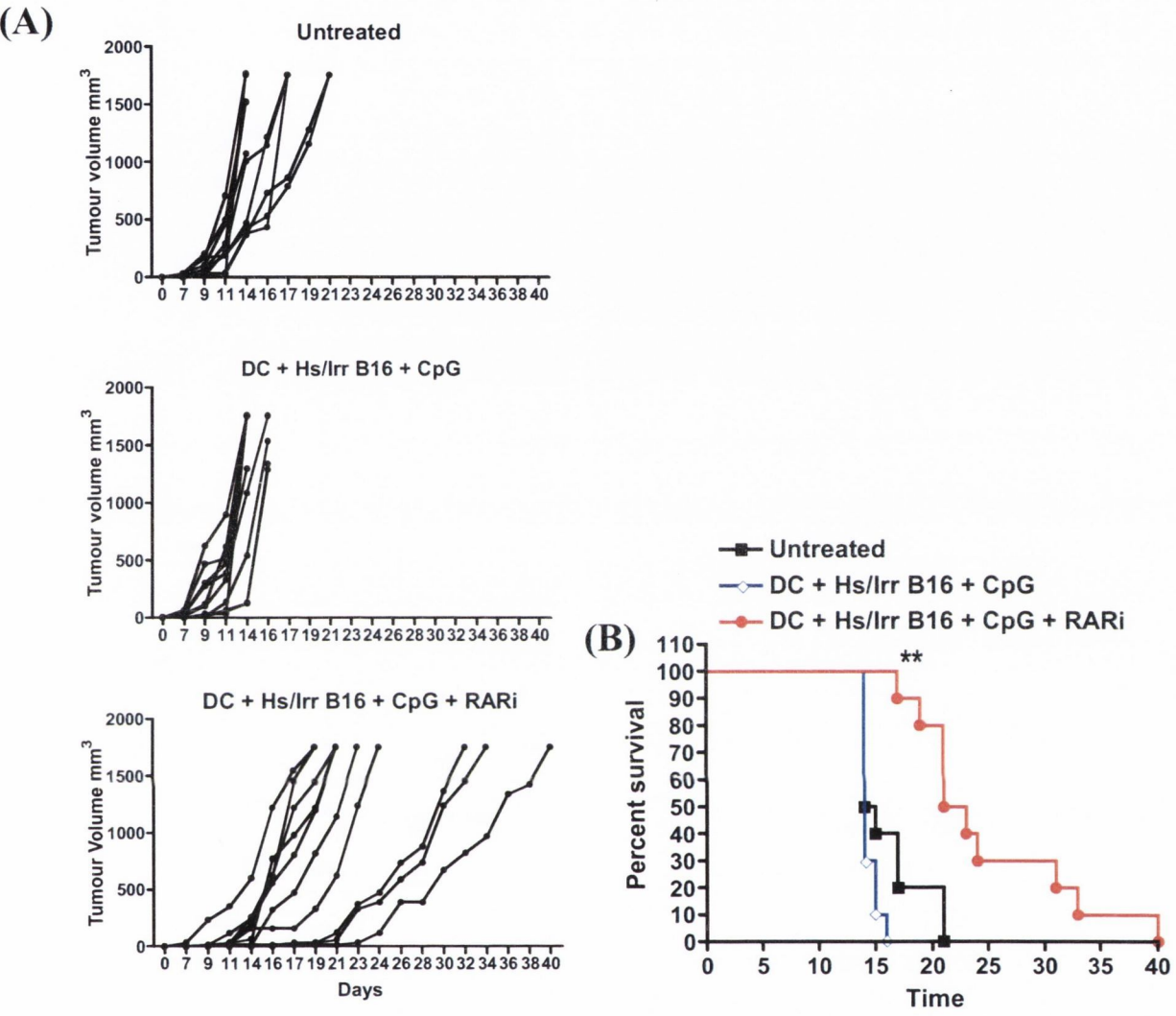


Figure 5.24 Therapeutic administration of 3 doses of a DC vaccine with RARi significantly enhanced survival in tumour bearing mice. C57BL/6 mice were injected s.c. with 2 x 10^5 B16 melanoma tumour cells. Mice were injected s.c. in the region of the tumour 3, 10 and 17 days later with 1–5 x 10^5 DC that were preincubated with medium only (control) or RARi (5 μM) for 15 minutes and then pulsed for 24 hours with hs/irr B16 tumour cells (ratio 1:1) and CpG (5 μg/ml). Tumour volumes were measured every second day from day 7. (A) Represents the tumour volumes of 15 individual mice per group, while (B) Represents the mean tumour volume +/- SEM for 10 mice per group. This experiment is a representative from 3 experiments. (**$p<0.05$; *** $p<0.001$ by two way ANOVA).

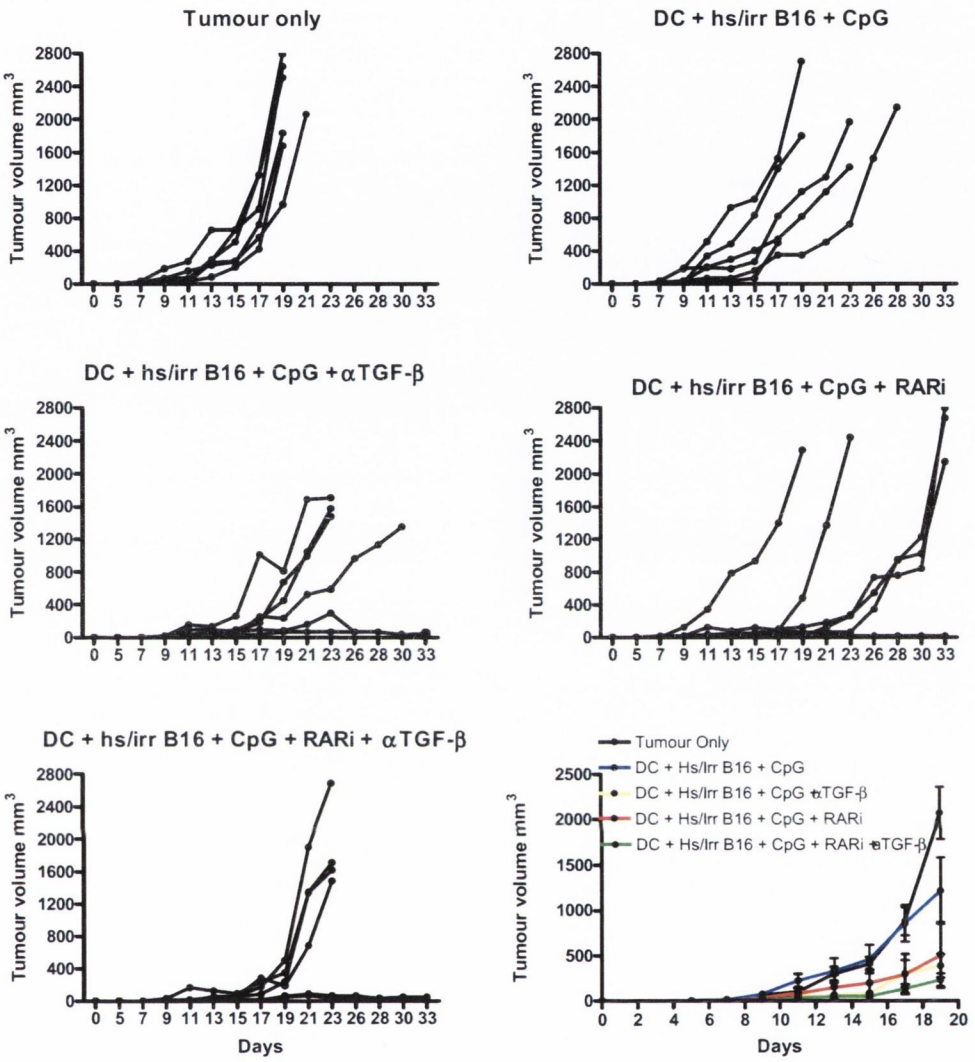


Fig 5.25 Neutralising TGF-β at the site of the tumour does not significantly improve the efficacy of the RARi DC based vaccine as a cancer immunotherapy. C57BL6 mice were challenged with 2 x 10^5 B16 tumour cells and injected s.c. in the region of the tumour 3, 10 and 17 days later with 1–5 x 10^5 DC that had been pulsed for 24 hr with hs/irr tumour cells (ratio 1:1), and CpG (5 μg/ml) in the presence or absence of the RARα antagonist (5 μg/ml). Mice were also injected every second day from day +1 with a neutralising TGF-β antibody or isotype control. Tumour volumes were measured every 2-3 days from day 7. (A) Represents the mean tumour volume +/- SEM for 6 mice per group. (** $p<0.01$, *** $p<0.001$ by two way ANOVA).

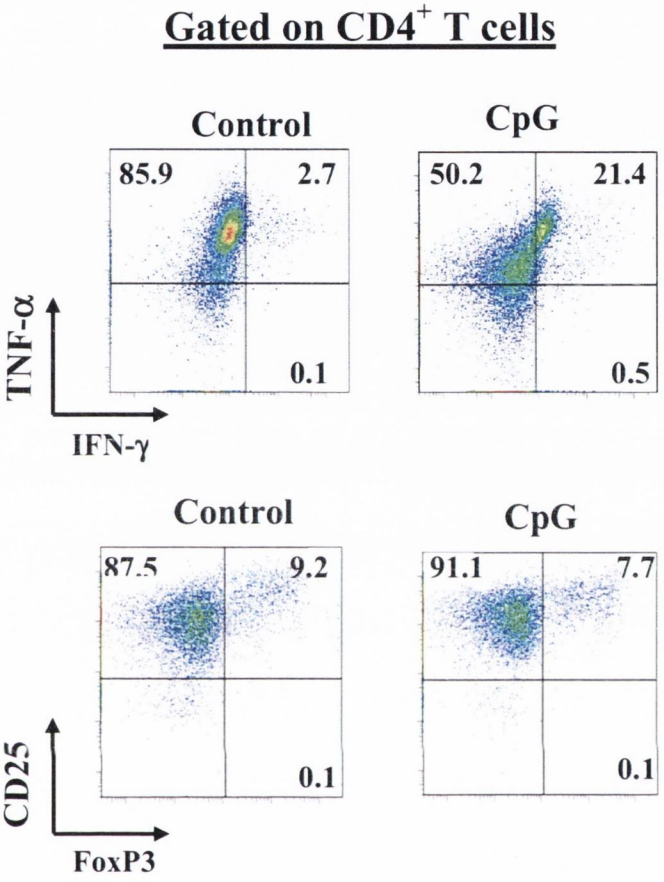


Fig 5.26 TLR-9 stimulated DC promote IFN-γ production from CD4$^+$ T cells in vitro. BMDC were activated with CpG (5 μg/ml). After 24 hours DC were washed and added to allogeneic αCD3 stimulated CD4$^+$ T cells at a T cell: DC ratio of 5:1. After 3 days cells were stimulated with PMA (50 ng/ml), Ionomycin (500 ng/ml) and BFA (5 μg/ml) for 6 hours. Cells were washed and stained for CD4, CD25, FoxP3, IFN-γ and IL-10.

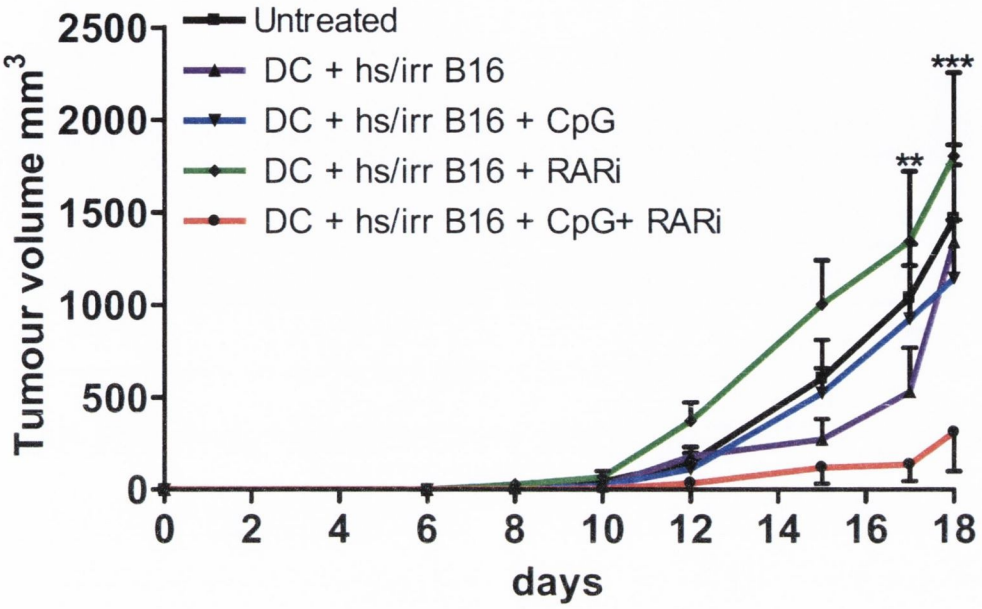


Figure 5.27 A combination of RARi and CpG augments the protective efficacy of a DC vaccine against B16 tumours growth. Mice were challenged with 2 x 10^5 B16 tumour cells and injected s.c. in the region of the tumour 3, 10 and 17 days later with 1–5 x 10^5 DC pulsed for 24 hr with hs/irr B16 cells (ratio 1:1) with and without CpG (5 μg/ml) and RARi (5 μM). Tumour volumes were measured every second day from day 6. (A) Represents the mean tumour volume +/- SEM for 6 mice per group. This experiment is a representative from 2 experiments. (**, $p<0.05$; ***, $p<0.001$ by two way ANOVA).

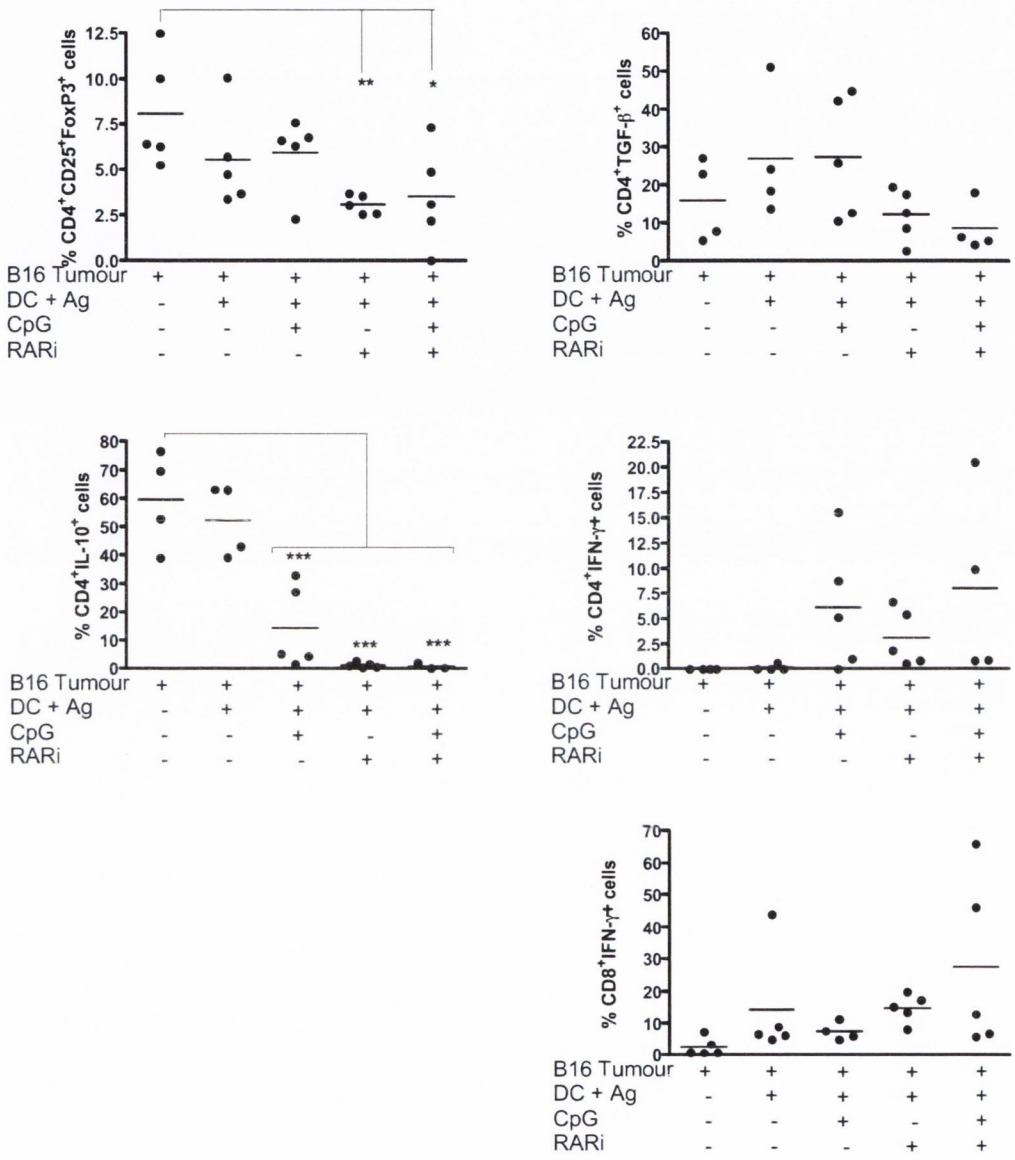


Fig 5.28 Blocking RARα in combination with a DC vaccine significantly inhibits tumour infiltrating Treg cells. C57BL/6 mice were inoculated with the B16F10 melanoma tumours and therapeutically treated as described in Fig 5.21. Subcutaneous tumours were removed on day 18 and tumour infiltrating lymphocytes were stained for CD4, CD8, CD25, FoxP3, TGF-β, IL-10 and IFN-γ. Data analysis was determined by flow cytometry.

Chapter 6

General Discussion

6. GENERAL DISCUSSION

The development of safe and effective therapies against cancer remains to be one of the fundamental challenges in the field of oncology. Over the past number of years, considerable progress has been made in the development of active immunotherapy, which has the potential to be a more promising and less aggressive approach than current cytotoxic drugs. The primary aim of active immunotherapy is to enhance Th1 and CTL responses and to limit the suppressive functions of Treg cells, as it is clear that tumour cells can exploit the tolerogenic and regulatory mechanisms of the immune system to evade and subvert antitumour immunity. Immune subversion by tumours, especially their ability to induce or activate Treg cells, is a major obstacle in the development of successful tumour immunotherapeutics.

The targeted inhibition of Treg cells has shown encouraging results in animal tumour models, which subsequently progressed to clinical trials. This lead to the development of ONTAK, a recombinant fusion protein composed of IL-2 and diphtheria toxin that binds $CD25^+$ cells and subsequently induces death via the diphtheria toxin portion [271]. Despite encouraging *in vivo* results in experimental models, ONTAK has had limited success in the clinic to date [272-273]. One explanation for the failure of this approach as a therapy is that CD25 is also upregulated on activated T cells, therefore targeted inhibition of the CD25 receptor also destroys activated effector T cells, and therefore compromises host immunity. Alternative strategies that inhibit Treg cell functions have also been developed, blocking negative regulators of T cell activation, such as CTLA4. CTLA4 is

constitutively expressed on Treg cells, inhibiting DC and the TCR function, with confirmed functions in antitumour immunity [55, 213]. A number of animal studies have demonstrated that anti-CTLA4 therapy augments antitumour immune responses and induces tumour rejection [274]. Furthermore, clinical trials in cancer patients with various malignancies, such as melanomas, bladder cancer and prostate cancer, have reported successful antitumour immune responses when treated with anti-CTLA4 antibodies, such as ipilimumab and tremelimumab. Despite encouraging antitumour immune responses, the beneficial response to treatment with anti-CTLA4 antibodies was associated with adverse side effects, such as dermatitis, colitis and hepatitis [216, 275]. Therefore, while anti-CTLA4 successfully induces antitumour immunity, the serious side-effects associated with these antibodies limits its success as a future immunotherapy.

While it is clear that inhibiting or limiting Treg cells in cancer patients has the potential to confer protective antitumour immunity, current monotherapies that limit Treg cell functions also compromise host immune tolerance against self-antigens, which can lead to the development of autoimmune disease. The present study investigated the effects of combination therapies, inhibiting immunosuppressive molecules involved in the development of *de nova* Treg cells while also boosting effector T cell responses via tumour-antigen loaded DC vaccines that promote the induction of antigen-specific effector T cells. A key finding of the study was the demonstration that treatment of tumour-bearing mice with TGF-β siRNA that transiently reduced local TGF-β gene expression,

circumventing the immunosuppressive nature of the tumour environment, and subsequently enhanced the efficacy of a DC vaccine against tumour growth *in vivo*. Furthermore, this study found that an RARi-treated antigen-pulsed and CpG-activated DC was shown to be a promising immunotherapeutic strategy that significantly inhibited tumour growth and enhanced survival *in vivo*, via blocking RA-iTreg cells and promoting effective CTL and Th1 responses.

The results from the present study showed that transiently reducing TGF-β gene expression in a tumour cell using siRNA resulted in a marked decrease in the ability of tumour cells to grow *in vivo*, supporting the concept that tumour-derived TGF-β can promote its own growth *in vivo* [229]. However, as a direct monotherapy TGF-β siRNA did not work therapeutically to significantly attenuate tumour growth *in vivo*. Given that the widespread lack of efficacy in cancer vaccines has been attributed, in part to tumour-derived immunosuppressive molecules, such as TGF-β, and tumour-induced Treg cells, the present study combined the administration of TGF-β siRNA with an antigen-pulsed and TLR-activated DC vaccine approach. The results revealed that transiently silencing TGF-β at the site of the tumour significantly enhanced the efficacy of the DC-based vaccine against the poorly immunogenic B16F10 melanoma *in vivo*. This study provided further evidence of the potent suppressive effects of TGF-β in compromising the capacity of immunotherapies to enhance antitumour immune responses *in vivo*. In addition, attenuation of tumour growth with the DC vaccine combined with TGF-β siRNA was associated with a reduction in tumour infiltrating

Treg cells. These findings lend further support to the hypothesis that tumour-induced Treg cells and tumour derived-TGF-β can significantly impair antitumour immune responses. The present study found that treatment with TGF-β siRNA or the DC therapy alone did not confer significant protection when administered alone, highlighting the benefits of a combined approach that suppresses Treg cells and enhances effector responses to protect against tumour growth *in vivo*. Since DC vaccines and siRNA technology have been well tolerated in clinical trials, this combined approach is likely to be similarly well tolerated in humans and if translated into the clinic could function as an effective anti-cancer immunotherapy.

In addition to demonstrating that TGF-β siRNA can reduce tumour growth *in vivo*, this study also showed that excessive TGF-β enhanced tumour growth *in vivo*. It has been demonstrated that infection with the helminth parasite *F. hepatica* is associated with expansion or recruitment of $FoxP3^+$ Treg cells, which suppress host immune responses to both the parasite and to unrelated autoantgens via a TGF-β-dependent mechanism [238]. The present study found that infection with *F. hepatica*, or it's secretory products promoted systemic immune suppression *in vivo*, and this resulted in exacerbation of tumour growth in lung and s.c. tumour models. The enhancement of tumour growth following systemic infection with a parasite or parasite products highlights the importance of immune surveillance in regulating the growth of neoplasms. The exacerbation of tumour growth by the parasite was associated with a dramatic enrichment of tumour infiltrating Treg cells, supporting the concept that a high frequency of Treg cells can compromise immune

surveillance and antitumour immune responses [141]. Moreover, the present study established that the exacerbation of tumour growth by *F. hepatica* ES products was completely reversed by neutralising TGF-β *in vivo*, demonstrating that parasite products can exacerbate tumour growth via TGF-β-dependent mechanisms. Parasite driven expansion of Treg cell function was clearly able to overwhelm the host's ability to control tumour growth via suppressing antitumour effector immune cells. This is consistent with studies which have shown that depletion of Treg cells from tumour-bearing animals strongly impeded tumour growth *in vivo*. Previous studies together with the data presented here, highlight the delicate balance between regulatory and effector T-cell responses in the control of tumour growth and provides evidence that manipulating this balance in favour of effector T cells has the potential to improve the efficacy of immunotherapies for cancer. Conversely, this study also highlighted that extreme caution must be taken when manipulating this pathway, as proposed treatments for autoimmunity could potentially promote tumour growth, whilst antitumour therapies must act locally or risk causing autoimmunity.

It is broadly accepted that conventional $CD4^+$ T cells can be induced to express $FoxP3^+$ in response to the growing tumour *in vivo* [143, 151]. In agreement with several reports, the present study found that RA enhanced TGF-β-mediated peripheral conversion of naïve cells into $Foxp3^+$ T cells, and blocking RARα inhibited this synergistic effect [91, 116-117]. Given that RA is an important morpohogen that regulates several biological processes, blocking the RA-RARα

axis in the host may not be generally applicable for the treatment of human tumours. Since RA is metabolized from vitamin A by DC, blocking RARα in an Ag-pulsed and TLR-activated DC vaccine may be a more desirable approach to improve the efficacy of cell-based immunotherapies, enhancing Ag-specific effector T cells and limiting RA-induced Treg cell responses.

The present found that blocking RARα significantly inhibited the production of immunosuppressive cytokines, such as TGF-β and IL-10, while enhancing IL-12p70 from CpG-activated DC pulsed with hs/irr B16 cells. In addition, blocking RARα in CpG-activated DC pulsed with hs/irr B16 cells significantly influenced the capacity of these DC to direct $CD4^+$ T cells responses, inhibiting TGF-β expression, while enhancing IFN-γ production from $CD4^+$ T cells. Taken together, this data suggests that antagonising the RA-RARα axis modulates CpG-activated DC away from regulation and towards a pro-inflammatory Th1 response.

Consistent with previous findings, treatment with CpG-activated DC did not confer protection against tumour growth *in vivo*. However, blocking RARα in CpG-activated DC pulsed with hs/irr B16 cells significantly improved the efficacy of the TLR-activated DC vaccine, significantly attenuating tumour growth and promoting survival *in vivo*. The reduction of tumour growth was associated with a substantial increase in the percentage of tumour infiltrating $CD4^+$ T cells, and IFN-γ producing $CD4^+$ and $CD8^+$ T cells and a corresponding significant reduction in tumour infiltrating $CD4^+CD25^+FoxP3^+$ T cells and $CD4^+IL10^+$ T cells. These findings

suggest that blocking RARα in a CpG-DC vaccine significantly circumvents the tolerogenic environment of the tumour, inhibiting RA-mediated peripheral conversion of iTreg cells via the DC, while driving antitumour immune response and conferring significant protection against tumour growth *in vivo*. As a potential cell-based therapy, the RARi-treated DC vaccine is promising, in that it should act locally at the tumour site in an antigen-specific fashion, preventing any systemic adverse events, inhibiting tumour-induced immune tolerance while simultaneously driving potent antitumour immune responses.

In summary, this study supports and highlights the beneficial effects of combining a cell-based vaccine approach that will boost effector T cell responses with strategies that will reduce Treg cells to enhance the efficacy of vaccines and immunotherapies for cancer. In the case of the RARi-treated DC vaccine, CpG was required as an adjuvant to enhance CTL and Th1 responses and confer protection against tumour growth *in vivo*. Similarly, treatment with TGF-β siRNA or Ag-pulsed and CpG-activated DC vaccines did not work therapeutically as monotherpaies, however, when combined a significant attenuation of tumour growth was conferred *in vivo*. Interestingly, the data from the *F. hepatica* infection model provides direct experimental evidence that altering the effector to Treg cell ratio, inducing excessive TGF-β and Treg cells in response to the parasite, significantly exacerbated tumour growth *in vivo*. This data highlights the delicate balance between regulatory and effector-T cells responses in controlling tumour growth and development, validating the capacity of Treg cells to suppress protective antitumour

immune responses, providing strong evidence that manipulating this balance has the potential to improve the efficacy of tumour immunotherapies.

The success of next generation therapies is dependent on developing safe and effective vaccines that overcome the immunosuppressive nature of the growing tumour to confer protective immunity, without breaking immune tolerance to self or innocuous antigens. Adding a vaccine approach to currently translated treatments that target Treg cells, such as ONTAK and anti-CTLA4, may increase the expansion of tumour specific CTL and Th1 cells, leading to an increased frequency of memory-specific tumour responses and enhanced efficacy of these therapies. Furthermore, by infrequent treatment with a vaccine, therapies such as ONTAK and anti-CTLA4 may not totally inhibit Treg cells because it is not chronic administration and thus may not compromise host immune tolerance to self-antigens, preventing the development of autoimmune disease.

Chapter 7

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