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Induction of anti-tumour immune responses by overcoming tumour immune subversion

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M.Sc.

A thesis submitted to
Trinity College Dublin
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2016

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Abstract

Immunotherapy has considerable potential to improve disease outcome and survival of patients with cancer. Tumour eradication by the immune system is largely dependent on pro-inflammatory signals and effector cell infiltration at the tumour site. However, tumours can overcome effector immune responses, for example by the secretion of immunosuppressive molecules (TGF-β, retinoic acid (RA)), the recruitment of immunosuppressive cells (regulatory T (Treg) cells, myeloid-derived suppressor cells (MDSCs)) and the expression of immune checkpoint ligands (PD-L1, PD-L2). This project aimed to investigate approaches for activating anti-tumour immune responses, mechanisms of tumour-mediated immunosuppression and strategies to overcome immunosuppression.

BAMLET, a bovine α-lactalbumin - oleic acid complex, is a novel and promising candidate for the treatment of cancer because of its capacity to kill cancer cells. While recent studies focused on direct cytotoxic effects of BAMLET, the potential of BAMLET to modulate immune responses against tumour cells was investigated in the present study. Surprisingly, BAMLET had immunomodulatory effects and activated dendritic cells (DCs), in addition to killing tumour cells. Treatment of tumour-bearing mice with BAMLET and the TLR7/8 agonist R848 conferred a high level of protection, which was associated with an increased frequency of IFN-γ-secreting T cells in lymphoid organs. These findings suggest that BAMLET contributes to tumour eradication by directly targeting tumour cells and activating anti-tumour immune responses.

TGF-β has been shown to induce the conversion of naïve CD4 T cells into Treg cells which is enhanced by RA, a Vitamin A metabolite. The present study investigated the capacity of a RA receptor-alpha antagonist (RARi) to block the induction of Treg cells and to enhance anti-tumour immunity. RARi inhibited RA and TGF-β-induced conversion of naïve $CD4^+$ T cells into Treg cells. Moreover, RARi promoted a pro-inflammatory phenotype of TLR-stimulated DCs *in vitro*. Blocking RARα alone had no effect on tumour growth *in vivo*. However, RARi enhanced the therapeutic efficacy of a TLR-activated antigen-pulsed DC vaccine, significantly attenuating tumour growth and enhancing survival.

Lastly, the role of the inhibitory receptor programmed cell death protein 1 (PD-1) was investigated in the context of immunosuppression in the tumour microenvironment. The results revealed an inverse correlation between Th1-type cytokine production and the

expression of FoxP3 and inhibitory receptors during the development of CT26 tumours in mice. In tumour draining lymph nodes, T cells expressed low levels of Foxp3 and the inhibitory receptors PD-1 and LAG-3 and secreted high concentrations of TNF and IFN-γ. In contrast, tumour-infiltrating T cells expressed high Foxp3, PD-1 and LAG-3 and secreted low concentrations of TNF and IFN-γ, suggesting that inhibitory receptors negatively regulated T cell effector function. Furthermore, blocking PD-1 *in vitro* reversed the suppressive effects of TGF-β on T cells, resulting in enhanced T cell proliferation, IFN-γ secretion and cytotoxic activity. Moreover, blocking PD-1 reduced TGF-β-induced Treg cell conversion.

Using the CT26 tumour model, therapeutic administration of an anti-PD-1 antibody alone had little effect on tumour growth. In contrast, treatment of mice with anti-PD-1 in combination with a cancer vaccine comprising heat-shocked and irradiated tumour cells and the TLR7/8 agonist R848 led to the eradication of established tumours and significantly prolonged survival. The rejection of tumours in mice treated with the anti-PD-1 vaccine combination was associated with a reduction of tumour-infiltrating Treg cells and enhancement of IFN-γ-secreting cytotoxic T cells. These findings demonstrate that the PD-1/PD-L1 axis is a major control mechanism that dampens anti-tumour T cell responses and blocking this axis can significantly enhance effector T cell responses and the efficacy of a therapeutic vaccine in a murine cancer model.

Publications

Dyck L, Wilk MM, Raverdeau M, Misiak A, Boon L, Mills KH. Anti-PD-1 inhibits Foxp3+ Treg cell conversion and unleashes intratumoural effector T cells thereby enhancing the efficacy of a cancer vaccine in a mouse model. Cancer Immunology Immunotherapy (2016) 65: 1491.

Galvin KC, **Dyck L**, Marshall NA, Stefanska AM, Walsh KP, Moran B, Higgins SC, Dungan LS, Mills KH. Blocking retinoic acid receptor-α enhances the efficacy of a dendritic cell vaccine against tumours by suppressing the induction of regulatory T cells. Cancer Immunology Immunotherapy (2013) 62: 1273.

List of abbreviations

ALDH	alcohol dehydrogenase
ALL	acute lymphoid leukemia
ANOVA	analysis of variance
APC	antigen-presenting cells
ATP	adenosine triphosphate
BAMLET	bovine alpha-lactalbumin made lethal to tumour cells)
BCG	*Bacillus Calmette-Guerin*
BFA	brefeldin A
BMDC	bone marrow derived dendritic cell
BMDM	bone marrow derived macrophage
BSA	bovine serum albumin
CAR	chimeric antigen receptor
CD	cluster of differentiation
CIS	cytokine-inducible SH2-containing protein
CLL	chronic lymphocytic leukemia
CML	chronic myelogenous leukemia
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
CTV	CellTrace Violet
D	diameter
DAMP	danger associated molecular pattern
d	day
DC	dendritic cell
dLN	draining lymph node
DMEM	Dulbecco's Modiefied Eagles Medium
E:T	effector:target
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
FDA	US food and drug administration
Foxp3	forkhead box P3
GALT	gut associated lymphoid tissues

h	hour
HBSS	Hank's balanced salt solution
HMGB1	high mobility group box 1 protein
hs/irr	heat shocked/ irradiated
HSP	heat shock protein
i.p.	intraperitoneal
i.v.	intravenous
IDO	indoleamine 2,3-dioxygenase
IFN-γ	interferon gamma
ImC	immature myeloid cells
irAE	immune related adverse effect
IRAK	interleukin-1 receptor-associated kinase
JNK	c-Jun N-terminal kinase
L/D aqua	live/dead stain aqua
LAL	limulus amebocyte Lysate
LAP	latency associated peptide
LCMV	lymphocytic choriomeningitis virus
LPS	lipopolysaccharide
LTBP	latent TGF-β-binding protein
mAb	monoclonal antibody
MACS	magnetic-activated cell sorting
MAPK	mitogen-activated protein kinase
mDC	myeloid DC
MDSC	myeloid derived suppressor cell
MHC	major histocompatibility complex
min	minute
M-MDSC	monocytic MDSCs
MMP	matrix metalloproteinase
MyD88	myeloid differentiation primary response gene 88
NFkB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK cells	natural killer cells
NKR	NK receptor
NSCLC	non-small cell lung cancer
O/N	overnight
OA	oleic acid
OD	optical density
OPD	o-phenylenediamine dihydrochloride

OVA	ovalbumin
PAMP	pathogen associated molecular pattern
PAP	prostatic acid phosphatase
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PD-1	programmed cell death protein 1
pDC	plasmacytoid DC
PD-L	programmed death-ligand 1
PFA	protein fatty acid
Pi3K	phosphoinositide 3-kinase
PMA	phorbol 12-myristate 13-acetate
PMN-MDSC	polymorphonuclear MDSCs
pTreg	peripherally-derived Treg
RA	retinoic acid
RALDH	retinol dehydrogenases
RAR	retinoic acid receptor
RARE	RA response elements
RARi	RARα antagonist (Ro-41-5253)
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT	room temperature
RXR	retinoid X receptor
SBE	SMAD-binding element
SLP	synthetic long peptide
SMI	small molecule inhibitor
TAA	tumour associated antigen
TAM	tumour-associated macrophage
TCR	T cell receptor
Tfh	T follicular helper
TGFBR	TGF-β receptor
TGF-β	transforming growth factor beta
Th	T helper
TIL	tumour infiltrating lymphocytes
TLR	Toll-like receptor
TNF	tumour necrosis factor
TRAF	TNF receptor associated factor
Treg	regulatory T cell

TRIF	TIR-domain-containing adapter-inducing interferon-β 1
tTreg	thymic Treg
v/v	volume per volume
VEGF	vascular endothelial growth factor
Vit-A	Vitamin A
w/v	weight per volume
α-LA	alpha-lactalbumin

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Chapter 1:

Introduction

1. Introduction

1.1. Cancer

Cancer is one of the leading causes of death worldwide. According to the World Health Organization, the number of cancer cases is expected to rise dramatically in the next 20 years [1]. Cancer is a group of diseases that is characterised by uncontrolled growth of cells that derive from the body. Different types of cancer are categorised according to the cell type and tissue of origin. In normal cells, growth is tightly controlled and cells have a limited capacity to proliferate. In order for a cell to transform into a tumour cell, many transformation steps have to occur.

Weinberg and Hannahan proposed the hallmarks of cancer which describe properties of transformed cells that lead to the development of cancer [2]. In their first landmark review in 2000, they proposed six properties shared by all tumour cells: 1. sustaining proliferative signalling, 2. evading growth suppressors, 3. activating invasion and metastasis, 4. enabling replicative immortality, 5. inducing angiogenesis and 6. resisting cell death [2]. These hallmarks represent the main prerequisites for tumours to become malignant. Since then, research on cancer has progressed and it became evident that not only tumour cells but also the tumour microenvironment plays an important role in the progression of cancer. Hallmarks that acknowledge a major role of the immune system in cancer have since been added to the model, which were summarised as "tumour-promoting inflammation" and "avoiding immune destruction" [3].

1.1.1. Chronology of cancer therapy

Cancer therapy has made remarkable developments in the last 100 years (Figure 1.1). Until the beginning of the 20th century, surgery was the only treatment for cancer. This changed with the discovery that cancer cells can by killed by radiation. Radiotherapy is still commonly used, especially for tumours that are not dissectible by surgery, such as brain tumours or tumours of the head and neck [4].

In 1949, nitrogen mustard was approved by the US food and drug administration (FDA) as the first chemotherapeutic drug, and this marked the starting point of the era of chemotherapy. Chemotherapeutics are a class of drugs that are cytotoxic towards quickly dividing cells, such as tumour cells but also affect immune cells and cells in mucosal tissues. Chemotherapeutic drugs interfere with different steps of the cell cycle

which causes cell death. These drugs can have severe side effects due to the lack of specificity. Since then, novel chemotherapeutic drugs were developed that specifically target tumour cells [5].

Traditionally, cancer therapy focused on directly killing tumour cells and was not taking into account the diversity of different types of cancer. In the 1990s, cancer therapy progressed into the era of targeted therapies. These therapies are based on the identification of specific mutations or molecular characteristics on patients' tumours. Targeted therapies include small molecule inhibitors (SMIs) and monoclonal antibodies (mAbs). Examples for targeted therapies are Gefitinib (epidermal growth factor receptor (EGFR) inhibitor) for the treatment of EGFR-overactive non-small cell lung cancer (NSCLC), Imatinib (tyrosine kinase inhibitor) for the treatment of Philadelphia chromosome-positive chronic myelogenous leukemia (CML), or Trastuzumab (mAb against Her2/neu) for the treatment of Her2-positive breast cancer [6].

Immunotherapy for cancer has been investigated since the 1970s, but rarely yielded results that could compete with chemotherapy or targeted therapies. However, the last 5 years have seen a paradigm shift in cancer therapy with the approval of pioneering immunotherapies. To date, four immune checkpoint inhibitors have been approved by the FDA which target the immune checkpoints cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed cell death protein 1 (PD-1) and PD-L1. Immune checkpoint inhibitors are approved for the treatment of melanoma, renal cancer, bladder cancer, and NSCLC and are expected to become the first line treatment for other cancers in the future [7]. It was recognised that immunotherapy can induce more potent and long-lasting anti-tumour immune responses while inducing less side effects [7]. This chapter will give an introduction into the interplay between tumours and the immune system and will review state-of-the-art immunotherapies at pre-clinical and clinical stages of development.

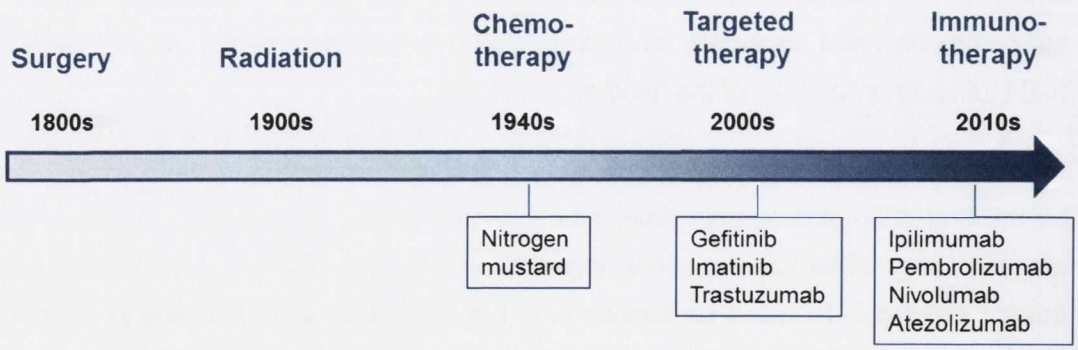


Figure 1.1: Chronology of cancer therapy

1.2. Immune responses against tumour cells

1.2.1. The concept of cancer immunosurveillance

The concept of cancer immunosurveillance was introduced in 1957 by Burnet and Thomas who provided the first description of the recognition of transformed cells by the immune system. It was proposed that transformed cells constantly accumulate in the body and get effectively cleared by the immune system without being clinically detectable. Lymphocytes are able to recognise cancerous cells due to the expression of altered antigens. This hypothesis has later been expanded, stating that the immune system can not only eradicate tumour cells but also contribute to tumour growth. The model of the three Es of cancer immunoediting describes three key processes of tumour progression: elimination, equilibrium and escape. The first phase is the elimination of tumour cells which is similar to the originally proposed concept of immunosurveillance. Infiltrating immune cells are able to recognise transformed cells and effectively induce cell death, for example by the secretion of interferon gamma (IFN-γ) or granzymes. Tumour cells that survive elimination by the immune system enter the equilibrium phase. During the equilibrium, resistant cells that overcome elimination by immune cells keep proliferating which increases the stability of the tumour against immune attack. Tumours can persist for years in this state of balance between slow proliferation of resistant cells and immune-induced cell death. However, this process ultimately generates a pool of immune insensitive tumour cells. The third phase, the escape of the tumour, marks the starting point of tumour cell proliferation that can no longer be controlled by the immune system. Many factors contribute to the escape of a tumour, including acquired genetic mutations that make the tumour cells resistant to cell death. Moreover, the heterogeneity of tumour cells through mutations and modifications of the tumour microenvironment, including infiltrating immune cells, can favour tumour growth [8].

1.2.2. Antigen-nonspecific anti-tumour immune responses

Innate immune cells represent the first line of defence against transformed cells. Inflammatory signals from tumour-surrounding tissues cause the infiltration of innate cells, including natural killer (NK) cells, γδ T cells and M1-like macrophages.

The infiltration of NK cells into tumours has been associated with better prognosis in cancer patients [9-11]. NK cells interact with other cells through activating and

inhibitory receptors. If activating signals dominate, for example through pro-inflammatory cytokines, lack of major histocompatibility complex (MHC) I expression or presentation of transformed-self antigens by MHC I, NK cells induce cell killing of the target cell. Cytotoxic mechanisms elicited by NK cells include the secretion of perforin/granzymes and death receptor interactions. Typically, NK cell cytotoxicity leads to controlled cell death by apoptosis [12, 13]. In addition, secretion of IFN-γ by NK cells has an anti-proliferative and cytotoxic effect on tumour cells and enhances MHC I expression [14]. IFN-γ also activates M1-like macrophages which contribute to tumour eradication by secretion of IL-12 which activates T cells and drives Th1 differentiation [15]. Moreover, IL-12 enhances NK cell function and contributes to NK-mediated tumour clearance [16].

Infiltration of γδ T cells has been observed in murine and human tumours. Tumour-promoting and anti-tumour properties have been reported for γδ T cells. γδ T cells are categorised based on their γ-chain expression and further divided into IFN-γ or IL-17-producing subsets. It is believed that IFN-γ-producing γδ T cells promote anti-tumour responses [17], while IL-17-producing γδ T cells enhance tumour growth through the promotion of myeloid derived suppressor cells (MDSCs) and Th17 cells [18, 19]. γδ T cells recognise tumour cells by their T cell receptor (TCR) or NK receptors (NKRs), including NKG2D, which senses stress molecules secreted by tumour cells. However, no γδ TCR-specific tumour antigens have yet been identified [20].

1.2.3. Antigen-specific anti-tumour immune responses

The mechanism of antigen-specific anti-tumour responses has been described as cancer-immunity cycle (Figure 1.2). Early innate responses, hypoxia, nutrient deprivation or cytotoxic drugs can induce tumour cell death which leads to the release of tumour antigens into the environment. These antigens are taken up by dendritic cells (DCs) which screen the periphery for danger. Upon antigen uptake, activated antigen-presenting DCs express CCR7 and migrate along a chemokine gradient to draining lymph nodes (dLN) where they encounter naïve T cells. T cells that recognise tumour antigens presented by MHC complexes on DCs get activated and undergo clonal expansion. Activated T cells migrate through the blood circulation along a chemokine gradient into the tumour bed. Chemokines that attract T cells, such as CXCL10 and CCL22, are secreted by tumour cells and surrounding tissues in response to IFN-γ and other inflammatory mediators. Upon recognition of specific antigens presented by MHC I by tumour cells, activated T cells release cytotoxic granules or pro-inflammatory

cytokines which kill tumour cells. Dying tumour cells release additional tumour antigens which increases the pool of T cells with different antigen specificities in subsequent cycles [21]. Importantly, defects in any of these steps can contribute to progressive tumour growth and weak responsiveness towards immunotherapy.

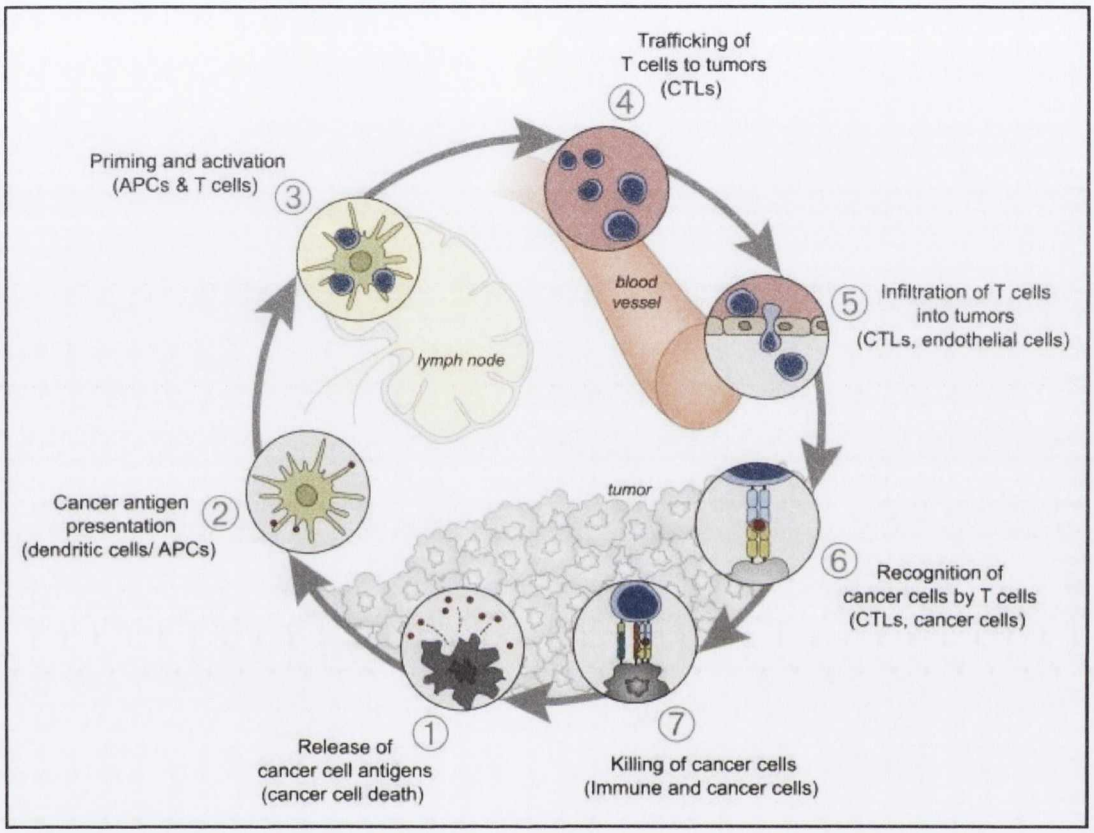


Figure 1.2: The cancer-immunity cycle
Dying tumour cells release antigens into the periphery (1). Antigen-presenting cells (APCs), such as DCs, take up cancer antigens and present them on their cell surface by MHC molecules (2). After migrating into dLN, APCs prime and activate tumour antigen-specific T cells (3). Activated T cells traffic through the bloodstream along a chemokine gradient (4) and infiltrate into the tumour (5). Upon recognition of cognate antigens through their TCR (6), T cells kill tumour cells (7). From Chen & Mellman, 2013 [21]

1.2.4. Dendritic cells

DCs are a class of APCs that were first characterised by Steinmann et al. in 1973 [22]. DCs are crucial for the initiation of antigen-specific anti-tumour responses. Common DC precursors from the bone marrow give rise to plasmacytoid DCs (pDCs) and myeloid DCs (mDCs). mDCs circulate through the bloodstream and migrate into lymphoid and non-lymphoid tissues as immature cells. In contrast, pDCs only migrate

into non-lymphoid tissues upon inflammation [23]. mDCs play an important role in immune responses against bacteria and fungi and potently cross-present foreign antigens to T cells. Moreover, mDCs contribute to anti-tumour responses by the secretion of IL-12 which induces T helper 1 (Th1) responses. pDCs, on the other hand, are important for anti-viral immune response and produce high levels of type 1 IFNs. Moreover, pDCs have been shown to have direct tumouricidal effects [24]. While both DC subsets have been implicated in anti-tumour immune responses, the remainder of this thesis will focus on mDCs [25].

The primary role of DCs is antigen presentation. The migration properties of DCs enable T cells to come in contact with otherwise unreachable peripheral antigens. DCs take up antigens from the periphery and present them to T cells after migrating to dLN. In addition, subsets of DCs that reside in LN can phagocytise antigens that enter the LN [26, 27]. An important feature of DCs is the ability to cross-present antigens through MHC I, which enables them to directly activate CD8 T cells [28]. Moreover, evidence is emerging that strongly activated DCs can have direct cytotoxic properties [24, 29]. Stary et al. reported the release of perforin and granzymes and direct anti-tumour cytotoxicity by mDCs and pDCs upon stimulation with the TLR7/8 agonist Imiquimod [29].

The maturation status of DCs has implications for DC responses in tumour immunity. Immature DCs are characterised by high intracellular expression of MHC II, high phagocytic potential, low expression of co-stimulatory molecules (e.g. CD40, CD80 and CD86) and low antigen presentation capacity. The stimulation of DCs with antigens in absence of further stimulation induces a tolerogenic DC phenotype [27, 30]. Presentation of antigens by tolerogenic DCs leads to T cell anergy or induction of regulatory T (Treg) cells in order to maintain tolerance under homeostatic conditions [31, 32]. However, tumour-infiltrating DCs were associated with a tolerogenic phenotype in several tumour mouse models and in cancer patients [33]. In mouse models of ovarian cancer, tumour infiltrating DCs were shown to suppress T cell responses [34] and to promote tumour growth [35]. Moreover, tolerogenic DCs have been identified in patients with colorectal, lung and breast cancer [33]. Early trials using a DC vaccine consisting of immature DCs induced weaker anti-tumour responses than mature DCs [36, 37]. Moreover, Dhodapkar et al. reported an induction of regulatory responses in humans in response to injection of immature DCs [38].

Mature DCs express high levels of MHC II and co-stimulatory molecules on the cell membrane and have little phagocytic potential. The ability of DCs to prime naïve T cells

constitutes a major feature of their functional maturation [31]. Mature DCs activate T cells by antigen presentation through MHC molecules and concurrent co-stimulation through interaction of CD80/86 with CD28. Moreover, DCs shape the type of T cell response by the secretion of polarising cytokines. The presence and phenotype of DCs plays a pivotal role in tumour immunity and cancer therapy. It has been reported that the presence of tumour-infiltrating mature DCs is associated with better prognosis and better efficacy of DC based therapies [25, 33].

1.2.5. DC maturation

The activation of DCs requires activation signals from danger associated molecular patterns (DAMPs), pathogen associated molecular patterns (PAMPS) which include toll-like receptor (TLR) agonists, pro-inflammatory cytokines or stimulatory ligands, such as CD40L. TLRs are evolutionary highly conserved proteins and innate sensors of PAMPs and DAMPs. There are 13 murine TLRs identified to date, 10 of which are also present in humans. TLRs recognise a plethora of pathogenic structures, including lipoproteins (TLR2), dsRNA (TLR3), lipopolysaccharide (LPS; TLR4), flagellin (TLR5), ssRNA (TLR7/8) or bacterial CpG DNA (TLR9) (Figure 1.3) [39].

TLR ligand binding initiates intracellular signalling cascades (Figure 1.3). All TLR agonists, apart from TLR3, induce the recruitment of the adapter protein myeloid differentiation primary response gene 88 (MyD88), resulting in the activation of the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and subsequent secretion of pro-inflammatory cytokines. TLR3 receptor-ligand interaction recruits the adapter molecule TIR-domain-containing adapter-inducing interferon-β 1 (TRIF1) and induces type 1 IFN secretion. Notably, TLR4 can also signal through TRIF1 by moving from the plasma membrane to endosomal compartments [40]. Important molecules in the TLR signalling pathways include interleukin-1 receptor-associated kinases (IRAKS), tumour necrosis factor (TNF) receptor associated factors (TRAFs), c-Jun N-terminal kinase (JNK), mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (Pi3K). These signalling molecules induce phenotypical changes and secretion of cytokines by DCs. Notably, different TLR agonists were shown to induce different profiles of secreted cytokines which has an impact on the type of immune response that is initiated [41].

In the tumour microenvironment, tumour cell death results in the release endogenous adjuvants, such as cyclic dinucleotide, adenosine triphosphate (ATP), heat shock proteins (HSPs) or high mobility group box 1 protein (HMGB1) that promote DC

maturation [21]. Moreover, pro-inflammatory cytokines such as IL-1, IFN-α and TNF have been shown to promote DC maturation [21, 25].

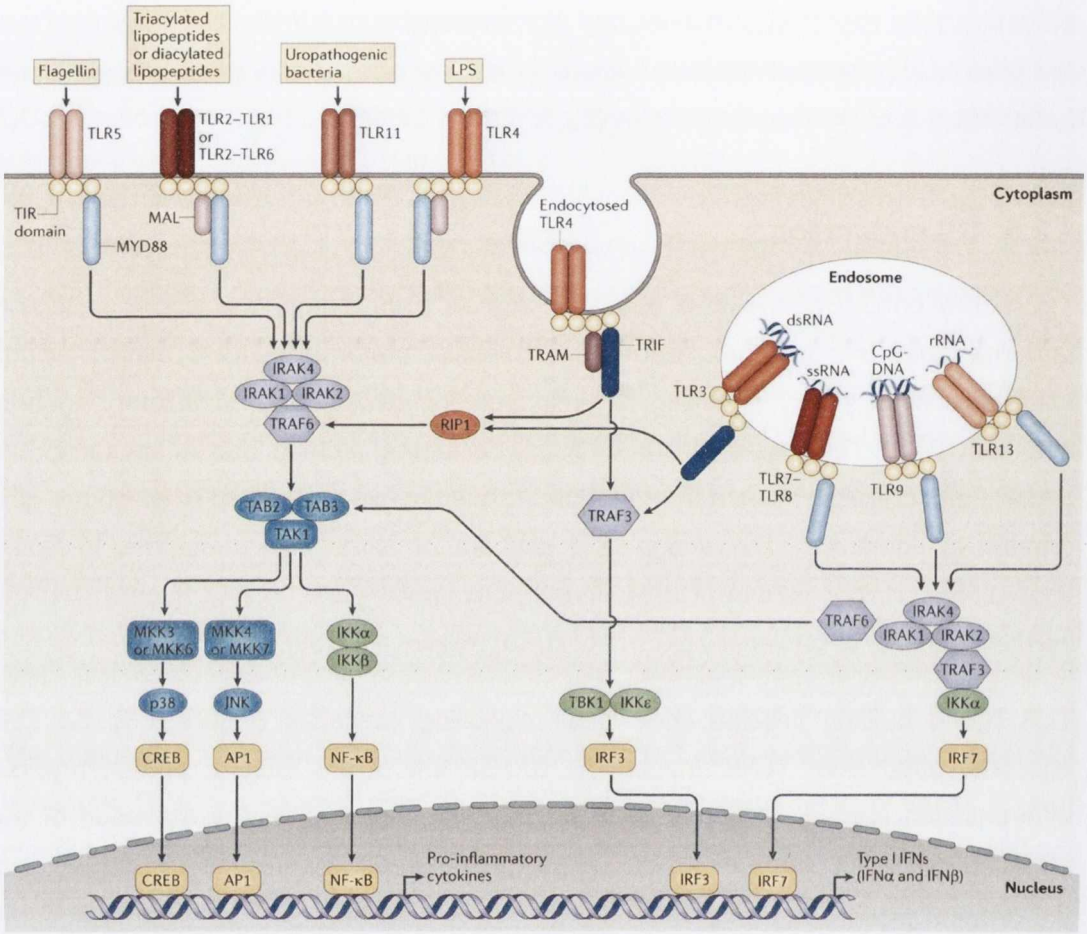


Figure 1.3: TLR-induced intracellular signalling cascades
TLRs are expressed on the cell surface (TLR1/2/4/5/6/10/11) or in endosomal compartments (TLR3/7/8/9/13). TLR4 is expressed on the plasma membrane but can also be endocytosed. After recognition of TLR agonists, the adapter proteins MyD88 or TRIF induce signalling cascades leading to the activation of transcription factors, such as CREB, AP1, NFκB, IRF3 and IRF7. From O'Neill, Golenbock & Bowie, 2013 [40]

1.2.6. CD8 T cells

T cells derive from lymphoid progenitors in the thymus from where they enter the blood and lymph circulation and reside in lymphoid organs. T cells expand and differentiate upon antigen encounter. Classically, APCs that have engulfed pathogens and processed antigens can induce T cell differentiation and clonal expansion by 3 signals. Signal 1 is the presentation of antigens through peptide-MHC complexes which are

recognised by the TCR. Each T cell has a TCR with a single unique peptide specificity which increases the likelihood of APCs interacting with a complementary T cell. The second signal is provided by co-stimulation through co-receptor interaction, for example the interaction of CD28 on T cells with CD80 and CD86 on DCs. The third signal is delivered by cytokines secreted by APCs, particularly DCs, which are critical signals for the outcome of T cell differentiation. Once a naïve T cell receives all 3 signals, it becomes an effector T cell [42, 43].

CD8 T cells, also called cytotoxic T lymphocytes (CTL), are one of the most important immune cell types in immunity to cancer. Upon recognition of their cognate antigen presented by MHC I, CD8 T cells can kill tumour cells by multiple mechanisms. The most important mechanism is the release of perforin and granzymes, which disrupt the plasma membrane of tumour cells leading to apoptosis. FASL expression on T cells has been shown to induce apoptosis on target cells by binding to FAS which induces a caspase-8 signalling cascade. In addition to direct cytotoxicity, activated CD8 T cells release IFN-γ, which contributes to tumour cell death by upregulating the expression of MHC I and by direct inhibition of tumour cell proliferation [44].

The significance of CD8 T cells in anti-tumour immunity has been demonstrated in numerous pre-clinical and clinical studies. It has been reported that the removal of CD8 T cells exacerbates tumour growth in murine tumour models [45-47]. The presence of CD8 T cells in tumours correlated with a better prognosis in cancer patients, including melanoma, colon cancer, breast cancer, renal cell carcinoma and ovarian cancer [48-53]. Moreover, patients with pre-existing CD8 T cell infiltration responded better to cancer immunotherapy [54, 55].

1.2.7. CD4 T cells

CD4 T cells are implicated in cancer defence in multiple ways. CD4 T cells are divided into different Th subtypes, which have different functions and roles in anti-tumour immunity. The cytokine milieu, which is mostly determined by DCs and other innate cells in the LN, influences the polarisation of naïve T cells to distinct subtypes (Figure 1.4). The presence of IL-12 promotes the differentiation of Th1 cells [56]. IL-4 has been shown to induce Th2 responses which are important for antifungal immunity and were shown to suppress Th1 cells [57]. Transforming growth factor beta (TGF-β) in combination with IL-6 induces the differentiation of Th17 cells, which are further activated and maintained by IL-1β and IL-23 [58, 59]. TGF-β and RA drive the differentiation of forkhead box P3 (Foxp3)-positive Treg cells which are able to

suppress effector T cell responses [60, 61]. More recently, naïve CD4 T cells were shown to differentiate into T follicular helper (Tfh) cells in presence of IL-6 and IL-21. Tfh cells activate B cells and promote antibody responses to infection. However, their role in the context of tumour immunity remains to be defined [62].

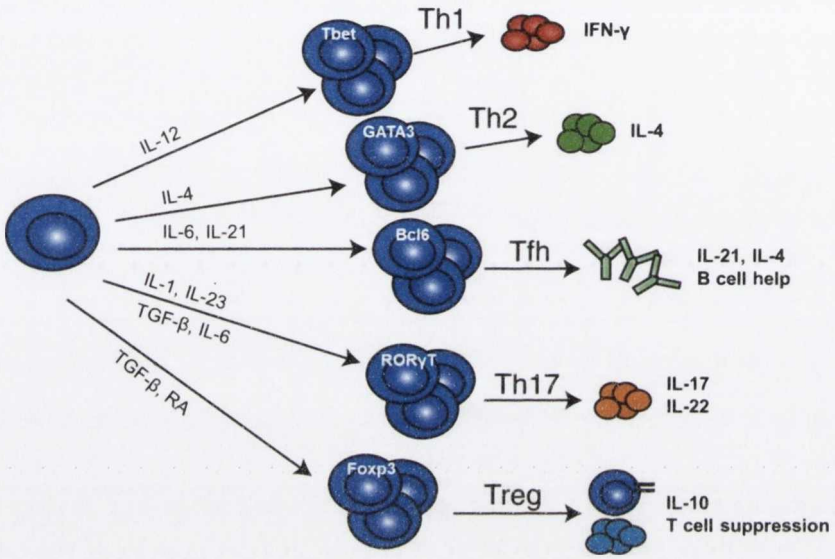


Figure 1.4: CD4 T cell differentiation

Th1 cells express the transcription factor T-bet and are induced in presence of IL-12. Th1 secrete high levels of pro-inflammatory IFN-γ and play a role in infection and cancer. Th2 cells are induced by IL-4 and express the transcription factor GATA3. Th2 cells secrete IL-4 and are important for the clearance of parasites. Tfh cells are induced by IL-6 and IL-21 and express the transcription factor Bcl6. Tfh cells secrete IL-21 and IL-4 and provide B cell help. Th17 cells can be induced by TGF-β and IL-6 and are activated by IL-1 and IL-23. Th17 cells express RORγT and produce IL-17 and IL-22. Th17 cells are important for the clearance of infections and are implicated in the induction of autoimmunity. Foxp3-expressing Treg cells differentiate in presence of TGF-β and RA. Treg cells suppress effector T cell responses by a range of mechanisms, including secretion of IL-10. Adapted from McKee et al., 2013 [63].

The most protective Th subtype in cancer are Th1 cells, which are characterised by the nuclear expression of the transcription factor T-bet. The most important role of Th1 cells is promoting the activation and proliferation of tumour-specific CD8 T cells by secretion of chemokines and cytokines and by interaction with MHC II-positive APCs. Moreover, Th1 cells orchestrate immune responses by secretion of high levels of IFN-γ and TNF which activate NK cells and macrophages. Furthermore, Th1 cells promote APC and NK cell recruitment to the tumour site [64, 65].

There is some evidence that MHC II-positive tumour cells can act as APCs and directly activate CD4 T cells [66-69]. A study by Quezada et al. reported that CD4 T cells are able to clear tumours in absence of CD8 T cells by developing cytotoxic activity [67]. Transfer of naïve tumour antigen-specific CD4 T cells into lymphopenic mice led to significant tumour rejection. CD4 T cell-mediated killing of tumour cells was dependent on MHC II-restricted antigen recognition by tumour-specific CD4 T cells which led to the secretion of granzyme granules [67]. This hypothesis is supported by a recent report showing that MHC II expression on melanoma cells in patients is associated with better response to immunotherapy [68]. These findings emphasise a diverse role of Th1 cells in the immune response against tumours.

1.3. Immune evasion elicited by the tumour microenvironment

Tumours utilise many mechanisms to escape immune responses (Figure 1.5). Two major obstacles to anti-tumour immunity are the lack of antigen presentation by tumour cells and the promotion of immune suppression.

The downregulation of MHC I is a typical feature of tumour cells. By downregulating MHC I, tumour cells reduce the presentation of endogenous antigens that can potentially be recognised by immune cells [70]. Tumour cells also downregulate pro-apoptotic proteins and up-regulate anti-apoptotic proteins, which increases their resistance to the induction of cell death [71]. In addition, the tumour microenvironment actively suppresses anti-tumour immune responses by secretion of anti-inflammatory molecules that suppress effector T cell function, such as TGF-β and IL-10. Tumour cells can express immunosuppressive enzymes, such as indoleamine 2,3-dioxygenase (IDO) [72]. Tumour cells recruit suppressive immune cells that promote tumour growth, including tumour-associated macrophages (TAM), MDSCs and Treg cells. Moreover, tumour cells can directly inhibit T cells, for example by expressing ligands for inhibitory receptors (Figure 1.5)[73].

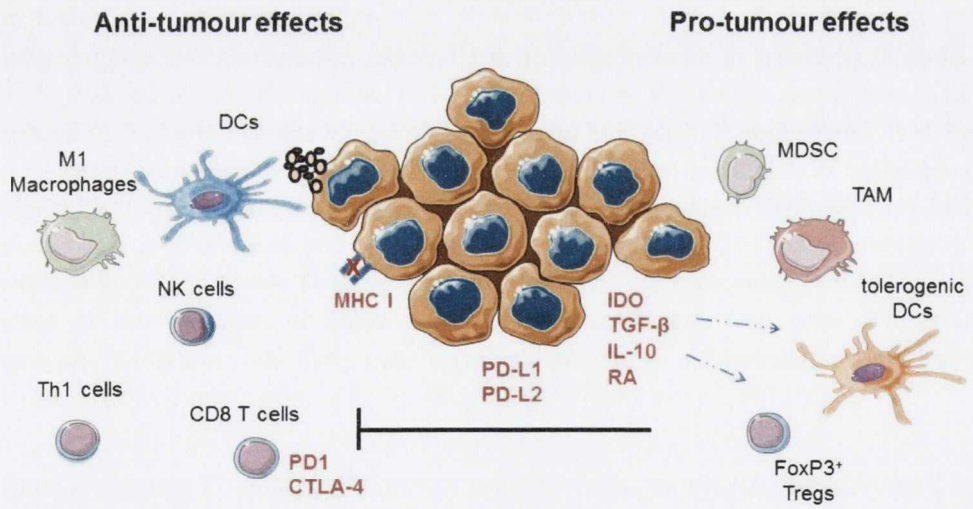


Figure 1.5: The immunosuppressive tumour microenvironment
Anti-tumour immune responses can be elicited by pro-inflammatory cells, such as DCs, M1 macrophages, NK cells, Th1 cells and CD8 T cells. Pro-tumour effects are mostly elicited by immunosuppressive cells, such as MDSCs, TAMs, tolerogenic DCs and Foxp3$^+$ Treg cells. Moreover, immunosuppressive molecules (IDO, TGF-β IL-10, RA), immune checkpoints (PD-L1/2, PD-1, CTLA-4) and downregulation of MHC I contribute to tumour-mediated immune suppression.

1.3.1. TGF-β

TGF-β is secreted by many tumour cell lines and has been identified in human tumours. TGF-β exists in three isoforms (TGF-β1, TGF-β2, TGF-β3) which differ in their affinity for the TGF-β receptor (TGFBR). TGF-β is produced as an inactive homo-dimeric pro-protein called latency associated peptide (LAP). Binding to latent TGF-β-binding protein (LTBP) induces the secretion of LAP. The LAP-LTBP complex needs to be processed extracellularly in order to release active TGF-β. Once released, active TGF-β binds to TGFBR2 which leads to hetero-dimerisation with TGFBR1 and induces canonical TGF-β signalling. In the canonical signalling pathway, TGF-β signals through TGFBR and recruits SMAD2 or SMAD3 which dimerise with SMAD4 and translocate into the nucleus, initiating the transcription of target genes. The non-canonical TGF-β signalling pathway only employs TGFBR2 and signals through various pathways, such as MAPK, RHO/ROCK, AKT, mTOR or p38 (Figure 1.6) [74].

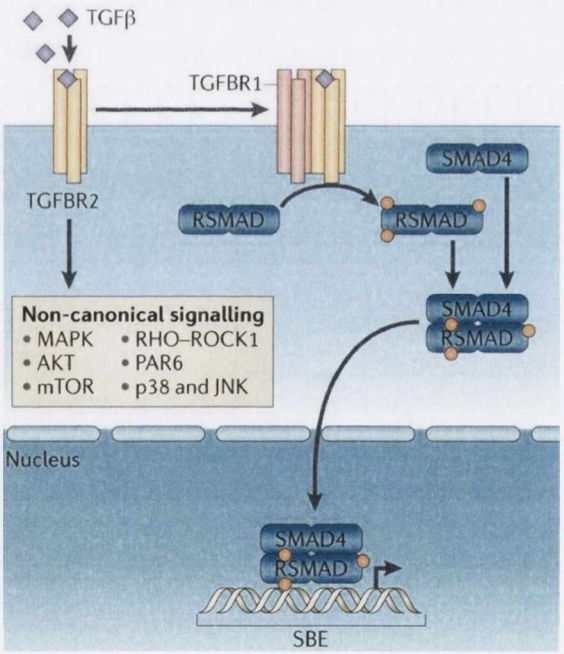


Figure 1.6: TGF-β signalling pathway
TGF-β signals through the TGFBR2 which dimerises with TGFBR1. Canonical signalling activates SMAD proteins which translocate into the nucleus. SMADs induce the transcription of TGF-β target genes by binding to SMAD-binding element (SBE). Non-canonical signalling is independent of SMAD and involves distinct signalling pathways, including MAPK, AKT and mTOR pathways. Adapted from Pickup, Novitskiy & Moses, 2013 [74]

The TGFBR is ubiquitously expressed and the presence of TGF-β affects tumour cells as well as immune cells in the tumour microenvironment. Direct effects on tumour cells are dichotomous. Early TGF-β signalling suppresses tumour cell proliferation by non-canonical TGF-β signalling. However, many tumours become resistant to TGF-β induced growth arrest. Instead, TGF-β contributes to tumour growth at later stages [74]. TGF-β promotes epithelial mesenchymal transformation and cellular migration of tumour cells which enables metastatic spread [75]. Moreover, TGF-β has pro-angiogenic effects, which are dependent on vascular endothelial growth factor (VEGF) [76].

In addition to direct effects on tumour cells, TGF-β mediates immunosuppression in the tumour microenvironment. TGF-β suppresses immune cells by SMAD-mediated inhibition of T cell proliferation and cytokine secretion [77]. Moreover, TGF-β has been shown to promote an M2 phenotype in macrophages [78]. Knockout of TGFR2 in mice induced a more pro-inflammatory DC phenotype, suggesting that TGF-β suppresses DC function [79]. TGF-β is also a major regulator of CD4 T cell differentiation. TGF-β promotes the conversion of naïve CD4 T cells into suppressive Treg cells by SMAD-binding to the Foxp3 promoter. In the presence of IL-6, however, the expression of Foxp3 is suppressed and CD4 T cells differentiate into RORγT-expressing Th17 cells [65].

In vivo studies further confirmed tumour-promoting activities of TGF-β. Knockdown of TGF-β by siRNA protected from tumour growth in the B16 model [80]. A study by Zhong et al. demonstrated that anti-TGF-β therapy with a TGFBRII antibody protected against tumour growth by interfering with tumour metastatic potential and by relieving TGF-β mediated immunosuppression [81]. A range of molecules targeting TGF-β have also been tested in clinical trials, but consistently led to mixed results due to pleiotropic effects of TGF-β [74].

1.3.2. IL-10

IL-10 is another cytokine which is thought to promote immunosuppression in cancer. Various tumours have been shown to secrete IL-10 and it has been shown that tumour cells recruit IL-10-producing cells and promote IL-10 production by tumour infiltrating cells [82].

The IL-10 receptor is expressed by T and B cells, macrophages and DCs. Immune suppressive effects have been well documented in the context of autoimmunity and

infection. *In vitro* studies have shown that IL-10 impairs APC function by downregulating MHC II and co-stimulatory molecules [83]. Moreover, IL-10 has been shown to inhibit the transcription of the p40 subunit of the pro-inflammatory cytokines IL-12 and IL-23 [84]. The inhibition of CD4 T cell activation and pro-inflammatory cytokine secretion by IL-10 is due to its suppressive effects on APCs [83]. Treg cells are a major source of IL-10 in the tumour microenvironment and IL-10 has been shown to maintain Treg homeostasis [85]. Moreover, a reduction of IL-10 secretion by DCs transferred to tumour-bearing mice improved the efficacy of DC vaccines [86-88].

Despite its suppressive effects on CD4 T cells and APC, evidence is emerging that IL-10 can promote anti-tumour immunity by CD8 T cell activation. In a model of chemical-induced skin carcinoma, IL-10 KO mice showed enhanced tumour growth and metastasis compared with wildtype controls [89]. Emmerich et al. reported that IL-10 in the tumour activates tumour-resident CD8 T cells and promotes cytotoxic T cell function [90]. This was independent from de novo infiltration of CD8 T cells from lymphoid organs [90]. These findings suggest distinct effects of IL-10 based on target cell and location.

1.3.3. Tumour-mediated T cell dysfunction

One very effective mechanism employed by tumours to evade tumour-directed immune responses is the suppression of T cell effector function. Indeed, the phenotype of T cells in the tumour microenvironment is often dysfunctional and shows similarities with exhausted T cells, which were first described in chronic infections. Exhausted T cells are characterised by a loss of IL-2 production, reduced proliferative capacity, reduced cytotoxic capacity and impairments in the production of pro-inflammatory cytokines [91]. Moreover, the expression of multiple inhibitory receptors has been associated with an exhausted T cell phenotype [92]. Inhibitory receptors expressed on T cells are often referred to as immune checkpoints and include PD-1, CTLA-4, LAG-3 and TIM-3 (Figure 1.7).

Many molecular mechanisms have been implicated in the induction of T cell exhaustion, however, persistent antigen exposure seems to be the main cause [93]. Studies of chronic lymphocytic choriomeningitis virus (LCMV) infection in mice demonstrated a correlation between antigen load and functional impairment in virus-specific CD8 T cells [94]. Moreover, lack of CD4 T cell help and inhibitory receptor ligation have been implicated in the induction of T cell exhaustion [93]. Interestingly,

blocking of the inhibitory receptor PD-1 restored CD8 T cell function in mice with chronic LCMV infection, indicating that T cell exhaustion is reversible [95].

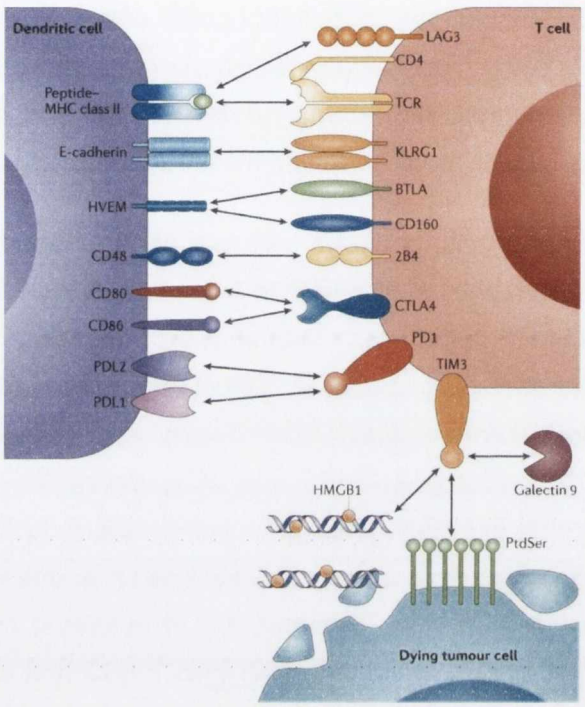


Figure 1.7: Inhibitory receptors on T cells
T cells express multiple inhibitory receptors including LAG-3, CTLA-4, PD-1 and TIM-3. Ligands for inhibitory receptors can be expressed by tumour cells and immune cells, such as DCs. Binding of cognate ligands to inhibitory receptors suppresses effector T cell function and can lead to T cell exhaustion. From Nguyen & Ohashi, 2015 [96]

The expression of inhibitory receptors is a regulatory mechanism to limit T cell responses, for example to prevent autoimmune responses during inflammation. However, the same receptors are targeted by the tumour microenvironment to protect tumour cells from anti-tumour immune response, leading to dysfunction or cell death of antigen-specific T cells. T cells that show an exhausted or dysfunctional phenotype, similar to chronic infections, have been identified in several cancers, including chronic lymphocytic leukemia (CLL), ovarian cancer, NSCLC and melanoma [97-100]. Moreover, the expression of multiple inhibitory ligands has been associated with reduced T cell effector function in melanoma and ovarian cancer [98, 101].

Inhibitory receptors have distinct ligands and mechanisms of action. CTLA-4 was one of the first inhibitory receptors investigated in T cell suppression [102]. CTLA-4 is constitutively expressed on Treg cells and its expression on effector T cells is induced

upon activation [103, 104]. CTLA-4 suppresses T cell function by multiple mechanisms. CTLA-4 is structurally similar to CD28 and binds to CD80 and CD86 at a higher affinity than CD28 [105]. It has been suggested that CTLA-4 expression interferes with T cell activation by reducing CD28 co-stimulation [103, 106]. Other mechanisms of CTLA-4-mediated suppression of T cell activation include the increase of T cell motility, inhibition of TCR and CD28-induced genes and reduction in CD25 expression [107-109]. It has also been shown that high CTLA-4 expression on CD4 T cells is crucial for the suppressive function of Treg cells [110]. Blocking CTLA-4 *in vivo* has been shown to promote anti-tumour immunity by acting on Treg and effector T cells [111]. Moreover, clinical blockade of CTLA-4 in cancer patients has achieved remarkable efficacy *in vivo* which has led to the FDA approval of the anti-CTLA-4 antibody Ipilimumab for the treatment of malignant melanoma in 2011 as the first approved immune checkpoint inhibitor [112].

1.3.4. The immune checkpoint PD-1

The inhibitory receptor PD-1 and its ligands gained significance in recent years with the approval of several antibodies that target PD-1 or its ligand PD-L1 [7]. PD-1 is expressed on T cells, B cells and certain myeloid cells, however, its role is best characterised in T cells. Two ligands of PD-1 have been identified so far, PD-L1 and PD-L2 [113, 114]. In addition to binding PD-1, PD-L1 has also been shown to interact with CD80, thereby delivering an inhibitory signal to CD80-expressing target cells [91]. PD-1 expression on T cells is induced by antigen stimulation [91]. Unlike CTLA-4, which limits early T cell activation, PD-1 mainly exerts its inhibitory effect on T cells in the periphery where T cells encounter PD-1 ligands [115].

The exact downstream effects of PD-1 engagement have not been well characterised, however, several mechanisms of PD-1-mediated suppression have been proposed (Figure 1.8). PD-1 has an intracellular ITIM and ITSM motif [116]. It has been shown that PD-1 ligation leads to the recruitment of the phosphatases SHP-1 and SHP-2 to the ITSM motif [116]. Chemnitz et al. reported that PD-1 ligation inhibits T cell activation only upon TCR engagement [116]. SHP-2 affects T cell function by several mechanisms. Sheppard et al. demonstrated that SHP-2 interferes with TCR downstream signalling by preventing ZAP-70 and CD3ζ phosphorylation [117]. Moreover, PD-1 ligation has been shown to interfere with signalling molecules, such as Pi3K and Ras that are important for T cell proliferation, cytokine secretion and metabolism [91, 118]. Analysis of HIV-specific T cells also demonstrated PD-1

dependent BATF upregulation, which inhibited T cell function [119]. Furthermore, Li et al. reported a correlation between SHP-2 overexpression with PD-1 expression in tumour-infiltrating lymphocytes (TIL) from patients with head and neck cancer, which limited Th1-type responses [120]. However, most of these mechanisms are based on *in vitro* or *ex vivo* experiments, therefore, it remains to be validated whether the same mechanisms are responsible for T cell exhaustion *in vivo* [93].

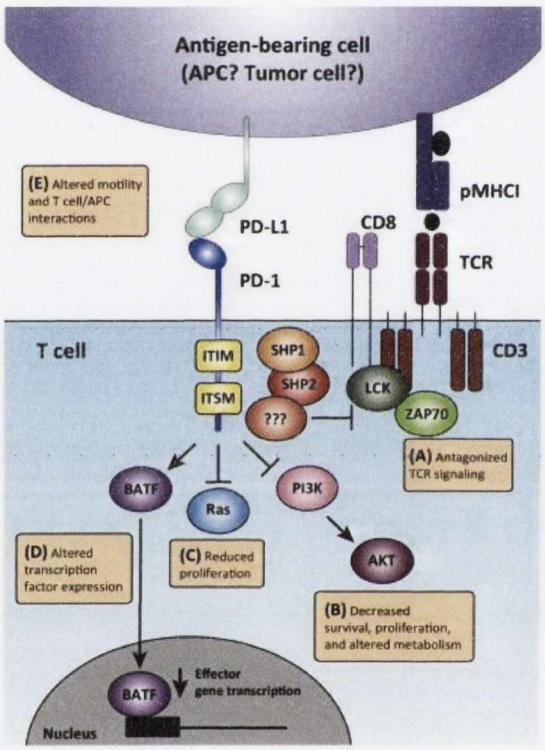


Figure 1.8: PD-1-mediated T cell suppression
Interaction of PD-L1 with PD-1 was suggested to inhibit T cell effector function through several mechanisms. PD-1 recruits phosphatases that prevent the phosphorylation of ZAP70 which directly antagonises TCR signalling (A). Inhibition of Pi3K reduces activation of AKT, resulting in decreased survival, proliferation and altered metabolism (B). The inhibition of Ras also leads to reduced proliferation (C). PD-1 alters the expression of transcription factors which affects transcription of effector genes (D). Moreover, PD-1 alters the motility and duration of T cell/APC interactions (E). From Pauken & Wherry, 2015 [91]

In addition to inhibiting effector T cell responses, the PD-1:PD-L1 axis has been implicated in the promotion of Treg cells. This hypothesis was supported by *in vitro* studies, showing enhanced Treg conversion in presence of PD-L1-expressing DCs or PD-L1-coated beads [121, 122].

There is increasing evidence from animal models and clinical data that PD-1 plays a crucial role in limiting anti-tumour T cell responses. A meta-analysis of 12 different epithelial cancers revealed significantly shorter overall survival for patients with PD-1-positive TIL [123]. Blockade of PD-1 improved anti-tumour immune responses in animal models for melanoma, colon cancer, ovarian cancer and many others [124-127]. Moreover, clinical success led to the FDA approval of anti-PD-1 antibodies for the treatment of malignant melanoma, NSCLC and RCC and an anti-PD-L1 antibody for patients with bladder carcinoma [7, 128].

1.3.5. Expression and regulation of PD ligands

To date, two ligands for PD-1 have been identified, PD-L1 and PD-L2, which are both capable of suppressing T cell responses [113, 114]. PD-L1 and PD-L2 differ greatly in their expression patterns [129]. PD-L1 is constitutively expressed on multiple hematopoietic cells, including T cells, B cells, DCs and macrophages [129]. Stimulation with TLR agonists has been shown to upregulate PD-L1 expression on DCs [130]. Non-hematopoietic cells in peripheral tissues upregulate PD-L1 in response to pro-inflammatory stimuli, such as IFN-γ and type I IFNs [115, 131]. PD-L1 expression has also been found on many tumour cells and was enhanced by stimulation with IFN-γ [96, 132]. Interestingly, hypoxia-induced HIF1α has been shown to upregulate the expression of PD-L1 on macrophages, DCs and tumour cells [133]. Moreover, a recent study demonstrated that tumour cells can induce PD-L1 expression on murine and human NK cells, which was dependent on IFN-γ and NKG2D [134].

The expression of PD-L2 is much less abundant than PD-L1. PD-L2 has been detected on DCs, macrophages and bone marrow mast cells upon stimulation with pro-inflammatory stimuli, such as GM-CSF, IL-4 and IFN-γ [129]. Although PD-L2 expression has been detected on some tumours, it is believed that PD-L2 only plays a minor role in the promotion of T cell exhaustion [96].

The expression of PD-L1, and possibly PD-L2, constitutes another mechanism by which tumours evade targeted immune responses and has been associated with a worse prognosis [123].

1.3.6. Suppressive myeloid cells

Another feature of the tumour microenvironment is the recruitment of suppressive immune cells. Macrophages constitute a major proportion of immune cells in the

tumour microenvironment [135]. For reasons of simplification, macrophages are often divided into two groups, M1 and M2, although there is strong plasticity among these two phenotypes, especially in the tumour microenvironment. M1 or classical activation of macrophages is driven by LPS and IFN-γ. M1 macrophages are pro-inflammatory and promote Th1 responses through IL-12 secretion and promote T and NK cell activation. This phenotype of macrophages is found early during tumour development [136].

Macrophages with an M2 phenotype constitute the majority of macrophages at later time points of tumour development and are induced by stimulation with IL-4 and IL-13. M2 macrophages promote tumour growth through a range of mechanisms which inhibit Th1 responses. M2 macrophages secrete IL-10, TGF-β and VEGF and promote Th2 responses. Moreover, M2 macrophages can directly inhibit T cells, for example through the expression of PD-L1 and PD-L2 [135, 136]. The infiltration of macrophages correlates with worse prognosis in some cancers, such as breast, cervix and bladder cancers [15].

MDSCs are a heterogeneous population of myeloid cells often found in the tumour microenvironment. MDSCs derive from the same myeloid progenitors as DCs or monocytes but persist in an immature state and are further divided into monocytic MDSCs (M-MDSCs) or polymorphonuclear MDSCs (PMN-MDSCs). PMN-MDSCs are more commonly found in peripheral lymphoid organs and have modest suppressive activity while M-MDSCs are strongly immunosuppressive and the main MDSC type in tumours. Moreover, M-MDSCs can differentiate into TAMs [137]. Co-culture experiments with MDSCs and T cells induced inhibition of T cell activation and proliferation [138-140]. Several mechanisms were suggested by which MDSCs inhibit effector T cell function. These include nutrient depletion, reactive oxygen species (ROS) production, inhibition of lymphocyte trafficking and induction of apoptosis [141]. Moreover, MDSCs have been shown to induce Treg cells in cancer [142, 143]. In addition to maintaining suppression in the tumour microenvironment, MDSCs were found promote angiogenesis and tumour cell invasion in a VEGF and matrix metalloproteinase (MMP)-dependent manner [144].

1.3.7. Treg cells

Treg cells are anti-inflammatory immunosuppressive CD4 T cells and are important for the prevention of excessive inflammation and autoimmunity. Several studies have shown that the removal of Treg cells, for example through depletion of CD25, leads to

spontaneous autoimmunity [145, 146]. Conversely, the presence of Treg cells is associated with a worse prognosis in cancer [147]. Treg cells are characterised by the expression of Foxp3 and high levels of the IL-2 receptor chain CD25. The transcription factor Foxp3 is a critical regulator of Treg cell development and function [148, 149]. Foxp3 controls the expression of typical Treg proteins, including CD25, GITR and CTLA-4 and inhibits the production of IL-2 and IFN-γ [150].

There are two types of $Foxp3^+$ Treg cells which are distinguished based on their origin. Thymic Treg (tTreg) cells, or natural Treg cells, develop during thymic T cell selection from self-reactive thymocytes [151]. In contrast to other T cell subsets, Treg cells leave the thymus as functionally mature cells and are able to suppress self-reactive T cells with no need for further antigen-priming. tTreg cells were reported to express higher levels of the markers Helios and Neurolipin-1 compared with peripherally-derived Treg (pTreg) cells, which are sometimes used to distinguish these Treg subtypes [151].

In the periphery, $Foxp3^+$ cells constitute about 10-15% of CD4 T cells, of which pTreg cells constitute about 5% in healthy mice [150, 152]. Naïve CD4 T cells can differentiate into $Foxp3^+$ cells in a suppressive microenvironment. Studies have shown that Treg cells can be induced from naïve CD4 T cells upon antigen stimulation in presence of TGF-β [153]. However, the presence of IL-6 shifts the differentiation towards a Th17 phenotype [61]. Retinoic acid (RA) has been shown to promote TGF-β-driven Treg conversion, while inhibiting IL-6-driven Th17 differentiation [61, 153, 154]. TGF-β signalling-induced SMAD3 can bind to the Foxp3 promoter and Foxp3 transcription is enhanced by RA binding to retinoic acid receptor (RAR)/ retinoid X receptor (RXR) [155]. The role of RA in promoting Treg cells has initially been described in the gut where $CD103^+$ DCs promoted tolerance by RA-induced Treg induction [154].

There is some evidence that tolerogenic DCs can induce the conversion of naïve CD4 T cells to Treg cells [32, 156]. It has been reported that DCs promote Treg cells through mild TCR stimulation and secretion of TGF-β and RA [156]. Moreover, it has been suggested that PD-L1 expression by DCs contributes to Treg conversion by binding to PD-1 on T cells [122].

1.3.8. Treg-mediated immunosuppression

Treg cells suppress immune responses by cell-contact dependent and independent mechanisms (Figure 1.9) [152]. The secretion of anti-inflammatory molecules, such as

IL-10 and TGF-β, has been implicated in the suppression of inflammatory lymphocytes as well as DCs [150, 157]. Moreover, Treg cells secrete high levels of IL-35 in the tumour microenvironment [158]. Turnis et al. observed IL-35-driven T cell exhaustion at the tumour site and depletion of IL-35 reduced tumour burden while enhancing tumour-specific memory populations [158]. Furthermore, IL-2 is required for the proliferation and homeostasis of most T cell subtypes, including Treg cells. However, Treg cells do not produce IL-2 themselves [150]. It has been suggested that high levels of IL-2 uptake by Treg cells deprive effector T cells of IL-2, which consequently results in apoptosis of effector T cells [159]. Treg cells can also have direct cytotoxic effects on effector T cells and DCs by the secretion of perforin and granzymes [152].

Cell contact-dependent mechanisms of suppression are mostly mediated by the checkpoint protein CTLA-4. Treg cells constitutively express the inhibitory receptor CTLA-4 which is involved in the downregulation of T cell effector function [152, 160]. Strikingly, CTLA-4 has also been shown to remove CD80/86 from DCs by trans-endocytosis which, as a consequence, lost their ability to activate T cells [161].

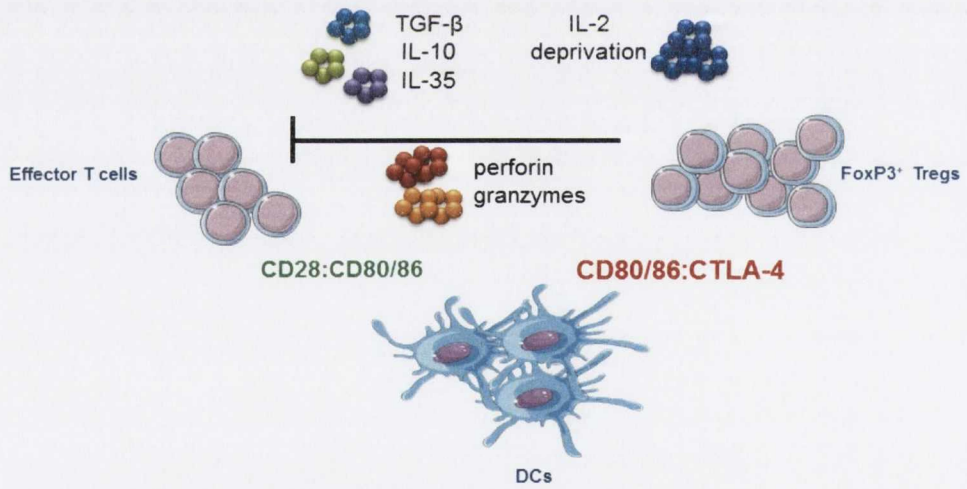


Figure 1.9: Mechanisms of Treg-mediated immunosuppression
Treg cells suppress T cell effector function through secretion of anti-inflammatory cytokines (TGF-β, IL-10, IL-35), secretion of cytotoxic granules (perforin, granzymes), IL-2 deprivation and competition for interaction with CD80/86.

1.3.9. Treg cells in cancer

Treg cell infiltration has been detected in mouse tumour models and in cancer patients. Depletion of Treg cells improved anti-tumour T cell responses and reduced tumour

growth in murine tumour models for melanoma and colon cancer [46, 162]. Moreover, a higher ratio of Treg cells to CD8 T cells has been associated with poorer disease outcome in cancer, suggesting that Treg cell-mediated T cell suppression is an important mechanism by which tumour cells evade immune responses [163-165].
The enrichment of Treg cells in the tumour is based on recruitment of Treg cells from the periphery and the expansion of tumour-resident Treg cells [166]. Tregs are attracted to the tumour bed by chemokines or VEGF which are secreted by tumour cells or immune cells in the tumour microenvironment. A range of chemokines have been shown to attract Treg cells in cancer, including CCL17, CCL22, CCL28 and CXCL12 [166, 167]. CCR4 appears to be the dominant chemokine receptor in the context of cancer and blockade of CCR4 depleted Treg cells and enhanced pro-inflammatory immune responses in mouse models and cancer patients [168, 169].

The immunosuppressive tumour microenvironment also promotes the expansion of Treg cells. Soluble molecules, such as IL-2, TGF-β, RA and IDO contribute to intra-tumoural Treg expansion [166]. Moreover, tolerogenic DCs in the tumour microenvironment have been implicated in the induction of Treg cells [156, 170]. A contributing factor for the activation of Treg cells in tumour is the expression of self-antigens by tumour cells which can induce tolerance. Indeed, Tregs cells from cancer patients recognised tumour-associated antigens, such as NY-ESO-1 and survivin [147, 171, 172]. Antigens that arise from mutations are called neoantigens and often contribute to tumour cell transformations. Interestingly, tumours with low neoantigen expression have been shown to attract more Treg cells, whereas high neoantigen expression correlated with increased infiltration of CD8 T cells and Treg depletion [165].

The primary effect of Treg cells in tumour is the suppression of effector T cells and NK cells. Treg cells contribute to tumour progression by additional mechanisms apart from immunosuppression. Treg cells form a suppressive network by interaction with MDCSCs and TAMs, which promote Treg infiltration [166]. Conversely, Tiemessen et al. observed Treg cell-induced alternative activation of macrophages towards an M2 phenotype [173]. Moreover, Treg cells promoted angiogenesis in the ID8 tumour model through the secretion of VEGF [167].

1.4. Cancer Immunotherapy

Immunotherapy has considerable potential to improve disease outcome and survival of patients with cancer, as highlighted by recent breakthroughs in this area. The primary aim of cancer immunotherapy is to activate anti-tumour immune responses. Many immunotherapeutic strategies have been investigated ranging from treatment with immune-stimulatory molecules to injection of activated immune cells (Figure 1.10).

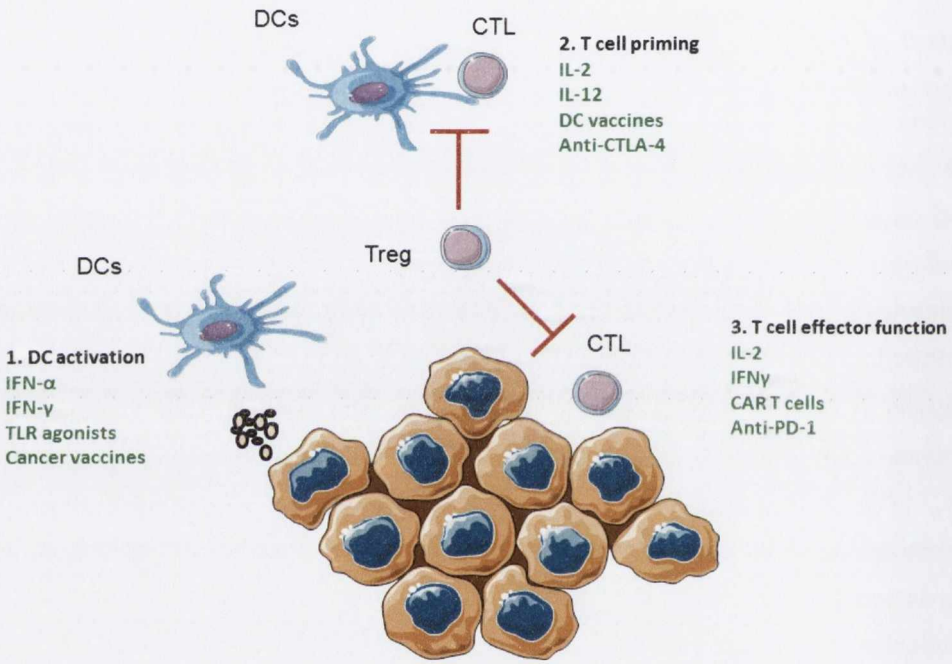


Figure 1.10: Cancer immunotherapy approaches

Current cancer immunotherapy strategies target the interaction of immune cells and tumour cells at different stages. Pro-inflammatory cytokines (IFN-α and IFN-γ), TLR agonists and cancer vaccines promote DC activation. IL-2, IL-12, DC vaccines and CTLA-4 blocking antibodies enhance T cell priming in dLN. Moreover, IL-2, IFN-γ, CAR T cells and PD-1 blocking antibody promote T cell effector responses.

1.4.1. Cytokines

The first immunotherapy approaches employed pro-inflammatory cytokines. To date, the only cytokines licensed for the treatment of cancer are IL-2 and IFN-α, which are both approved for melanoma. IL-2 is required for the homeostasis, function and

proliferation of T cells. However, treatment with high-dose recombinant IL-2 (Aldesleukin) only showed low response rates and was associated with significant risks of developing systemic inflammation [174-176]. Moreover, high-dose IL-2 promoted suppressive Treg cells in melanoma patients which correlated with a worse clinical outcome [177]. A caveat of IL-2 therapy is that Treg cells have a higher affinity to IL-2 than effector T cells which deprives effector T cells of essential IL-2 [159]. Novel approaches in the IL-2 field include selective IL-2-antibody complexes that preferentially bind to the IL-2R on effector T cells over Treg cells [178]. Moreover, Levin et al. designed an IL-2 'superkine', which has an increased binding affinity for IL-2Rβ and shows enhanced stimulation of CD8 T cells and not Treg cells. *In vivo* experiments demonstrated that the IL-2 'superkine' induced significantly better clearance of B16 tumours than recombinant IL-2 [179].

IFNα was approved for the treatment of high-risk melanoma in 1995. Although showing some overall survival benefit in patients with resected tumours at high risk of recurrence, IFNα treatment was frequently associated with high-grade adverse effects [180] .

Many immunologic and non-immunologic anti-tumour effects have been described for IFN-γ in pre-clinical studies, suggesting that IFN-γ could be a very potent drug against malignancies [181]. IFN-γ activates macrophages and promotes Th1 responses while inhibiting Treg cells and Th2 cells. Moreover, IFN-γ increases the immunogenicity of tumour cells by increasing MHC I expression and inhibiting proliferation of melanoma cells. However, IFN-γ has not been approved as a single agent to date, due to failures or inconclusive outcomes from clinical trials. It has been suggested that IFN-γ can also have pro-tumour effects. It has been reported that IFN-γ-induced immune exhaustion could contribute to tumour cell proliferation, enhanced Treg development and the induction of resistance to CTL and NK responses [14].

In vitro and *in vivo* studies have shown that IL-12 is a very potent activator of NK cells and CD8 T cells. Moreover, IL-12 promotes Th1 differentiation and IFN-γ secretion by T cells, making it a promising candidate for cancer immunotherapy. However, clinical trials revealed only modest effects, due to immunosuppressive effects elicited by the tumour microenvironment [182]. Moreover, treatment of patients with high dose IL-12 led to severe toxicities [182]. Combinations of low-dose IL-12 with drugs that target immunosuppressive networks in cancer might bring a comeback for IL-12 therapy in the future.

The outcomes from clinical trials using cytokines suggest that cytokines alone are rarely effective enough to counteract immune suppression in the tumour microenvironment. Instead, their use as adjuvants in combination with other immune-therapeutics could be more favourable.

1.4.2. TLR agonists

TLR agonists are either used as monotherapies or as adjuvants in cancer vaccines. The reasoning behind using TLR agonists in cancer therapy is their capacity to activate and induce the maturation of DCs. In addition, TLR agonists promote T cell polarising cytokine secretion by DCs, including IL-1, IL12 and IL-23. In this way, TLR agonists activate immature DCs in the tumour microenvironment and thereby induce T cell responses.

To date, three TLR agonists have been approved by the FDA in the context of cancer. In 1990, *Bacillus Calmette-Guerin* (BCG) was approved as monotherapy against non-invasive transitional bladder carcinoma. BCG contains ligands for TLR2, TLR4 and TLR9 [183]. The second TLR agonist to be approved was Imiquimod. Imiquimod is a member of the chemical family of imidazoquinolines and binds to TLR7 in mice and TLR7 and TLR8 in humans. It is approved as an adjuvant in several skin cancers and genital warts and used as a topical cream (Aldara^{TM}) [184]. Since its approval in 1997, many clinical trials have been initiated testing Imiquimod as single agent or in combination therapies in various other cancers, including glioblastoma, NSCLC and actinic keratosis [185]. However, recent data suggests that the beneficial effect of Aldara might be independent of TLR7 signalling [186]. The most recently approved TLR agonist is MPL, which is a less toxic derivative of LPS. LPS is a ligand for TLR4 and has been shown to work as a vaccine adjuvant in various settings in animal models. However, the toxicity of LPS *in vivo* excluded its use in human vaccines, leading to the generation of safer derivatives. MPL is safe in humans and is a component of Cervarix, which is a vaccine against HPV 16 and 18 [185, 187]. MPL promotes Th1 responses and induces the secretion of IL-12, IL-1 and TNF [188, 189]. However, the MPL-adjuvanted HPV vaccine prevents the spread of a potentially cancer-causing virus rather than being a direct cancer vaccine. In addition, Picibanil, a *Streptococcus pyogenes* preparation that contains TLR agonists, has been approved as cancer therapy in Japan [190].

Current research on TLR agonists mainly focuses on BCG, Imiquimod and MPL and similar agonists, due to the existing FDA approvals. Additional TLR agonists in clinical

trials include R848 (TLR7/8), poly:IC and hiltonol (TLR3), and motolimod (TLR8) which are mostly used as adjuvants in cancer vaccines [190].

R848, also called Resiquimod, is an analogue of Imiquimod and is more potent and soluble than Imiquimod, making it a promising adjuvant candidate [191, 192]. R848 induces pro-inflammatory cytokines and promotes Th1 responses [193, 194]. Current clinical trials are investigating the use of R848 in melanoma, glioma and nodular basal cell carcinoma [195]. R848 has mostly been tested as a topical gel against skin cancers and warts. In current trials, R848 has also been injected under the skin of cancer patients and was found to be safe [196]. Moreover, R848 can induce disease regression and enhance T-cell effector functions in cutaneous T cell lymphoma [197].

Many pre-clinical *in vivo* studies have reported strong anti-tumour responses induced by TLR agonists. However, testing of TLR agonists as immunotherapeutics has been declining over the years due to unsuccessful results from clinical trials. The underlying reasons include immune regulatory effects, such as the secretion of anti-inflammatory IL-10 and TGF-β and the induction of PD-L1 and PD-L2 on DCs [198]. Several approaches in animal models have addressed this problem and have tried to reverse the regulatory DC phenotype, for example by using p38 or Pi3K inhibitors, which led to reduced secretion of IL-10 and enhanced secretion of IL-12 by DCs [86, 87]. Moreover, limited availability of clinical grade TLR agonists and the strong focus on checkpoint inhibitors are other reasons why the interest in TLR agonists has declined [185, 190]. Currently, the trend seems to reverse and there has been an increase again of the use of TLR agonists in clinical trials since 2014, probably because of the invigorated interest in immunotherapy since the approval of checkpoint inhibitors. The results from clinical trials using TLR agonists in combination with checkpoint inhibitors are eagerly awaited [190].

1.4.3. Cancer Vaccines

Cancer vaccines aim to generate tumour-specific T cell responses *in vivo* by injecting tumour antigens in combination with adjuvants that stimulate antigen-presenting mature DCs. An ideal cancer vaccine induces proper DC activation, strong cytotoxic T cell responses, and little Treg activation and overcomes immunosuppression by the tumour microenvironment. The right choice of immunogenic antigens and adjuvants is key to effective vaccines. Different sources of antigens have been tested, including peptides, proteins, tumour lysates or nucleic acids [199].

1.4.4. Peptide vaccines

Cancer cells have a high degree of heterogeneity within a tumour and between patients. Therefore, tumour vaccines are often formulated using tumour associated antigens (TAA), which are antigens that are typically expressed in tumours and scarcely expressed by healthy tissues. Many early attempts with peptide vaccine failed mostly because short peptides were used which have a short half-life *in vivo* and were poorly immunogenic without adjuvants. The failure to overcome immunosuppression is another hurdle for cancer vaccination. Recently, however, peptide vaccine strategies have improved by using longer peptides or whole proteins in combination with immune stimulatory adjuvants [199].

There are several classes of TAAs that can be used in vaccines: viral proteins, tumour-specific mutated antigens and self-antigens. Viral proteins only protect from virus-associated cancers which are rare. Tumour-specific mutated antigens are highly specific and effective, however, require patient-specific identification. Self-antigens include tissue differentiation antigens, tumour germline antigens and normal proteins overexpressed by cancers [200]. The problem with non-mutated self-antigens in vaccines is the induction of weak T cell responses. Firstly, most T cells that recognise these antigens would have been depleted during the induction of central tolerance. Second, T cells that recognise self-antigens and were not depleted only have a low affinity TCR. Moreover, Treg cells are often specific for self-antigens which could potentially lead to tolerance [201]. Despite these challenges, anti-tumour T cell responses have been generated with some of these self-antigens, including MAGE-A3, gp100, NY-ESO-1 and TRP-2 [202].

One of the most frequently used antigens in cancer vaccines is the melanoma antigen gp100. gp100 in combination with IL-2 increased the overall clinical response and progression-free survival in melanoma patients compared to patients treated with IL-2 only [203]. Similarly, a DC vaccine loaded with gp100 and tyrosinase induced a 29% response rate and relatively long median overall survival in metastatic uveal melanoma patients [204].

Another peptide under investigation as vaccine antigen is the melanoma differentiation antigen tyrosinase-related protein 2 (TRP-2). TRP-2 is an enzyme expressed in melanocytes and is involved in the generation of melanin pigments. The TRP-2 protein is expressed by mouse and human melanocytes and shows 83% identity and 90% similarity between the two species [205]. TRP-2 is a promising melanoma target

antigen because of its restricted expression in melanoma cells, melanocytes and retina but not in other human tissues [206]. Moreover, TRP-2 is present in the commonly used B16 murine melanoma cell line which makes it possible to study TRP-2 vaccines in mouse tumour models. Indeed, several pre-clinical trials using TRP-2 peptides demonstrated successful induction of tumour-specific CTL [207, 208]. The investigation of TRP-2 was not restricted to animal models. An ongoing clinical trial is testing a DC vaccine in melanoma patients where the DCs are transfected with full length TRP-2 mRNA (NCT01456104).

To improve the stability of a peptide and to induce stronger DC activation, Melief and colleagues designed long synthetic peptides (SLP) covalently linked to a TLR (SLP-TLRA). One of the advantages of SLP-TLRA is that DCs encounter long peptides together with PAMP stimulation, consequently maximising T cell responses [209]. Vaccination trials using SLP in patients with HPV16-positive vulvar lesions resulted in clinical responses in 79% of patients and complete responses in 47% of patients [210]. In line with many other immunotherapies, the success or failure of the vaccination correlated with the induction of Treg cells [211].

1.4.5. Tumour lysate vaccines

Autologous tumour vaccines are prepared from tumour lysates from a patient's tumour sample. The advantage of using tumour lysates over peptides is that numerous tumour antigens, including immunogenic neoantigens, will be presented to T cells. This potentially increases the pool of T cells with different specificities and enhances the likelihood of generating tumour-specific T cell responses. A meta-analysis including a wide range of cancers has demonstrated that patients treated with DC vaccines using tumour lysates as antigen source responded better to vaccines than patients treated with DC vaccines loaded with molecularly defined antigens [212]. A negative aspect of tumour lysates is that the vaccine has to be individually generated for each patient, which makes it more laborious and costly. Moreover, the presence of non-mutated endogenous antigens could increase the risk of developing autoimmunity. Nevertheless, the results from clinical trials showed that vaccination with autologous tumour lysates is safe in prostate cancer, melanoma and mesothelioma patients [213-215].

Tumour lysates can be generated by different methods. Irradiation of tumour cells by gamma (ionising) or UVB radiation (non-ionising) has been shown to induce apoptosis. Other approaches involve oxidisation of tumour cells prior to vaccination or freeze-

thawing of tumour cells [216]. Freeze-thawing has been shown to induce necrosis. A study comparing irradiation versus freeze-thawing revealed that apoptotic tumour cells are more immunogenic and induce better anti-tumour immune responses *in vivo* than necrotic tumour cells [217]. Moreover, hyperthermia induced by heat-shocking tumour cells has been shown to increase the immunogenicity of tumour cells and enhance antigen expression and production of HSPs. Heat-shocked cells secrete danger signals including HSPs and HMGB1 which promote DC activation [216]. Importantly, precautions have to be taken to make sure autologous cells are dead to prevent spread of live tumour cells which has been reported in older literature [218].

Direct vaccination using tumour lysates induced potent anti-tumour immune responses in mouse models of cancer. In a mouse model for glioblastoma, immunization of mice with tumour lysates and CpG led to tumour rejection in up to 55% of mice which was accompanied by improved DC and T cell responses [219]. Another study demonstrated potent CD8 T cell activation and tumour rejection in response to immunization with tumour lysates and baculovirus as adjuvant in the CT26 tumour model [220].

Several vaccines using whole tumour lysates are currently under investigation in phase I studies in cancer patients with glioma, recurrent ovarian fallopian tube cancer or primary peritoneal cancers. These pilot studies include combinations with TLR agonists such as polyI:C, Imiquimod or radiotherapy (NCT01312389, NCT01678352, NCT01400672, NCT02549833). In addition, whole tumour lysates are frequently used as antigen source in the context of DC vaccines [216].

1.4.6. DC-based therapies

DCs vaccines consist of DCs that are isolated from a patient and stimulated with tumour antigens and adjuvants *ex vivo*. Fully mature, activated tumour antigen-presenting DCs are then transferred back to the patient by i.v. infusion. There are two crucial parameters in designing effective DC vaccines: proper DC activation and immunogenicity of antigens. Similar to classical cancer vaccines, antigen sources can be recombinant peptides, proteins, tumour lysates or transfected nucleic acids. Non-mutated antigens have shown limited success because they often provoked Treg responses, while mutated antigens induced better effector T cell responses [221]. Moreover, clinical trials have demonstrated that mature DCs induced better anti-tumour responses that immature DCs [36, 222]. Adjuvants that could be used in DC therapies include TLR agonists, STING agonists and cytokines [25, 223].

The biggest success of DC therapy has been the approval of the DC vaccine Sipuleucel-T (Provenge) in 2010 for the treatment of prostate cancer. Sipuleucel-T is the only approved cellular immunotherapy against cancer to date. Peripheral blood mononuclear cells (PBMCs) from prostate cancer patients are cultured *ex vivo* with PAP - GM-CSF, consisting of the prostate cancer antigen prostatic acid phosphatase (PAP) linked to GM-CSF, which promotes DC differentiation. Clinical trials demonstrated an increase in the overall survival of 4.1 months compared to patients in the placebo control group [224].

In vivo DC loading represents another approach to promote DC-driven anti-tumour responses. The advantages of *in vivo* DC loading over *ex vivo* DC vaccines are that therapeutics targeting DCs *in vivo* are easier to generate. Studies by Steinman and others identified markers for different DC subsets which have been exploited to target specific DC subsets *in vivo,* such as DEC-205. For *in vivo* DC loading, tumour antigens are bound to antibodies directed against surface antigens on DCs [225-227]. Binding of the antibody to DCs leads to endocytosis of the antigen. A problem with this strategy is that antigen priming of DCs in absence of an adjuvant could lead to the induction of tolerance [226]. Dhodapkar et al. developed a complex comprising the tumour antigen NY-ESO-1 fused to DEC-205 (CDX-1401) [196]. In a phase I clinical trial, patients with advanced malignancies were treated with CDX-1401 in combination with the TLR agonists R848 (TLR7/8) and Hiltonol (TLR3). This treatment was safe, induced NY-ESO-1-targeted immune responses and led to tumour regression in two patients [196].

A novel strategy employed tumour antigens and adjuvants incorporated into micro- and nanoparticles which bind to DCs through antibody targeting. This method reduces the risk of adjuvant-mediated side effects since only targeted DCs get in contact with the adjuvant. Kwon et al. reported effective activation of CTL responses *in vivo* by targeted nanoparticles [228]. Moreover, Cruz et al. demonstrated successful targeting of human DCs by targeted nanoparticles [229]. This method has been further advanced in a recent study by Kranz et al. who developed RNA-lipoplexes that target DCs in an anti-body-independent manner [230]. The researchers found that adjustment of the net charge of lipoplexes led to specific targeting of macrophages and DCs in lymphoid organs. Moreover, RNA-lipoplexes induced tumour rejection in mouse models for melanoma and colon carcinoma. A clinical trial starting this year will reveal whether this method is safe and effective in patients [230].

1.4.7. T cell transfer

There has been a strong increase in the testing of autologous T cells in cancer therapy in recent years. The advantages of adoptively transferring T cells are that tumour-reactive T cells can be specifically selected, pre-activated and largely expanded *ex vivo* [231]. On the other hand, one of the big challenges is the identification of cells that selectively recognise tumour cells but not healthy cells to avoid autoimmune responses [231].

Early T cell transfer studies were using the host's own TIL. For this approach, TIL were taken from the tumour and tumour-reactive T cells expanded *ex vivo* and transferred back to the patient [232]. This method has been tested in several clinical trials, mostly in melanoma patients, revealing a positive outcome and increased survival rates in a small percentage of patients [233-236].

In addition to the expansion of the host's own TIL, researchers have developed genetically engineered T cells with tumour antigen specificity. There are two different types of genetically engineered T cells, which either carry TCRs or chimeric antigen receptors (CARs) [221]. The advantages of using CARs over TCRs are that CARs can recognise antigens that are expressed on the cell surface, while TCRs only recognise antigens presented by MHCs [231]. CAR T cells contain variable regions of antibody heavy and light chains linked to intracellular CD3ζ. New generations of CAR T cells also include intracellular co-stimulatory domains, such as CD28 and/or 4-1BB, to enhance T cell activation [237]. Another advancement of CAR T cell technology is the addition of switches that control T cell activation and expansion *in vivo* [238]. Rodgers et al. developed CAR T cells that target B cell lymphomas through switches that specifically bind to CD19. Thus, CAR T cells only get activated in the presence of switches, which allows for dosing and changes in targeted antigens [238]. Moreover, Liu et al. developed CAR T cells that are transfected with a PD-1-CD28 construct, which reverses immunosuppression mediated by PD-1:PD-L1 and instead leads to T cell activation in presence of PD-L1. This method led to the eradication of large solid tumours in mouse models [239].

CAR T cells have demonstrated impressive clinical efficacy in certain cancers. Most trials have been in hematologic malignancies, including CLL, acute lymphoid leukemia (ALL), and multiple myeloma with CD19-specific CAR T cells [237]. The most impressive results have been achieved in phase I clinical trials with ALL patients where complete response rates of up to 90% have been reported [237, 240]. CAR T cells

have been less effective in solid tumours, due to higher levels of heterogeneity and resistance by the immunosuppressive tumour microenvironment. Modifications of CAR T cells are currently investigated which might improve CAR T cell therapy for solid tumours in the future [237, 239].

1.4.8. Immune checkpoint blockade

A major mechanism by which tumours overcome effector T cell responses is the stimulation of inhibitory receptors, or checkpoints, on T cells. Receptors that are associated with suppression include CTLA-4, PD-1, TIM-3 and LAG-3 (Figure 1.7) [241]. Checkpoint inhibitors are monoclonal antibodies against immune checkpoints on immune cells and block the interaction with the respective checkpoint ligands (Figure 1.11). The primary aim of checkpoint blockade is to reduce suppression of effector T cells, especially CD8 T cells, and thereby to improve specific anti-tumour immune responses. Antibodies have been developed for most of these inhibitory receptors and tested in pre-clinical models and clinical trials [242]. The huge potential of checkpoint inhibitors is mirrored by a large amount of ongoing clinical trials in a range of different cancer types. A search on clinicaltrials.gov listed more than 200 open studies for "PD-1" and "cancer" and 73 open studies for "CTLA-4" and "cancer".

CTLA-4 was the first immune checkpoint that was targeted in the context of cancer. Pre-clinical studies demonstrated that blocking of CTLA-4 reduced tumour growth in mouse models of melanoma, colon carcinoma and many others [124, 243]. The efficacy of CTLA-4 therapy in pre-clinical models was associated with increased T cell infiltration into tumours [124]. Based on these findings, antibodies targeting CTLA-4 were tested in clinical trials. One of these antibodies, Ipilimumab, was the first checkpoint inhibitor that was approved by the FDA in 2011. Ipilimumab has been approved for metastatic melanoma and has shown great success in patients, increasing the median overall survival by 10 months [112]. In a parallel study, treatment with Ipilimumab and Dacarbazine led to superior survival rates for 1-, 2- and 3- year survival compared with Dacarbazine alone [244]. Pooled analysis from 12 clinical trials involving Ipilimumab revealed survival of up to 10 years in some melanoma patients [245]. Ipilimumab has also been tested in other malignancies such as NSCLC, renal cancer and prostate cancer. However, these trials did not meet the projected endpoints [7].

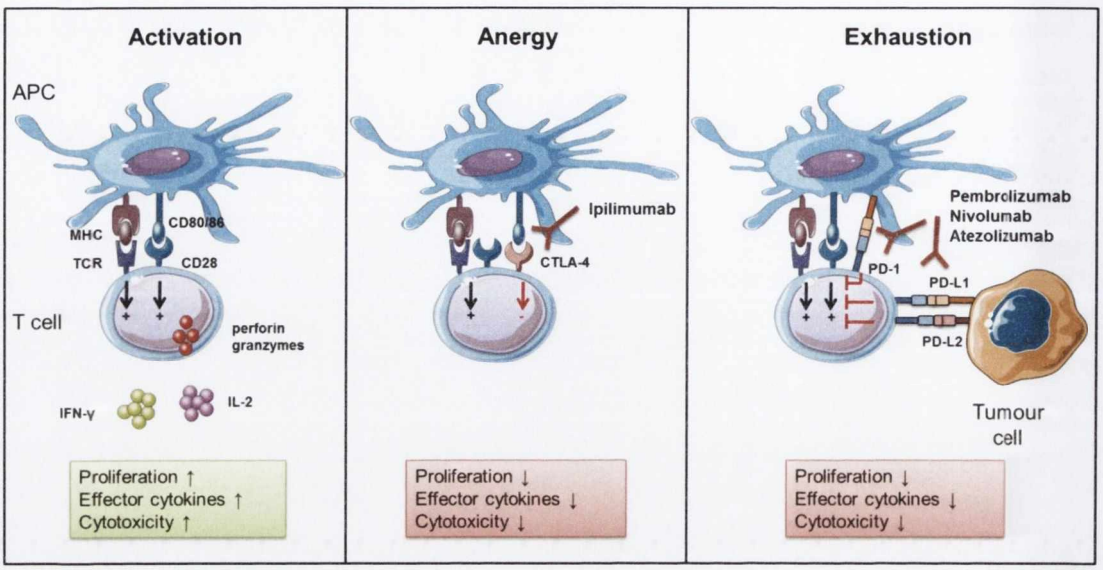


Figure 1.11: Mechanisms of immune checkpoint blockade

(A) During T cell activation, APCs present antigens to T cells by MHC-TCR interaction and co-stimulate T cells through CD80/86 binding to CD28. Activated T cells proliferate, secrete effector molecules (IFN-γ, IL-2, perforin, granzymes) and gain cytotoxic activity. (B) CTLA-4 expression on T cells can lead to anergy by binding to CD80/86, which limits co-stimulation through CD28. Anergic T cells have limited effector function. Ipilimumab, a CTLA-4 blocking antibody, inhibits CTLA-4 binding to CD80/86 and promotes T cell activation. (C) PD-1 expressed on T cells can be bound by PD-L1 and PD-L2, typically expressed by APCs or tumour cells. PD-1 ligation interferes with TCR downstream signalling events which suppresses T cell function and can lead to T cell exhaustion. Antibodies blocking PD-1:PD-L1/2 interaction restore T cell effector function.

The efficacy of Ipilimumab in a cohort of patients has been a proof-of-concept that re-activation of T cells could lead to anti-tumour responses in patients. The approval of Ipilimumab also marked a paradigm shift in cancer immunology research. According to PubMed, the publications per year on checkpoint inhibitors more than doubled from 2011 to 2012 and the number is still rising each year. More impressively, the publications on PD-1 were doubling almost every year since 2012. Pre-clinical studies with PD-1 or PD-L1-blocking antibodies have demonstrated a reduction in tumour growth in several murine tumour models [124, 125, 127, 246]. Mouse models using PD-1 antibodies revealed that protection was associated with increased CD8 T cell infiltration and function [124, 127, 247]. Moreover, the ratio of CD8 T cells to Treg cells has been elevated when PD-1 was blocked [127]. Furthermore, blocking PD-1 enhanced T cell infiltration to tumours by increasing the secretion of IFN-γ-inducible chemokines [248].

PD-1 and PD-L1-blocking antibodies have been tested in the clinic and remarkable responses were observed for patients with melanoma and NSCLC. To date, three antibodies have been approved by the FDA which target the PD-1:PD-L1 axis. The first antibody targeting PD-1 Pembrolizumab (Merck) was approved in September 2014. It was approved for the treatment of advanced or unresectable melanoma in patients failing other treatments and later became the first line treatment [249]. It is now also approved for NSCLC [250]. A second PD-1 blocking antibody Nivolumab (Bristol-Myers Squibb) has also been approved for the treatment of melanoma, NSCLC and renal cancer [7]. Moreover, Atezolizumab has been approved by the FDA in May 2016 for the treatment of bladder cancer [128]. Other cancer types that have shown some evidence of activity with PD-1 therapy include head and neck cancers, breast cancer, ovarian cancer and gastric cancer [7].

A study in melanoma patients that directly compared Ipilimumab (anti-CTLA-4) with Pembrolizumab (anti-PD-1) revealed better responses in the Pembrolizumab-treated cohort [251]. Patients that received Pembrolizumab had 12- month survival rates of 74 % (every two weeks) and 68 % (every 3 weeks) compared to 58 % in patients treated with Ipilimumab. Moreover, the combination of Ipilimumab and Nivolumab induced significant responses in untreated melanoma patients and was more effective than either treatment alone [252]. A recent study reported that patients that showed prolonged benefit from Ipilimumab also showed improved outcomes from subsequent Pembrolizumab [253]. These findings suggest non-redundant functions CTLA-4 and PD-1, which exert their function at different stages of T cell activation. CTLA-4 mainly works at T cell priming stage in the LN whereas PD-1 affects T cells mainly in the periphery. This would suggest that CTLA-4 blockade increases the pool of activated T cells in the LN, but does not prevent immune suppression in the tumour microenvironment. PD-1 blocking antibodies, on the other hand, prevent T cell exhaustion at the tumour site.

Frequent side effects of treatments with checkpoint inhibitors are autoimmune reactions, such as skin toxicities, colitis and hepatitis [7]. Blockade of CTLA-4 and PD-1 induced similar adverse effects, however, high-grade adverse effects were less frequent in patients treated with Pembrolizumab (10.1 – 13.3 %) compared with Ipilimumab (19.9 %) [251].

The field of checkpoint inhibition is progressing very quickly. Current clinical trials are also investigating novel checkpoint inhibitors that target TIM-3 and LAG-3. Two trials with anti-TIM-3 antibodies are currently ongoing in advanced malignancies and

metastatic solid tumours (NCT02609268, NCT02817633). Moreover, IMP321 (anti-LAG-3) is being tested in melanoma (NCT02676869), renal cancer (NCT00351949), and breast cancer (NCT02614833). Studies using BMS-986016 (anti-LAG-3) alone or in combination with anti-PD-1 are currently recruiting patients with advanced solid tumours and hematologic malignancies (NCT01968109, NCT02061761). All of these studies are still in phase 1 and testing the safety in a small cohort of patients with advanced malignancies. The next years will show whether these novel immune checkpoint inhibitors are able to compete with the results that were achieved with PD-1 blocking antibodies and whether combined immune checkpoint inhibitors work in synergy.

1.4.9. Checkpoint inhibitors targeting NK cells

Inhibitory receptors have been shown to dramatically impair T cell responses. Recently, several immune checkpoints were also identified on NK cells which dampen anti-tumour immune responses. Da Silva et al. reported that TIM-3 is an exhaustion marker on NK cells. NK cells from melanoma patients overexpressed TIM-3 and were functionally impaired, which was reversed *ex vivo* with an anti-TIM-3 antibody [254]. Moreover, KIRs and NKG2A, which bind to MHC I molecules, were shown to suppress NK cell function upon ligation. Blocking antibodies for both receptors are currently under investigation in phase I/II clinical trials for the treatment of solid tumours [255]. In addition, Delconte et al. identified cytokine-inducible SH2-containing protein (CIS) as a novel regulator of NK cell function [256]. CIS was induced upon IL-15 stimulation of NK cells and inhibited IL-15 secretion and IL-2R expression. Moreover, CIS-deficient mice displayed decreased tumour burden in several mouse tumour models [256].

1.4.10.Combination therapies

Albeit significantly prolonging the survival in some patients, the majority of patients has not benefited from monotherapies with CTLA-4 or PD-1-blocking antibodies. The observation that combined treatment with Ipilimumab and Nivolumab induced better responses than either treatment alone paved the way for the search of effective checkpoint therapy combinations [252]. Several combination strategies are now under investigation. Experiments in murine tumour models have demonstrated that different checkpoint inhibitors can work synergistically, due to non-redundant mechanisms. This has been shown for combinations of anti-PD-1 with anti-CTLA-4, anti-TIM-3 and anti-LAG-3 [124, 127, 246, 247]. Moreover, combination of anti-PD-1 with an activating

antibody against the co-stimulatory receptor 4-1BB improved anti-tumour responses in a mouse model for colon carcinoma [257].

Combinations of checkpoint blockade with immune activators, such as cancer vaccines, could be particularly favourable for patients that lack T cell infiltration into the tumours. There is some pre-clinical evidence supporting this hypothesis. Curran et al. demonstrated that blocking PD-1 and CTLA-4 significantly improved the efficacy of a vaccine comprising irradiated Flt3-ligand-secreting B16 cells [127]. The reduction in tumour growth was associated with an elevated CD8/Treg ratio in B16 tumours [127]. Similarly, combination of a GM-CSF-CT26 vaccine with anti-PD-L1 and anti-CTLA-4 induced significant tumour reduction in the CT26 tumour model [124]. Moreover, Ali et al. demonstrated that anti-CTLA-4 and anti-PD-1 each improved the efficacy of a vaccine comprising tumour lysates, GM-CSF and CpG in the B16 tumour model [126]. Anti-PD-1 and anti-CTLA-4 significantly elevated the levels of infiltrating IFN-γ-secreting CD8 T cells compared with mice that were treated with the vaccine alone [126].

Checkpoint blockade has also shown to improve the efficacy of therapies that do not directly target the immune system, such as radiotherapy and targeted therapies [258-260]. According to the database on clinicaltrials.gov, current clinical trials focus on checkpoint inhibitors in a variety of combinations. The outcome of these trials will give more insight into which combinations are suitable for which patients.

1.4.11.Prognostic markers for immunotherapy

With the advances made in immunotherapy and the remarkable responses observed for some patients upon immunotherapy, the search for prognostic markers for immunotherapy, and particularly checkpoint blockade, has become a major focus of research. Factors associated with poor immunotherapeutic responses to checkpoint blockade include age, HLA type, genetics and metabolism while T cell infiltration is associated with improved responses [261].

Pre-existing T cells in tumours of patients were suggested to be a prerequisite for successful immunotherapy with checkpoint inhibitors [262, 263]. Ji et al. analysed tumour samples from patients before and after Ipilimumab treatment and found that signs of pre-existing immune infiltration were associated with prolonged survival [264]. Moreover, Spranger et al. identified a correlation between WNT/β-catenin signalling and the absence of T cell infiltration in samples of human metastatic melanoma [265]. It

was demonstrated that β-catenin suppressed the recruitment of DCs in a murine tumour model, while reconstitution with activated pDCs restored responses to checkpoint blockade [265].

The expression of the checkpoint ligand PD-L1 is under investigation as a prognostic marker for immune checkpoint therapy. A meta-analysis of 20 clinical trials revealed that PD-L1 expression was associated with better clinical responses to treatment with PD-1 or PD-L1-blocking antibodies in metastatic melanoma and non-squamous NSCLC patients [266]. Dunne et al. observed a correlation between PD-L1 expression and immune cell infiltration in colorectal cancer patients which also correlated with better survival [267]. Moreover, aberrant PD-L1 expression on tumour cells has been associated with immune escape in multiple cancers [268].

Surprisingly, a proportion of patients with PD-L1-negative tumours also responded to anti-PD-1 therapy [266]. There are several possible explanations for this observation. The first reason could be that current techniques for PD-L1 detection by immunohistochemistry are not sensitive enough and wrongly categorised patients as PD-L1-negative. Second, structural variances of PD-L1 could lead to a failure in detecting PD-L1 on tumour samples [268]. Third, PD-1:PD-L1 interactions might play a role outside the tumour, such as LN during T cell priming. Fourth, PD-L1 expression on non-tumour cells might impair T cell function.

The expression of neoantigens has been associated with increased responsiveness to immunotherapy. Cancers with a high frequency of neoantigens, such as melanoma and NSCLC, responded best to checkpoint blockade [269, 270]. Interestingly, patients with lung cancer that were current or former smokers had a higher load of somatic mutations and responded better to checkpoint blockade than non-smokers. On the other hand, patients with colorectal, prostate or ovarian cancer displayed low levels of somatic mutations and responded poorly to immunotherapy [269].

The microbiome has recently sparked the interest of immunologists in many areas. It has been reported that the microbiome plays a crucial role in immunity to infections, autoimmunity and in immune responses against tumour cells [271]. In a recent landmark study, Vétizou et al. found that certain families of bacteria in the gut microbiome of patients were associated with improved responses to anti-CTLA-4 therapy [272]. Moreover, the composition of the microbiome has also been associated with the induction of side effects [273].

1.5. Aims

The aim of this project was to improve the understanding of immune evasion mechanisms elicited by tumours and to develop potent immunotherapeutic approaches to overcome immunosuppression. This project investigated combinations of different therapies that simultaneously activate DCs and antigen-specific CD8 T cells while reducing immunosuppressive mechanisms, such as induction of Treg cells or induction of T cell dysfunction.

Specifically, the use of TLR agonists in anti-tumour therapy was investigated. Albeit showing strong efficacy in activating DCs, research on TLR agonists has seen some setbacks in recent years, mostly due to the failure of overcoming immunosuppression. This thesis investigated the use of TLR agonists in different combination therapies, trying to identify combinations that improve TLR-induced responses. TLR agonists were tested in combination with a chemotherapeutic agent, as an adjuvant in a DC vaccine in combination with RARα inhibitor, and as an adjuvant in a cancer vaccine in combination with a checkpoint inhibitor.

The specific aims were:

- To examine anti-tumour and immune-modulatory effects of the cytotoxic agent BAMLET (bovine alpha-lactalbumin made lethal to tumour cells)
- To investigate anti-tumour activity of BAMLET with a TLR agonist in murine tumour models
- To examine the role of RARα in suppression of anti-tumour immune responses
- To investigate therapy combinations of a TLR-adjuvanted vaccine and a RARα antagonist in murine tumour models
- To examine the role of PD-1 in suppression of anti-tumour immune responses
- To investigate therapy combinations of TLR-adjuvanted vaccines and checkpoint blockade in murine tumour models

Chapter 2:

Materials and Methods

2.1. Materials

2.1.1. Buffers, media and solutions

Ammonium chloride lysis solution

0.77 g Ammonium chloride (NH_4Cl)
Dissolved in 100 ml dH_2O

cRPMI

Roswell Park Memorial Institute-1640 medium (RPMI; Sigma-Aldrich)
10% heat-inactivated fetal calf serum (FCS; Sigma-Aldrich)
100 mM L-glutamine (Sigma-Aldrich)
100 U/ml penicillin / 100 µg/ml streptomycin (Sigma-Aldrich)

cDMEM

Dulbecco's Modiefied Eagles Medium (DMEM; Sigma-Aldrich)
10% heat-inactivated FCS (Sigma-Aldrich)
100 mM L-glutamine (Sigma-Aldrich)
100 U/ml penicillin / 100 µg/ml streptomycin (Sigma-Aldrich)

ELISA blocking buffer

10 % (w/v) skimmed milk (Marvel) in phosphate buffered saline (PBS)
or
1 % (w/v) bovine serum albumin (BSA; Sigma-Aldrich) in PBS

ELISA washing buffer

0.5 l of 20x PBS
9.5 l dH_2O
5 ml Tween20 (Sigma-Aldrich)

ELISA substrate solution

25 ml phosphate citrate buffer
1 o-phenylenediamine dihydrochloride (OPD) tablet (10 mg; Sigma-Aldrich)
15 µl 1 M H_2O_2 (30 %; Sigma-Aldrich)

ELISA stop solution

26.74 ml 18 M sulfuric acid (H_2SO_4; Sigma-Aldrich)
473.26 ml dH_2O

MACS buffer

500 ml PBS
2.5 ml FCS
2 mM ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich)

Phosphate citrate buffer

20.23g anhydrous citric acid ($C_6H_8O_7$)
73.8g sodium phosphate dibasic ($Na_2HPO_4.12H_2O$)
made up to 2 l with dH_2O
pH 5

PBS 20x

800 g sodium chloride (NaCl)
116 g sodium phosphate (Na_2HPO_4)
20 g potassium phosphate (KH_2PO_4)
20 g potassium chloride (KCl)
5 l dH_2O
pH 7

Saponin buffer

0.5% (w/v) saponin (Sigma-Aldrich) in PBS

2.1.2. Cytokine ELISA

Cytokine	Blocking	Top working standard	Supplier
IFN-γ	10% milk	10 ng/ml	R&D
IL-10	1% BSA	2000 pg/ml	BD Pharmingen
IL-12p40	10% milk	5000 pg/ml	R&D
IL-12p70	1% BSA	1500 pg/ml	BD Pharmingen
IL-17a	1% BSA	1000 pg/ml	BD Pharmingen
IL-1β	1% BSA	1000 pg/ml	BD Pharmingen
IL-23	1% BSA	2500 pg/ml	BD Pharmingen
IL-6	10% milk	5000 pg/ml	R&D
TGF-β	1% BSA	2000 pg/ml	BD Pharmingen
TNF	1% BSA	2000 pg/ml	BD Pharmingen

2.1.3. FACS antibodies

Specificity	Fluorochrome	Clone	Ig subtype	Supplier
CD11b	PerCP-Cy5.5	M1/70	Rat IgG2b, κ	eBioscience
CD11c	PE-Cy5	N418	AH IgG	eBioscience
CD11c	BV786	N418	AH IgG	Biolegend
CD11c	PE-Cy7	N418	AH IgG	eBioscience
CD19	PE-Cy7	eBio1D3	Rat IgG2a, κ	eBioscience
CD19	FITC	1D3	Rat IgG2a, κ	eBioscience
CD19	PE-CF594	1D3	Rat IgG2a, κ	BD Biosciences
CD25	PE-Cy7	PC61.5	Rat IgG1, λ	eBioscience
CD3	A780	17A2	Rat IgG2b, κ	eBioscience
CD3	e450	145-2C11	AH IgG	eBioscience
CD3	BV650	17A2	Rat IgG2b, κ	Biolegend
CD4	A700	RM4-5	Rat IgG2a, κ	eBioscience
CD4	A780	RM4-5	Rat IgG2b, κ	eBioscience
CD40	FITC	03/23	Rat IgG2a, κ	BD Biosciences
CD45	BV605	30-F11	Rat IgG2b, κ	Biolegend
CD45	PE	30-F11	Rat IgG2b, κ	eBioscience
CD45.2	PE	104	Mouse IgG2a, κ	eBioscience
CD80	PE	16-10A1	AH IgG	Biolegend
CD86	PE-Cy5	GL1	Rat IgG2a, κ	eBioscience
CD86	APC-Cy7	GL1	Rat IgG2a, κ	eBioscience
CD86	A700	GL1	Rat IgG2a, κ	eBioscience
CD8a	A780	53-6.7	Rat IgG2a, κ	eBioscience
CD8a	FITC	53-6.7	Rat IgG2a, κ	eBioscience
F4/80	PE-Cy5.5	BM8	Rat IgG2a, κ	Biolegend
Foxp3	APC	FJK-16s	Rat IgG2a, κ	eBioscience
Foxp3	PE	NRRF-30	Rat IgG2a, κ	eBioscience
Granzyme B	FITC	NGZB	Rat IgG2a, κ	eBioscience
IFN-γ	PE-Cy7	XMG1.2	Rat IgG1, κ	eBioscience
IFN-γ	PerCP-Cy5.5	XMG1.2	Rat IgG1, κ	eBioscience
IL-10	PE	JES5-16E3	Rat IgG2b, κ	eBioscience
IL-10	BV421	JES5-16E3	Rat IgG2b, κ	Biolegend
IL-17	PerCP-Cy5.5	eBio17B7	Rat IgG2a, κ	eBioscience
Ki-67	e450	SolA15	Rat IgG2a, κ	eBioscience
LAG-3	PE-Cy7	eBioC9B7W	Rat IgG1, κ	eBioscience
MHC II	e450	M5/114.15.2	Rat IgG2b, κ	eBioscience

MHC II	APC	M5/114.15.2	Rat IgG2b, κ	eBioscience
PD-1	BV785	29F.1A12	Rat IgG2a, κ	Biolegend
PD-L1	APC	10F.9G2	Rat IgG2b, κ	Biolegend
PD-L2	PE	TY25	Rat IgG2a, κ	Biolegend
RORγT	PE	B2D	Rat IgG1, κ	eBioscience
T-bet	PE-Cy7	eBio4B10	Mouse IgG1	eBioscience
T-bet	PE-Cy5.5	eBio4B10	Mouse IgG1	eBioscience
TNF	PE-Cy7	TN3-19.12	AH IgG	eBioscience
TRP-2 Tetramer	A647	-	-	NIH tetramer facility

2.1.4. Real-time PCR primers

Gene	Protein	Product code	Supplier
18sRNA	-	4319413E	Applied Biosystems
Arg-1	Arginase 1	Mm00475988_m1	Applied Biosystems
ICAM-1	ICAM-1	Mm00516023_m1	Applied Biosystems
NOS2	iNOS	Mm00435175_m1	Applied Biosystems
PTGS2	PTGS2	Mm00478374_m1	Applied Biosystems
RARα	RARα	Mm00436264_m1	Applied Biosystems
RARβ	RARβ	Mm01319677_m1	Applied Biosystems
RARγ	RARγ	Mm00441091_m1	Applied Biosystems
VEGFa	VEGFa	Mm01281449_m1	Applied Biosystems
Chi313	YM-1	Mm00657889_mH	Applied Biosystems

2.1.5. TLR agonists

TLR agonist	TLR	Dose in vitro	Dose in vivo	Supplier
CpG	9	5 μg/ml	N/A	Sigma-Aldrich
LP1569	2	1 μg/ml	50 μg	EMC microcollections
LPS	4	100 ng/ml	N/A	Alexis
R848	7/8	100 ng/ml	40 μg	Enzo Life Sciences

2.1.6. Antibodies

Antibody	Clone	Isotype	Dose in vitro	Dose in vivo	Supplier
Anti-CD28	37.51	SH IgG2, λ1	3 – 5 µg/ml	-	BD Pharmingen
Anti-CD3e	145-2CII	AH IgG	1 – 3 µg/ml	-	BD Pharmingen
Anti-IFN-γ	R4-6A2	Rat IgG1,κ	10 µg/ml	-	BD Pharmingen
Anti-PD-1	RMP1-14	Rat IgG2a	1 – 10 µg/ml	200 µg	Bioceros
Anti-β-GAL	GL117	Rat IgG2a	1 – 10 µg/ml	200 µg	Bioceros

2.1.7. Antigens

Antigen	Sequence	Dose in vitro	Dose in vivo	Supplier
Ovalbumin (OVA) protein	-	-	100 µg	Hyglos
$OVA_{323-339}$	ISQAVHAAHAEINEAGR	1 µg/ml	-	New England Peptide
TRP-2_{58-78}	Ac-RGQCAEVQTDTRPWSGPYIL R-amide	-	100 µg	GenScript
TRP-$2_{148-165}$	Ac-AKKSIHPDYVITTQHWLG-amide	-	100 µg	GenScript
TRP-$2_{180-188}$	SVYDFFVWL	1 – 25 µg/ml	100 µg	GenScript

2.1.8. Recombinant cytokines

Cytokine	Dose in vitro	Supplier
IL-2	10 ng/ml	Immunotools
IFN-γ	100 – 500 ng/ml	BD Pharmingen
TGF-β	4 – 100 ng/ml	Immunotools
GM-CSF	20 ng/ml	J558 supernatants
M-CSF	20 % (v/v)	L929 supernatants

2.1.9. Other reagents

Reagent	Dose in vitro	Dose in vivo	Supplier
GDC0941 (GDC)	1 µM	N/A	LC laboratories
Retinoic acid (RA)	100 nM	200 µg	Enzo Life Sciences
Ro-41-5253 (RARi)	5 µM	400 µg	Enzo Life Sciences
SB203580 (SB203)	1 µM	N/A	LC laboratories

2.1.10.BAMLET

BAMLET was kindly provided by Dr. N. Harte and Prof. K. H. Mok from the School of Biochemistry and Immunology, Trinity College Dublin. BAMLET was generated as previously described [274]. BAMLET was used *in vitro* at a concentration of 0.1 mg/ml where not otherwise stated. BAMLET was used *in vivo* at a dose of 1 mg per mouse per injection in 10 % DMSO in PBS.

2.1.11.Oleic acid (OA)

To obtain an OA stock solution (≙ 141.61 mM), 200 mg OA (224 µl; Sigma-Aldrich) was mixed with 500 µl of 100 % EtOH by vortexing and topped up with 5 ml 10 mM Tris (pH 8.5). The solution was subsequently sonicated with a probe sonicator (50 % intensity, 5 minutes (min), on ice). Vehicle controls were sonicated as described for OA. OA was used *in vitro* at concentrations ranging from 4-100 µM.

2.2. Mice

Female BALB/c mice were purchased from Harlan Laboratories, Bicester, UK. Female C57BL/6 mice were purchased from Harlan Laboratories, Bicester, UK or bred in house in the Comparative Medicine Unit in Trinity College Dublin. Ovalbumin (OVA) TCR transgenic (DO11.10), C3H/HeN and C3H/HeJ mice were bred in house in the Comparative Medicine Unit in Trinity College Dublin. Mice were bred and housed under specific pathogen-free conditions and maintained according to European Union regulations. Animal experiments and maintenance were approved and regulated by the university ethics committee and the Health Products Regulatory Authority Ireland. Mice were age-matched for each experiment and typically used at 6-12 weeks of age. Mice were sacrificed using a CO_2 chamber and cervical dislocation.

2.3. Methods

2.3.1. Cell culture

The B16.F10 murine melanoma and CT26 murine colon carcinoma cell lines (ATCC, Manassas, VA) were typically cultured in 50 ml cDMEM in T175 culture flasks. Cells were grown until they reached 70-90% confluency and passaged every 2-3 days. Medium was removed and cells gently washed with PBS. After incubation with 4 ml trypsin/EDTA (0.25%; Sigma-Aldrich) for 5 min at 37° C, trypsin was neutralised by addition of cDMEM. Subsequently, cells were split in a ratio of 1:10 in cDMEM.

2.3.2. Freezing of cells

Cells were grown to a confluency of 70%, detached with 4ml trypsin/EDTA and resuspended in freezing medium (cDMEM + 5% DMSO) at a concentration of 1-2 x 10^6 cells/ml. Cells were frozen in cryotubes with 1 ml cells/tube and brought down to -80°C using a freezing container. Cryotubes were stored in liquid nitrogen.

2.3.3. Cell counting

Cells were counted using a hemocytometer. Cells were diluted in trypan blue (0.4%; Sigma-Aldrich) in a final volume of 100 µl. The hemocytometer chamber was loaded with 10 µl per sample. All squares were counted and the cell number determined using the following formula:

#cells / #squares * dilution factor * 10^4 / ml

2.3.4. Tumour models

To induce tumours, B16.F10 cells were injected into C57Bl/6 mice and CT26 cells were injected into BALB/c mice. Typically, cells were cultured to a confluency of 70 % and harvested during the log growth phase. Harvested cells were washed 3 times with sterile PBS, counted and the cell concentration adjusted to 2 x 10^6 (B16) or 3 x 10^6 (CT26) cells/ml in PBS. Mice were shaved and 100 µl of the tumour cell suspension was injected s.c. in the right flank. Tumour growth was monitored and tumour size measured every 2-3 days using callipers, measuring the diameters (D) of the tumour. Tumour size was calculated using the following formula: $(D1)^2$ x (D2/2), D1 being the smaller value. Mice were sacrificed when the tumour size exceeded 15 mm or tumours

became ulcerous. Tumour-bearing mice were randomised before therapeutic treatments.

2.3.5. Prophylactic tumour vaccine

For prophylactic vaccination, mice were immunized with TRP-$2_{180-188}$ (100 µg/mouse) and R848 (40 µg/mouse) 5 weeks and 1 week prior to tumour induction. Where indicated, mice were subsequently injected intraperitoneally (i.p.) with anti-PD-1 (200 µg/mouse) or isotype control (anti-β-GAL; 200 µg/mouse) on days 3, 10 and 17 post tumour induction.

2.3.6. Therapeutic tumour vaccine

Tumour peptide vaccines comprised TRP-$2_{180-188}$ (100 µg/mouse) and where indicated TRP-2_{58-78} (100 µg/mouse), TRP-$2_{148-165}$ (100 µg/mouse) and R848 (40 µg/mouse). Unless otherwise stated, the vaccine was injected s.c. in a volume of 100 µl into the tumour site 3, 10 and 17 days after tumour induction. Anti-PD-1 (200 µg/mouse) was injected i.p. 2, 9 and 16 days after tumour induction.

Tumour lysate vaccines comprised heat shocked and irradiated (hs/irr) B16.F10 or CT26 tumour cells and R848. Tumour cells were heat-shocked (43° C for 1 hour (h)), then γ-irradiated (200 Gy) and washed 3 times with PBS. R848 (40 µg/mouse) and hs/irr CT26 cells (10^6 cells/mouse) were injected s.c. in a volume of 100 µl into the tumour site 3, 10 and 17 days after tumour induction. Anti-PD-1 (200 µg/mouse) was injected i.p. 2, 9 and 16 days after tumour induction.

2.3.7. Therapeutic treatment with BAMLET

C57BL/6 mice were injected with B16.F10 tumour cells. Mice were injected s.c. in the area of the tumour with vehicle (10% DMSO in PBS), BAMLET (1 mg/mouse) and/or R848 (40 µg/mouse) on days 3, 7, 10 and 14 post tumour induction.

2.3.8. TRP-2 immunization

Mice were injected with TRP-$2_{180-188}$ (100 µg) and/or R848 (40 µg) into the footpads. On day 7 after immunization, popliteal LN were dissected and single-cell suspensions prepared by filtering the LN through a 70 µm nylon mesh. To detect antigen-specific T cells, LN cells were stained by tetramer staining and analysed by flow cytometry. For

antigen recall, cells were counted and plated on a 96-well plate at a concentration of 10^6 cells/ml. TRP-$2_{180-188}$ peptide was added at increasing concentrations (1-25 µg/ml). After 5 days, supernatants were removed and the concentration of secreted cytokines quantified by ELISA.

2.3.9. Tumour cell stimulation

Tumour cells were seeded in 96-well flat bottom plates at a concentration 5 x 10^4 cells in 200 µl cDMEM per well. Cells were incubated overnight (O/N) to attach, medium removed and cells treated as required in fresh cDMEM. For ELISA, supernatants were removed after 24 h. For flow cytometric analysis, non-adherent cells were removed first by repeat pipetting. Adherent cells were detached using trypsin/EDTA for 5 min at 37°C and pooled with non-adherent cells.

2.3.10.Tumour killing assay

The tumour killing assay was performed in order to determine the cytotoxic activity of immune cells against tumour cells. In this assay, cultured tumour cells were harvested and stained with CellTrace Violet (CTV). CTV-stained tumour cells were co-cultured with effector cells in different effector:target (E:T) ratios (2:1, 10:1, 50:1). After 5 h or 24 h, cells were harvested from the plate. Live tumour cells were detached from the plate by incubation with trypsin/EDTA for 5 min at 37° C. Harvested cells were washed and stained with 7-AAD (BD Pharmingen) or L/D aqua to detect dead cells. Cell death was analysed by flow cytometry.

2.3.11.CTL assay

The CTL assay was performed in order to determine if tumour-specific T cells from tumour bearing mice can be expanded with the TRP-$2_{180-188}$ peptide. For this, 3-5 mice were injected with 2 x 10^5 B16 tumour cells/mouse. On day 14, spleens were isolated from tumour-bearing and naïve mice. Isolated splenocytes (1.5 x 10^7 cells) were cultured in 7 ml cRPMI containing 10 ng/ml IL-2 with or without TRP-21_{80-188} (20 µg/ml) in T25 cell culture flasks at 37°C. After 5 days of stimulation, cells were washed, counted and co-cultured with B16 tumour cells at different E:T ratios (25:1, 50:1, 100:1). The tumour killing assay was performed as described above.

2.3.12.Generation of GM-CSF from J558 cell line

GM-CSF was obtained from culture supernatants of the GM-CSF transfected J558 plasmacytoma cell line. Thawed J558 cells were resuspended at a concentration of 0.5-1 x 10^6 cells/ml and in recovery medium containing 1 mg/ml of the selection antibiotic G418 (Geneticin; Sigma-Aldrich). Cells were expanded until they were 80 % confluent and passaged twice in medium containing G418 (1 mg/ml). After 2-3 passages the cells were expanded to produce GM-CSF. Cells were seeded at 2 x 10^5 cells/ml in T175 flasks without G418. After 3 days, conditioned medium was collected and cells re-seeded at 2 x 10^5 cells/ml without G418. After 3 days, the conditioned medium was collected as above. Conditioned medium was collected from a total of 3 passages and the concentration of GM-CSF quantified by ELISA.

2.3.13.Bone marrow derived dendritic cell (BMDC) culture

Femurs and tibiae were isolated from naïve C57BL/6 mice and surrounding tissues removed. Ends of bones were cut open and bone marrow flushed with cRPMI using a 27 G needle. Cell aggregates were broken up by gently pipetting up and down. Cells were spun down at 1250 rpm for 5 min and resuspended in 2 ml of filter-sterilised lysis buffer. After 2 min, 18 ml of cRPMI was added and cells spun down. Supernatants were decanted and the pellet resuspended in 10 ml of medium and counted. Cells were seeded at a concentration of 0.5-0.7 x 10^6 cells /ml in T175 tissue culture flasks (25 ml per flask) in cRPMI containing 20 ng/ml GM-CSF. On day 3, 25 ml cRPMI + 20 ng/ml GM-CSF were added per flask. On day 6, supernatants were gently removed and 25 ml of fresh cRPMI + 20 ng/ml GM-CSF was added to each flask. On day 8, 25 ml cRPMI + 20 ng/ml GM-CSF was added per flask. On day 10, loosely adherent cells were removed by gently repeat pipetting and spun down. Supernatants were decanted and cell pellets from all flasks pooled in 10 ml of medium. Cells were counted, plated out a concentration of 10^6 cells/ml in cRPMI + 10 ng/ml GM-CSF and treated the next day in fresh medium without GM-CSF and as required. Alternatively, cells were plated out without GM-CSF, left to adhere for at least 30 min and subsequently treated as required on the same day.

2.3.14.Bone marrow derived macrophage (BMDM) culture

For the generation of BMDMs, bone marrow cells were cultured in M-CSF which was obtained from culture supernatants of the M-CSF transfected L929 cell line. Bone

marrow from femurs and tibiae of naïve C57BL/6 mice were flushed using a 27 G needle and cell aggregates broken up. Cells were spun down at 1250 rpm for 5 min and resuspended in 2 ml of filter-sterilised lysis buffer. After 2 min, 18 ml of cRPMI was added and cells spun down. Supernatants were decanted and the pellet resuspended in 10 ml of medium and counted. Bone marrow cells were seeded at a concentration of 10^6 cells/ml in 10 ml cRPMI containing 20% M-CSF/L929 supernatants in 10 cm petri dishes. On day 3, 2 ml of M-CSF/L929 supernatants was added to each petri dish. On day 6, culture supernatants were poured off and 5 ml fresh medium added. Cells were gently scraped off the petri dishes and transferred to a 50 ml tube. Cells were spun down at 1250 rpm for 5 min and resuspended in 10 ml cRPMI + 10% M-CSF/L929 supernatants, counted and plated at a concentration of 10^6 cells/ml. The next day, BMDM were treated as required.

2.3.15.Magnetic-activated cell sorting (MACS)

CD4 T cells were isolated from spleens by MACS using the $CD4^+$ T cell negative isolation kit (Miltenyi Biotec) according to manufacturer's instructions. Briefly, cells were resuspended in 40 µl MACS buffer and 10 µl antibody cocktail per 10^7 total cells. The suspension was mixed and incubated for 5 min at 4° C. Subsequently, 30 µl MACS buffer and 20 µl anti-Biotin MicroBeads per 10^7 total cells were added, cells mixed and incubated for 10 min at 4° C. An LS column was placed in a MACS separator and rinsed with 3 ml of MACS buffer. The cell suspension was added to the column and the flow-through containing enriched CD4 T cells was collected. The column was washed with 3 ml of MACS buffer and the flow-through collected and pooled with the first flow-through. The purity of isolated CD4 T cells was analysed by flow cytometry and was typically higher than 80% (Figure 2.1).

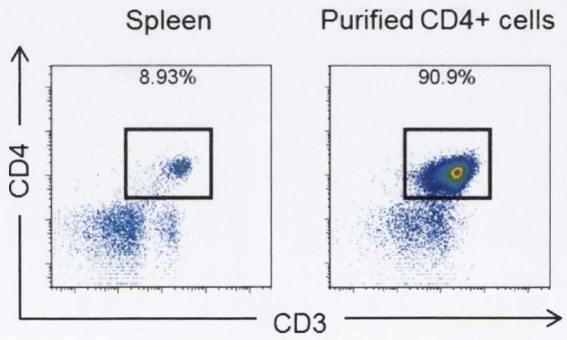


Figure 2.1: Purity of isolated CD4 T cells analysed by flow cytometry

2.3.16.Treg conversion assay

CD4 T cells were purified from spleens of naïve mice by MACS. Cells were cultured with plate-bound anti-CD3 (1 µg/ml), soluble anti-CD28 (5 µg/ml) and IL-2 (10 ng/ml) and, where indicated, TGF-β (5 ng/ml) and RA (100 nM). Where indicated, cells were pre-treated with RARi (5 µM) 15 min prior to stimulation. Alternatively, spleen cells from naïve mice were cultured with plate-bound anti-CD3 (1 µg/ml) and soluble anti-CD28 (5 µg/ml), and where indicated, TGF-β (5 ng/ml) and RA (100 nM) in presence or absence of anti-PD-1 (10 µg/ml) and anti-IFN-γ (10 µg/ml). After 3 days, cells were harvested and Foxp3 expression analysed by flow cytometry

2.3.17.Treg conversion of OVA-immunized LN cells

OVA TCR transgenic mice were immunized with OVA protein (100 µg) and CpG (50 µg) into the footpads and popliteal LN were removed 7 days post-immunization. LN cells were cultured with $OVA_{323-339}$ peptide (1 µg/ml) and IL-2 (10 ng/ml) and, where indicated, TGF-β (5 ng/ml) and RA (100 nM). After 3 days, cells were harvested and Foxp3 expression analysed by flow cytometry

2.3.18.DC and CD4 T cell co-culture

BMDCs were pre-stimulated with RARi (5 µM) or vehicle for 15 min and subsequently treated with CpG (5 µg/ml) and/or RA (100 nM). After 24 h, DCs were harvested, washed, counted and co-cultured with MACS-purified CD4 T cells at a ratio of 1:1 with plate-bound anti-CD3 (1 µg/ml), soluble anti-CD28 (5 µg/ml), IL-2 (10 ng/ml) and TGF-β (5 ng/ml).

2.3.19.T cell polarisation

CD4 T cells were purified from spleens of naïve mice by MACS. T cells were stimulated in 96-well plates with plate-bound anti-CD3 (3 µg/ml) and soluble anti-CD28 (3 µg/ml). Th1-polarised cells were stimulated with IL-12 (10 ng/ml). Th17-polarised cells were treated with IL-1β (10 ng/ml) and IL-23 (10 ng/ml). After 72 h, supernatants were removed for ELISA. Intracellular cytokines were analysed by flow cytometry.

2.3.20.Surface and intracellular cytokine staining for flow cytometry

For the analysis of intracellular cytokines, cells were stimulated with phorbol 12-myristate 13-acetate (PMA; 10ng/ml; Sigma-Aldrich), ionomycin (500 ng/ml; Sigma-Aldrich) and brefeldin A (BFA; 5 µg/ml; Sigma-Aldrich). After stimulation for 4.5-6 h, cells were washed with PBS and incubated with 50 µl LIVE/DEAD Fixable Aqua Stain (L/D aqua; 1:600 in PBS; Life Technologies) for 20 min at room temperature (RT). Subsequently, cells were washed with PBS and stained with 50 µl surface antibodies (1:200 – 1:600 in PBS) and Fcγ block (1:200 in PBS; BD Pharmingen) for 20 min at RT. Cells were washed with PBS and then fixed with 50 µl paraformaldehyde (2 %; Thermo Fisher Scientific) for 5-10 min at RT. Fixed cells were washed with PBS and resuspended in 200 µl saponin buffer and spun down for 5 min. Supernatants were decanted and cells resuspended in 50 µl of intracellular cytokine antibodies (1:200) in saponin buffer. Cells were incubated for 20 min at RT and subsequently washed in saponin buffer and then in PBS for membrane closure. Cells were resuspended in PBS and analysed by flow cytometry.

If no cytokine staining was performed, cells were washed with PBS and stained with L/D aqua (1:600 in PBS) and surface antibodies (1:200 – 1:600 in PBS) and Fcγ block (1:200 in PBS) as described above. Cells were washed and directly analysed by flow cytometry or fixed with 2% paraformaldehyde in PBS and stored at 4°C until analysis. Flow cytometry results were analysed using FlowJo software.

2.3.21.Intranuclear staining for flow cytometry

Transcription factor staining was performed using the Foxp3 staining buffer set (eBioscience). Cells were washed in PBS and stained with 50 µl L/D aqua (1:600 in PBS) for 20 min at RT and subsequently washed with PBS. Surface antibodies were added to each sample (50 µl; 1:200-1:600 in PBS). After incubation for 20 min at RT, cells were washed with PBS and resuspended in 200 µl Fix/Perm solution. After incubation for 30 min at RT or at 4°C O/N, plates were centrifuged at 1250 rpm and supernatants decanted. Cells were resuspended in perm buffer and spun down at 1250 rpm. Subsequently, cells were stained with 50 µl of antibodies staining transcription factors (1:100 in perm buffer) at RT. If required, antibodies staining intracellular cytokines were added at this step. After 30 min incubation, cells were washed with perm buffer, then PBS and kept at 4°C until flow cytometric acquisition.

2.3.22.In vivo staining of circulating leukocytes

For analysis of tumour-resident or tumour-circulating immune cells, mice were injected with CD45.2-PE (1.5 µg/mouse in 200 µl PBS) intravenously (i.v.) into the tail veins and killed 10 min later. With this method, only leukocytes in the circulation were stained, since the antibodies do not penetrate the tissue within this time frame. This method was described first in a publication by Christensen et al [275].

2.3.23.TIL analysis

Tumours, spleens and LN were removed from tumour-bearing mice. Spleens and LN were processed through a 70 µm nylon mesh with cRPMI. Tumours were chopped into small pieces and digested with DNAse I (20 U/ml; Roche) and collagenase D (1mg/ml; Sigma-Aldrich) in Hank's balanced salt solution (HBSS; Sigma-Aldrich) or cRPMI for 1 h. Subsequently, tumour cells were filtered through a 100 µm nylon mesh and topped up with cRPMI. Single cell suspensions from spleens, LN and tumours were stimulated with PMA (10 ng/ml), ionomycin (500 ng/ml) and BFA (5 µg/ml) for 4.5-6 h and subsequently stained for intracellular cytokines. Alternatively, cells were directly stained for surface markers and analysed by flow cytometry.

2.3.24.Tetramer staining

Tetramer staining was performed to detect antigen-specific T cells [276]. The present study used a tetramer consisting of four MHC I molecules carrying TRP-$2_{180\text{-}188}$ peptides bound to fluorescently-labelled streptavidin. For tetramer staining, cells were washed and resuspended in PBS containing tetramers (1:500) and incubated for 30 min at RT. Cells were subsequently washed and stained with FACS antibodies as required.

2.3.25.CTV staining

Cells were stained with CTV using the CellTrace Violet Cell Proliferation Kit (Life Technologies) according to manufacturer's instructions. Briefly, cells were washed and resuspended in PBS at a concentration of 10^6 cells/ml. The appropriate amount of CTV reagent (stock concentration: 5mM) was added while vortexing to obtain a final concentration of 1 µM. After incubation for 20 min at 37°C in the dark, five times the original volume of pre-warmed complete medium were added and incubated for 5 min

at RT. Cells were spun down and resuspended in pre-warmed medium and counted for cell culture.

2.3.26.ALDEFLUOR assay

The ALDEFLUOR assay was performed to detect ALDH activity using the ALDEFLUOR™ Kit (Stemcell Technologies) according to manufacturer's instructions. Briefly, cells were stimulated as required in a 96-well plate. After stimulation, cells were centrifuged at 1250 rpm for 5 min and supernatants discarded. Cells were resuspended in 200 µl ALDEFLUOR assay buffer containing ALDEFLUOR reagent (1.5 µM) with or without DEAB (15-75 µM). Cells were incubated for 30-60 min at 37° C and subsequently centrifuged. Supernatants were discarded and cells resuspended in 200 µl of cold ALDEFLUOR assay buffer and stored at 4°C until flow cytometry measurements. Alternatively, cells were washed in ALDEFLUOR assay buffer after ALDEFLUOR incubation and stained with FACS antibodies before flow cytometry measurements.

2.3.27.Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was performed to quantify cytokines from cell stimulations. High-binding 96-well ELISA plates were coated with 50 µl diluted coating antibodies in PBS and incubated O/N at 4° C or for 2 h at 37° C. Plates were then aspirated and washed 4 times with washing buffer. Plates were blocked by adding 200 µl of blocking solution to each well and incubated at RT for 2-3 h. Plates were subsequently washed 4 times with washing buffer. 50 µl of sample or standards in 1 % BSA in PBS were added per well and incubated for 2 h at RT or O/N at 4°C. After washing the plates 4 times, 50 µl of the detection antibody diluted in 1 % BSA was added to each well and incubated for 1-2 h at RT. Plates were washed 4 times and 50 µl of Streptavidin-HRP diluted in 1 % BSA was added to each well and incubated for 20 min at RT in the dark. Plates were washed 4 times and 50 µl of substrate solution was added to each well and incubated for approximately 20 min at RT in the dark. To stop the reaction, 50 µl of stop solution was added to each well. The optical density (OD) of each well was measured using a microplate reader at a wavelength of 490 nm. Cytokine concentrations were calculated from OD values using SoftMaxPro software.

2.3.28.RNA isolation with TRIzol

Tissues and cells were resuspended in 1 ml of TRIzol (Life Technologies) in sterile RNAse-free tubes and homogenised. At this stage, samples were stored at -80°C or isolation was continued. After addition of 200 µl of chloroform, tubes were vortexed and let stand at RT for 3 min. Samples were centrifuged at 12000 rpm for 15 min at 4°C. The aqueous phase was transferred to a new tube and 500 µl of cold isopropanol added, mixed and let stand at RT for 15 min. Samples were centrifuged at 12000 rpm for 15 min at 4°C and supernatants decanted. The pellet was washed twice with 600 µl of cold 75% EtOH. During the washing steps, samples were centrifuged at 7600 rpm for 5 min. EtOH was completely removed and pellets resuspended in 20 µl of RNAse-free water and samples stored at -80°C. RNA concentrations were measured using a Nanodrop.

2.3.29.Reverse transcription of RNA

RNA was transcribed into cDNA using the High Capacity cDNA reverse transcription kit (Applied BioSystems) according to manufacturer's instructions. Briefly, 1-2 µg of RNA were diluted in water in a final volume of 10 µl and mixed with 10 µl of 2 X RT master mix. Following thermal cycling conditions were used for the PCR reaction:

	Step 1	Step 2	Step 3	Step 4
Temperature	25°C	37°C	84°C	4°C
Time	10 min	120 min	5 min	∞

After the PCR cycle, 130 µl of RNAse free water was added to each sample.

2.3.30.Real-time PCR

Real-time PCR was performed using TaqMan Fast Universal PCR Master Mix (Applied BioSystems) according to manufacturer's instructions. Briefly, 4.5 µl of cDNA was mixed with 5.5 µl of master mix consisting of 4.5 µl master mix reagent + 0.5 µl reaction primers + 0.5 µl endogenous primers. The reaction was performed on triplicate samples and water instead of cDNA was used as negative control. The ΔΔCt method

was used to calculate relative changes in gene expression which were normalised to 18S rRNA expression.

	Step 1	Step 2 + Step 3 (45 cycles)	
Temperature	95°C	95°C	60°C
Time	20 sec	3 sec	30 sec

2.3.31.LAL (limulus amebocyte lysate) assay

The LAL assay was performed using the Pierce LAL Chromogenic Endotoxin Quantitation Kit (Thermo Scientific) according to manufacturer's instructions. Briefly, 50 µl of different BAMLET dilutions or standard samples were dispensed into a pre-warmed 96-well microplate and incubated at 37° C. After 5 min, LAL was added to each well and incubated for 10 min at 37° C. Subsequently, 100 µl of the substrate solution was added to each well and incubated at 37° C. After 6 min, 50 µl of Stop Reagent (25% acetic acid) was added to the wells and the absorbance measured at 405-410 nm on a microplate reader. Endotoxin concentrations were determined by comparison with a standard curve.

2.3.32.Polymixin B purification

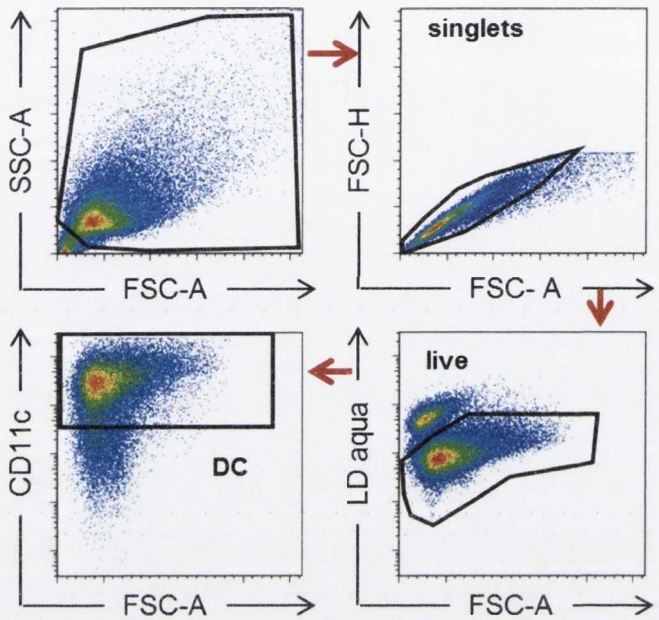
BAMLET was purified using Detoxi-Gel Endotoxin Removing Columns (Thermo Fisher Scientific), consisting of immobilized polymixin B gel, according to manufacturer's instructions. Briefly, the Detoxi-Gel resin was washed with 5 ml of 1 % sodium deoxycholate, followed by 5 ml of PBS to remove the detergent. The Detoxi-Gel resin was equilibrated with 5 ml PBS and 1 ml of the BAMLET sample was applied. Aliquots of PBS were added and the flow-through was collected. The protein concentration of the flow-through was determined using a Nanodrop.

2.3.33.Statistical analysis

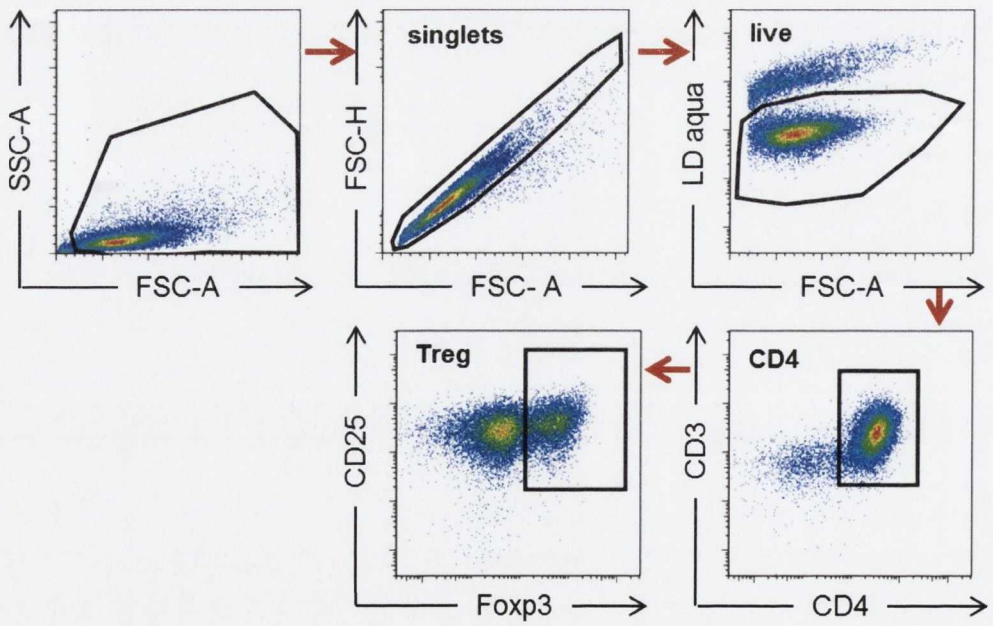
Statistical analysis was performed using GraphPad Prism 5. Statistical analysis between two groups was performed by Student's t test. Analysis of 3 groups or more was performed by analysis of variance (ANOVA). Tumour growth curves were compared by 2-way ANOVA. Bonferroni-post tests were used as post-hoc test to determine significance.

2.4. Flow cytometry gating strategies

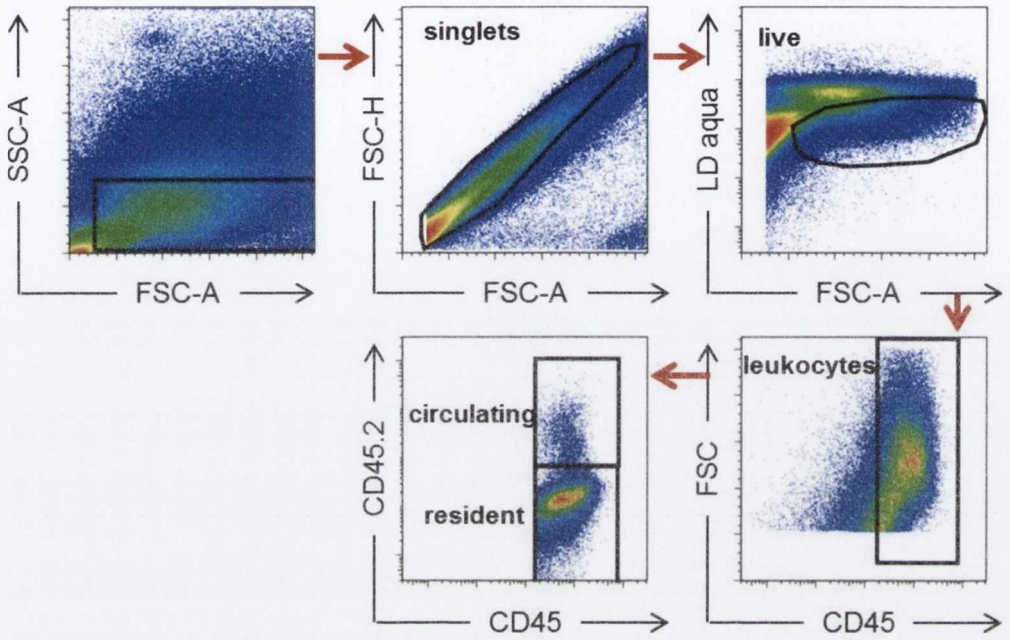
2.4.1. DC maturation



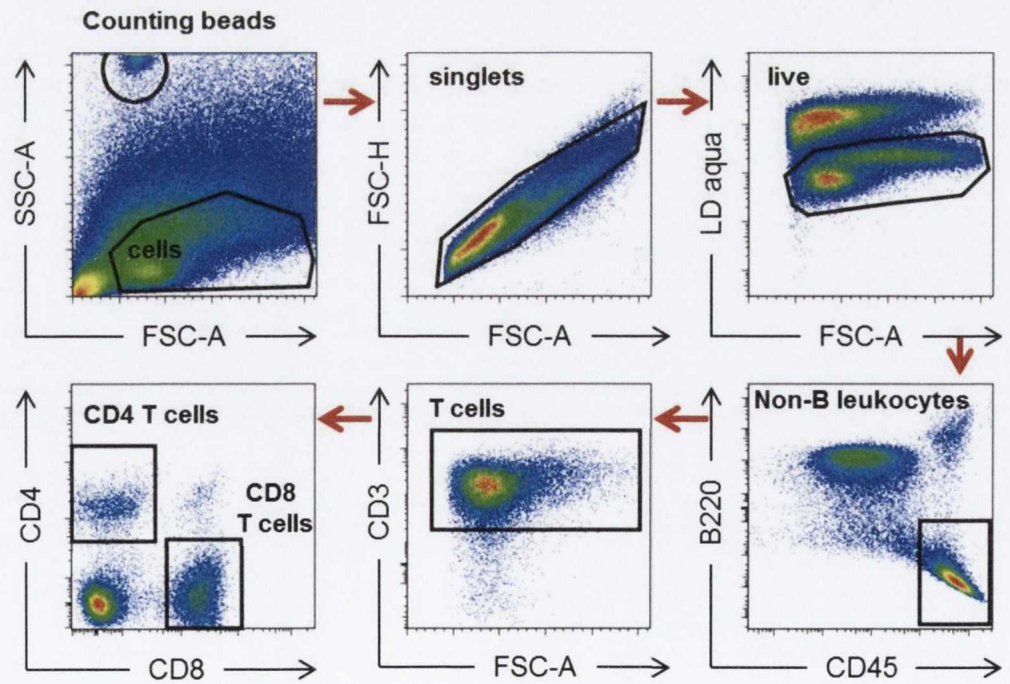
2.4.2. Treg conversion assay



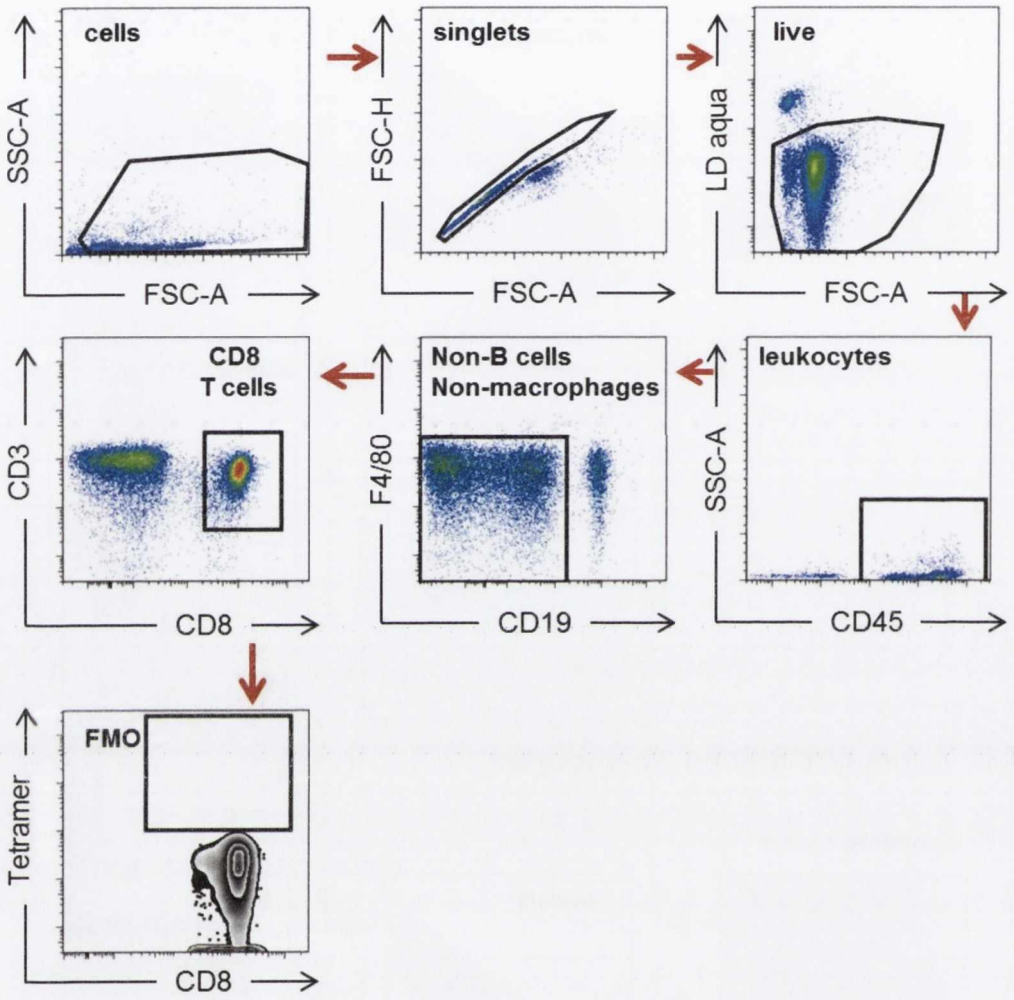
2.4.3. Tumour-resident and tumour-circulating leukocytes



2.4.4. Tumour-infiltrating lymphocytes



2.4.5. Tetramer staining



Chapter 3:

Anti-tumour and immune-modulatory properties of BAMLET

3.1. Introduction

Chemotherapy is the first line treatment for many cancers and employs drugs that kill tumour cells. Treatment of patients with chemotherapy leads to severe side effects, because it non-specifically targets rapidly dividing cells, including healthy cells. This has prompted the search for more specific drugs that kill tumour cells while leaving healthy cells unharmed. Strategies include targeted therapies, immunotherapies or the use of novel tumour-specific chemotherapeutic agents, such as protein fatty acid (PFA) complexes.

BAMLET is a PFA complex consisting of alpha-lactalbumin (α-LA) and oleic acid (OA) and is under investigation for the therapy of cancer. It is the bovine counterpart of HAMLET which was first discovered in human milk by Svanborg and colleagues in 1995 and was found to be cytotoxic against a multitude of tumour cells [277]. Human and bovine α-LA share 76% homology and divergence was found in regions that were not essential for fatty acid binding. Moreover, no apparent differences were found in their efficiency in inducing cell death [278]. It was reported that native α-LA has no cytotoxic activity and it was believed that the complex gained cytotoxic activity through a change in the protein conformation, which was achieved by removing the calcium normally bound to α-LA and adding the fatty acid OA [278, 279]. However, more recent studies suggested that OA forms the active component of the complex and that α-LA is required as a carrier or solubiliser [280-282].

The most promising property of BAMLET and related PFA complexes was the selectivity in only killing tumour cells, while leaving normal cells and tissues unharmed. This hypothesis was quickly challenged with the observation that primary cells and erythrocytes were also sensitive to BAMLET [280, 282]. Nevertheless, positive effects of HAMLET on tumour growth have been described in animal models and in clinical trials. In a rat xenograft model for glioblastoma, HAMLET infusions led to a significant reduction in tumour growth and apoptosis was detected by TUNEL staining [283]. In the first human clinical trial, patients with bladder cancer were treated with intravesical instillations of HAMLET. After 2 h, shedding of apoptotic tumour cells was observed in the urine of HAMLET-treated patients [284]. Furthermore, topical treatment of skin papillomas with HAMLET was associated with a reduction in lesion volume in 75% of patients, in contrast to 15% of patients in the vehicle-treated group [285]. These studies demonstrated *in vivo* anti-tumour activity of HAMLET.

Since its discovery in 1995, many studies have investigated the molecular mechanisms behind HAMLET and BAMLET-induced cell death, which remain a subject of debate [286]. However, no immune-modulatory effects of BAMLET have been described to date. The first aim of this chapter was to investigate anti-tumour and potential immune-modulatory effects of BAMLET *in vitro*.

Another strategy to specifically target tumour cells is immunotherapy. Immunotherapy involves the manipulation of the immune system to promote immune-mediated clearance of tumours and has become a promising approach for the treatment of cancer. In the majority of cases, combination immunotherapy yielded better results in clinical trials than single therapeutic agents [287]. Thus, current studies of immunotherapies focus on combinations of immune stimulators. Different approaches have been tested in targeting the immune system for cancer therapy. These include adoptive transfer of immune cells, cancer vaccination, checkpoint inhibition or stimulation of immune responses with cytokines or TLR agonists. TLR agonists have been widely tested in the context of immunotherapy, either as monotherapy or as adjuvants in cancer vaccines. TLR agonists mostly exert effects by activating APCs. TLR agonists upregulate the expression of co-stimulatory molecules (CD40, CD80, CD86) on DCs and induce the secretion of pro-inflammatory cytokines that can promote T cell mediated anti-tumour responses [198].

The second aim of this chapter was to investigate anti-tumour properties of two TLR agonists, the novel TLR2 agonist LP1569 and the TLR7/8 agonist R848. LP1569 is derived from *B.pertussis* and was recently discovered as a novel TLR2 agonist by Dunne and colleagues [288]. It has been demonstrated that LP1569 induced potent Th1 and Th17 responses *in vitro* and *in vivo*, conferring protection against *B.pertussis* infection in mice [288]. R848 is a more potent and soluble analogue of the imidazoquinoline Imiquimod [191, 192]. R848 is under investigation in cancer therapy due to the induction of pro-inflammatory cytokines and Th1 responses [193, 194].

The present study investigated the hypothesis that potent anti-tumour immune responses can be generated by combining a cytotoxic drug, which induces tumour cell death, with a TLR agonist, which stimulates the immune system. The effects BAMLET and TLR agonist stimulation on innate and adaptive immune cells were investigated *in vitro*. Moreover, the efficacy of a combination therapy consisting of BAMLET and a TLR agonist was evaluated *in vivo*.

3.2. Results

3.2.1. BAMLET induces rapid B16 tumour cell death in a dose-dependent manner

Effective immunotherapy requires the release of tumour antigens from dying tumour cells in order to generate tumour-specific immune responses. Tumour cell death can occur naturally, for example through a lack of nutrients or oxygen. More effectively, tumour cell death can be induced by external measures, for example by chemotherapy, targeted therapy or radiotherapy. This chapter focuses on the mechanisms by which the α-LA-OA complex BAMLET can mediate anti-tumour responses. First, the ability of BAMLET to directly induce tumour cell death was investigated *in vitro*. B16 tumour cells were incubated with increasing concentrations of BAMLET and cell death was visualised by PI staining. Dose-dependent cell death of B16 cells was observed within 5 h of stimulation and 50% cell death was achieved with a concentration of 0.5 mg/ml (Figure 3.1 A,B). After 24 h, the highest concentrations of 0.5 mg/ml and 1 mg/ml led to a significant reduction of live cells in the culture, while lower BAMLET concentrations showed no significant differences. These findings suggest that high doses of BAMLET induce rapid cell death and that after 24 h tumour cell proliferation exceeded BAMLET-induced cell death at lower concentrations (Figure 3.1B).

3.2.2. BAMLET-induced cell death is not tumour cell specific

Initial studies suggested that α-LA-OA complexes specifically kill tumour cells while leaving differentiated and normal cells unharmed [286]. In order to test this hypothesis, two different tumour cell lines, spleen cells and BMDCs were treated with increasing concentrations of BAMLET for 5 h and cell death was determined by PI staining. BAMLET induced dose-dependent cell death in both B16 and CT26 tumour cells, however, CT26 cells were more resistant to cell death than B16 cells. While a dose of 0.5 mg/ml killed 60% of B16 cells, the same dose induced cell death in only 20% of CT26 cells. Interestingly, spleen cells and BMDCs were more susceptible to BAMLET-induced cell death than tumour cells, showing nearly 100% cell death at a BAMLET concentration of 0.25 mg/ml (Figure 3.2 A,B). These results suggest that BAMLET-induced cytotoxicity is not tumour- specific and also targets spleen cells and BMDCs.

3.2.3. TLR agonists induce BMDC maturation marker expression and pro-inflammatory cytokine secretion which is enhanced in presence of BAMLET

Having shown that BAMLET was capable of promoting cell death, it was then assessed whether the presence of BAMLET could alter immune responses. First, the effects of BAMLET stimulation on BMDCs were examined. DCs are important for the priming and activation of effector T cells. In order to prime effector T cells, DCs have to be activated and undergo maturation. The best known activators of DCs are TLR agonists. BMDCs were stimulated with two different TLR agonists, LP1569 and R848, in presence or absence of BAMLET. BAMLET was used at a concentration of 0.1 mg/ml and BMDC viability was over 90% at the end of the culture. Both TLR agonists LP1569 and R848 enhanced the expression of MHC II and the co-stimulatory molecules CD40, DC80 and CD86 on BMDCs. Interestingly, stimulation of BMDCs with BAMLET alone had the same effect as TLR stimulation and led to enhanced expression of MHC II, CD40, CD80 and CD86 (Figure 3.3A).

LP1569 and R848 promoted the secretion of the pro-inflammatory cytokines IL-12p70, IL-12p40, TNF and IL-6 and low concentrations of the anti-inflammatory cytokines TGF-β and IL-10 by BMDCs. The concentration of secreted cytokines was higher when BMDCs were stimulated with R848 than LP1569, suggesting that R848 is a more potent DC activator. When BMDCs were stimulated with BAMLET alone, secretion of IL-12p70, IL-12p40, TNF and IL-6 was detected at concentrations similar to stimulations with R848 alone. Interestingly, the concentration of all secreted cytokines tested here was significantly increased when LP1569 or R848 were used in combination with BAMLET. Moreover, the combination of BAMLET with R848 had a strong synergistic effect on IL-12p70 secretion (Figure 3.3B). These findings suggest that BAMLET can promote DC activation similar to TLR agonists and enhance TLR-induced cytokine secretion.

3.2.4. OA alone does not induce BMDC maturation or cytokine secretion

Recent studies suggested that OA is the active component of α-LA-OA complexes [280-282]. Moreover, it was shown that OA alone can induce cell death in a micellar conformation in absence of complex formation [281]. The next experiment was set out to determine whether OA alone can stimulate DC maturation and cytokine secretion. OA or a vehicle control were sonicated according to the protocol for the preparation of BAMLET. BMDCs were stimulated with OA or vehicle for 24 h and BMDC maturation

and cytokine secretion were measured. R848 was used as a positive control and induced the expression of MHC II, CD40, CD80 and CD86 on BMDCs (Figure 3.4A). Consistent with previous results, R848 also promoted the secretion of IL-12p40, TNF and IL-6 (Figure 3.4B). BMDCs stimulated with OA displayed the same expression levels of MHC II and co-stimulatory molecules as the medium or vehicle controls (Figure 3.4A). Moreover, no cytokine secretion was detected by BMDCs stimulated with OA (Figure 3.4B). These findings suggest that OA alone is not sufficient to promote DC activation and that the complex of OA and α-LA is required to induce DC maturation and cytokine secretion.

3.2.5. BAMLET-induced cytokine secretion is partially dependent on p38 and Pi3K- signalling

A study by Storm et al. observed p38 induction in lung carcinoma cells in response to HAMLET stimulation [289]. Interestingly, the signalling molecules p38 and Pi3K play a role in TLR-induced cytokine secretion [86]. The next experiment was designed to investigate whether p38 or Pi3K signalling were involved in BAMLET-induced cytokine secretion in BMDCs. BMDCs were pre-treated with the Pi3K-inhibitor GDC or the p38-inhibitor SB203 and subsequently stimulated with the TLR agonist R848 or different concentrations of BAMLET. As expected, GDC and SB203 pre-treatment increased R848-induced IL-12p70 secretion and reduced R848-induced IL-10 secretion. GDC and SB203 had no effect on R848-induced IL-12p40, TNF and IL-6 secretion and only SB203 elevated the concentration of R848-induced IL-1β. Pre-treatment with GDC or SB203 revealed a similar result for BAMLET-stimulated BMDCs. Both inhibitors reduced BAMLET-induced IL-10 and elevated the concentration of IL-12p70. SB203 also increased BAMLET-induced IL-1β secretion and no effects on IL-6, IL-12p40 and TNF were observed (Figure 3.5). These results suggest that p38 and Pi3K signalling play a partial role in BAMLET-induced cytokine secretion in BMDCs.

3.2.6. TLR-stimulated BMDCs induce CD4 T cell proliferation and cytokine secretion which is moderately enhanced by BAMLET

Having shown that BAMLET can stimulate BMDC maturation and cytokine secretion, co-culture experiments were set out in order to examine the effects of BAMLET-stimulated BMDCs on CD4 T cells. BMDCs were stimulated with BAMLET, R848 or LP1569 alone or in combination. After 24 h, cell culture supernatants were removed and DCs washed to remove residual BAMLET. Subsequently, stimulated DCs were co-

cultured with CTV-labelled naïve CD4 T cells in presence of soluble anti-CD3. After 4 d, CD4 T cell proliferation was analysed by flow cytometry and cytokine secretion measured by ELISA (Figure 3.6A). CD4 T cells cultured alone did not proliferate. In presence of medium-stimulated BMDCs, the CTV signal in CD4 T cells was diluted and appeared as separate peaks, indicating that CD4 T cells proliferated. R848-stimulated BMDCs induced marginally more proliferation, while LP1569-stimulated BMDCs strongly elevated the proportion of proliferating CD4 T cells compared with medium-BMDCs. BMDCs stimulated with BAMLET also only slightly increased CD4 T cell proliferation compared to BMDCs stimulated with medium only. However, the addition of BAMLET to R848-stimulated BMDCs induced more CD4 T cell proliferation than BMDCs stimulated with R848 alone. No differences were observed when CD4 T cells were co-cultured with LP1569-stimulated BMDCs compared with BAMLET/LP1569-stimulated BMDCs (Figure 3.6B).

The secretion of effector cytokines was measured in order to assess CD4 T cell activation by BMDCs. The secretion of IFN-γ was marginally enhanced in co-cultures of CD4 T cells with BMDCs pre-treated with BAMLET compared to untreated BMDCs or BMDCs treated with TLR agonists alone. The concentration of IL-10 was slightly elevated by BAMLET in absence or presence of TLR stimulation. No significant effects of BAMLET pre-stimulation of BMDCs on IL-17 or TGF-β secretion during co-cultures were observed (Figure 3.6C). In conclusion, while pre-stimulation of BMDCs with BAMLET before co-culture with CD4 T cells did not reveal significant changes, a trend towards increased CD4 T cell proliferation and cytokine secretion was evident.

3.2.7. BAMLET induces IL-10 and TNF secretion by BMDMs and enhances the expression of M2 markers

Having established that BAMLET promoted DC activation, immune-modulatory effects of BAMLET on macrophages were investigated. Macrophages constitute a high percentage of tumour infiltrating immune cells in many cancers. Tumour associated macrophages are mostly of a M2-like phenotype and contribute to tumour growth through the secretion of anti-inflammatory cytokines, promotion of vascularisation and effector cell inhibition. These macrophages are phenotypically different from immune-stimulatory, or M1, macrophages that are often found during infections. Macrophages with an M1-like phenotype are rare in tumours and were suggested to be tumour-killing [135]. *In vitro*, these two phenotypes of macrophages can be induced by stimulation with LPS and IFN-γ (M1 phenotype) or IL-4 (M2 phenotype). Stimulation of BMDMs

with LPS and IFN-γ induced the secretion of IL-12p40, TNF and IL-10 but not IL-12p70 or TGF-β. No induction of cytokine secretion was observed when BMDMs were stimulated with IL-4. Stimulation of BMDMs with BAMLET alone induced low levels of IL-12p40 and a significant increase in TNF and IL-10 secretion ($p<0.001$). Interestingly, when BMDMs were pre-treated with BAMLET and stimulated with LPS and IFN-γ, the secretion of IL-12p40 and TNF was reduced compared with BMDMs stimulated with LPS and IFN-γ alone (Figure 3.7A).

In addition, the markers *PTGS2*, *NOS2*, *YM-1* and *Arg1* are often used to distinguish M1 and M2 macrophages. M1 macrophages are associated with an increased expression of *PTGS2* and *NOS2*. M2 macrophages, on the other hand, have elevated levels of *YM-1* and *Arg1*. LPS and IFN-γ stimulation of BMDMs increased mRNA expression of the M1 markers *PTGS2* and *NOS2* while IL-4 promoted the mRNA expression of the M2 markers *YM-1* and *Arg1* (Figure 3.7B). Stimulation with BAMLET alone did not significantly alter the expression of these markers on BMDMs. However, BAMLET stimulation significantly reduced LPS/IFN-γ-induced *NOS2* expression and upregulated IL-4 induced *YM-1* and *Arg1* mRNA (Figure 3.7B). These findings suggest that BAMLET stimulation promotes an M2-like phenotype in BMDMs but only the presence of other signals (IL-4 or LPS + IFN-γ).

3.2.8. Stimulation of CD4 T cells with BAMLET increases the secretion of IL-10 but not IL-17 or IFN-γ

Having demonstrated that BAMLET has immune-stimulatory effects on BMDCs and BMDMs, it was then assessed whether BAMLET could directly modulate T cell responses. Purified naïve CD4 T cells were stimulated with anti-CD3/anti-CD28 alone or in combination with Th1 (IL-12) or Th17 (IL-1β + IL-23) polarising cytokines in presence or absence of BAMLET. After 3 d of stimulation, secreted cytokines were analysed by ELISA. In absence of TCR stimulation by anti-CD3/anti-CD28, no cytokines were secreted by CD4 T cells. CD4 T cells activated through their TCR and stimulated with medium control produced high levels of IFN-γ. Stimulation with IL-12, but not IL-1β and IL-23, significantly enhanced IFN-γ secretion. Conversely, IL-12 stimulation reduced IL-17 secretion while IL-1β and IL-23 increased IL-17 secretion by CD4 T cells. The presence of BAMLET significantly reduced IFN-γ of Th0 or Th17 but not Th1-polarised CD4 T cells. The secretion of IL-17 was not altered by BAMLET. However, BAMLET significantly upregulated the secretion of IL-10 in all polarising

conditions (Figure 3.8). These findings suggest that BAMLET can promote anti-inflammatory properties of CD4 T cells.

3.2.9. The TLR7/8 agonist R848 shows better anti-tumour efficacy when used as mono-therapy compared with the TLR2 agonist LP1569

It was demonstrated that both TLR agonists LP1569 and R848 were potent activators of DCs and induced the secretion of pro-inflammatory cytokines. However, R848 was more potent in inducing IL-12p70 compared with LP1569, especially in combination with BAMLET. In order to investigate the potential of these two TLR agonists to induce anti-tumour responses *in vivo,* two syngeneic tumour models were used. B16 or CT26 tumour cells were injected s.c. into the right flank of C57BL/6 or BALB/c mice, respectively. Tumour-bearing mice were injected with the TLR agonists LP1569 or R848 in the area of the tumour on days 3, 10 and 17 post tumour induction and tumour growth was monitored. Treatment of B16 tumour-bearing mice with LP1569 had no effect on tumour growth and LP1569 only marginally reduced tumour growth in CT26 tumour-bearing mice (Figure 3.9A). In line with the observations made *in vitro*, R848 showed the best efficacy *in vivo* compared with LP1569 and strongly reduced B16 but not CT26 tumour growth when used as monotherapy (Figure 3.9B). Therefore, R848 was selected for the combination with BAMLET for further *in vivo* studies.

3.2.10. Combination of BAMLET and R848 confers protection against B16 tumour growth and prolongs survival

In vitro analysis of immune-modulatory effects of BAMLET revealed that BAMLET directly modulated the activity and cytokine secretion of BMDCs, BMDMs and CD4 T cells. Moreover, BAMLET was capable of potently inducing tumour cell death. The next experiment was set out to investigate the anti-tumour effects of BAMLET *in vivo*. In order to stimulate anti-tumour immune responses, BAMLET was combined with the TLR7/8 agonist R848 which induced strong IL-12 secretion by BMDCs *in vitro* and was effective as monotherapy *in vivo*. Naïve C57BL/6 mice were inoculated with B16 tumour cells and BAMLET or R848 alone or in combination were injected s.c. in the area of the tumour 3, 7, 10 and 14 days post tumour induction. Treatment of mice with BAMLET or R848 alone induced some reduction in tumour growth and led to tumour rejection in 14% of BAMLET-treated mice and 29% of R848-treated mice. However, the combination of BAMLET with R848 induced the best response *in vivo* and significantly reduced tumour growth. Moreover, 57% of mice treated with the combination rejected

the tumour (Figure 3.10 A,B). In a total out of 7 mice per group, one mouse treated with BAMLET alone, 2 mice treated with R848 alone and 4 mice treated with the combination were tumour-free for at least 50 days while all control mice reached the maximum tumour size within 26 days (Figure 3.10C). Overall, mice that were treated with the combination of BAMLET and R848 showed the lowest tumour incidence compared with either treatment alone (Figure 3.10D).

Mice that rejected the tumour were re-challenged with B16 tumour cells and tumour growth was compared with naïve mice that were challenged for the first time. All mice that had previously rejected the tumour showed a delay in onset of tumour growth. Interestingly, mice previously treated with BAMLET and R848 showed the strongest reduction in tumour growth and one mouse was able to reject the second tumour (Figure 3.10E). These data reveal a novel and effective anti-tumour effect of BAMLET and R848 in the B16 tumour model.

3.2.11. BAMLET and R848 do not significantly alter the frequency of DCs or Treg cells in lymphoid organs

In order to examine the mechanism of R848 and BAMLET-induced tumour rejection, the treatment was repeated and mice sacrificed on day 17 post tumour induction. dLN and spleens were dissected in order to characterise immune cells by flow cytometry. Analysis of TIL was not possible since no tumours were detectable in mice that received the combination of BAMLET and R848.

The frequency of DCs was analysed in dLN and spleens. Overall, very low levels of DCs were detected which constituted less than 2% of cells in dLNs and less than 1% of cells in spleens. Albeit not reaching significance, treatment with R848 alone or in combination with BAMLET showed a trend towards increasing the frequency of DCs in dLN and reducing the frequency of DCs in spleens (Figure 3.11). This observation suggests that R848 promoted homing of DCs into dLN.

The frequency of $Foxp3^+$ Treg cells was below 5% in dLN and spleens for all mice. While treatment with BAMLET slightly reduced the frequency of Tregs in dLN and spleens, the opposite effect was observed when mice were treated with R848. Treatment with R848 increased the percentage of $Foxp3^+$ CD4 T cells of live cells in dLN and spleens which was not altered by the addition of BAMLET (Figure 3.12).

3.2.12.Treatment with BAMLET and R848 increased the frequency of IL-10$^+$ and IFN-γ$^+$ T cells in dLN and spleens

The stimulation of CD4 T cells with BAMLET *in vitro* suggested that BAMLET is capable of modifying T cell cytokine secretion. In order to investigate whether BAMLET had an effect on T cell cytokine secretion *in vivo*, T cells in dLN and spleens from BAMLET and R848-treated mice were stained by intracellular cytokine staining and analysed by flow cytometry.

Treatment with BAMLET alone significantly increased the frequency of IL-10$^+$ CD4 T cells in the dLN. Treatment with R848 alone had no effect on the frequency of IL-10$^+$ CD4 T cells. However, mice treated with the combination of BAMLET and R848 displayed the highest frequency of IL-10$^+$ CD4 T cells in dLN and spleens compared with vehicle-treated mice. In contrast, CD8 T cells produced negligible levels of IL-10 (Figure 3.13).

Treatment of mice with BAMLET or R848 alone only moderately increased the frequency of IFN-γ$^+$ CD4 T cells in dLN and spleens compared with vehicle-treated mice. However, treatment of mice with the combination of BAMLET and R848 significantly increased the frequency of IFN-γ$^+$ CD4 T cells in dLN and spleens. Similarly, mice treated with R848 alone or in combination with BAMLET displayed the highest frequency of IFN-γ$^+$ CD8 T cells compared with mice treated with vehicle or BAMLET alone (Figure 3.14).

Overall, the combination of BAMLET with R848 demonstrated strong anti-tumour efficacy in the B16 model. The protection was probably due to BAMLET-induced cell death and the activation of DCs by BAMLET and R848 which in turn activated a Th1 response against the tumour.

3.2.13.BAMLET induces the expression of MHC II, CD40, CD80 and CD86 in TLR-defective BMDCs

Since several steps of the BAMLET manufacturing process were performed under non-sterile conditions, the BAMLET preparation could have been contaminated with LPS (endotoxin) which can activate DCs through TLR4. The LAL assay was performed in order to determine the endotoxin concentration in the BAMLET preparation. Indeed, traces of endotoxin were detected which translated to 5 pg of LPS per 1 µg of BAMLET. This means that the LPS concentration in the BMDC culture amounted to 0.5 ng/ml and a dose of 1 ng LPS was injected in *in vivo* experiments.

To rule out that the stimulatory effects of BAMLET on BMDCs were due to LPS contaminations, experiments were repeated using BMDCs from C3H/HeJ (HeJ) mice and C3h/HeN (HeN) mice as controls. HeJ mice carry a spontaneous point mutation in the TLR4 gene and are consequently non-responsive to TLR4 stimulation, whereas HeN mice have a functional TLR4 [290]. In HeN BMDCs, stimulation with LPS or BAMLET increased the expression of MHC II and the co-stimulatory molecules CD40, CD80 and CD86. In TLR4-defective HeJ BMDCs, BAMLET still upregulated the expression of co-stimulatory molecules and MHC II. In contrast, LPS did not induce the expression of co-stimulatory molecules on BMDCs from HeJ mice (Figure 3.15). These findings suggest that BAMLET-induced BMDC maturation was independent of TLR4 signalling and not the result of LPS contamination.

3.2.14.BAMLET induces the secretion of IL-12p40, TNF and IL-6 by TLR4-defective BMDCs

Having shown that BAMLET enhanced DC maturation independently of TLR4, the role of TLR4 in BAMLET-induced cytokine secretion was assessed. BMDCs from HeN or HeJ mice were stimulated with different concentrations of BAMLET or LPS as internal control, and cytokine secretion was quantified by ELISA. In HeN BMDCs, BAMLET induced secretion of IL-12p70, IL-12p40 and IL-6 and low levels of TNF, TGF-β and IL-10. The same secretion pattern was observed when HeN BMDCs were stimulated with LPS. As expected, stimulation with LPS did not induce cytokine secretion in HeJ BMDCS due to a dysfunctional TLR4. However, BAMLET at a concentration of 0.1 mg/ml significantly upregulated the secretion of IL-12p40 ($p<0.01$), TNF ($p<0.01$) and IL-6 ($p<0.001$), suggesting that BAMLET is capable of driving TLR4-independent cytokine secretion in BMDCS (Figure 3.16).

3.2.15.Polymyxin-B purified BAMLET loses the ability to induce BMDC cytokine secretion and to promote tumour cell death

The results so far indicated that the effects of BAMLET on BMDCs were not a result of an LPS contamination. In attempt to get rid of residual LPS in the sample, BAMLET was run through a polymyxin B column, which binds negatively charged LPS. Proteins have a positive charge and therefore run through the column. After running BAMLET through the column, the previously opaque solution became clear, indicating that OA was stripped off the protein and that only α-LA was present in the solution. BMDCs were subsequently stimulated with purified or non-purified BAMLET and cytokine

secretion was analysed by ELISA. In line with previous experiments, pre-column BAMLET induced the secretion of IL-12, TNF, IL-6 and IL-10 by HeJ and HeN BMDCs. In contrast, LPS only induced the secretion of IL-12, TNF and IL-6 in HeN BMDCs but not HeJ BMDCs. BAMLET that was purified with a polymyxin B column did not induce any cytokine secretion by BMDCs, regardless of the presence of a functional TLR4 (Figure 3.17A).

Moreover, B16 tumour cells were stimulated with pre- or post-column BAMLET and cell viability was measured. While pre-column BAMLET induced dose-dependent cell death, no cell death was observed when B16 cell were stimulated with polymyxin B-purified BAMLET (Figure 3.17B).

These results suggest that polymyxin B disrupted the protein-OA complex and thereby inhibited BAMLET-induced cytokine secretion and tumour cell killing. Therefore, polymyxin B purification was not a suitable method to gain higher purity BAMLET.

3.3. Discussion

This chapter studied anti-tumour and immune-modulatory properties of BAMLET, a PFA complex consisting of α-LA and OA. The present study demonstrated that BAMLET can rapidly induce cell death in tumour cells. In addition, the results revealed a novel role for BAMLET in modulating immune responses. BAMLET induced maturation and cytokine secretion by BMDCs and synergised with TLR agonists to increase the secretion of DC cytokines. Moreover, BAMLET was found to directly modulate cytokine secretion by macrophages and T cells. Lastly, treatment of B16 tumour-bearing mice with BAMLET in combination with the TLR agonist R848 induced tumour rejection in 57% of mice. Mice treated with this combination displayed an increased frequency of IFN-γ-secreting T cells in lymphoid organs. These findings suggest a dual role for BAMLET in promoting tumour rejection by targeting tumour cells and activating immune cells.

The present study confirmed previous reports showing that BAMLET and related PFA complexes induce cell death in a range of tumour cells *in vitro* and *in vivo* [274, 277-279, 283, 284, 291]. The mechanism behind BAMLET-induced cell death was not a subject of this study but has been investigated by others. While HAMLET and BAMLET have been discovered over two decades ago, there is still no consent on which exact mechanisms are responsible for the induction cell death. The induction of apoptosis-like cell death by α-LA fatty acid complexes has been observed in some studies [277, 278, 283, 284]. However, blockade of caspases did not prevent BAMLET-induced cell death. Instead, lysosomal cell death has been suggested as the main cytotoxic mechanism [292]. Aits et al. observed induction of autophagy by HAMLET and increased survival of HAMLET-treated cells when autophagy was inhibited [293]. In contrast, inhibition of autophagy did not affect BAMLET-induced cell death [292]. A recent study by Wen et al. suggested that the OA moiety of the complex can cause membrane disruption, thereby inducing cell death [282]. Moreover, Fang et al. identified 17 targets of an α-LA-OA complex which are involved in disruption of cytoskeleton stability and cell motility, and inhibited DNA, lipid and ATP synthesis [294]. Overall, BAMLET and other PFA complexes seem to exert their anti-tumour activity by a combination of several mechanisms.

The present study found that OA was crucial for the activity of BAMLET. Purification of BAMLET using a polymyxin B column removed OA from α-LA which, as a result, lost its ability to induce cell death or activate BMDCs. This is in line with previous studies suggesting that the protein part of the complex is not required for BAMLET-induced cell

death. It has been suggested that α-LA solubilises OA [286] and that OA in a micellar conformation induced by sonication can promote cell death in absence of a carrier protein [281]. This hypothesis was supported by the fact that OA complexed with other proteins, such as cytochrome C, were just as effective as BAMLET in inducing cell death [295].

Until recently, BAMLET has been defined as a tumour cell-specific cytotoxic agent. However, the present study and recent publications found that non-cancerous cells can also be targeted by BAMLET. In the present study, BMDCs were more sensitive to BAMLET-induced cell death than B16 or CT26 tumour cells. Brinkman et al. suggested that some primary cell types were more sensitive to treatment with BAMLET than cancer cell lines, which agrees with the results presented here [280]. Moreover, Wen et al. reported that BAMLET caused erythrocyte hemolysis through membrane destabilisation [282]. Other non-cancer cell lines, including Cho-K1 and NIH/3T3, were reported to respond to BAMLET similar to tumour cells [281]. These observations prompted the analysis of the effects of BAMLET on other cell types, specifically on immune cells.

The present study revealed novel effects of BAMLET on immune cells. BAMLET modulated the secretion of cytokines by BMDCs, BMDMs and T cells and induced the expression of co-stimulatory molecules on BMDCs. To date, little is known about potential effects of PFA complexes on the immune system. It has been suggested that HAMLET, the human counterpart of BAMLET, spared healthy cells from cell death by stimulating innate immunity through cytokine secretion. HAMLET-treatment of healthy kidney cells stimulated the secretion of IL-6, IL-8 and TNF which required ion channel activation. In contrast, no cytokine secretion was observed in kidney carcinoma cells [289]. Effects on ion channels and ion influx may represent a mechanism by which BAMLET-induced cytokine secretion by immune cells.

Other possible mechanisms by which BAMLET affected immune cells could be receptor-ligand interactions on the surface of immune cells which triggered downstream events. Alternatively, BAMLET could have been taken up by cells and directly interfered with signalling cascades involved in cytokine secretion or expression of co-stimulatory molecules. Recent literature strongly suggested that OA is the crucial cell death-inducing component [280-282]. The same could be true for BAMLET's properties as an immune modulator. It is conceivable that OA activated TLRs on DCs, since BAMLET stimulation induced effects similar to TLR agonists. Zeyda et al. reported that polyunsaturated fatty acids interfered with DC activation and function but

observed no effect on DCs by using the monounsaturated fatty acid OA [296]. However, in their study OA was not sonicated and possibly OA has to be stabilised in a protein complex or micellar conformation to exert its effects on DCs, as it has been reported for the induction of cell death [281]. Other fatty acids, including stearic acid and palmitic acid, have been shown to induce TLR4 signalling. It was suggested that TLR4 signalling was activated indirectly by fatty acids through a yet unknown endogenous ligand [296]. The results of the present study showed that BAMLET was capable of inducing DC maturation and cytokine secretion in BMDCs that lack a functional TLR4, suggesting a TLR4-independent mechanism.

Storm et al. reported p38 activation in response to HAMLET in carcinoma cells and blockade of p38 reduced cell death [289]. It has previously been reported that p38 and Pi3K signalling mediated TLR-induced secretion of IL-10 [86, 87]. Indeed, inhibition of p38 or Pi3K reduced BAMLET-induced secretion of IL-10 and increased BAMLET-induced secretion of IL-12p70. These findings suggest that p38 and Pi3K activation may be mechanisms by which BAMLET induced or modulated cytokine secretion by immune cells.

The most interesting finding of this study was the observation that BAMLET together with R848 induced substantial protection against B16 tumour growth. The analysis of lymphocytes in dLN and spleens revealed that T cells from mice that were treated with this combination produced significantly more IL-10 and IFN-γ which indicates improved T cell effector function. While increased secretion of IFN-γ can be beneficial against tumour growth, IL-10 has been shown to suppress immune responses [82]. The presence of IL-10 did not seem to interfere with the therapy efficacy. However, it would be interesting to assess whether blocking IL-10 with antibodies or by using IL-10 KO mice could further improve the anti-tumour efficacy of the combination of BAMLET and R848.

The present study demonstrated that BAMLET induced tumour cell death and modulated immune responses. The contribution of each of these two mechanisms to tumour rejection needs to be further elucidated. It is possible that BAMLET strongly induced cell death and that its effects on the immune system only played a minor role. Conversely, anti-tumour effects elicited by BAMLET could have been solely mediated by the immune system. Xenograft tumour models could be used to study whether BAMLET is able to kill tumour cells in absence of immune responses. Moreover, TUNEL staining of BAMLET-treated tumours could indicate whether BAMLET induced apoptosis *in vivo*.

While the present study made the observation that BAMLET modulated immune responses, more work needs to be carried out to reveal the underlying molecular mechanisms. This was not possible within the scope of this project due to difficulties with the supply of functional BAMLET and the issues of LPS contaminations. Due to non-sterile conditions during the manufacturing process, traces of LPS were found in the BAMLET preparation. Experiments performed in HeJ BMDCs showed that BMDCs still undergo maturation and secrete cytokines in absence of a functional TLR4. However, it cannot be excluded that the anti-tumour effects seen in the *in vivo* tumour model are a result of synergistic effects of BAMLET, R848 and LPS. For future work, BAMLET needs to be purified and tested in combination with R848. It also needs to be evaluated whether the presence of an additional TLR agonist is required for BAMLET-mediated anti-tumour effects. Since LPS is not safe for the use in humans, safe TLR agonists such as CpG (TLR9) or Polyl:C (TLR3) could be tested.

Overall, the results of this chapter suggest the following mechanism: BAMLET induces cell death of tumour cells by a combination of several mechanisms. Dying tumour cells release antigens that are taken up by DCs. Subsequently, DCs present fragments of the tumour antigens on their cell surface and undergo maturation which is enhanced by the presence of BAMLET and the TLR agonist R848. At the same time, DCs migrate along a chemokine gradient into dLN, where they present tumour antigens to T cells. Tumour-specific T cells get activated by interaction with DCs and the presence of pro-inflammatory IL-12 which promotes Th1 cells. Activated T cells proliferate in the dLN and migrate into the tumour bed where they secreted IFN-γ and induced the killing of tumour cells (Figure 3.18).

Taken together, the present study suggests a novel mechanism of BAMLET-driven anti-tumour activity by inducing cell death and activating immune cells.

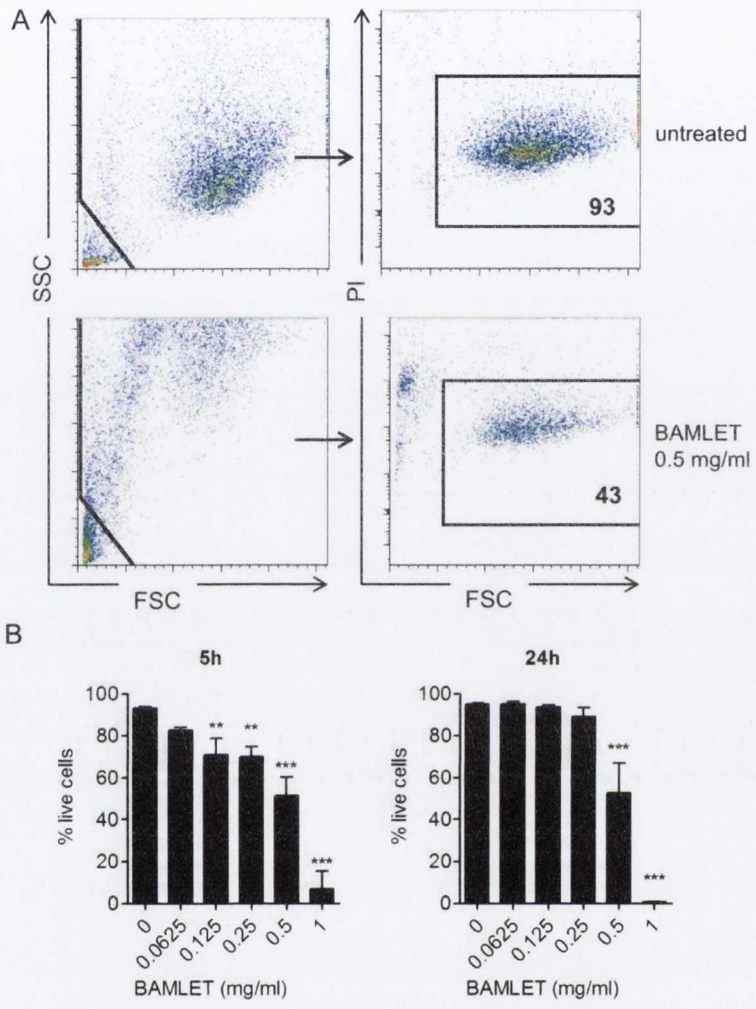


Figure 3.1

BAMLET induces rapid B16 tumour cell death in a dose-dependent manner

Cultured B16 cells were treated with increasing concentrations of BAMLET (0.0625-1 mg/ml). After 5 h or 24 h, cells were stained with PI and cell death was measured by flow cytometry. Results are shown as representative FACS plots after 5h (A) or mean ± SD from triplicate samples (B). **p<0.01 , ***p<0.001 versus medium control by 1-way ANOVA. Data are representative of at least 3 experiments.

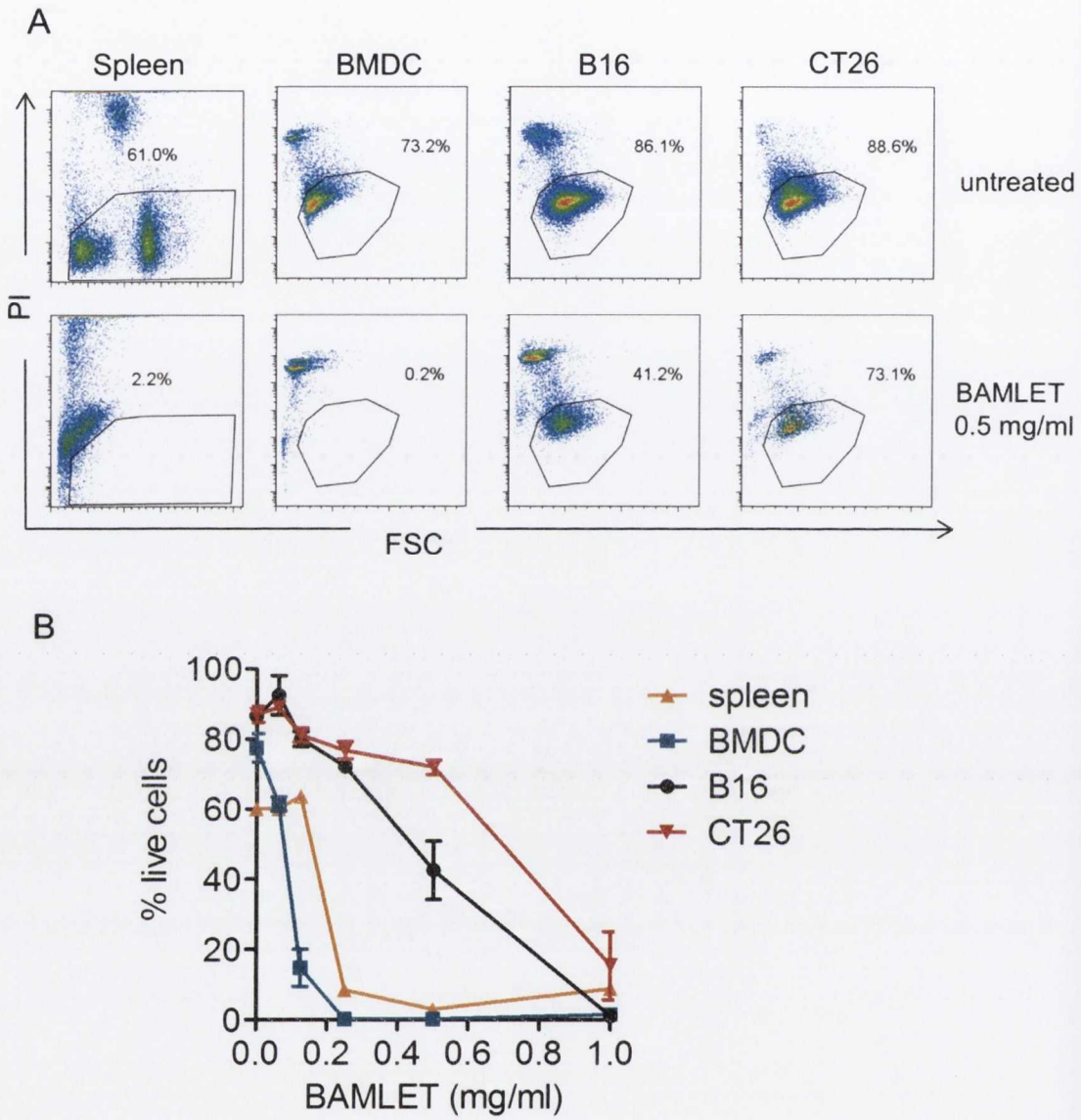


Figure 3.2
BAMLET-induced cell death is not tumour cell specific

Cultured spleen cells, BMDCs, B16 or CT26 cells were treated with increasing concentrations of BAMLET (0.0625-1 mg/ml). After 5 h, cells were stained with PI and cell death was measured by flow cytometry. Results are shown as representative FACS plots (A) or means ± SD from triplicate samples (B).

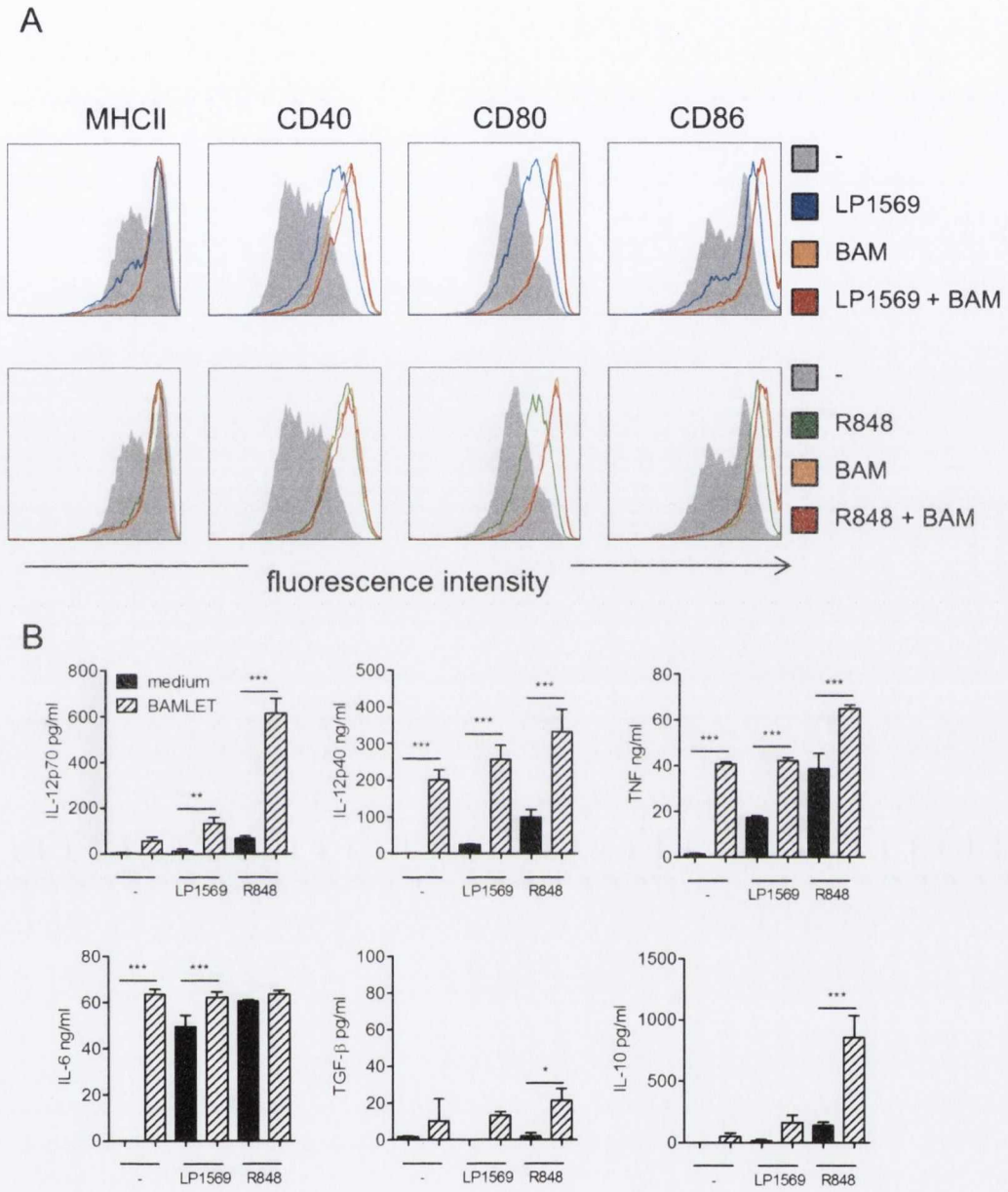


Figure 3.3
TLR agonists induce BMDC maturation marker expression and pro-inflammatory cytokine secretion which is enhanced in presence of BAMLET

BMDCs were pre-treated with BAMLET (BAM, 0.1 mg/ml) for 30 min and subsequently stimulated with R848 (80 ng/ml) or LP1569 (1 µg/ml). After 24h, expression of MHC II, CD40, CD80, CD86 was analysed by surface staining and flow cytometry, gating on $CD11c^+$ cells (A). Cytokine levels in culture supernatants were quantified by ELISA and results are shown as mean + SD from triplicate samples (B). BMDC viability was over 90 % after 24 h. *p<0.05, **p<0.01 ***p<0.001 by 1-way ANOVA. Data are representative of at least 3 experiments.

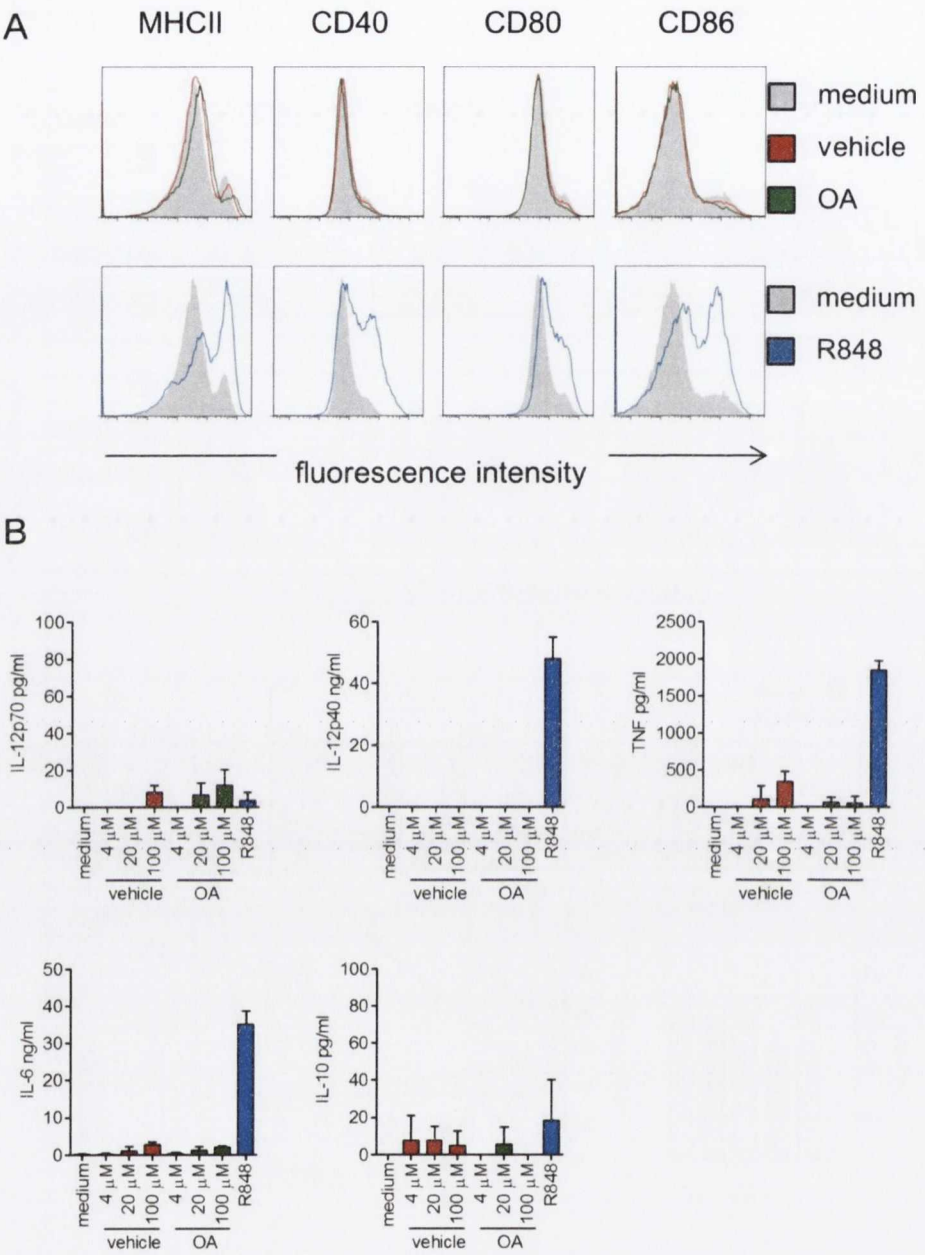


Figure 3.4
OA alone does not induce BMDC maturation or cytokine secretion

BMDCs were stimulated with medium, sonicated vehicle control (vehicle), sonicated OA (100 µM or as indicated) or R848 (100 ng/ml). After 24 h, expression of MHC II, CD40, CD80, CD86 was analysed by surface staining and flow cytometry, gating on $CD11c^+$ cells (A). Cytokine levels in culture supernatants were quantified by ELISA and results are shown as mean + SD from triplicate samples (B). Data are representative of 2 experiments.

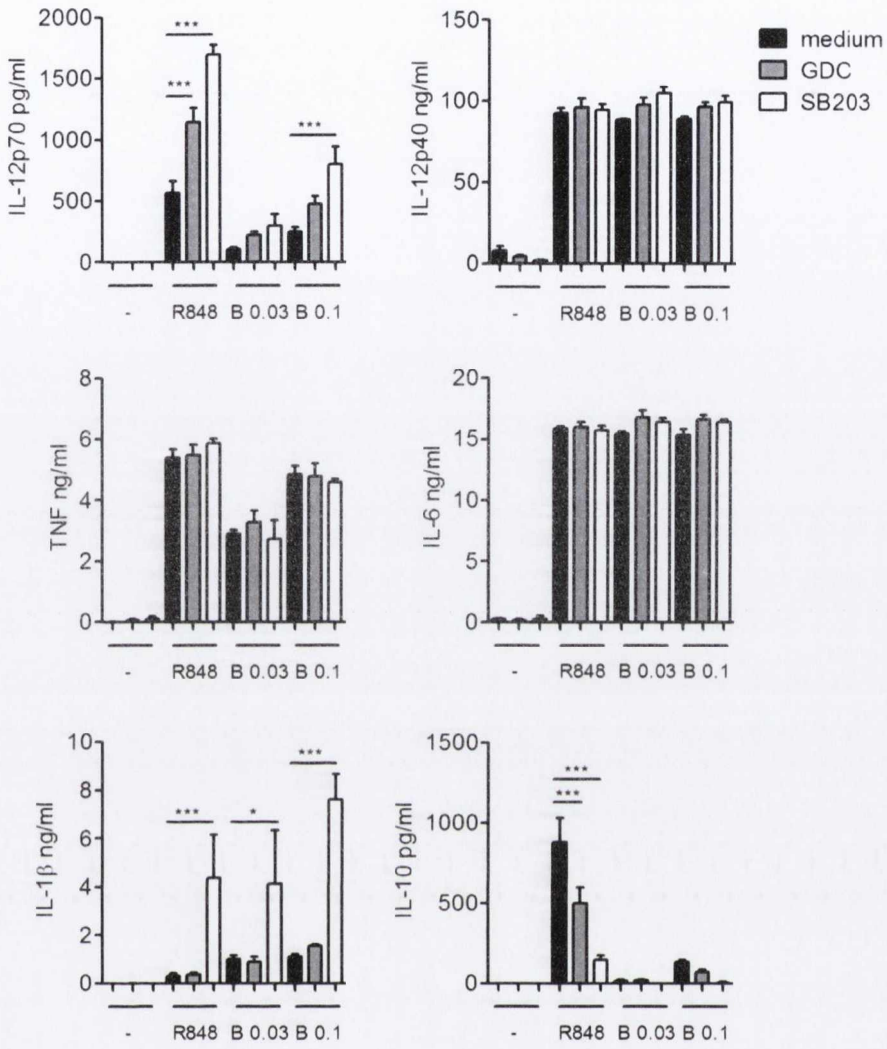


Figure 3.5
BAMLET-induced cytokine secretion is partially dependent on p38 and Pi3K-signalling

BMDCs were pre-treated with medium, GDC (1 μM) or SB203 (1 μM) for 30 min and subsequently stimulated with medium (-), R848 (50 ng/ml) or BAMLET (B; 0.03-0.1 mg/ml) for 24 h. Cytokine levels in supernatants were quantified by ELISA and results are shown as mean + SD from triplicate samples. *$p<0.05$, ***$p<0.001$ by 1-way ANOVA.

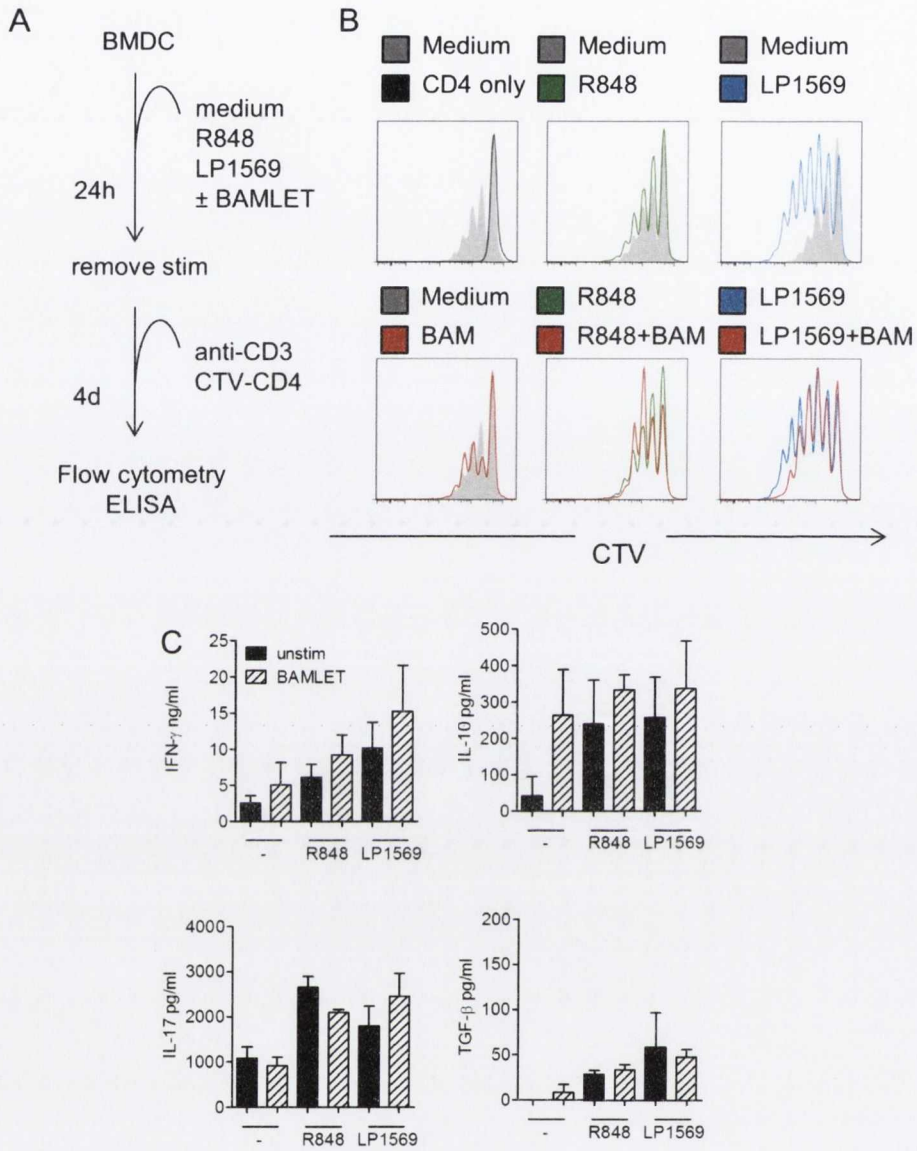


Figure 3.6
TLR-stimulated BMDCs induce CD4 T cell proliferation and cytokine secretion which is moderately enhanced by BAMLET

BMDCs were stimulated with medium (-), R848 (80 ng/ml) or 1569 (1 µg/ml) in presence or absence of BAMLET (0.1 mg/ml). After 24 h, supernatants were removed and BMDCs washed. Stimulated BMDCs and naïve CTV-labelled CD4 T cells were co-cultured at a DC:T cell ratio of 1:10 in presence of soluble anti-CD3 (1 µg/ml) (A). After 4 days, CD4 T cell proliferation was measured as CTV fluorescence intensity by flow cytometry, gating on $CD4^+$ cells. Figure headers indicate BMDC stimulation conditions. CD4 only group indicates CD4 cultured in absence of BMDCs (B). Cytokine levels in co-culture supernatants were quantified by ELISA and results are shown as mean + SD from triplicate samples (C).

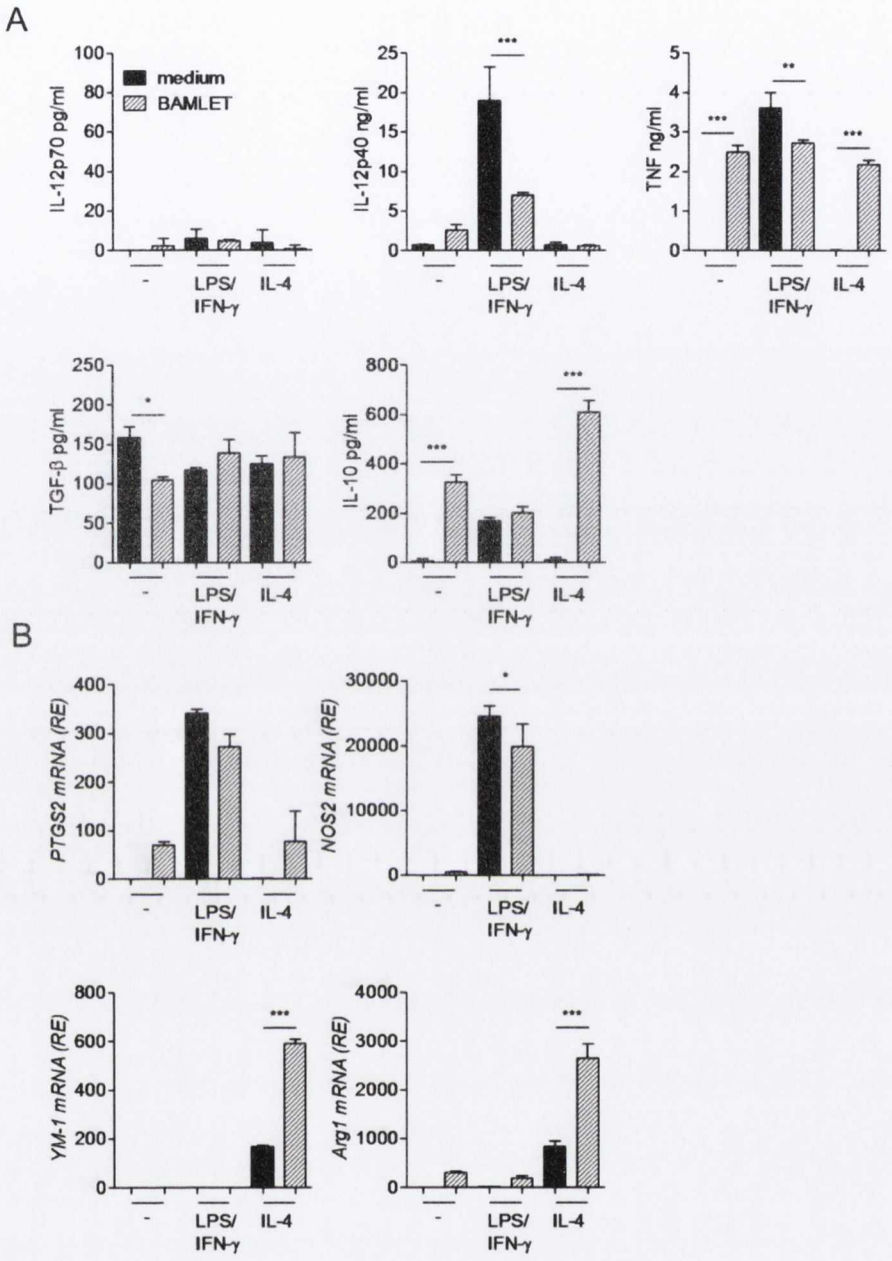


Figure 3.7
BAMLET induces IL-10 and TNF secretion by BMDMs and enhances the expression of M2 markers

BMDMs were pre-treated with medium or BAMLET (0.1 mg/ml) for 30 min and subsequently stimulated with medium (-), LPS (100 ng/ml) and IFN-γ (20 ng/ml) or IL-4 (20 ng/ml) for 24 h. Cytokine levels in supernatants were quantified by ELISA and results are shown as mean + SD from triplicate samples (A). Relative expression of *PTGS2*, *NOS2*, *YM-1* and *Arg1* mRNA was analysed by RT-PCR and normalised against *18sRNA*. Results are shown as mean + SD from triplicate samples (B). *p<0.05. **p<0.01, ***p<0.001 by 1-way ANOVA. Data are representative of 2 experiments.

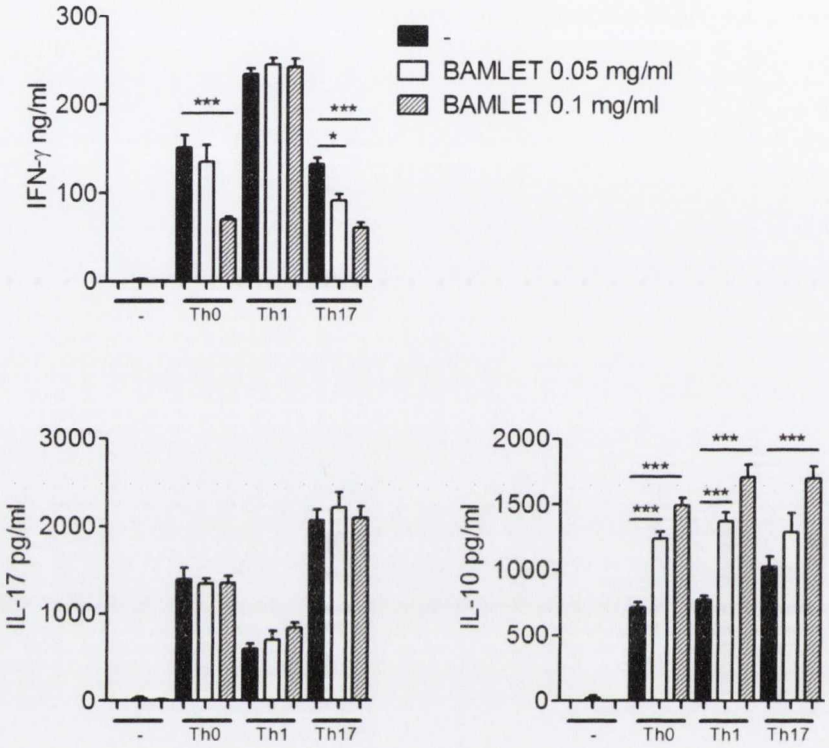


Figure 3.8
Stimulation of CD4 T cells with BAMLET increases the secretion of IL-10 but not IL-17 or IFN-γ

CD4 T cells were isolated from spleens of naïve C57BL/6 mice by MACS. CD4 T cells were either unstimulated (-) or activated with plate-bound anti-CD3 (3 μg/ml) and soluble anti-CD28 (3 μg/ml) and stimulated with medium (Th0) or polarised towards Th1 (10 ng/ml of IL-12) or Th17 (10 ng/ml of IL-1β + 10 ng/ml of IL-23) in presence of absence of BAMLET (0.05 - 0.1 mg/ml). After 3 d, supernatants were removed and cytokine levels quantified by ELISA. *$p<0.05$, ***$p<0.001$ by 1-way ANOVA.

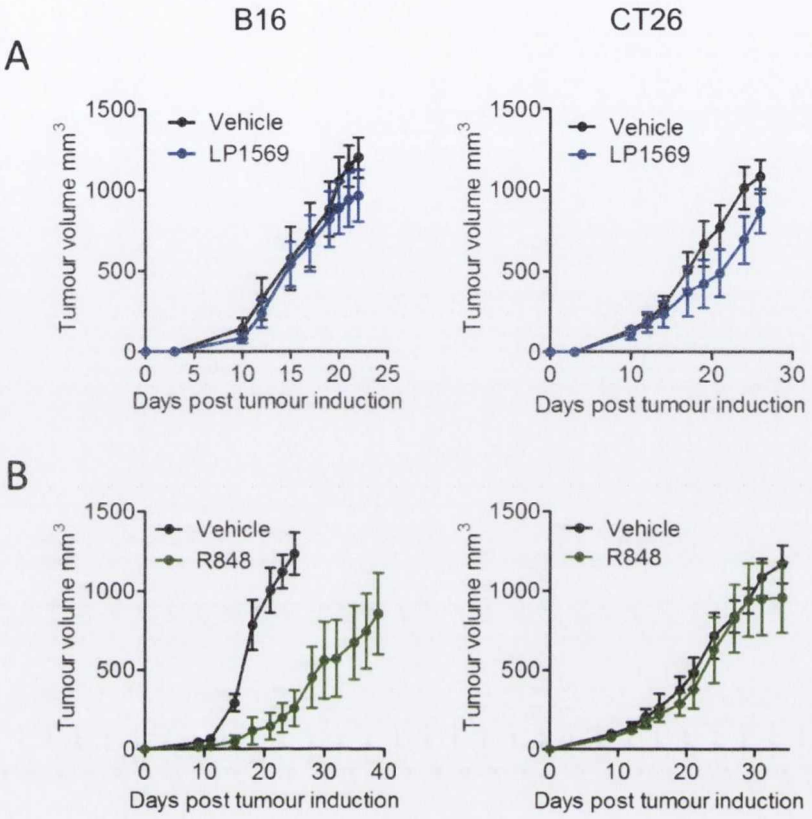


Figure 3.9
The TLR7/8 agonist R848 shows better anti-tumour efficacy when used as mono-therapy compared with the TLR2 agonist LP1569

B16 ($2x10^5$ cells/mouse) or CT26 ($3x10^5$ cells/mouse) tumour cells were injected s.c. into naïve C57Bl/6 or BALB/c mice, respectively. Mice were injected s.c. with LP1569 (50 µg/mouse; A) or R848 (40 µg/mouse; B) on days 3, 10 and 17 post tumour induction. Data are representative of at least 2 experiments for each graph. (n=7 mice per group)

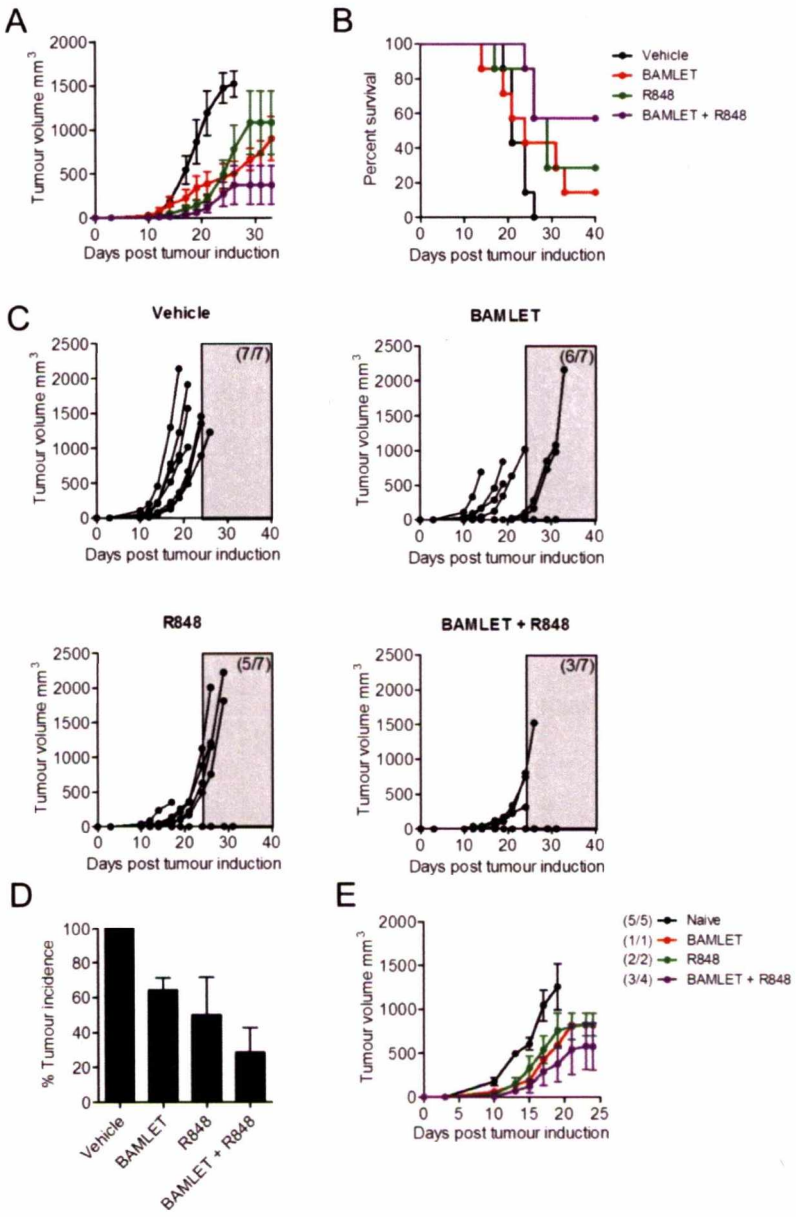


Figure 3.10
Therapeutic treatment of mice with BAMLET and R848 confers protection against B16 tumour growth and prolongs survival

Naïve C57BL/6 mice were injected with B16 tumour cells ($2x10^5$ cells/mouse, 7 mice/group) and treated with vehicle, BAMLET (1 mg/mouse), R848 (40 µg/mouse) alone or in combination on days 3, 7, 10 and 14 post tumour induction. Results are shown as mean tumour growth ± SEM (A), percent survival (B), tumour growth curves of individual mice (C) or tumour incidence by day 17 (D). Tumour-free mice were re-challenged with B16 tumour cells and tumour growth was monitored for 25 days. Naïve mice were used as internal control. Growth curves represent mean values ± SEM (E). Numbers in brackets indicate tumour incidence. Data are representative of 2 experiments.

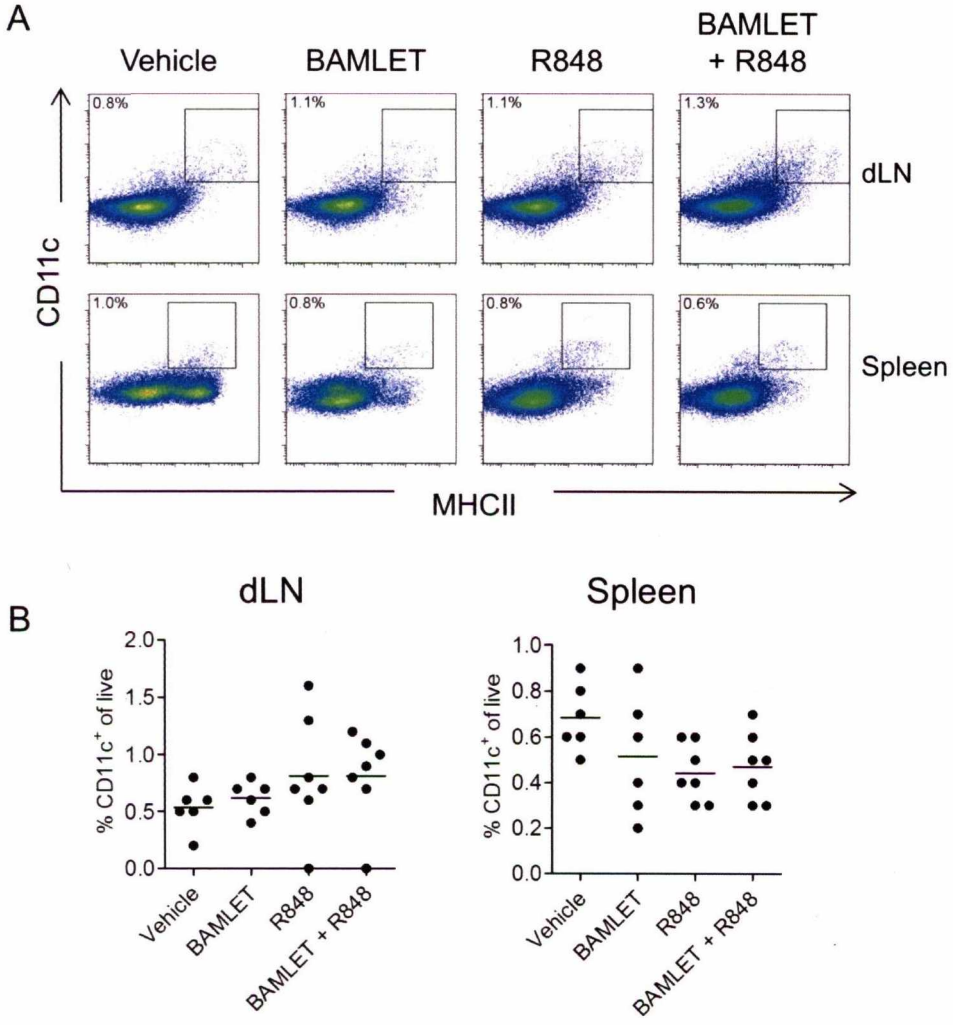


Figure 3.11
Therapeutic treatment with BAMLET and R848 does not significantly alter the frequency of DCs in lymphoid organs

Naïve C57BL/6 mice were injected with B16 tumour cells ($2x10^5$ cells/mouse, 7 mice/group) and treated with vehicle, BAMLET (1 mg/mouse), R848 (40 µg/mouse) alone or in combination on days 3, 7, 10 and 14 post tumour induction. The experiment was terminated on day 17 and CD11c expression in dLN and spleens analysed by surface staining and flow cytometry. Results are shown as representative FACS plots (A) or mean values (B).

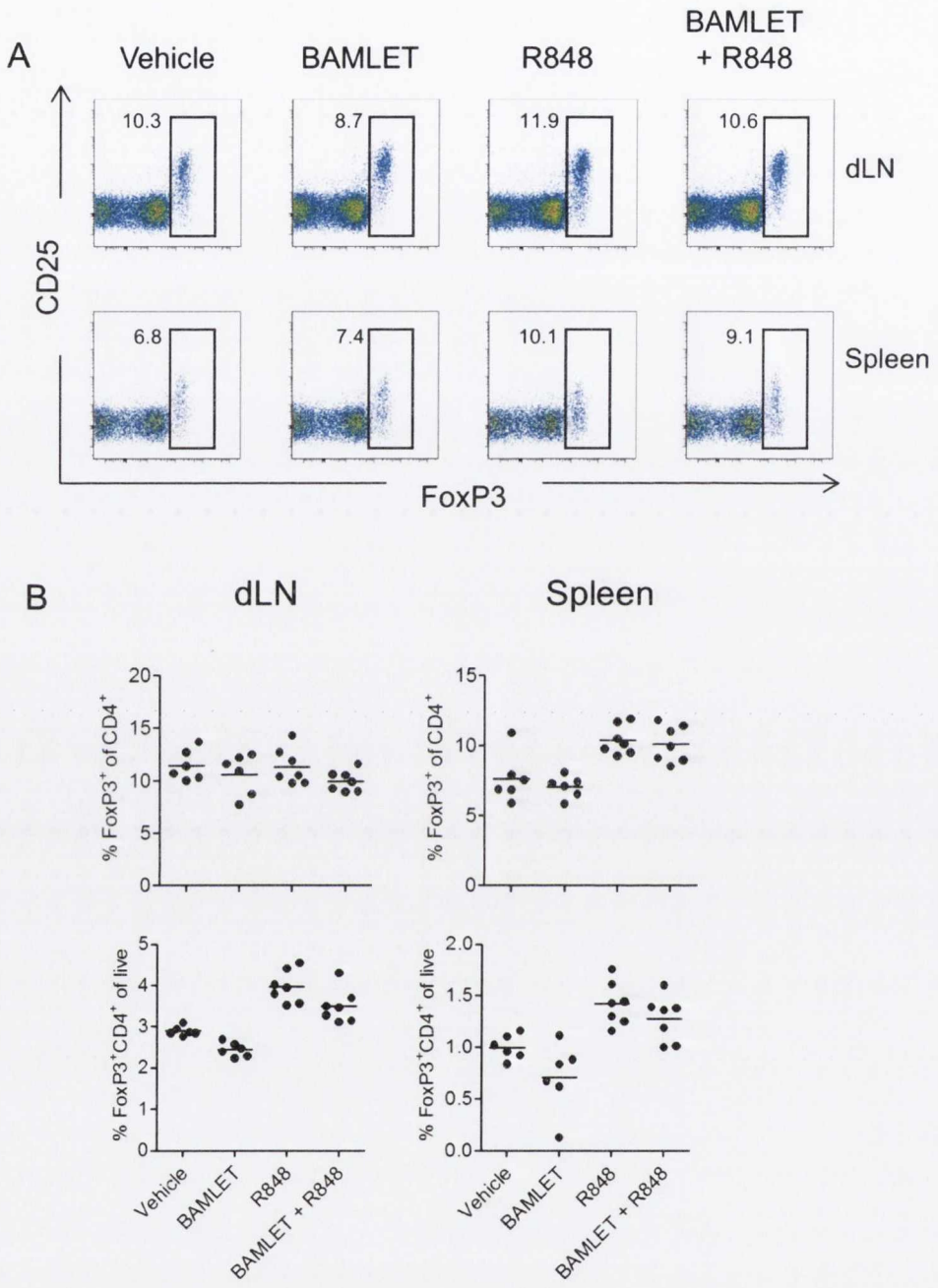


Figure 3.12
Therapeutic treatment with BAMLET and R848 moderately increases the percentage Foxp3$^+$ cells in lymphoid organs

Naïve C57BL/6 mice were injected with B16 tumour cells ($2x10^5$ cells/mouse, 7 mice/group) and treated with vehicle, BAMLET (1 mg/mouse), R848 (40 µg/mouse) alone or in combination on days 3, 7, 10 and 14 post tumour induction. The experiment was terminated on day 17 and Foxp3 expression in dLN and spleens analysed by intranuclear staining and flow cytometry, gating on CD4$^+$ cells. Results are shown as representative FACS plots (A) or mean values (B).

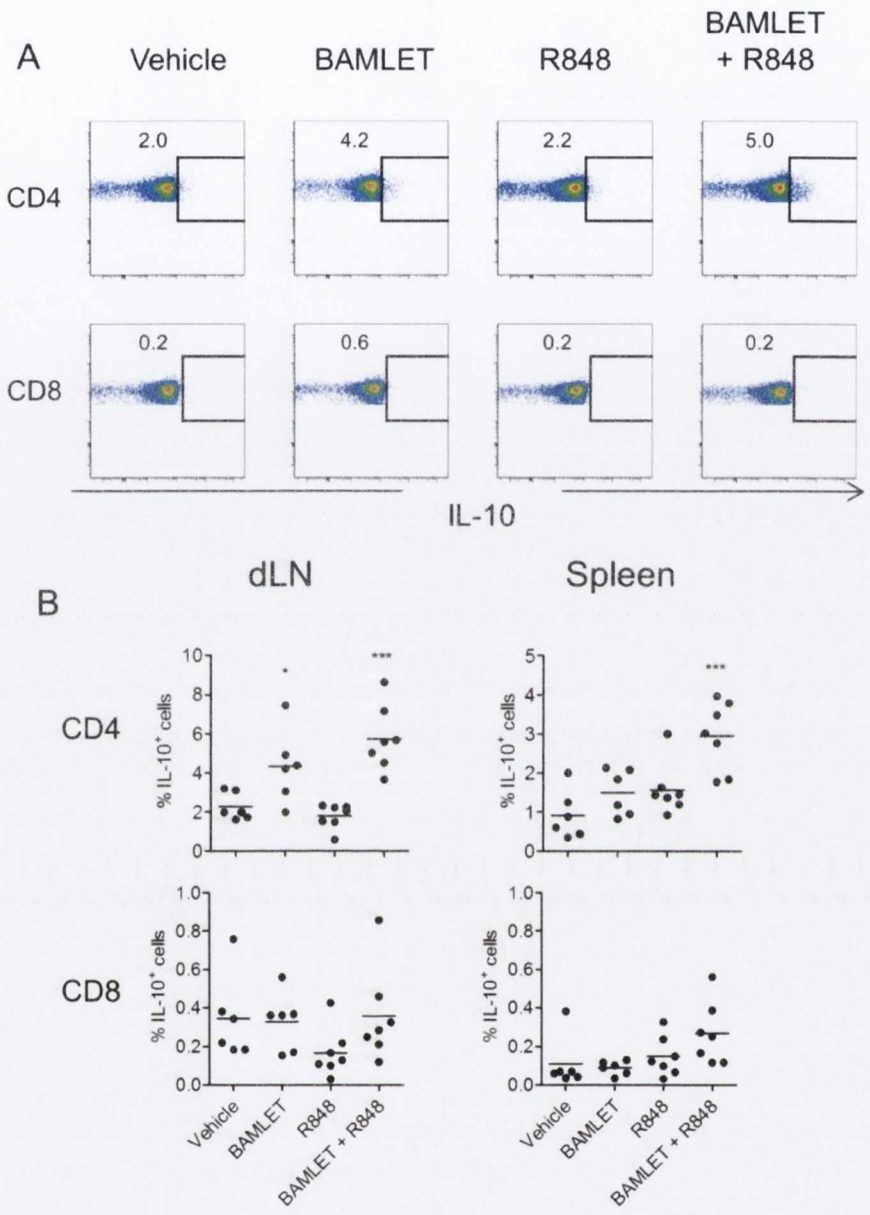


Figure 3.13
Therapeutic treatment with BAMLET and R848 increases the frequency of IL-10$^+$ CD4 T cells in dLN and spleens

Naïve C57BL/6 mice were injected with B16 tumour cells ($2x10^5$ cells/mouse, 7 mice/group) and treated with vehicle, BAMLET (1 mg/mouse), R848 (40 µg/mouse) alone or in combination on days 3, 7, 10 and 14 post tumour induction. The experiment was terminated on day 17 and IL-10 expression in dLN and spleens analysed by intracellular staining and flow cytometry, gating on CD4$^+$ or CD8$^+$ cells. Results are shown as representative FACS plots (A) or mean values (B). *$p<0.05$, ***$p<0.001$ versus vehicle control by 1-way ANOVA.

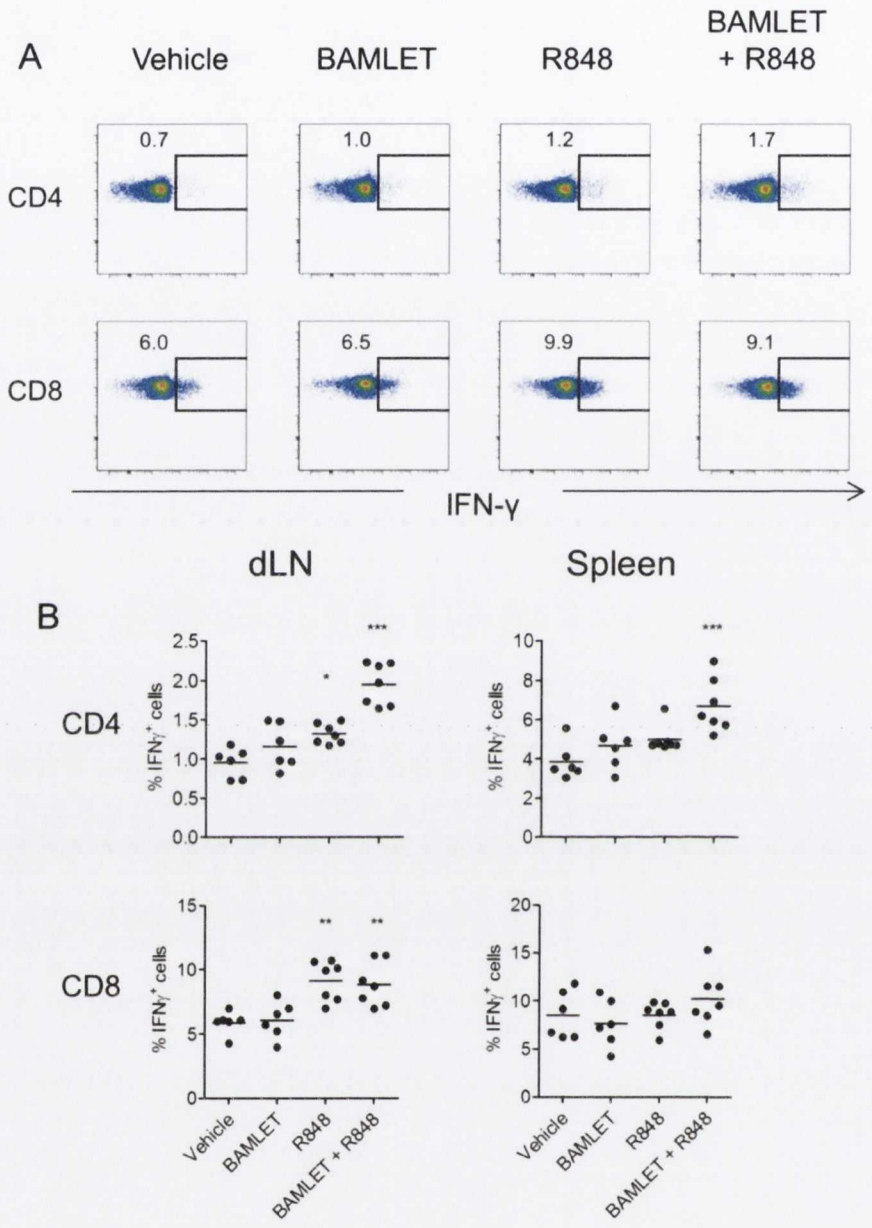


Figure 3.14
Therapeutic treatment with BAMLET and R848 increase the frequency of IFN-γ$^+$ T cells in dLN and spleens

Naïve C57BL/6 mice were injected with B16 tumour cells ($2x10^5$ cells/mouse, 7 mice/group) and treated with vehicle, BAMLET (1 mg/mouse), R848 (40 µg/mouse) alone or in combination on days 3, 7, 10 and 14 post tumour induction. The experiment was terminated on day 17 and IFN-γ expression in dLN and spleens analysed by intracellular staining and flow cytometry, gating on CD4$^+$ or CD8$^+$ cells. Results are shown as representative FACS plots (A) or mean values (B). *p<0.05, **p<0.01 , ***p<0.001 versus vehicle control by 1-way ANOVA.

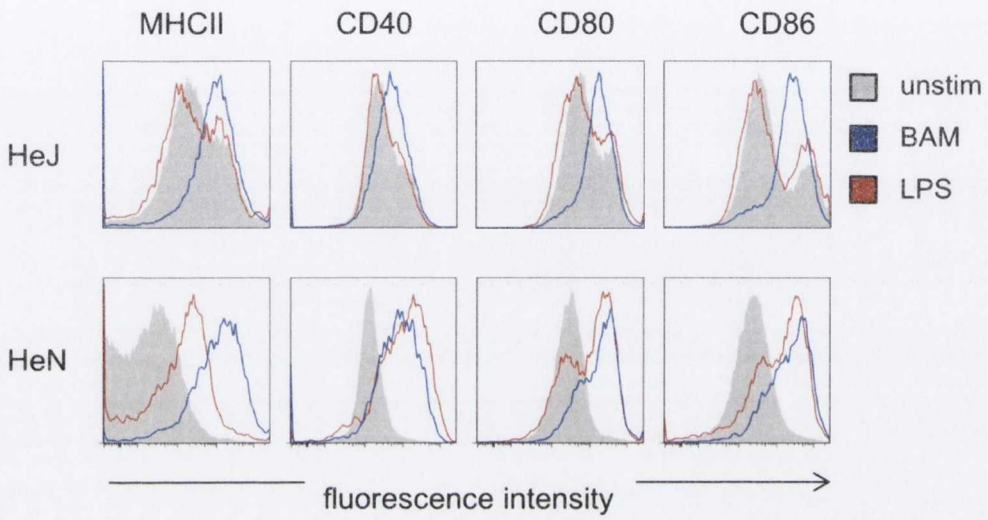


Figure 3.15
BAMLET induces the expression of MHCII, CD40, CD80 and CD86 in TLR4-defective BMDCs

C3H/HeJ or C3H/HeN BMDCs were stimulated with medium only (unstim), BAMLET (BAM; 0.01-0.1 mg/ml) or LPS (100 ng/ml). After 24 h, expression of MHC II, CD40, CD80, CD86 was analysed by surface staining and flow cytometry, gating on $CD11c^+$ cells. DC viability was over 90% after 24 h. Data are representative of 3 experiments.

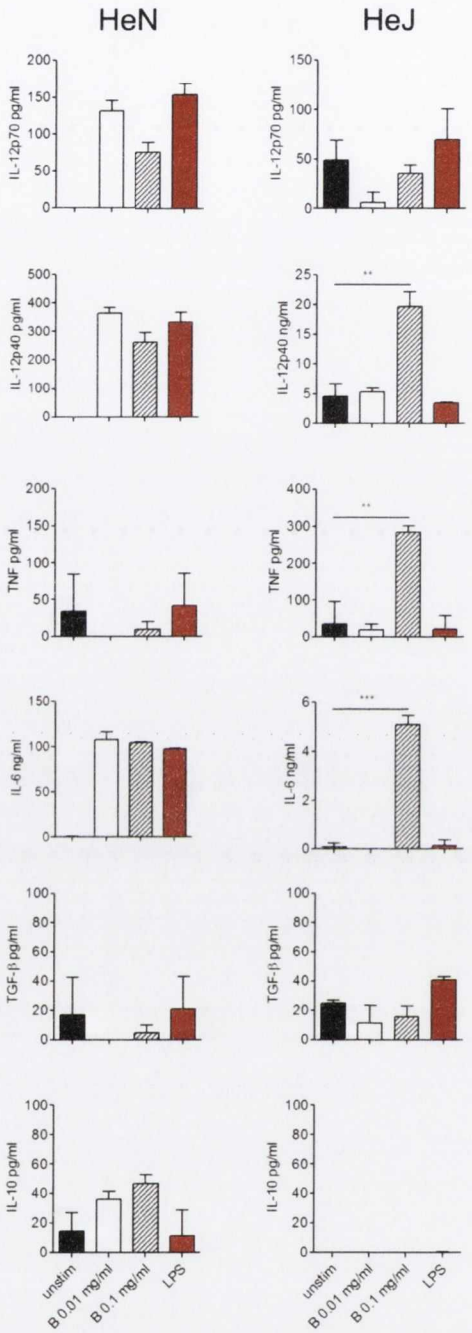


Figure 3.16
BAMLET induces the secretion of IL-12p40, TNF and IL-6 by TLR4-defective BMDCs

C3H/HeJ or C3H/HeN BMDCs were stimulated with medium only (unstim), BAMLET (B; 0.01-0.1 mg/ml) or LPS (100 ng/ml) for 24 h. Cytokine levels in culture supernatants were quantified by ELISA and results are shown as mean + SD from triplicate samples. BMDC viability was over 90% after 24 h. **p<0.01, ***p<0.001 by 1-way ANOVA. Data are representative of at least 3 experiments.

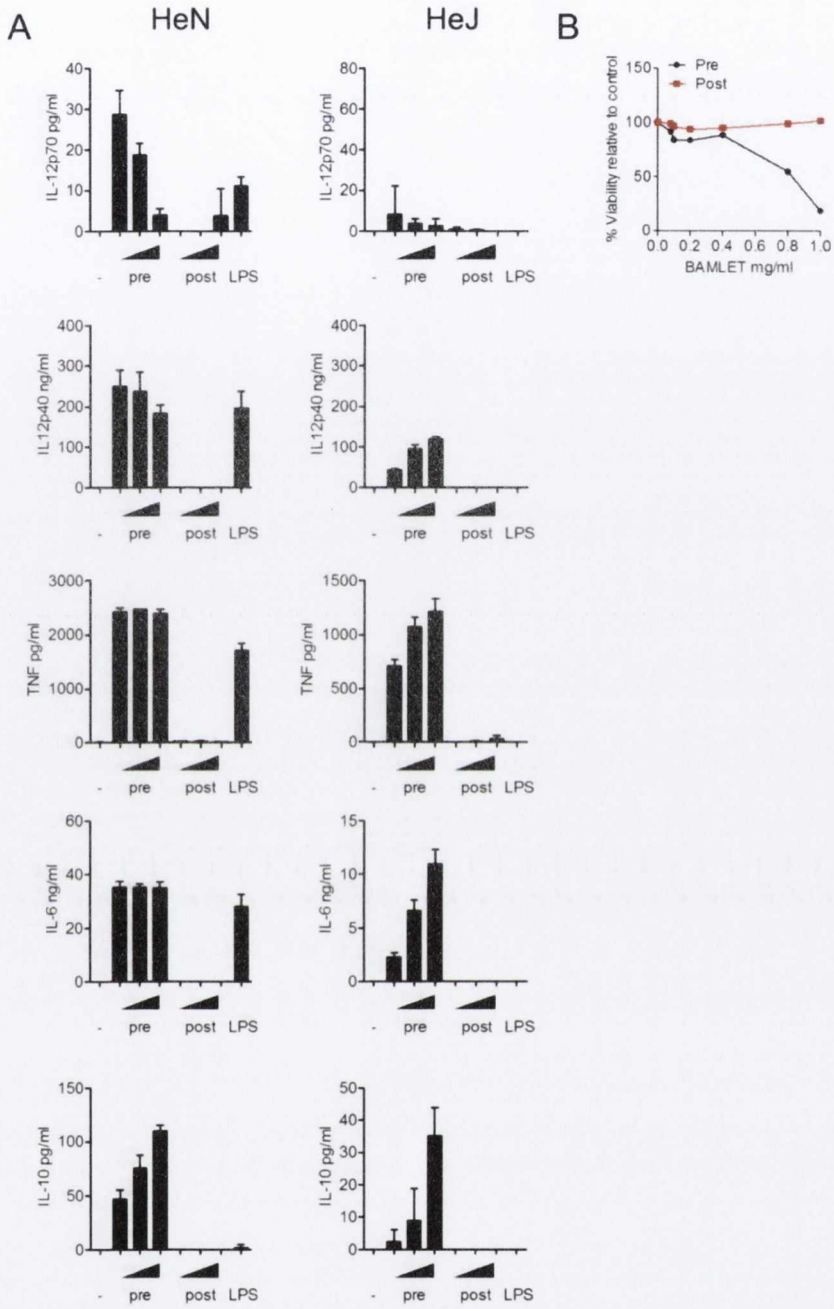


Figure 3.17
Polymyxin B-purified BAMLET loses ability to induce BMDC cytokine secretion and to promote B16 tumour cell death

BAMLET was purified using a polymyxin B column. C3H/HeJ or C3H/HeN BMDCs were stimulated with medium (-), or increasing concentrations of non-purified (pre) or purified (post) BAMLET (0.05-0.2 mg/ml) or LPS (100 ng/ml) for 24h. Cytokine levels in supernatants were quantified by ELISA and results are shown as mean ± SD from triplicate samples (A). B16 tumour cells were cultured in presence of increasing concentrations of non-purified (pre) or purified (post) BAMLET (0.0625-1 mg/ml) for 24 h and cell viability was measured using the alamar blue assay (B).

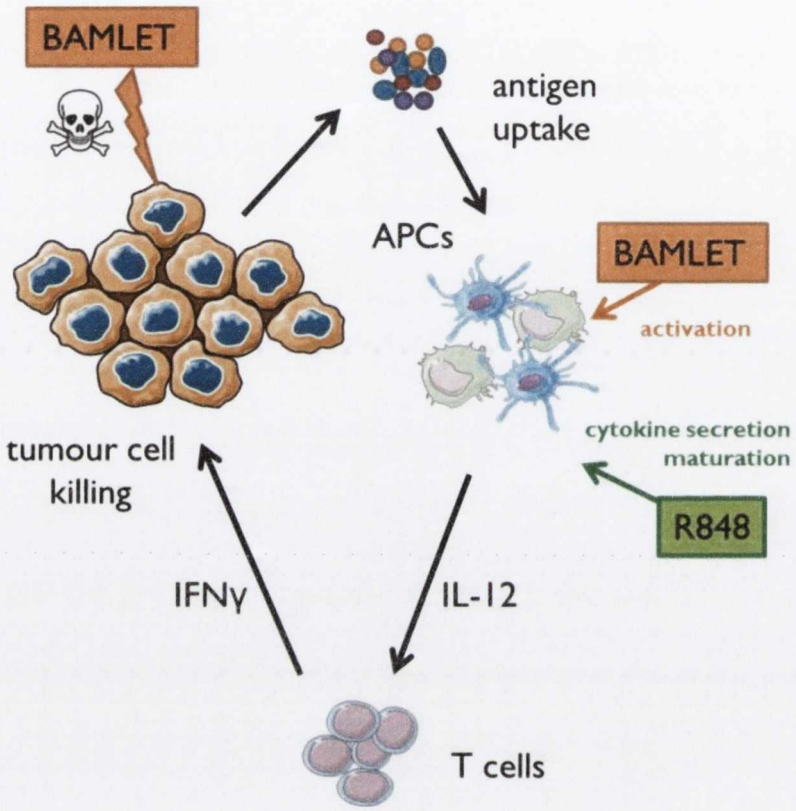


Figure 3.18
Proposed mechanism of anti-tumour activity mediated by BAMLET and R848

BAMLET induces cell death of tumour cells. Tumour antigens are taken up by APCs and presented to T cells. Stimulation of APCs with BAMLET and the TLR7 agonist R848 promotes maturation and pro-inflammatory cytokine secretion. Secreted IL-12 promotes IFN-γ-secreting T cells which have anti-tumour activity.

Chapter 4:

Manipulation of anti-tumour immune responses by targeting RARα

4.1. Introduction

Vitamin-A (Vit-A) is an essential nutrient which is important for human health, growth, reproduction and the immune system. An early indicator of Vit-A deficiency is nyctalopia (night blindness), due to the role of Vit-A in the phototransduction in the eye [297]. Impairments in the immune defence against pathogens during Vit-A deficiency also suggest an important role of Vit-A in shaping immune responses [298]. Vit-A, also called retinol, is metabolised into retinal by alcohol dehydrogenases (ALDH) or retinol dehydrogenases (RALDH). In a second irreversible step, RALDH1-3 catalyse the oxidation of retinal into retinoic acid. Retinoic acid is the functionally active form of Vit-A and exists in two isoforms, all-trans retinoic acid (RA) and 9-cis retinoic acid, of which RA represents the most common isoform in mammalian tissues [299]. RA acts as a transcription factor by binding to its nuclear receptors. Typically, it binds to a RAR/RXR heterodimer which then induces the transcription of target genes by binding to RA response elements (RARE) on the DNA. Three different RARs have been described, RARα, RARβ and RARγ. However, RARα is the pre-dominant receptor which RA signals through. The transcription of RA target genes is tightly regulated and also depends on the interaction with different co-activators and co-repressors [300]. While ligand bound RAR/RXR recruits co-activators, unbound RAR/RXR suppresses the transcription of target genes by recruiting co-repressors [301]. RA has hundreds of target genes which are involved in key processes of the human body, including the development and function of the immune system.

A role for RA in modulating the immune system has initially been established in the gut, where it promotes tolerance by maintaining immunosuppression under homeostatic conditions. RA secreted by myeloid cells in gut associated lymphoid tissues (GALT), such as DCs or macrophages, induces homing of T cells into the gut by prompting the expression of α4β7 and CCR9 [302]. Moreover, RA secreted by gut $CD103^+$ DCs contributes to TGF-β-induced expression of $Foxp3^+$ in CD4 T cells [154, 303]. The enhancement of TGF-β driven Treg induction by RA has been confirmed in numerous *in vitro* studies [61, 88, 154]. RA exerts its effects through SMAD3 and RAR/RXR induction of enhancers in the Foxp3 promoter region [155, 304, 305]. Conversely, the differentiation of pro-inflammatory Th17 cells induced by TGF-β and IL-6 can be suppressed by RA, demonstrating a reciprocal regulation of Treg and Th17 cells [61, 306]. In addition to promoting TGF-β-induced Treg conversion, RA also confers Treg lineage stability in presence of IL-6 by downregulating IL-6R expression [307]. A contribution of RA to immune tolerance was also demonstrated in mice with DSS-

induced colon inflammation. RA promoted IL-22 secretion by γδ T cells and innate lymphoid cells, which significantly attenuated gut inflammation and colon shortening [308]. Moreover, RA was shown to reduce intestinal inflammation in Vit-A deficient mice [309]. While gut $CD103^+$ DCs constitutively express genes encoding RALDH, DCs in the periphery have been shown to express negligible levels of RALDH in a steady state. However, peripheral DCs can be induced to express RALDH, for example by stimulation with GM-CSF, IL-4 or TLR agonists [310-312].

The immunological effects of RA are highly tissue and context-dependent, as demonstrated by pleiotropic effects on the differentiation and plasticity of CD4 T cell subtypes. Recent publications demonstrated that RA can also promote inflammation under certain conditions. In presence of pro-inflammatory IL-15, RA can act as an adjuvant and reduce Treg cells while enhancing Th17 and Th1 responses [313]. In another study, RA has been found to be critical for Th1 lineage commitment by repressing Th17 trans-differentiation, thereby preventing Th17-mediated autoimmunity [314]. These studies demonstrate that the effect of RA on T cells is controlled by the microenvironment and cytokine milieu.

RA has also been shown to be critical for the differentiation and function of DCs. Mice fed with a Vit-A deficient diet had a reduction in splenic DCs. RA was found to be crucial for the differentiation of pre-DCs into $CD8^+$ DCs and a Notch-dependent expansion of DCs was observed when mice were fed with a diet with increased amounts of Vit-A [315]. Moreover, RA-stimulated mDCs acquired a "mucosal DC" phenotype which led to enhanced secretion of IL-6 and TGF-β and induction of gut homing receptors on T cells [316].

In the context of cancer, the function of RA is also context dependent. Treatment with retinoids was protective in leukaemia patients, however, retinoids failed to induce protection against other cancers [317, 318]. RA is generally considered to be anti-proliferative and an inducer of cell differentiation. Consistent with this, studies using the B16 murine melanoma model found that RA reduced tumour cell proliferation *in vitro* and *in vivo* in combination with the chemotherapeutic agent cisplatin [319]. The enrichment of RA in the tumour microenvironment also correlated with the expansion and survival of tumour-reactive CD8 T cells, suggesting that RA has tumour suppressor properties [320]. However, strong RA signalling and ALDH1A expression was linked to poorer patient survival in breast cancer patients [321]. Moreover, ALDH positive melanoma cells appear more tumourigenic and more resistant to chemotherapeutic

drugs than ALDH negative cells, suggesting a tumour-promoting role of RA signalling [322].

Apart from direct effects of RA on tumour cell proliferation and survival, the capability of RA to shape immune responses has implications for anti-tumour immunity. Potent activation of DCs and T cells is fundamental for the induction of tumour directed responses. DCs are required for the priming and activation of T cells. This step requires three signals from DCs: antigen presentation through MHCs, the interaction between co-stimulatory molecules on DCs and CD28 on T cells and the secretion of cytokines. In situations where PAMPs/DAMPs are present, such as during an infection, DCs mature and are able to activate T cells. Under steady state conditions, immature DCs present antigens to T cells but induce apoptosis or T cell anergy in order to maintain tolerance [323]. Tolerogenic DCs, which include most immature DCs, can induce naïve T cells to differentiate into Treg cells, for example through low levels of antigen presentation or secretion of suppressive molecules [156].

While the role of RA has been extensively studied in the context of homeostasis and autoimmunity, little is known about the effect of RA in modulating anti-tumour immune responses. The aim of this chapter was to investigate the effect of RA signalling on T cells and DCs in the context of cancer and to use those findings to develop an immunotherapy approach that is effective in reducing tumour growth. More specifically, the potential of a RARα antagonist in anti-cancer therapy alone or in context of a DC vaccine was examined. This study investigated the hypothesis that immunosuppressive effects elicited by RA can be reversed by blocking RARα.

4.2. Results

4.2.1. B16 and CT26 tumour cells produce the suppressive molecules RA, TGF-β and IL-10

In the course of tumour progression, tumour cells acquire mechanisms that suppress anti-tumour immune responses to promote their survival and cell growth. In order to characterise such mechanisms, two murine tumour cell lines were cultured *in vitro* and the production of suppressive molecules was analysed. The ALDEFLUOR assay detects the activity of ALDH, an enzyme that converts retinol into RA. In the presence of ALDH, the ALDEFLUOR substrate is converted into a fluorescent product which can be detected by flow cytometry. A significant proportion of B16 and CT26 tumour cells was positive for ALDEFLUOR which was blocked by using the ALDH inhibitor DEAB, indicating that B16 and CT26 tumour cells have active ALDH and produce RA (Figure 4.1A). Moreover, increasing levels of secretion of TGF-β and IL-10 were detected over time by cultured tumour cells, indicating a constant suppressive phenotype of both tumour cell lines (Figure 4.1B).

4.2.2. Retinoic Acid Receptors are expressed in murine tissues and immune cells

Having shown that B16 and CT26 tumour cells are potent producers of RA, the next experiment was designed to identify potential RA targets. The expression of the three isotypes RARα, RARβ and RARγ was analysed in different murine tissues and immune cells by RT-PCR. Low levels of all RARs were detected in the liver. The lung tissue expressed all three RARs. The highest expression levels of all three RARs were found in BMDMs. BMDCs and naïve CD4 T cells expressed high levels of RARα but no RARβ and only low levels of RARγ (Figure 4.2). Overall, RARα was the most abundant RAR found in immune cells.

4.2.3. RA enhances the conversion of naïve CD4 T cells into Foxp3$^+$ Treg cells which can be blocked by RARi

It has previously been demonstrated that RA plays a role in promoting the induction of Treg cells [61]. This characteristic of RA was further investigated, by stimulating purified naïve CD4 T cells in presence of IL-2 and TCR stimulation with anti-CD3 and anti-CD28 *in vitro*. TGF-β increased the frequency of Foxp3-expressing cells from

naïve CD4 T cells. The addition of RA significantly enhanced the conversion of naïve CD4 T cells into Treg cells over a range of concentrations. Importantly, the presence of TGF-β was crucial for the conversion, as RA alone did not have an effect on the expression of Foxp3. The effect of RA on Treg cell induction was reported to be mediated through the receptor isotype RARα [306, 324]. The addition of a RARα antagonist (RARi) significantly reduced Foxp3 expression in CD4 T cells induced by TGF-β alone or in combination with RA (Figure 4.3 A,B).

Moreover, RA significantly inhibited the secretion of IFN-γ by CD4 T cells stimulated with anti-CD3 and anti-CD28. TGF-β alone did not affect IFN-γ secretion but amplified RA-induced suppression of IFN-γ production. The presence of RARi did not restore RA-suppressed IFN-γ secretion (Figure 4.3C). These findings suggest that RA suppresses CD4 T cell effector function by promoting Treg conversion and suppressing IFN-γ secretion.

4.2.4. RA enhances the conversion of antigen-stimulated CD4 T cells into Foxp3$^+$ Treg cells

The previous experiment investigated Treg conversion from naïve CD4 T cells. The next experiment was designed to elucidate the ability of RA to induce the conversion of Treg cells from committed CD4 T cells. OVA-TCR transgenic mice were immunized with OVA protein and CpG into the footpad and draining lymph nodes were removed 7 days later. LN cells were restimulated with OVA peptide in presence or absence of TGF-β and RA and Foxp3 expression was examined by flow cytometry. Similar to naïve CD4 T cells, RA with TGF-β significantly increased the frequency of Foxp3$^+$ Treg cells, however, RA and TGF-β showed little effect on their own. Under these conditions, RARi was not able to reduce Treg cell conversion (Figure 4.4).

Immunization of OVA TCR transgenic mice with OVA and CpG induced a Th1 response which was detected by intranuclear staining of T-bet in CD4 T cells. Upon re-stimulation with OVA peptide, the majority of CD4 T cells were T-bet$^+$Foxp3$^-$. When TGF-β and RA were added to the culture, the frequency of T-bet$^+$Foxp3$^+$ and Foxp3$^+$Tbet$^-$ cells was increased (Figure 4.5).

The effects of TGF-β, RA and RARi stimulation on cytokine production by OVA-restimulated T cells were examined after 3 d of OVA-stimulation. TGF-β and RA decreased the percentage of IFN-γ-producing cells, indicating that TGF-β and RA suppress T cell effector function. The addition of RARi did not restore IFN-γ production

(Figure 4.6 A,B). Moreover, only low levels of IL-17-producing T cells were detected, which were not significantly altered by any of the treatments (Figure 4.6 A,C).

Taken together, the results suggest that stimulation of T cells from OVA and CpG-immunized mice with RA and TGF-β induces a shift from a Th1 to Treg response.

4.2.5. RA does not significantly affect growth of B16 tumours in vivo

An examination of the effects of RA on the development of Treg cells *in vitro* revealed that RA has the potential to enhance Treg responses. However, recent publications have suggested that RA can also enhance effector T cell responses in an inflammatory milieu, while maintaining anti-inflammatory responses in a steady state [313]. To address the effects of RA on tumour growth *in vivo*, mice were implanted with B16 melanoma cells and treated with RA either early (d -1, 1, 3 and 5 post tumour induction) or late (d 7, 9, 11 and 13 post tumour induction). The results demonstrated that neither treatment regimen significantly altered B16 tumour growth *in vivo* (Figure 4.7).

To determine whether treatment with RA had an effect on T cell differentiation *in vivo*, tumour infiltrating CD4 T cell populations were analysed by flow cytometry. CD4 T cells were analysed for differentiation into Treg cells ($Foxp3^+$), Th17 cells ($ROR\gamma T^+$) and Th1 cells ($T\text{-}bet^+$). In contrast to the findings *in vitro* where RA enhanced the expression of Foxp3, the opposite effect was observed *in vivo*. Early treatment had no effect and late treatment with RA significantly reduced the frequency of $Foxp3^+$ cells in the dLN and tumours. Interestingly, a trend towards reduced RORγT and T-bet levels was also observed in tumour and dLN when mice were treated with RA (Figure 4.8). These findings suggest that RA inhibits CD4 T cell polarisation *in vivo*, however, without a visible impact on tumour growth.

4.2.6. Blocking RARα *in vivo* does not significantly affect B16 tumour growth

Since blocking RARα reduced the induction of $Foxp3^+$ Treg cells *in vitro*, it was investigated whether RARi could have a therapeutic effect against tumours *in vivo*. B16 tumours were implanted into mice and RARi was injected at early (d -1, 1, 3 and 5 post tumour induction) or late (d 7, 9, 11 and 13 post tumour induction) time points beside the tumour. Administration of RARi into the tumour site did not significantly alter tumour growth (Figure 4.9).

In order to investigate whether RARi affected T cell responses *in vivo*, tumours and dLN were analysed for infiltrating lymphocytes by flow cytometry. In the dLN, no differences in the frequencies of CD4 T cells, CD8 T cells or Treg cells were detected. Moreover, RARi did not alter the production of IFN-γ, IL-17 or IL-10 by CD4 and CD8 T cells in the dLN (Figure 4.10). In contrast, differences in lymphocyte populations were detected in the tumours. Both early and late administrations of RARi induced a modest reduction in the percentage of $Foxp3^+$ CD4 T cells. In addition, RARi reduced the production of the anti-inflammatory cytokine IL-10 and the pro-inflammatory cytokines IL-17 and IFN-γ by both in CD4 and CD8 T cells (Figure 4.11). This suggests that RARi modulated pro- as well as anti-inflammatory cytokine secretion by T cells *in vivo*, potentially counter-balancing pro and anti-tumour effects. This may explain why, despite the change in tumour infiltrating lymphocytes, no significant effects on tumour growth were detected *in vivo*.

Blocking RARα enhances the efficacy of a DC vaccine by reducing Treg cells

The results so far showed that blocking RARi can reduce the induction of Tregs *in vitro*. Direct treatment of tumour-bearing mice with RARi, however, failed to induce potent anti-tumour responses *in vivo*. The results shown in figure 4.2 demonstrated that RARα is expressed ubiquitously, demonstrating that other immune cells in the tumour microenvironment may be RA targets. Previous work in our lab has investigated RARi in the context of a DC vaccine. The DC vaccine comprised BMDCs pulsed with antigens (hs/irr B16 tumour cells) and primed with the TLR9 agonist CpG [88]. Antigen-pulsed and TLR-primed BMDCs were injected into mice on day 3, 10 and 17 post tumour inoculation. The results demonstrated that pre-incubation of BMDCs with RARi significantly improved the efficacy of the DC vaccine and led to a further reduction of tumour growth and prolonged survival. This effect was associated with a decrease of tumour infiltrating Treg cells and an increase of IFN-γ-producing CD4 T cells (Figure 4.12). These findings revealed that blocking RARα can significantly enhance the efficacy of a DC vaccine by reducing Treg cells and enhancing Th1 responses.

4.2.7. CpG induces maturation marker expression on BMDCs

The mechanism underlying the beneficial effects of blocking RARα in a DC vaccine was investigated further. DCs can shape T cell responses through several mechanisms, including the expression of co-stimulatory or co-inhibitory ligands and the secretion of cytokines or other soluble mediators. The effects of RARi on these phenotypic properties of DCs were assessed *in vitro*.

The DC vaccine employed in the study described in Figure 4.12 utilised BMDCs pulsed with tumour antigens by incubation with hs/irr B16 cells which are taken up by the BMDCs to be presented to T cells. During the antigen pulse, CpG was added to the BMDCs as an immune stimulator. The results show that CpG potently increased the expression of MHC II and the co-stimulatory molecules CD40, CD80 and CD86 on BMDCs (Figure 4.13). When BMDCs were pre-incubated with RARi before stimulation with hs/irr B16 and CpG, the expression of MHC II, CD80 and CD86 was moderately increased. This effect was less evident when DCs were unstimulated or stimulated with hs/irr B16 or CpG alone (Figure 4.14). These findings suggest that blocking RARi promotes a more mature phenotype of BMDCs that are stimulated with hs/irr B16 and CpG.

4.2.8. Blocking RARα does not affect TLR-induced PD ligand expression on BMDCs

Having shown that RARi increases the expression of co-stimulatory molecules on BMDCs, the effect of RARi on co-inhibitory ligand expression was examined. PD-L1 and PD-L2 are two ligands that have been shown to bind to the inhibitory receptor PD-1, which is expressed on activated T cells. Ligand binding to PD-1 can suppress T cell function and blocking this interaction has been shown to improve anti-tumour immune responses in pre-clinical tumour models [124, 125, 246, 247] and in clinical trials [249, 251, 252]. Unstimulated BMDCs expressed low basal levels of PD-L1 and PD-L2. Interestingly, stimulation of BMDCs with hs/irr B16 and to a bigger extent with CpG increased the expression of PD-L1 and PD-L2 (Figure 4.15A). These findings suggest that DAMPs and PAMPs strongly upregulate the expression of PD ligands on BMDCs.

It was then investigated whether RA signalling plays a role in the induction of PD-L1 and PD-L2 expression on BMDCs. However, neither the presence of RA nor RARi had an effect on PD-L1 or PD-L2 expression levels (Figure 4.15B), demonstrating that PD ligand induction in BMDCs is independent of RA signalling.

4.2.9. RARi inhibits anti-inflammatory cytokine secretion by BMDCs in response to hs/irr B16 cells and CpG

It is well established that TLR stimulation of BMDCs is able induce the secretion of a range of pro- as well as anti-inflammatory cytokines. To investigate the role of RARα on the induction of cytokine production, BMDCs were pre-incubated with RARi and subsequently stimulated with hs/irr B16 and CpG and cytokine secretion was quantified

by ELISA. CpG alone or in combination with hs/irr B16 induced the secretion of the pro-inflammatory cytokines IL-12p70, IL-12p40, TNF, IL-6, IL-23 and IL-1β and the anti-inflammatory cytokines IL-10 and TGF-β. RARi reduced the secretion of IL-12p70 and IL-12p40 and moderately enhanced the secretion of TNF by DCs stimulated with CpG alone or in combination with hs/irr B16. The secretion of IL-23 and IL-1β was not significantly altered by RARi. Importantly, blocking RARα significantly inhibited the secretion of IL-10, TGF-β and IL-6 by BMDCs stimulated with hs/irr B16 and CpG (Figure 4.16).

4.2.10. ALDH expression in BMDCs is enhanced by TLR9 stimulation with CpG

The results so far have demonstrated that RA promotes the induction of Treg cells but also affects the DC phenotype. In the tumour microenvironment, RA can have multiple sources, such as tumour cells, stromal cells or infiltrating leukocytes. A study by Villablanca et al. showed that RA can also have autocrine effects in DCs, causing its own secretion through a positive feedback loop [325]. RA production by BMDCs was investigated using the ALDEFLUOR assay. Around 30% of unstimulated BMDCs were positive for ALDEFLUOR and the activity was inhibited by the ALDH inhibitor DEAB. Stimulation of BMDCs with CpG, but not with hs/irr B16 cells significantly increased ALDH activity (Figure 4.17). These findings reveal that BMDCs are potent inducers of RA and this activity is enhanced by TLR stimulation.

In order to investigate a possible positive feedback effect of RA signalling on RA production, BMDCs were pre-stimulated with a RARα antagonist or a RAR pan reverse agonist, followed by stimulation with hs/irr B16, CpG or RA. ALDH activity was measured using the ALDEFLUOR assay. Blocking RARα significantly reduced ALDH activity in unstimulated DCs and DCs stimulated with hs/irr B16, CpG or RA. Blocking all RA receptors using a pan inverse agonist reduced ALDH activity to an even greater extent. Surprisingly, stimulation with RA also significantly reduced ALDH activity (Figure 4.18 A,B). It was investigated whether a reduction of ALDH activity could be due to cytotoxicity, however, neither of the stimulants induced cell death of DCs (Figure 4.18C). These findings suggest that there is a positive feedback effect of endogenous RA on ALDH activity, but high dose of exogenous RA induced the opposite effect inhibiting RA production.

4.2.11.RARi stimulated BMDCs reduce TGF-β-induced Treg conversion

Having shown that RARi reduced the secretion of anti-inflammatory cytokines and RA by BMDCs, it was then investigated whether the presence of RARi-treated BMDCs can interfere with Treg conversion. Purified CD4 T cells were cultured alone or in presence of previously stimulated BMDCs. RARi significantly reduced TGF-β and RA-enhanced induction of Foxp3-expressing Treg cells when CD4 T cells were cultured alone (Figure 4.19 A,C). When unstimulated BMDCs were added to TGF-β-stimulated CD4 T cells, the frequency of Treg cells was lower compared to CD4 T cell cultured alone (Figure 4.19 B,D). Interestingly, BMDCs previously stimulated with CpG suppressed the induction of Treg cells which was even more reduced when BMDCs were pre-stimulated with both RARi and CpG. In contrast, RA pre-stimulated BMDCs marginally increased the frequency of Treg cells in the culture (Figure 4.19 B,D).

Moreover, pre-stimulation of BMDCs with RARi increased the concentration of IL-12p70 in co-cultures and stimulation with CpG significantly enhanced the levels of secreted IL-12p40 (Figure 4.20). These findings demonstrate that BMDCs activated with CpG and stimulated with RARi acquire a pro-inflammatory phenotype capable of suppressing the induction of Treg cells and secreting Th1-polarising cytokines.

Overall, the results suggest that blocking RARα enhanced the efficacy of a DC vaccine by enhancing the expression of co-stimulatory molecules and reducing the secretion of anti-inflammatory molecules by BMDCs which are able to reduce the generation of Treg cells.

4.3. Discussion

RA is the active metabolite of Vit-A and regulates hundreds of genes, including genes that are important for the development and function of the immune system. The role of RA has been extensively studied in the context of homeostasis and autoimmunity. However, little is known about the role of RA in anti-tumour immunity. The observation that cells of the tumour microenvironment produce RA suggests that RA can have profound implications for anti-tumour immune responses and immunotherapy [320].

The present study has demonstrated that RA is a key molecule in the differentiation of Foxp3-expressing Treg cells from naïve CD4 T cells. The results presented here show that the presence of TGF-β is crucial for RA-mediated Treg conversion, suggesting that RA signalling in CD4 T cells mediates TGF-β-induced Foxp3 expression. These results are consistent with previous studies showing the involvement of RA in the generation of Treg cells [61, 154, 303, 306, 326]. Interestingly, blocking RA with a RARα antagonist also reduced TGF-β-induced Treg conversion of purified CD4 T cells in absence of RA. These findings suggest that endogenous RA signalling is involved in TGF-β-driven Treg conversion and that RA is secreted by CD4 T cells in an autocrine manner which promotes Foxp3 expression.

The present study also provided evidence that exogenous RA promotes the expression of Foxp3 in committed Th1 cells, suggesting that Th1 cells can trans-differentiated into Treg cells. This observation was surprising since RA has been implicated in the maintenance Th1 lineage stability in a study by Brown et al. [314]. Endogenous RA signalling through RARα prevented Th1 to Th17 trans-differentiation and loss of RARα promoted Th1 plasticity. Moreover, no differences in Foxp3-expressing CD4 T cells were observed when RARα was knocked out [314]. In contrast to the study by Brown et al., the present study investigated the effects of high-dose exogenous RA which enhanced the frequency of $Foxp3^+$ CD4 T cells. This suggests that the intensity of RA signalling may be a crucial factor for the outcome of RA signalling. Moreover, blocking RARα did not inhibit RA-induced Treg conversion of CD4 T cells from OVA-immunized mice, indicating that signalling of RA through other RARs could have played a role. Furthermore, Treg cells constitute about 10-15% of peripheral CD4 T cells which would have been present in LN of OVA-immunized mice that were stimulated with RA in the present study [152]. It needs to be further elucidated whether TGF-β and RA converted previously committed Th1 cells or induced the proliferation of existing Treg cells.

The present study demonstrated that TGF-β and RA inhibited IFN-γ secretion by activated CD4 T cells. This effect could have been mediated by Treg cells that were induced in these conditions or directly by TGF-β or RA signalling in CD4 T cells. It has been reported that TGF-β can directly inhibit IFN-γ secretion in CD4 T cells through distinct mechanisms. At the priming stage, TGF-β was found to suppress IFN-γ secretion through STAT4 inhibition [327]. However, at the recall stage STAT4 suppression was dispensable for TGF-β-mediated IFN-γ suppression, which was instead dependent on T-bet suppression [327]. In the present study, RA inhibited IFN-γ production in absence of TGF-β, which indicates that this effect is mediated directly by RA and not by Treg cells. The literature on the effect of RA on IFN-γ production by CD4 T cells is contradictory. In mice, stimulation of CD4 T cells with RA did not interfere with IL-1β and IL-23-induced IFN-γ secretion [328]. In contrast, RA inhibited PMA/Ionomycin-induced IFN-γ production by human CD4 T cells [329]. This indicates that the effect of RA on IFN-γ secretion by CD4 T cells may be dependent on the cell origin or type of stimulus.

While RA enhanced TGF-β-induced Treg conversion *in vitro,* which was blocked with a RARα antagonist, no major differences in tumour growth were observed *in vivo* following treatments with RA or RARi. This was probably due to the pleiotropic nature of RA-mediated immune regulation and direct effects of RA on tumour cells. RARα is ubiquitously expressed and therefore RA treatment could affect a multitude of cells which induce pro- as well as anti-tumour effects. Indeed, much of the literature on RA in cancer has demonstrated beneficial effects of RA against tumour growth. It has been suggested that RA has anti-tumour effects by directly inhibiting tumour growth and promoting melanoma cell differentiation [330, 331]. RA also prolonged effects of several anti-tumour treatments *in vivo* by promoting the differentiation of immature myeloid cells (ImC) which can suppress effector T cells [332]. Mirza et al. observed a reduction of ImC in patients with metastatic renal cell carcinoma that were treated with RA. However, subsequent treatment with IL-2 eliminated the positive effect of RA on ImC [333]. Moreover, RA has been shown to contribute to inflammation in an inflammatory environment [313]. This property of RA could potentially promote anti-tumour immune responses. Another study by Guo et al. reported that endogenous RA production in the microenvironment promoted CD8 T cell survival and anti-tumour activity which was absent in mice where CD8 cells lacked RARα signalling [320]. These findings are at variance with the view that RA signalling in the tumour microenvironment promotes immunosuppression though Treg induction. However, in the present study neither RA administration nor general blockade of RARα had a

significant impact on tumour growth *in vivo*. These findings lead to the conclusion that RA signalling may play a dual role in mediating immune responses which are strongly dose-, context-, and tissue- dependent.

The study also examined the effects of a RARα in the context of a DC vaccine. The approval of the DC vaccine Sipuleucel-T for the treatment of prostate cancer demonstrated the feasibility of DC vaccines in humans [224]. One of the challenges of *ex vivo* DC vaccines is proper priming of DCs that leads to the generation of mature antigen-presenting DCs that preferentially promote Th1 responses. TLR agonists have been used as adjuvants in DC vaccines to induce DC maturation. However, it has been shown that T cell activation can be inhibited by anti-inflammatory cytokines, such as IL-10 and TGF-β, which are commonly secreted by TLR-stimulated DCs [160, 334]. Thus, the present study aimed to improve the pro-inflammatory phenotype of DCs. Indeed, blocking RARα in antigen-pulsed and TLR-activated DCs enhanced the efficacy of a DC vaccine in the B16 tumour model. By blocking RARα during *in vitro* DC priming, the infiltration of Treg cells was significantly reduced, while Th1 responses were enhanced *in vivo* [88].

Follow up experiments studied the effects of RARi on BMDCs during the *in vitro* priming stage. The present study revealed the novel finding that blocking RARα in DCs reduced the secretion of anti-inflammatory cytokines, indicating that TLR-induced cytokine secretion is mediated by autocrine secretion of RA. Indeed, TLR9 stimulation with CpG enhanced ALDH activity, suggesting that CpG-induced RA feeds back and promotes the secretion of IL-10 and TGF-β. Moreover, blocking RARα increased the expression of maturation markers on activated BMDCs. This is in line with previous studies that reported that RA promotes a regulatory DC phenotype. Jin et al. observed that RA stimulation of human monocyte-derived DC reduced the expression of maturation markers and induced the secretion of IL-10 [335]. DCs from cord blood monocytes were shown to produce IL-10 in response to RA which was blocked with a RARα antagonist. Moreover, RA-stimulated DCs have been shown to promote Treg conversion and to induce IL-10 secretion by CD4 T cells [325, 336, 337].

The present study demonstrated that blocking RARα significantly reduced ALDH activity in BMDCs, indicating that endogenous RA also feeds back on RA production. Moreover, DCs pre-stimulated with RARi inhibited the conversion of naïve CD4 T cells into Tregs while increasing IL-12 production. This is in agreement with reports of RA-DC mediated Treg conversion [325, 336, 337]. Villablanca et al. demonstrated that RA induces a positive feedback loop on its own synthesis [325]. These findings suggest

that blocking endogenous RA signalling suppresses regulatory and promotes Th1 responses by DCs. These *in vitro* effects of RARi on the DC phenotype could explain the improved efficacy of the RARi pre-treated DC vaccine.

The present study demonstrated that TLR stimulation increased the expression of the co-inhibitory molecules PD-L1 and PD-L2 on DCs which were shown to suppress T cell effector function [113, 114]. This suggests that a combination of a TLR-primed DC vaccine with immune checkpoint inhibitors targeting PD-1:PD-L1/2 interaction could lead to further improvements of the vaccine. Clinical data on the combination of DC vaccines with immune checkpoint inhibitors is scarce, however, this combination is expected to be tested in the future with the recent approvals of several checkpoint inhibitors for the treatment of cancer [7]. A pilot study using a DC vaccine pulsed with the tumour antigen MART in combination with a CTLA-4 blocking antibody resulted in objective durable anti-tumour responses in melanoma patients demonstrating the feasibility of such a combination [338]. Moreover, Rosenblatt et al. reported PD-L1 expression on an autologous dendritic cell/myeloma fusion vaccine which showed immunological responses in clinical trials [339]. They demonstrated that PD-1 blockade enhanced *ex vivo* T-cell responses to the vaccine suggesting that PD-L1 expression could have reduced the clinical efficacy of the vaccine [339].

Taken together, the present findings demonstrate a complex role of RA in the regulation of immune responses against cancer and highlight the difficulty of translating *in vitro* experiments with RA into physiological responses *in vivo*. While blocking RARα inhibited the conversion of naïve CD4 T cells *in vitro,* RA administration or RARα blockade did not affect tumour growth or T cell responses *in vivo*. This suggested that targeting RA in cells *ex vivo* was a better and more controlled approach. Indeed, blocking RARα improved the efficacy of an antigen-pulsed and CpG-activated DC vaccine *in vivo* which enhanced anti-tumour immune responses while reducing immunosuppression.

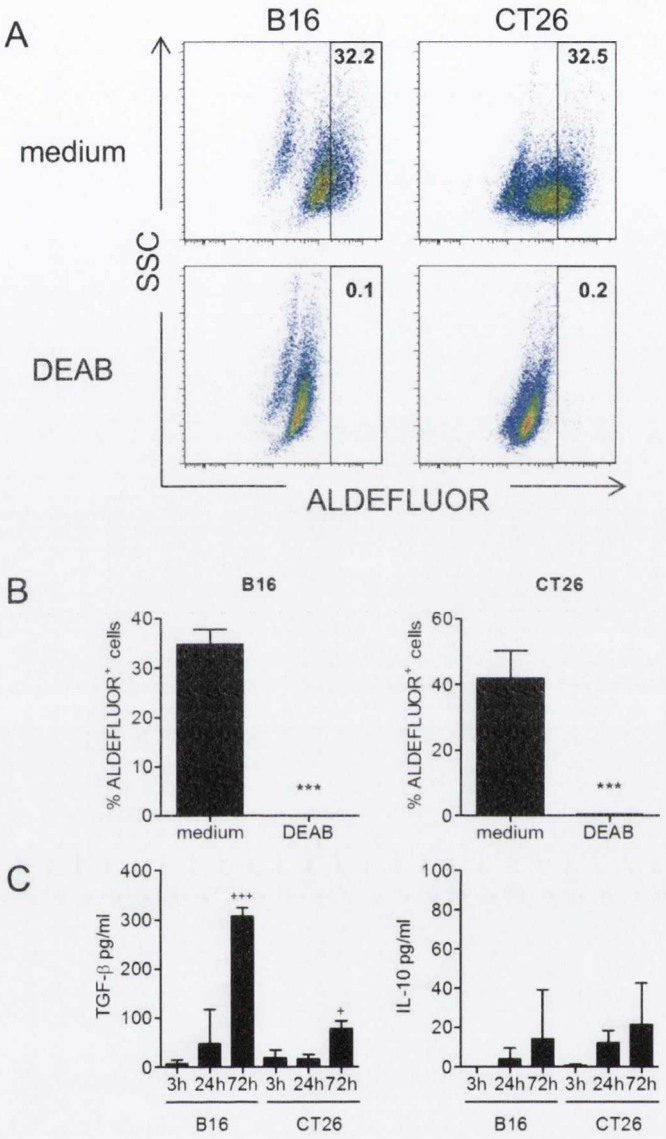


Figure 4.1
B16 and CT26 tumour cells produce the suppressive molecules RA, TGF-β and IL-10

B16 and CT26 cells were cultured *in vitro* for 24h. Subsequently, cells were stimulated for 1 h in presence or absence of the ALDH inhibitor DEAB and ALDH activity was measured using the ALDEFLUOR assay. ALDEFLUOR activation was analysed by flow cytometry and results are shown as representative FACS plots (A) and mean values ± SD for triplicate samples (B). Culture supernatants were collected after 3h, 24h and 72h. TGF-β and IL-10 concentrations in cell culture supernatants were quantified by ELISA. Results are shown as mean values ± SD for triplicate samples (C) ***$p<0.001$ versus medium control, $^{+}p<0.05$, $^{+++}p<0.001$ versus 3h by 1-way ANOVA. Data are representative of 2 experiments.

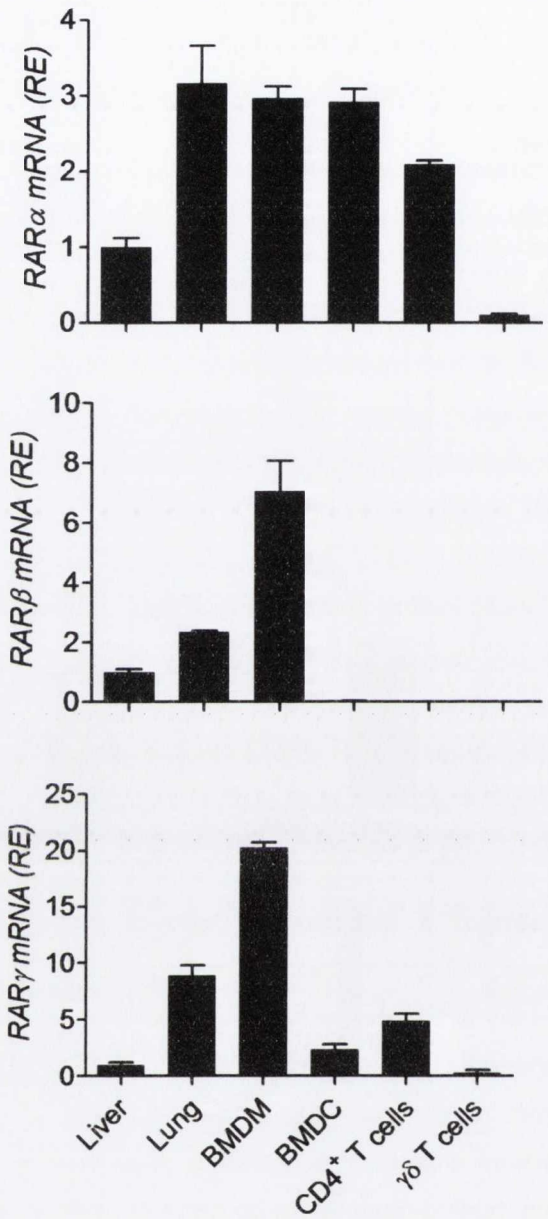


Figure 4.2
Retinoic acid receptors are expressed in murine tissues and immune cells

mRNA was isolated from livers, lungs, BMDMs, BMDCs or purified CD4 and γδ T cells from naïve C57BL/6 mice. cDNA was generated by reverse transcription and relative expression levels of RARα, RARβ and RARγ were measured by RT-PCR. Expression levels were normalised to 18sRNA. Results are shown as mean ± SD values for triplicate samples.

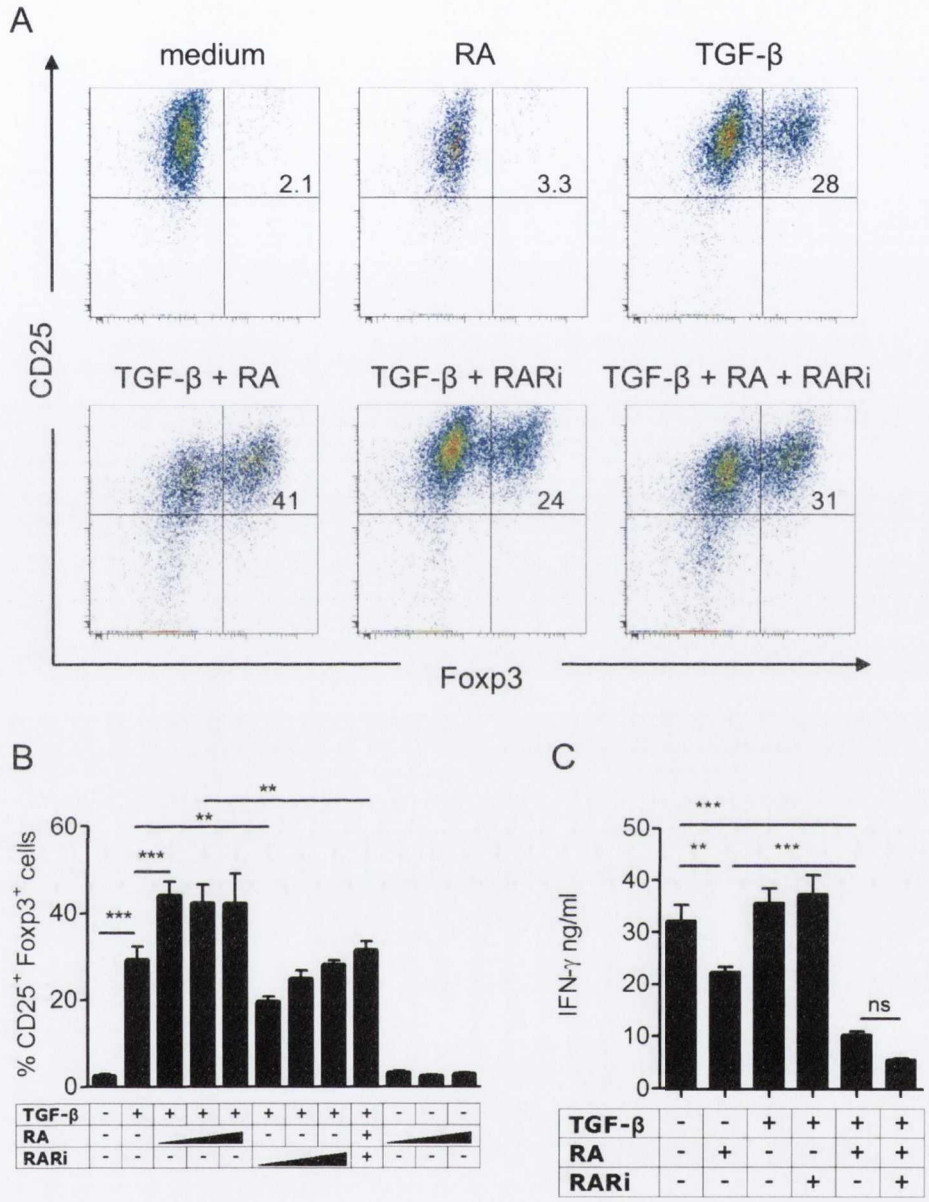


Figure 4.3

RA enhances the conversion of naïve CD4 T cells into Foxp3+ Treg cells which can be blocked by RARi

Naïve murine CD4 T cells were cultured in anti-CD3-coated wells (1 µg/ml) in medium containing IL-2 (10 ng/ml), soluble anti-CD28 (5 µg/ml) with TGF-β (5 ng/ml), RA (50, 100 or 200 nM) and RARi (2.5, 5, or 10 µM) as indicated. After 3 d, the frequency of Treg cells was detected by flow cytometry, gating on CD3+CD4+CD25+Foxp3+ cells. Results are shown as representative FACS plots (A) or mean values ± SD for triplicate samples (B). IFN-γ levels in supernatants were quantified by ELISA (C) **p<0.01, ***p<0.001 by 1-way ANOVA. Data are representative of at least 3 experiments.

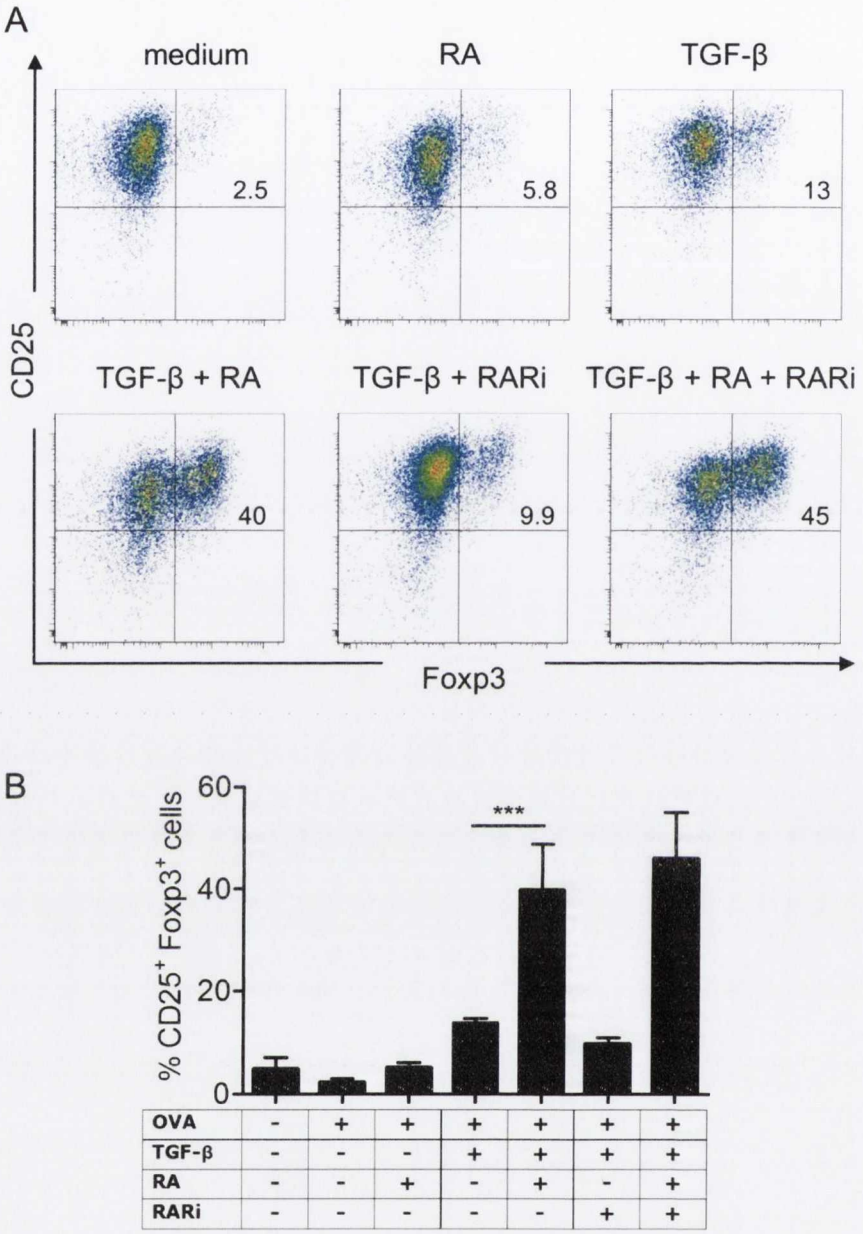


Figure 4.4

RA enhances the conversion of antigen-experienced CD4 T cells into Foxp3+ Treg cells

LN cells from OVA TCR transgenic mice immunized with OVA protein and CpG were re-stimulated with OVA peptide (1 µg/ml) and stimulated with TGF-β (5 ng/ml), RA (100 nM) and RARi (5 µM) as described. After 3 d, Foxp3 and CD25 expression was analysed by intranuclear staining and flow cytometry after gating on CD4+ cells. Results are shown as representative FACS plots (A) or mean ± SD values from triplicate samples (B). **p<0.01 by 1-way ANOVA. Data are representative of 2 experiments.

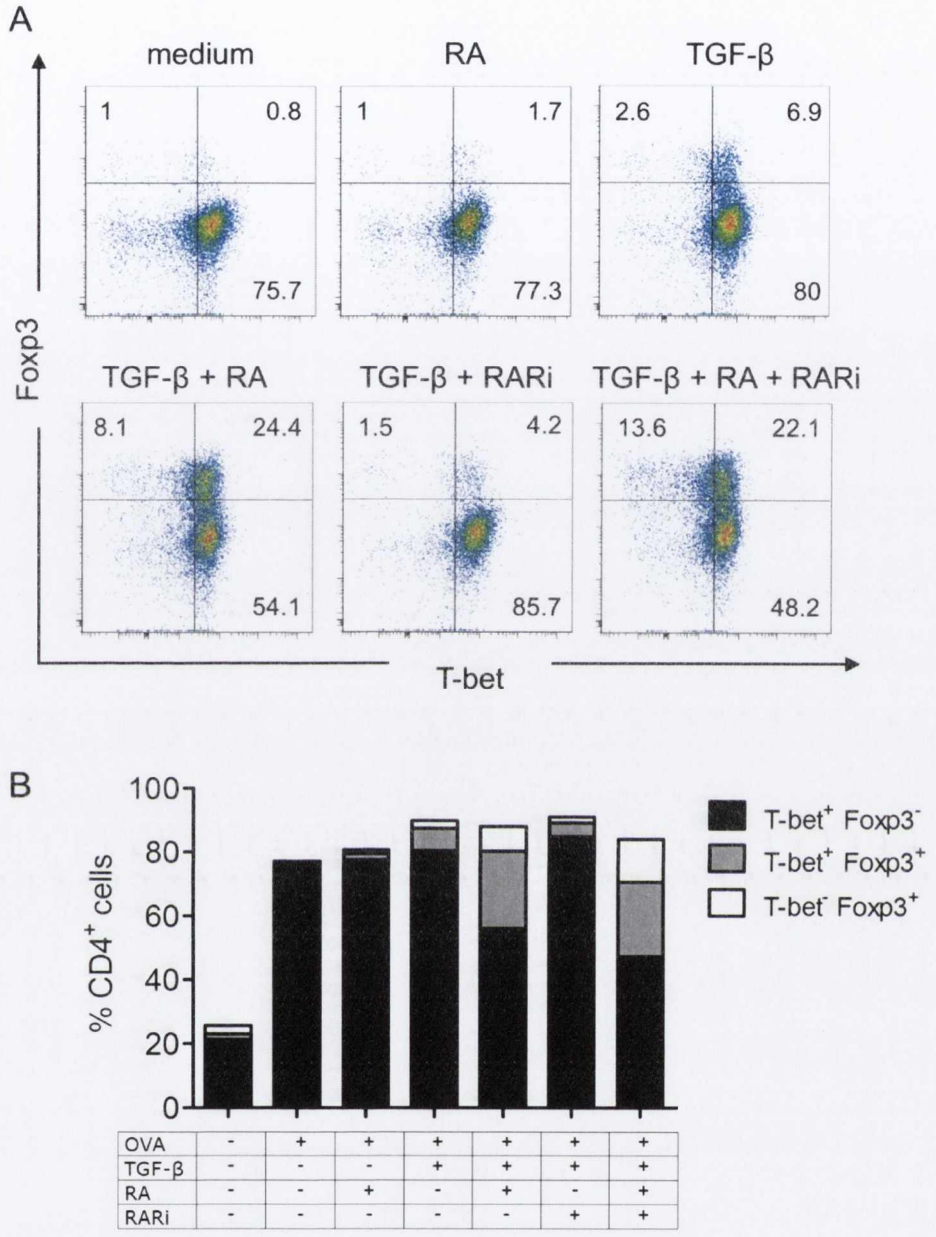


Figure 4.5
Immunization of OVA TCR transgenic mice with OVA and CpG induces a Th1 phenotype that is shifted towards Foxp3$^+$ by TGF-β and RA

LN cells from OVA TCR transgenic mice immunized with OVA protein and CpG were re-stimulated with OVA peptide (1 µg/ml) and stimulated with TGF-β (5 ng/ml), RA (100 nM) and RARi (5 µM) as indicated. After 3 d, Foxp3 and T-bet expression were analysed by intranuclear staining and flow cytometry, gating on CD4$^+$ cells. Results are shown as representative FACS plots (A) or mean values from triplicate samples (B). Data are representative of 2 experiments.

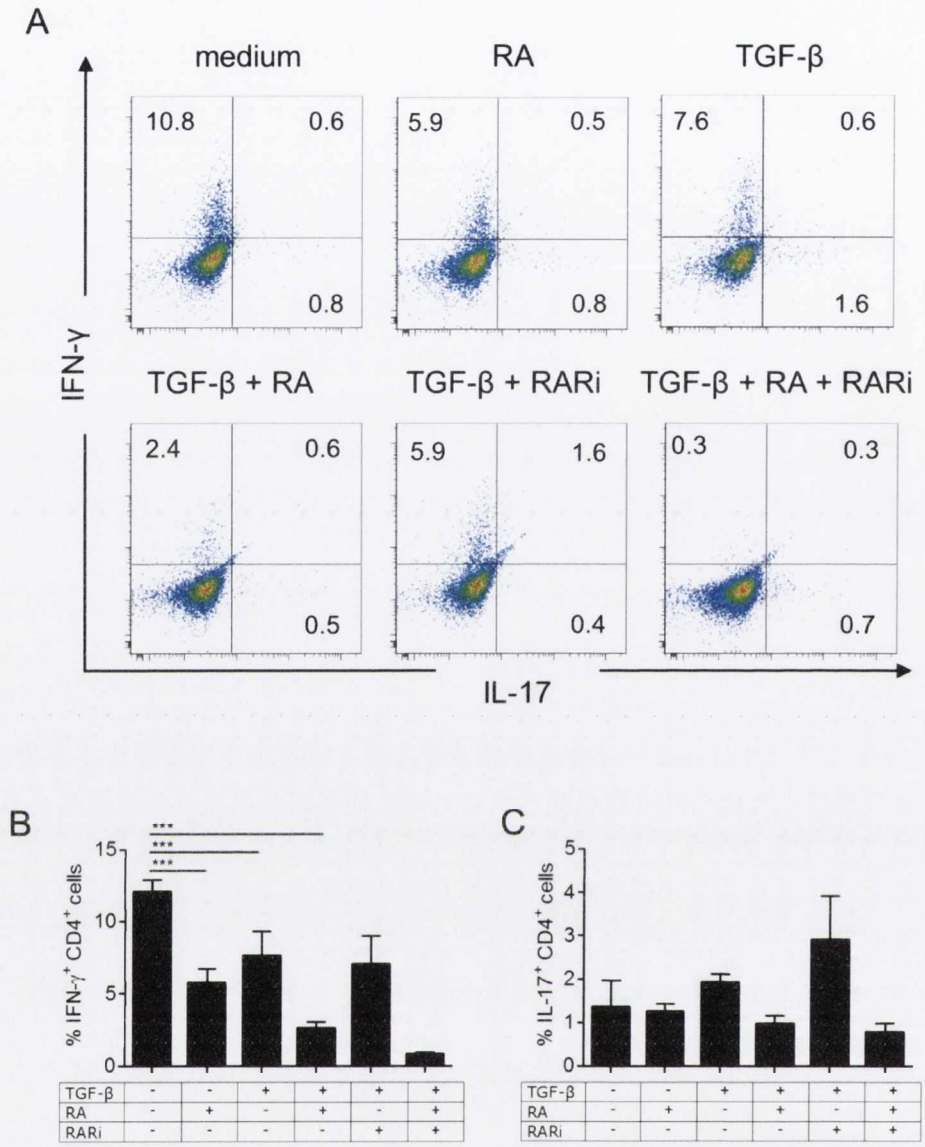


Figure 4.6
TGF-β and RA decrease IFN-γ production by antigen-specific CD4 T cells

LN cells from OVA TCR transgenic mice immunized with OVA protein and CpG were re-stimulated with OVA peptide (1 µg/ml) and stimulated with TGF-β (5 ng/ml), RA (100 nM) and RARi (5 µM) as indicated. After 3, cells were stimulated with PMA/Ionomycin/BFA for 4 h and analysed by intracellular cytokine staining and flow cytometry for expression of IFN-γ and IL-17, gating on $CD4^+$ cells. Results are shown as representative FACS plots (A) or mean ± SD values from triplicate samples (B,C). ***p<0.001 by 1-way ANOVA. Data are representative of 2 experiments.

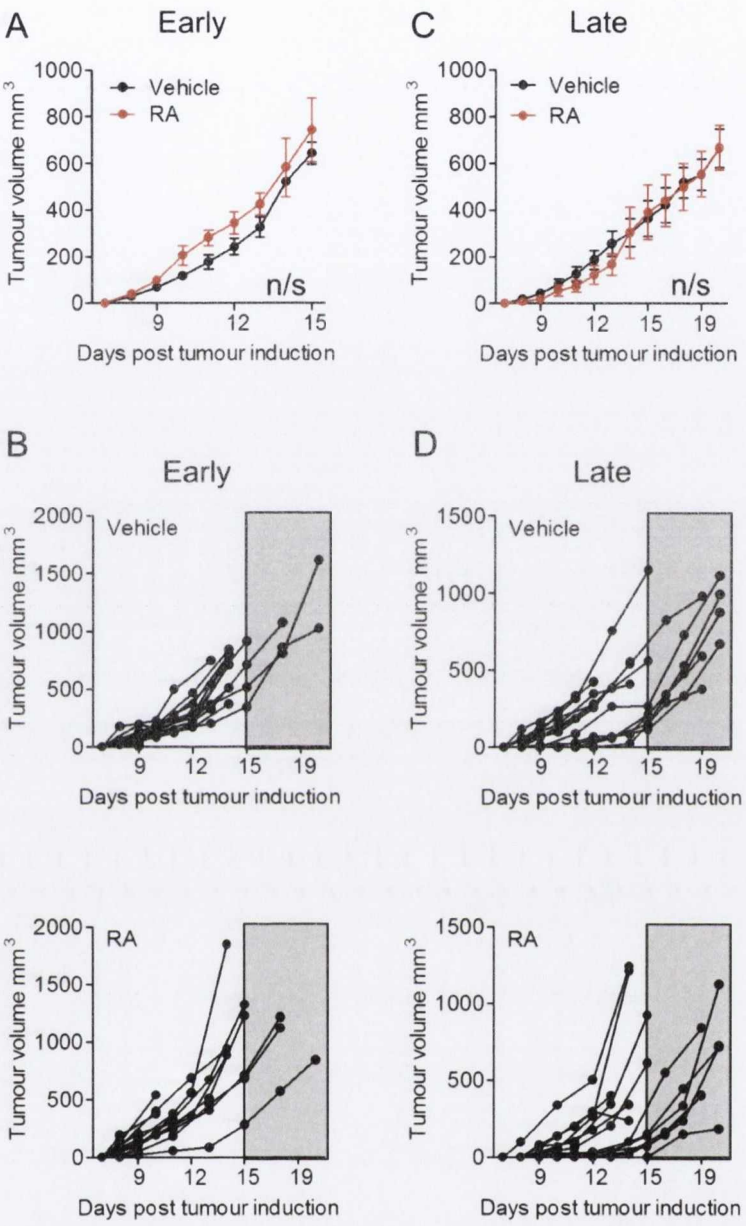


Figure 4.7
Treatment of mice with RA does not significantly affect B16 tumour growth

C57BL/6 mice were injected with B16 tumour cells (2 x 10^5 cells/mouse) s.c. into the flank. RA (200 µg/mouse) or a vehicle control were injected s.c. in the region of the tumour on either days -1, 1, 3 and 5 (early treatment; A,B) or on days 7, 9, 11 and 13 (late treatment; C,D) post tumour induction. Results are shown as mean tumour growth ± SEM (A,C) or tumour growth curves of individual mice (B,D) from 2 combined experiments (n = 14 mice per group).

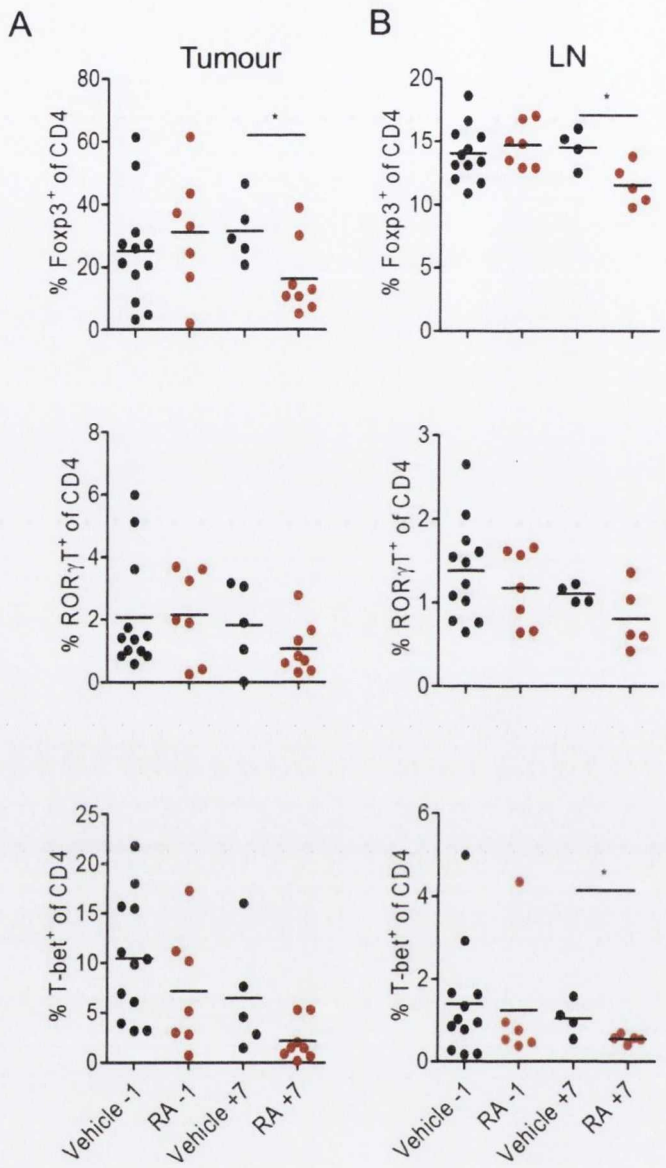


Figure 4.8
Treatment of mice with RA reduces CD4 T cell polarisation to Treg cells *in vivo*

C57BL/6 mice were injected with B16 tumour cells. RA or a vehicle control were injected s.c. in the region of the tumour on days -1, 1, 3 and 5 (Vehicle -1 and RA-1) or on days 7, 9, 11 and 13 (Vehicle +7 and RA +7) post tumour induction. Mean frequencies of CD4 T cell subtypes from tumours (A) or draining lymph nodes (B) were analysed by flow cytometry gating on $CD4^+$ cells. Results are shown as mean frequencies from 2 experiments (n = 5-12 mice per group). *$p<0.05$ by unpaired t-test.

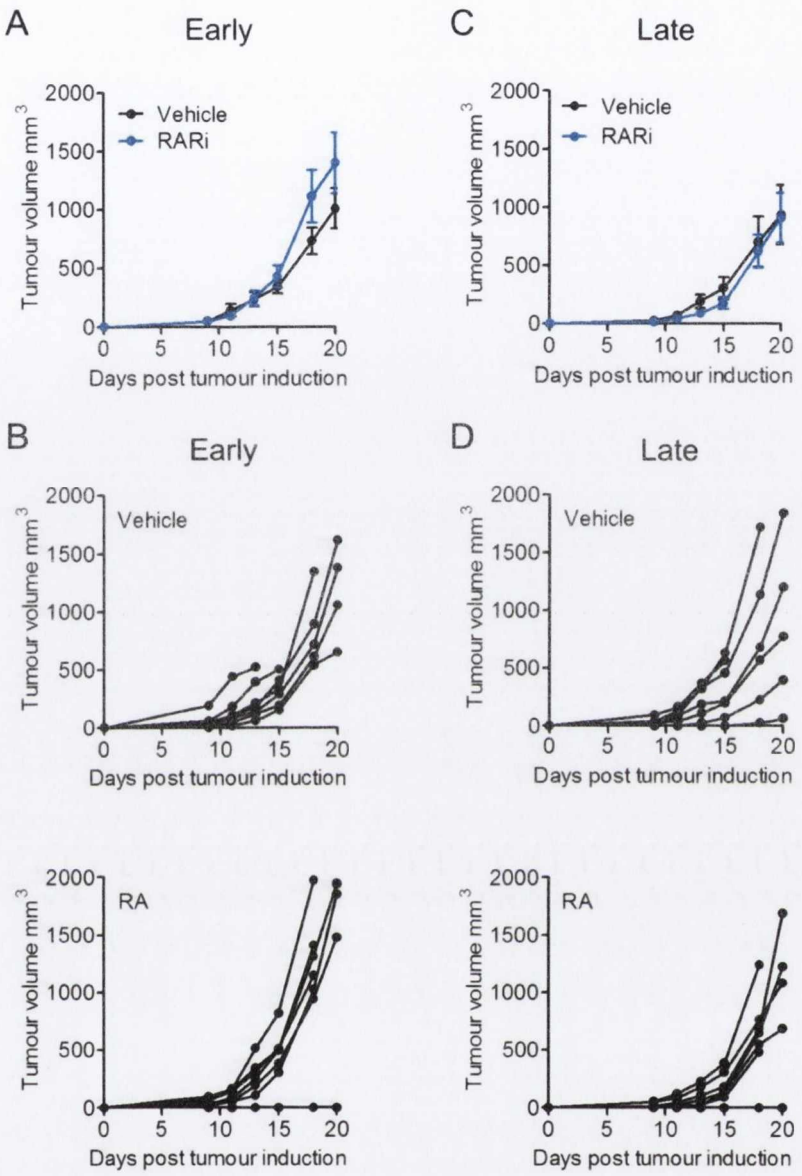


Figure 4.9
Blocking RARα *in vivo* does not significantly affect B16 tumour growth

C57BL/6 mice were injected with B16 tumour cells (2 x 10^5 cells/mouse) s.c. into the flank. RARi (400 µg/mouse) or a vehicle control were injected s.c. in the region of the tumour on either days -1, 1, 3 and 5 (early treatment, A,B) or on days 7, 9, 11 and 13 (late treatment, C,D) post tumour induction. Results are shown as mean tumour growth ± SEM (A,C) or tumour growth curves of individual mice (B,D). (n = 7 mice per group)

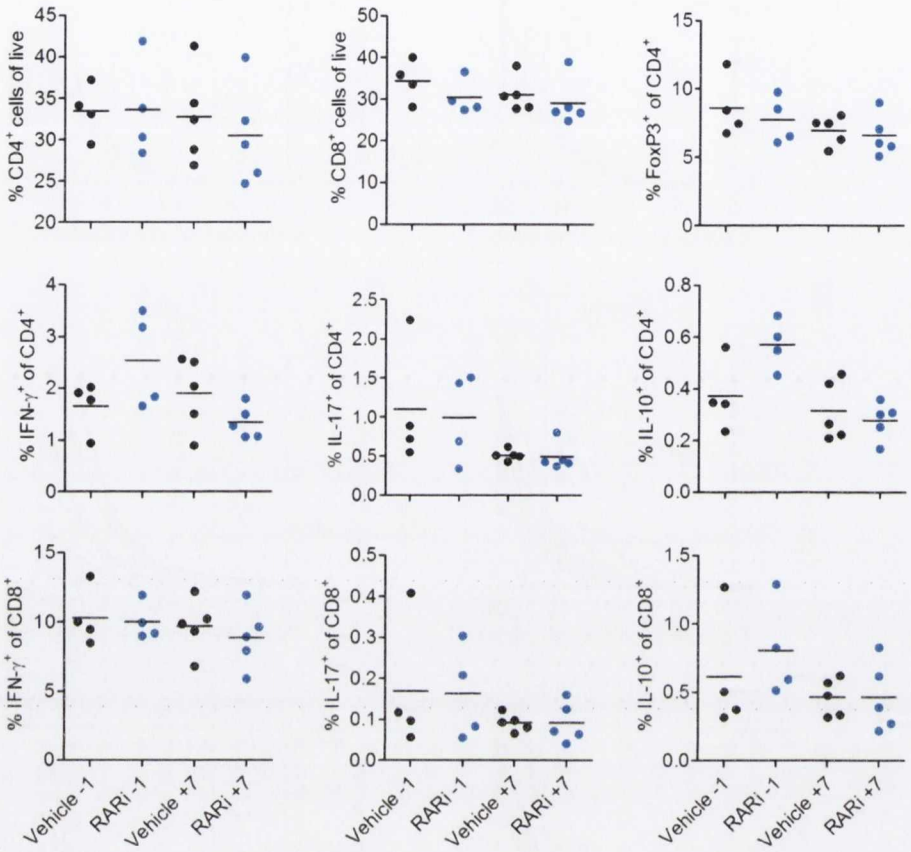


Figure 4.10
Blocking RARα *in vivo* does not have an effect on cytokine production by T cells in tumour dLN

C57BL/6 mice were injected with B16 tumour cells. RARi or a vehicle control were injected s.c. in the region of the tumour on either days -1, 1, 3 and 5 (Vehicle -1 and RARi -1) or on days 7, 9, 11 and 13 (Vehicle +7 and RARi +7) post tumour induction. Mean frequencies and cytokine production from T cell populations in dLN were analysed by intracellular cytokine staining and flow cytometry, gating on $CD4^+$ or $CD8^+$ cells. Results are shown as mean frequencies from 4-5 mice per group.

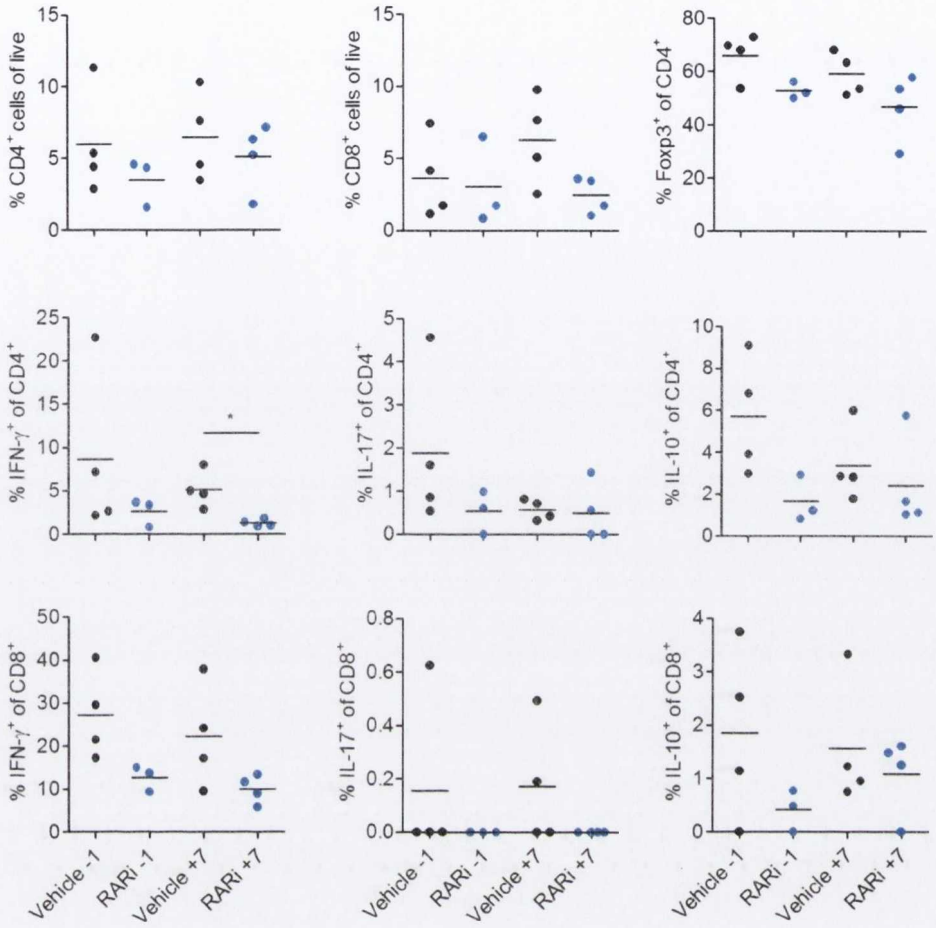


Figure 4.11
Blocking RARα *in vivo* reduces pro- and anti-inflammatory cytokine production by tumour infiltrating lymphocytes

C57BL/6 mice were injected with B16 tumour cells s.c. into the flank. RARi or a vehicle control were injected s.c. in the region of the tumour on either days -1, 1, 3 and 5 (Vehicle -1 and RARi -1) or on days 7, 9, 11 and 13 (Vehicle +7 and RARi +7) post tumour induction. Frequencies and cytokine production from T cell populations within tumours were analysed by intracellular cytokine staining and flow cytometry, gating on $CD4^+$ or $CD8^+$ cells. Results are shown as mean frequencies from 3-4 mice per group. *$p<0.05$ by unpaired t-test.

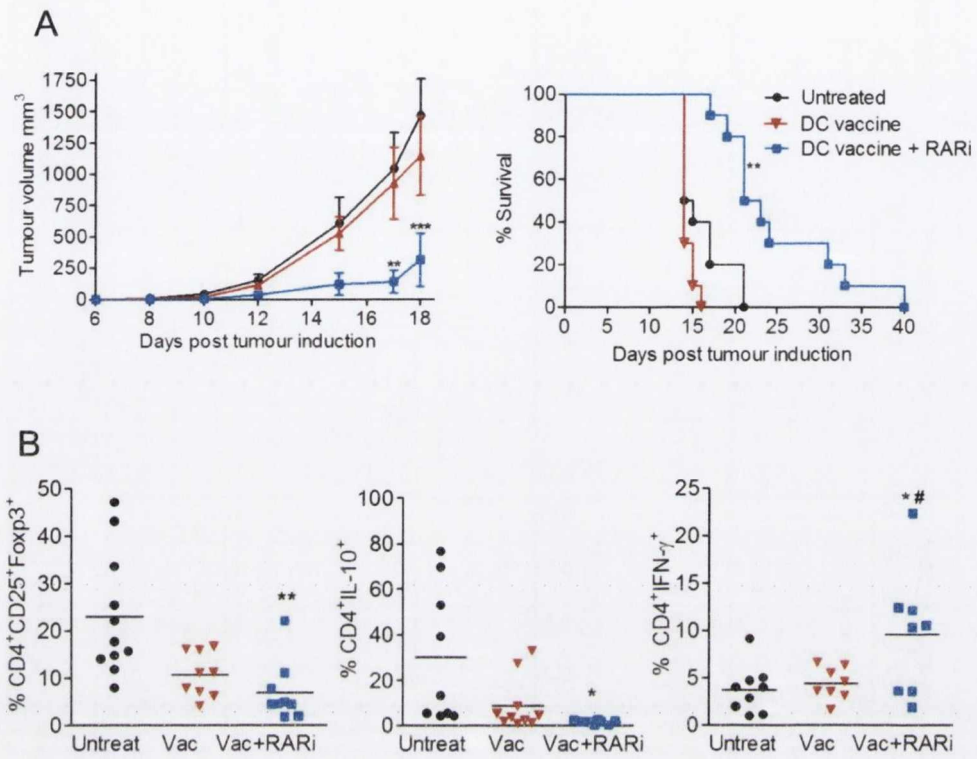


Figure 4.12
Blocking RARα enhances the efficacy of a DC vaccine by reducing regulatory T cells (Experiment performed by K. Galvin)

C57BL/6 mice were injected with B16 tumour cells (2 x 10^5 cells/mouse) s.c. into the flank. Mice were injected s.c. in the region of the tumour on 3, 10 and 17 days post-tumour induction with 1-5 x 10^5 DC that were pre-incubated with medium only (control) or RARi (5 μM) for 15 min and then pulsed for 24 h with hs/irr B16 tumour cells (ratio 1:1) and CpG (5 μg/ml). (n = 8 mice per group); **p<0.01, ***p<0.001 by 2-way ANOVA (A). Mean frequency of tumour-infiltrating $CD4^+CD25^+Foxp3^+$ T cells, $CD4^+IL\text{-}10^+$ T cells, $CD4^+IFN\text{-}\gamma^+$ T cells and $CD8^+IFN\text{-}\gamma^+$ T cells from each treatment group (n = 8 from 2 experiments, *p<0.05, **p<0.01 versus untreated; #p<0.05 versus DC vaccine, by 1-way ANOVA) (B).

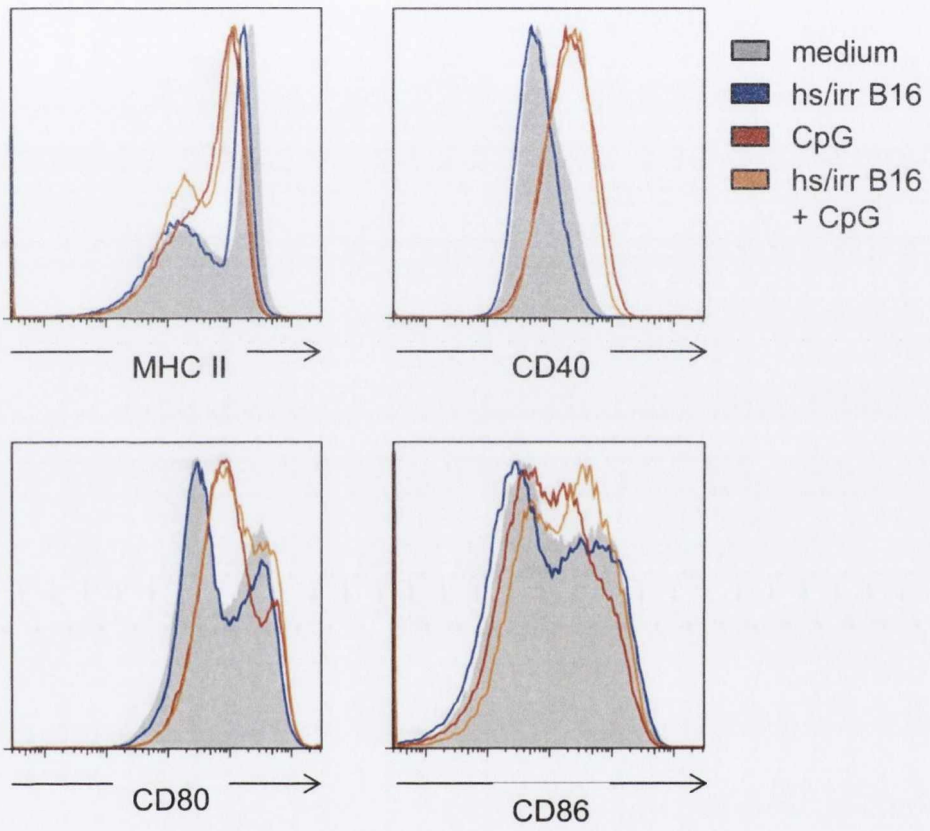


Figure 4.13
CpG induces maturation marker expression on BMDCs

BMDCs were incubated with medium, hs/irr B16 cells (1:1) alone or in combination with CpG (5 µg/ml) for 24 h. Subsequently, the expression of MHC II, CD40, CD80 and CD86 was analysed by surface staining and flow cytometry, gating on $CD11c^+$ cells. Results are shown as overlay histograms of fluorescence intensities.

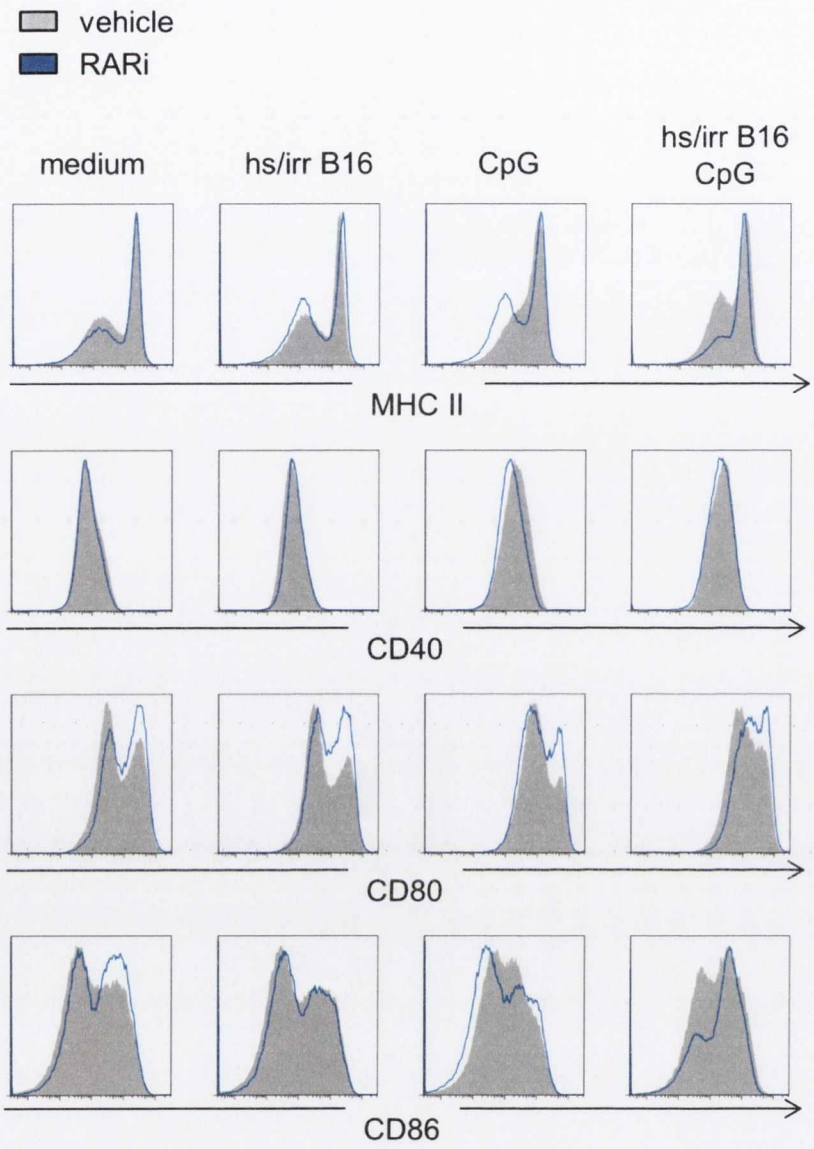


Figure 4.14
Blocking RARα moderately enhances maturation marker expression on antigen-pulsed and CpG-stimulated BMDCs

BMDCs were pre-incubated with RARi (5 µM) or vehicle for 15 min and subsequently stimulated with medium, hs/irr B16 (1:1) alone or in combination with CpG (5 µg/ml) as indicated. After 24 h, the expression of MHC II, CD40, CD80 and CD86 was analysed by surface staining and flow cytometry, gating on $CD11c^+$ cells. Results are shown as overlay histograms of fluorescence intensities.

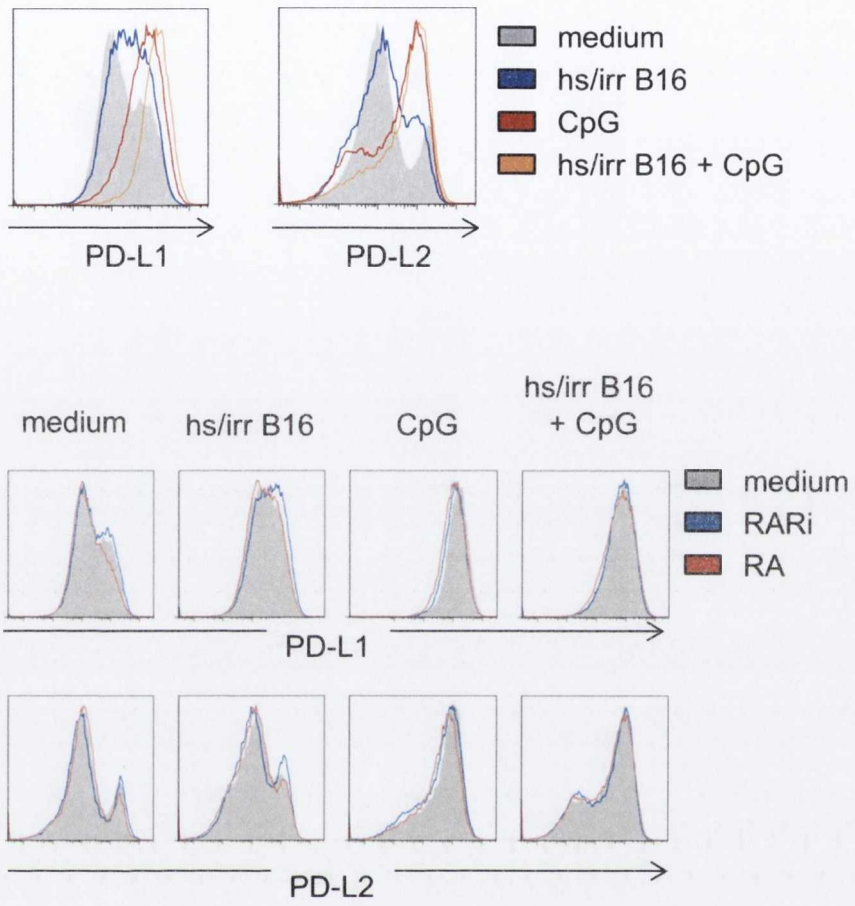


Figure 4.15
Blocking RARα does not affect TLR-induced PD ligand expression on BMDCs

BMDCs were pre-incubated with medium or RARi for 15 min and subsequently stimulated with medium, RA, hs/irr B16 alone or in combination with CpG (5 µg/ml) as indicated. After 24 h, the expression of PD-L1 and PD-L2 was analysed by flow cytometry gating on $CD11c^+$ cells. Data is shown as overlay histograms of fluorescence intensities.

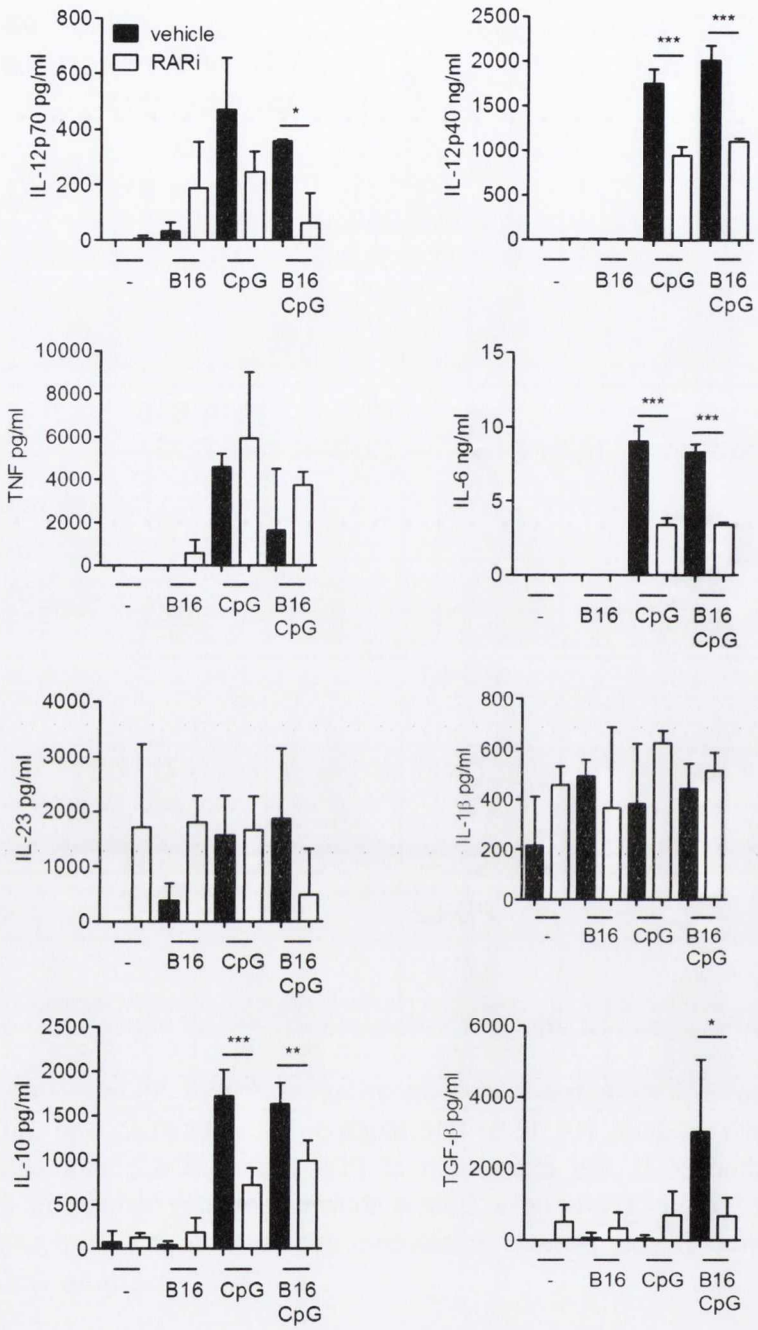


Figure 4.16
RARi inhibits anti-inflammatory cytokine secretion by BMDCs in response to hs/irr B16 cells and CpG

BMDCs were pre-incubated with RARi (5 μM) or vehicle for 15 min and subsequently stimulated with medium, hs/irr B16 alone or in combination with CpG (5 μg/ml) as indicated. Cytokine levels in supernatants were quantified by ELISA. Results are shown as mean + SD values for triplicate samples. *p<0.05 , **p<0.01, ***p<0.001 by 1-way ANOVA. Data are representative of 3 experiments.

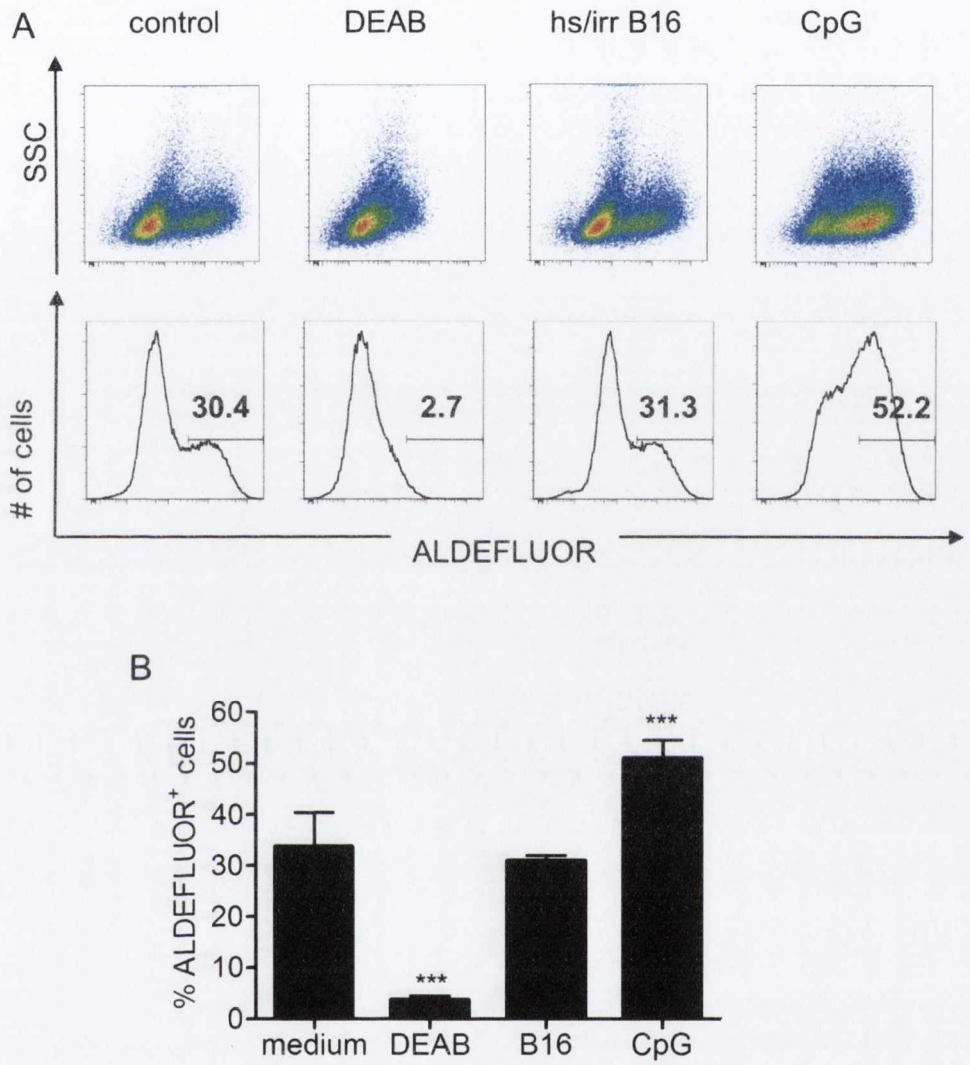


Figure 4.17
ALDH expression in BMDCs is enhanced by TLR9 stimulation with CpG

BMDCs were cultured with medium, hs/irr B16 cells (1:1) or CpG (5 µg/ml) for 24h. Cells were washed and incubated with ALDEFLUOR alone or with DEAB (ALDH inhibitor). After 1 h, cells were stained with CD11c and ALDEFLUOR levels analysed by flow cytometry, gating on $CD11c^+$ cells. Results are shown as representative FACS plots (A) or mean values ± SD for triplicate samples (B). ***$p<0.001$ versus medium control by 1-way ANOVA. Data are representative of 3 experiments.

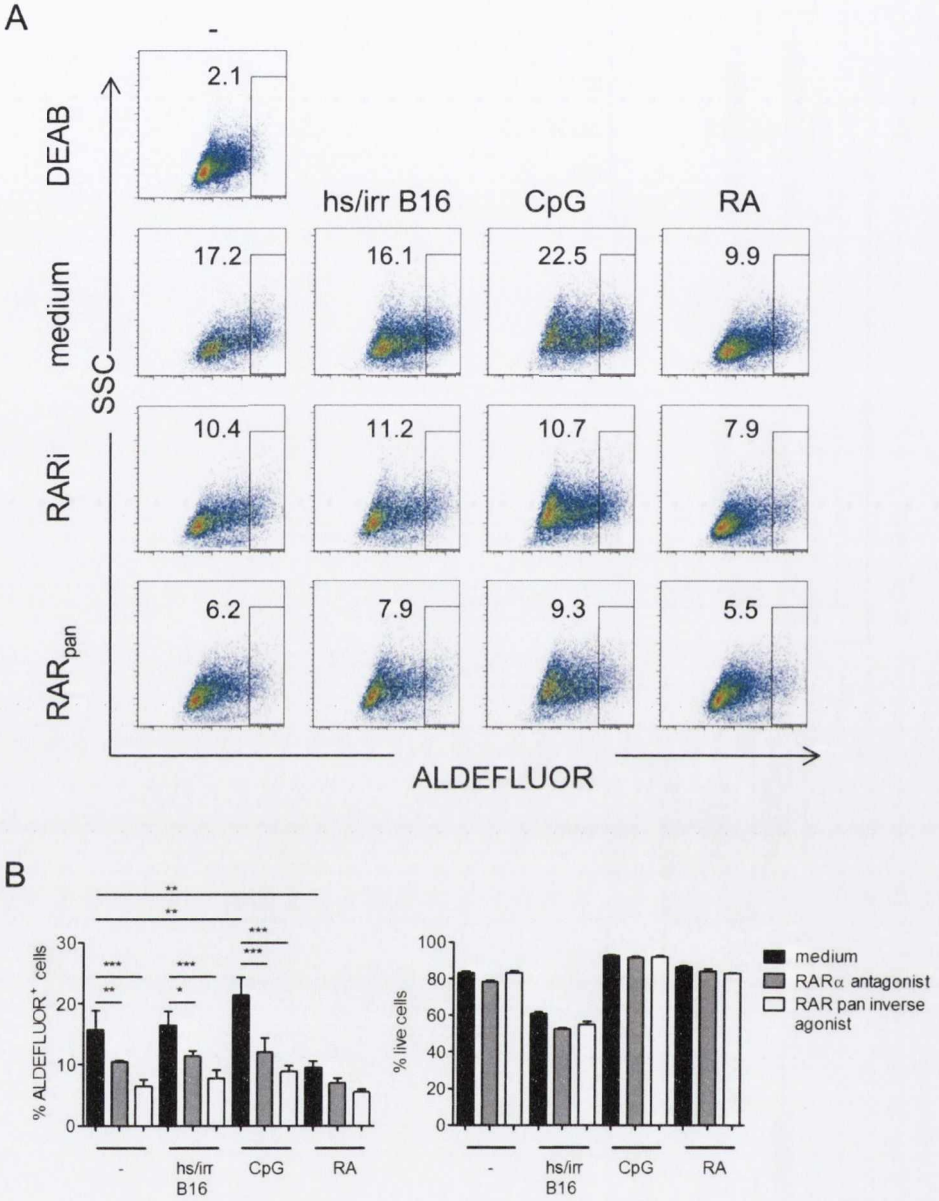


Figure 4.18
ALDH expression in BMDCs is reduced by blocking RAR

BMDCs were pre-treated with medium, a RARα antagonist (RARi; 5 μM) or a RAR pan inverse agonist (RAR_{pan}; 5 μM) for 15 min and subsequently stimulated with medium, hs/irr B16 alone or in combination with CpG (5 μg/ml) as indicated. Cells were washed and incubated with ALDEFLUOR alone or with DEAB (ALDH inhibitor). After 1 h, cells were stained with CD11c and ALDEFLUOR levels analysed by flow cytometry gating on $CD11c^+$ cells. Results are shown as representative FACS plots (A) or mean + SD values for triplicate samples (B). ***p<0.001 versus medium control by 1-way ANOVA.

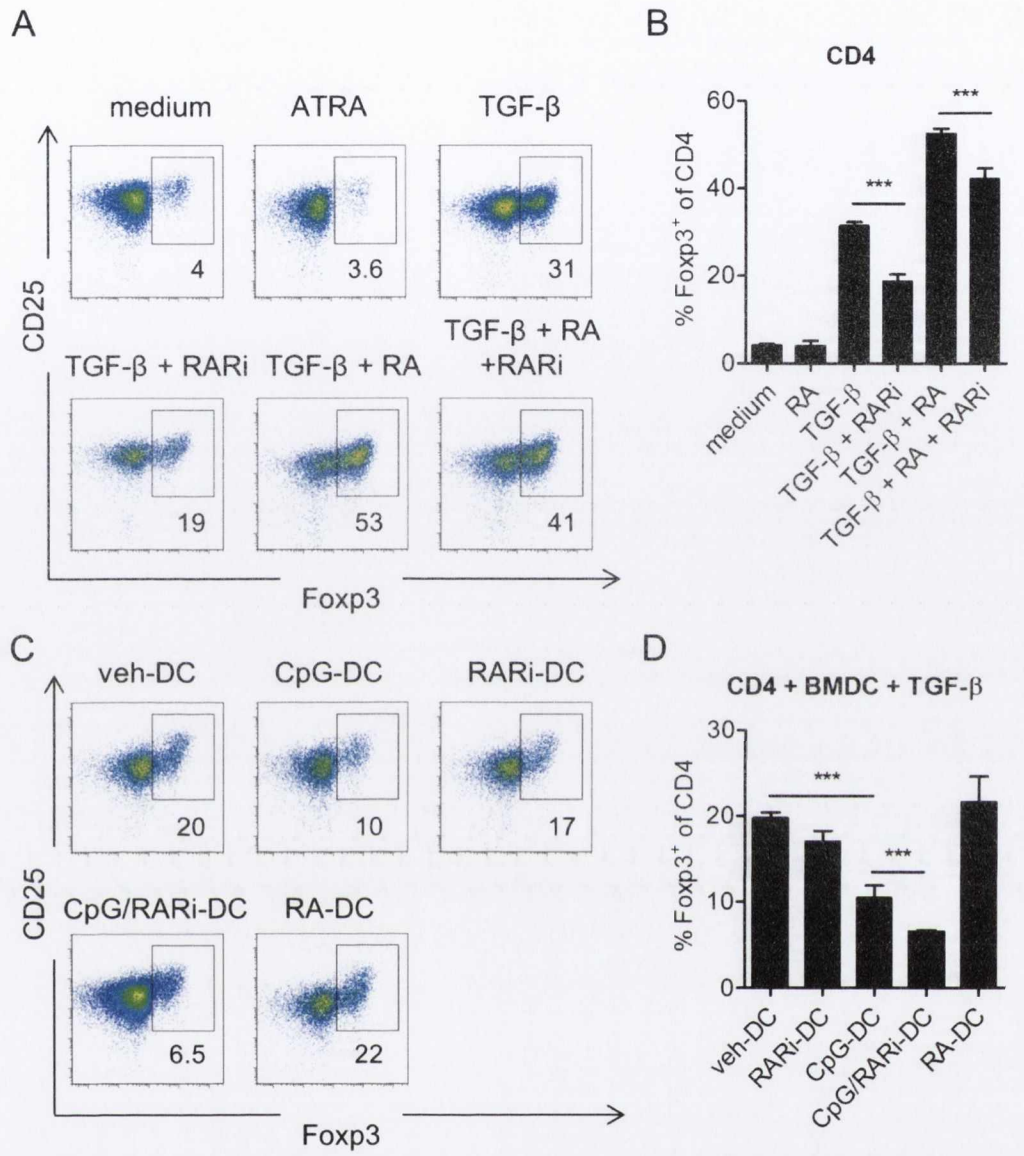


Figure 4.19
RARi-stimulated BMDCs reduce TGF-β-induced Treg conversion

Naïve murine CD4 T cells were cultured in anti-CD3-coated wells (1 µg/ml) in medium containing IL-2 (10 ng/ml), soluble anti-CD28 (5 µg/ml) with TGF-β (5 ng/ml), RA (100 nM) and RARi (5 µM)(A,B). BMDCs were pre-incubated with RARi (5 µM) or vehicle for 15 min and subsequently stimulated with medium, RA (100 nM) or CpG (5 µg/ml). After 24 h, BMDC were washed and co-cultured with naïve CD4 T in anti-CD3-coated wells (1 µg/ml) in medium containing IL-2 (10 ng/ml), soluble anti-CD28 (5 µg/ml) and TGF-β (5 ng/ml). After 3 d, the frequency of Treg cells was detected by flow cytometry, gating on $CD3^+$ $CD4^+$ $Foxp3^+$ $CD25^+$ cells. Results are shown as representative FACS plots (A, C) or as mean ± SD values for triplicate samples (B,D). ***$p<0.001$ by 1-way ANOVA.

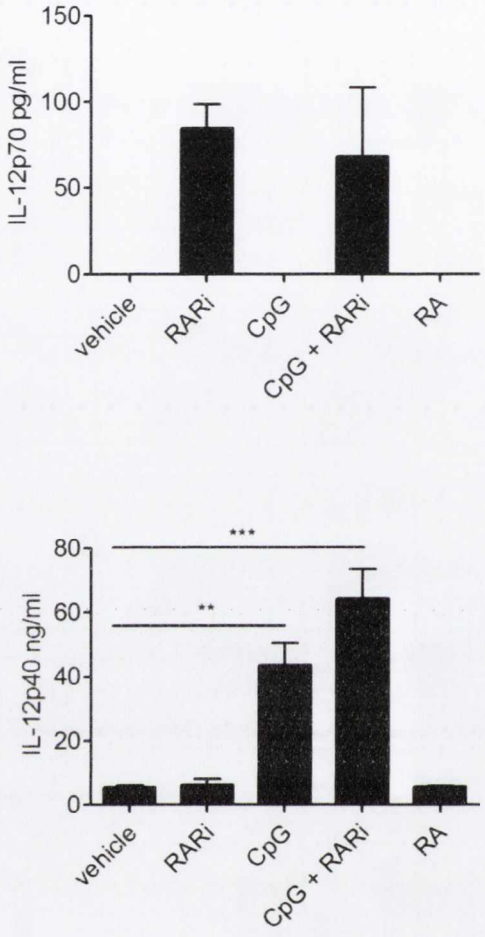


Figure 4.20
RARi pre-treatment enhances production of IL-12 in BMDC-CD4 co-cultures

BMDCs were pre-incubated with RARi (5 µM) or vehicle for 15 min and subsequently stimulated with medium, RA (100 nM) or CpG (5 µg/ml) as indicated. After 24 h, BMDC were washed and co-cultured with naïve CD4 T in anti-CD3-coated wells (1 µg/ml) in medium containing IL-2 (10 ng/ml), soluble anti-CD28 (5 µg/ml) and TGF-β (5 ng/ml). After 3 d, cytokine levels in supernatants were quantified by ELISA. Results are shown as mean ± SD values for triplicate samples . **$p<0.01$, ***$p<0.001$ by 1-way ANOVA.

Chapter 5:

Effect of a combination therapy on anti-tumour immunity using a cancer vaccine and an immune checkpoint inhibitor

5.1. Introduction

The present chapter focuses on the interaction between three central cell types involved in tumour elimination: tumour cells, DCs and cytotoxic T cells. Chen et al. described their interaction as a cancer-immunity-cycle [21]. According to this model, dying tumour cells release cell-specific antigens, which are taken up by DCs that constantly screen their environment for dangers. DCs process tumour antigens and express them on their cell surface through MHC molecules while undergoing maturation. At this stage, DCs migrate to dLN where they present tumour antigens to naïve T cells leading to T cell activation, priming and proliferation. Primed T cells migrate through the blood stream to the tumour bed. Once they recognise their specific antigens on the cell surface of tumour cells, cytotoxic T cells induce killing of tumour cells.

The role of CD8 T cells in cancer has been extensively studied in animal models where it was demonstrated that CD8 T cells play a pivotal role in preventing tumour growth. Depletion of CD8 T cells led to accelerated tumour growth in cancer models for melanoma and colon carcinoma [46, 47]. Koebel et al. demonstrated that the adaptive immune system keeps tumour cells in an equilibrium state of poor proliferation which they escape once T cells are depleted [340]. Moreover, the results from the previous chapters demonstrated that successful immunotherapy was associated with an increase of IFNγ-producing T cells. T cell infiltration into the tumour has also been associated with better clinical outcome and improved responses to immunotherapies in melanoma patients [54]. Thus, approaches to induce potent CD8 T cell responses are investigated further in the present study.

Mature DCs are crucial for potent activation of tumour-homing CD8 T cells. DCs that lack proper expression of co-activating proteins, such as CD40 or CD80/86, can induce a state of anergy in T cells [44]. Moreover, weak activation of CD4 T cells can lead to the generation of suppressive Treg cells [199, 341]. As demonstrated in chapters 3 and 4, TLR agonists have the potential to activate DCs and to induce anti-tumour responses *in vivo*. While TLR agonists are a potent tool to activate DCs and T cells, many TLR agonists failed in the clinic because dysfunction of T cells at later stages was not prevented [198].

In addition to investigating the role of T cells and DCs in the tumour microenvironment, this study aimed to develop a potent cancer vaccine. The results in chapter 4 demonstrated that potent anti-tumour responses can be achieved using an antigen-

primed TLR-activated DC vaccine. The present chapter focuses on direct immunization approaches with an antigen-adjuvant combination. Cancer vaccines have shown some success in cancers with low levels of immunosuppression, such as premalignant lesions [210, 211, 342]. However, many cancer vaccine regimes failed to induce potent T cell responses in the clinic. The biggest obstacles in the design of cancer vaccines were the right choice of antigens or adjuvants and immune-suppressive mechanisms elicited by tumours that vaccines failed to overcome [199].

The tumour microenvironment has been shown to maintain immunosuppression. Suppressive cytokines (TGF-β, IL-10) or cell types (Tregs, MDSCs, TAM) have been associated with impaired CTL responses. In recent years, much attention has been drawn to the role immune checkpoints in the context of tumour-induced immunosuppression. Immune checkpoints are co-inhibitory molecules expressed on lymphocytes and their role is best characterised in chronic infections. Constant antigen exposure, and thus constant TCR stimulation, induces destructive changes on T cells which as a result become 'exhausted'. T cell exhaustion is a complex process and is characterised by enhanced expression of co-inhibitory receptors, reduced cytokine secretion and loss of effector function [93]. It is believed that T cells in tumours undergo a similar process through constant exposure to tumour antigens similar to chronic infections.

PD-1 has been shown to be particularly important in mediating T cell dysfunction. PD-1 is expressed mostly by T and B cells and can be bound by its ligands PD-L1 and PD-L2 [113, 114]. PD-1- ligand interaction mostly occurs in the periphery. PD-L1 was shown to be expressed by myeloid cells and by many tumours. The exact molecular mechanisms of PD-1 downstream signalling are still not very well characterised. However, it has been shown that ligand binding to PD-1 triggers a signalling cascade and induces SHP-2. SHP-2 binds to the TCR and prevents TCR signalling. Moreover, it interferes with a range of signalling molecules involved in survival, proliferation and cytokine secretion [343]. Three antibodies targeting the PD-1:PD-L1 interaction have recently been approved for the treatment of multiple cancers [7]. The combination of Ipilimumab (anti-CTLA-4) and Nivolumab (anti-PD-1) has revealed significant responses in untreated melanoma patients and was more effective than either treatment alone [252]. While immune checkpoint inhibitors were shown to induce very potent T cell responses in some patients, there were still a significant proportion of patients that did not respond [252]. Predictive biomarkers and combinations of immunotherapies are currently a major topic of investigation.

The aim of this chapter was to characterise suppressive mechanisms, particularly immune checkpoints, in tumour models of cancer and to design combination therapies that activate tumour-specific T cells and diminish immune evasion. The hypothesis that a vaccine consisting of tumour antigens and a TLR agonist can improve the generation of tumour-specific T cells by activating DCs and by delivering tumour antigens to be presented to T cells was tested. Two different approaches of vaccine design were compared, namely the use of tumour peptides versus the use of tumour lysates as antigen source. Moreover, the role of immune checkpoints in tumour models of cancer and the potential of anti-PD-1 to improve the efficacy of tumour vaccines were investigated in murine tumour models. The main objective of this chapter was to investigate whether better anti-tumour immune responses can be generated when both immune activators and immune suppressors are targeted.

5.2. Results

5.2.1. R848 enhances the frequency and total number of TRP-2 peptide-specific CD8 T cells

There are two basic approaches to overcome immune-inhibition by tumour cells, namely enhancement of anti-tumour responses and inhibition of immunosuppression. In this chapter, immunotherapy combinations were investigated combining both approaches by targeting the interaction of tumour cells, DCs and T cells. DCs are crucial for the activation of antigen-specific T cells. TLR agonists are amongst the most potent activators of DCs. Chapter 3 demonstrated that stimulation with the TLR7/8 agonist R848 enhanced the expression of co-stimulatory molecules on DCs and treatment of mice with R848 induced protection *in vivo*. Thus, the ability of R848 to act as a vaccine adjuvant was investigated in the present chapter. Previous studies have reported that the TRP-$2_{180-188}$ peptide sequence comprises a restricted MHC I epitope recognised by CD8 T cells [344]. Moreover, the TRP-$2_{180-188}$ protein is expressed by human and mouse melanoma cells, including the murine melanoma cell line B16, making it a potential vaccine candidate [344]. To investigate the adjuvanticity of R848, mice were injected with TRP-$2_{180-188}$ peptide and R848 into the footpad and dLN cells were removed 7 days later. TRP-$2_{180-188}$-specific CD8 T cells in LN were quantified by tetramer staining using flow cytometry. While immunization with TRP-$2_{180-188}$ alone resulted in very low levels of tetramer positive cells, immunization of TRP2-$_{180-188}$ in the presence of R848 significantly enhanced the frequency and total number of TRP-$2_{180-188}$-specific T cells (Figure 5.1 A,B). Moreover, LN cells from mice that were immunized with TRP-$2_{180-188}$ alone or in combination with R848 secreted significant concentrations of IFN-γ in response to re-stimulation with TRP-$2_{180-188}$ (Figure 5.1C). These findings confirm that TRP-$2_{180-188}$ is specifically recognised by CD8 T cells and show that R848 improves the generation of tumour-specific T cells *in vivo*.

5.2.2. TRP-$2_{180-188}$ specific T cells induce tumour cell killing *in vitro*

The capability of TRP-$2_{180-188}$ specific CD8 T cells to kill tumour cells was investigated *in vitro*. Spleen cells from B16 tumour-bearing mice were cultured with TRP-$2_{180-188}$ peptide in order to expand TRP-$2_{180-188}$-specific CD8 T cells. After 5 days, expanded cells were co-cultured with B16 tumour cells and B16 cell death was analysed by flow cytometry (Figure 5.2A). After 4h and 24h of co-culture, spleen cells previously cultured in presence of the TRP-$2_{180-188}$ peptide induced higher levels of cell death than

unstimulated spleen cells (Figure 5.2B). These findings support the hypothesis that TRP-$2_{180-188}$-specific T cells are able to target and kill B16 tumour cells.

5.2.3. Anti-tumour effects of a TRP-$2_{180-188}$ peptide vaccine in combination with R848 *in vivo*

Having established that the combination of R848 with the tumour peptide TRP-$2_{180-188}$ induces specific cytotoxic T cell responses, the next step was to test whether this combination protects against tumour growth *in vivo*. Mice were immunized s.c. with TRP-$2_{180-188}$ and R848 alone or in combination 5 weeks and 1 week prior to B16 tumour challenge. Prophylactic immunization with TRP-$2_{180-188}$ or R848 alone did not significantly reduce tumour growth (Figure 5.3 A,C). However, the combination of TRP-$2_{180-188}$ with R848 modestly reduced tumour growth (Figure 5.3 A,C) and prolonged survival (Figure 5.3B).

The combination of TRP-$2_{180-188}$ with R848 was then investigated in a therapeutic setting. Mice were immunized as described above on days 3, 10 and 17 post tumour challenge. In an effort to improve the vaccine, two TRP-2 peptides were included which were previously shown to be recognised by CD4 T cell epitopes [345]. Immunization with the three peptides without adjuvant had little effect on tumour growth (Figure 5.4 A,D). Treatment of mice with R848 alone induced some reduction in tumour growth, however, the combination of TRP-2 with R848 showed the best efficacy in terms of reducing tumour growth and prolonging survival (Figure 5.4 A,B). The addition of two TRP-2 peptides recognised by CD4 T cells did not further improve the vaccine efficacy over that seen with the CD8 epitope alone (Figure 5.4 A,D). Mice treated with R848 alone or in combination with TRP-2 that rejected the tumour during the therapeutic treatment were re-challenged with B16 cells 60 days post initial tumour challenge. However, no differences in tumour growth were observed compared to naïve mice that were challenged for the first time (Figure 5.4C). These findings suggest that the combination of TRP-$2_{180-188}$ with R848 induced anti-tumour immune responses leading to a reduction tumour growth but failed to induce memory T cell responses.

In order to study the underlying mechanism of protection, T cell populations in dLN and tumours were characterised on day 17 past tumour challenge (Figure 5.5). Mice treated with R848 alone or in combination with TRP-$2_{180-188}$ had reduced levels of Foxp3-expressing CD4 T cells in tumours but not in dLN (Figure 5.5A). The frequency of IFN-γ-producing CD8 and CD4 T cells was highest in dLN and tumours from mice treated with the peptide-R848 combination, albeit not significant when compared with control

mice (Figure 5.5A). Interestingly, when the frequency of IFN-γ-producing T cells was compared to tumour size, it was apparent that the frequency of IFN-γ-producing T cells decreased with increasing tumour size and this was independent of the therapy (Figure 5.5B). These findings demonstrate that the reduction in tumour growth induced by TRP-$2_{180-188}$ with R848 is associated with an increase of IFN-γ-producing T cells.

5.2.4. High expression of inhibitory ligands and receptors on tumour-infiltrating immune cells

Immune checkpoints have the potential to limit anti-tumour immune responses [93].The expression of the immune checkpoint protein PD-1 and its receptor PD-L1 was analysed in tumour-bearing mice that were immunized as described above. Tumours from mice treated with R848 alone or in combination with TRP-$2_{180-188}$ had an increased number of infiltrating immune cells, characterised by the frequency of $CD45^+$ cells (Figure 5.6). However, the expression of PD-L1 on $CD45^+$ cells was greater in vaccinated mice compared to vehicle treated mice, indicating that vaccination also modulates inhibitory ligand expression (Figure 5.6). Interestingly, less than 10% of CD4 and less and 3% of CD8 T cells expressed PD-1 in dLN which increased to around 70% in tumours, with no significant differences between treatment groups (Figure 5.6). These findings suggest that the combination of TRP-$2_{180-188}$ and R848 conferred moderate protection against B16 tumour growth through the induction of cytotoxic T cell responses and suggest that the efficacy of the vaccine could have been dampened through T cell exhaustion promoted by PD-1:PD-L1 interaction.

5.2.5. TLR agonists upregulate PD-L1 and PD-L2 expression on BMDCs

The results in chapter 3 demonstrated PD-L1 and PD-L2 induction on BMDCs by the TLR9 agonist CpG. The next experiment was designed to test whether the adjuvant R848 has a similar effect on DCs. Stimulation of BMDCs with CpG or R848 vastly enhanced the cell surface expression of PD-L1 and PD-L2 while stimulation with hs/irr B16 cells only marginally increased PD-L1 and PD-L2 expression (Figure 5.7A). Interestingly, BMDCs with a mature phenotype ($CD40^+CD86^+$) were found to co-express high levels PD-L1 and PD-L2 (Figure 5.7 B,C). These findings suggest that TLR agonists induce DC maturation but also promote expression of co-inhibitory molecules.

5.2.6. Immunization with TRP-$2_{180-188}$ stimulates PD-1 expression on CD8 T cells

Previous studies reported that chronic antigen-stimulation induces the expression of PD-1 on T cells [93]. To test whether immunization of mice induces PD-1 expression, mice were immunized with TRP-$2_{180-188}$ and R848 into the footpad and CD8 and CD4 T cells from dLN were stained for PD-1 on day 7 day post immunization. Immunization with TRP-$2_{180-188}$ significantly enhanced the frequency of PD-1-expressing CD8 T cells independent of the presence of R848 (Figure 5.8 A,B). The enhancement of PD-1 expression was antigen-specific since no changes in PD-1 expression were observed for CD4 T cells (Figure 5.8C). These findings demonstrate that TCR stimulation by antigens promote the expression of PD-1.

5.2.7. IFN-γ enhances the expression of MHC I and PD-L1 on tumour cells

To investigate whether B16 and CT26 tumour cells could dampen T cell responses through PD-1 engagement, expression of PD-1 ligands were analysed by flow cytometry. Basal expression of PD-L1, but not PD-L2, was found on both tumour cell lines *in vitro* (Figure 5.9) and *in vivo* (Figure 5.10). Kleffel et al. recently reported that cell-intrinsic PD-1 expression on B16 tumour cells promotes tumour growth. However, PD-1 was not found to be expressed on B16 or CT26 tumour cells (Figure 5.9).

IFN-γ is produced by activated cytotoxic T cells. IFN-γ stimulation of B16 and CT26 tumour cells greatly enhanced the expression of MHC I (Figure 5.9). In addition, IFN-γ strongly upregulated the expression of PD-L1 on both tumour cell lines (Figure 5.9). These findings show that IFN-γ has opposing effects on tumour cells by enhancing antigen presentation through MHC I and mediating T cell suppression through PD-L1.

5.2.8. Inverse correlation between expression of pro-inflammatory cytokines and inhibitory receptors on tumour-infiltrating lymphocytes

The data shown in figure 5.5 demonstrated that B16 tumours were infiltrated by a high proportion of immune cells expressing Foxp3, PD-1 and PD-L1 which could prevent the host from eradicating the tumour. B16 tumours are poorly immunogenic with limited T cell infiltration and were shown to respond poorly to immunotherapy [346]. Therefore, the infiltration of T cells was also characterised in the highly immunogenic CT26 colon carcinoma model. On day 23 post tumour induction, a PE-labelled CD45.2 antibody

was injected into the tail veins of tumour-bearing mice 10 min prior to culling. With this method, all circulating leukocytes were stained and thus could be distinguished from tumour-resident immune cells. Lymphocytes were subsequently analysed in dLN, tumour-draining blood vessels and the tumour bed by flow cytometry (Figure 5.11).

The proportion of $Foxp3^+$ CD4 T cells was around 18% in dLN and tumour circulation. Inside the tumour, the frequency of $Foxp3^+$ CD4 T cells was significantly higher with an average of 38%, suggesting that a significant proportion of CD4 T cells converted to Treg cells within the tumour bed or that significantly more Treg cells than effector T cells infiltrated the tumour. Similarly, expression levels of PD-1 on CD4 and CD8 T cells and LAG-3 on CD8 T cells were significantly higher in tumours when compared with dLN or tumour circulation. Moreover, $CD45^+$ cells (leukocytes) showed elevated levels of PD-L1 expression in the circulation and tumour compared to dLN and almost 40% of $CD45^-$ cells (tumour and stromal cells) expressed PD-L1 (Figure 5.11). These findings highlight a strong immunosuppressive environment within the tumour.

The proportion of IFN-γ-secreting CD8 T cells, but not CD4 T cells, was significantly lower in the tumour circulation and the tumour bed compared with dLN. Moreover, CD4 and CD8 T cells expressed high levels of intracellular TNF in dLN which was nearly completely absent in tumour-resident T cells (Figure 5.11).

Overall, these findings strengthen the hypothesis that the tumour microenvironment exerts immuno-suppression through the induction of Treg cells and co-inhibitory molecules.

5.2.9. High PD-1 expression on CD4 and CD8 T cells in the tumour microenvironment

The results in figure 5.11 highlighted an inverse correlation between the presence of Treg cells and the expression of inhibitory receptors on the one hand and IFN-γ and TNF secretion by T cells on the other hand. PD-1 is one of the co-inhibitory proteins found on dysfunctional T cell that have limited effector function. Previous studies reported that receptor ligand interaction recruits SHP-2 which binds to the CD3 chain of the TCR and inhibits downstream TCR signalling [347]. Moreover, PD-1 signalling has been shown to reduce T cell proliferation through RAS inhibition and to reduce survival through inhibition of the anti-apoptotic protein Bcl-xL [343]. However, PD-1 is also expressed on activated T cells and expression of PD-1 is not sufficient to confer dysfunction on T cells [93]. The next experiment was set out to analyse whether

differences in expression levels of PD-1 correlate with functional deficits. As shown in figure 5.11, PD-1 expression was elevated on T cells infiltrating the tumour compared with those from the dLN (Figure 5.12A). Furthermore, PD-1 expression was more abundant on CD8 than on CD4 T cells (Figure 5.12 A). When dividing T cells into PD-1 negative, intermediate or high expressing cells, both CD4 and CD8 T cells were mostly of a PD-1 negative phenotype in dLN (Figure 5.12B). In the tumour, the majority of CD4 T cells were PD-1 intermediate or negative while more than 80% of CD8 T cells in the tumour were PD-1 high (Figure 5.12 B). This suggests that CD8 T cells could be more strongly affected by the presence of PD-L1 expressing cells.

An examination of IFN-γ production revealed that PD-1 negative CD4 and CD8 T cells displayed the lowest levels of IFN-γ production in the dLN (Figure 5.13). The highest IFN-γ producers were PD-1 intermediate T cells and the frequency of IFN-γ producing cells was lower in PD-1 high CD4 and CD8 T cells. In the tumours, IFN-γ production was low in all T cells regardless of PD-1 expression. These findings indicate that T cells express intermediate levels of PD-1 as they become activated in the dLN and lose some of their capability to produce IFN-γ with increasing expression of PD-1 which is then strongly reduced once they enter the tumour.

Since T cells upregulate PD-1 upon activation in the dLN, it was not surprising that PD-1 intermediate and high expressing T cells were the most proliferating cells, as detected by the expression of Ki-67. Interestingly, in the tumour a notable proportion of CD4 and CD8 T cells expressing PD-1 were still positive for Ki-67, indicating residual proliferative capacity of PD-1 expressing cells (Figure 5.13).

The proportion of T cells expressing Foxp3 was also analysed with respect to PD-1 expression (Figure 5.13). In the dLN, mostly PD-1 intermediate cells were positive for Foxp3. In the tumours, PD-1 intermediate and high expressing cells included the highest proportion of Foxp3 expressing CD4 T cells, indicating a possible link between PD-1 and Foxp3 expression in CD4 T cells.

5.2.10.Blocking PD-1 augments IFN-γ and IL-10 secretion by TGF-β-stimulated spleen cells and restores CD8 T cell proliferation

The results so far have shown that a vaccine-TLR combination can induce moderate T cell activation and anti-tumour activity *in vivo*. However, it became apparent that high levels of PD-1 expression on T cells and extensive PD-L1 expression on tumour and immune cells could be limiting T cell driven anti-tumour responses. Therefore, it was

hypothesised that blocking the PD-1/PD-L1 axis could enhance the efficacy of a tumour vaccine.

First, the effects of blocking PD-1 interaction with its ligands were assessed *in vitro*. In order to mimic the tumour microenvironment, spleen cells were activated through their TCR with anti-CD3/anti-CD28 in presence of the suppressive molecules TGF-β and RA which induced $Foxp3^+$ Treg cells (Figure 5.14A). The presence of TGF-β strongly suppressed anti-CD3/anti-CD28-induced IFN-γ secretion which was further reduced by RA (Figure 5.14B). IFN-γ secretion was significantly restored in presence of anti-PD-1 in a dose-dependent manner (Figure 5.14B). Gating on the different T cell subtypes within the spleen, IFN-γ secretion by both CD4 and CD8 T cells was inhibited by TGF-β and partly reversed in presence of anti-PD-1 (Figure 5.14C).

Secretion of the anti-inflammatory cytokine IL-10 was analysed in the same stimulation context. TGF-β and RA strongly reduced IL-10 secretion by spleen cells (Figure 5.15A). Surprisingly, blocking PD-1 significantly enhanced IL-10 levels (Figure 5.15A). This effect was observed for both CD4 and CD8 T cells, suggesting that blocking PD-1 elevates T cell cytokine secretion independent of a pro- or anti-inflammatory T cell phenotype (Figure 5.15B).

Furthermore, TGF-β alone or in combination with RA suppressed the proliferation of CD8 T cells (Figure 5.16). The proliferation arrest was reversed by blocking PD-1, indicating that blocking PD-1 can maintain or restore CD8 T cell proliferation and cytokine secretion in a suppressive environment.

5.2.11. Blocking PD-1 reduces Treg conversion in TGF-β-stimulated spleen cell cultures

The observation that blocking PD-1 reversed suppressive effects of TGF-β on T cells strongly suggested that TGF-β and PD-1 signalling are linked. Since initial experiments were performed in mixed spleen samples, this link could either be direct interaction of PD-1 and TGF-β signalling within the same cell or indirect effects elicited by other cells.

First, the hypothesis that TGF-β and RA-induced Treg cells play a role in PD-1-mediated suppression of CD8 T cells was tested. Addition of anti-PD-1 strongly reduced TGF-β-induced conversion of naïve CD4 T cells into Foxp3+ Treg cells (Figure 5.17). In contrast, blocking IFN-γ increased the frequency of Treg cells (Figure 5.17). This suggests that PD-1 signalling plays a role in TGF-β-induced Treg conversion,

strengthening the hypothesis of a link between PD-1 and TGF-β signalling in CD4 T cells. Moreover, these findings demonstrate that IFN-γ interferes with the induction of Treg cells.

It was then investigated whether TGF-β affected the expression of PD-1 or PD-L1 on CD4 T cells, thereby altering their responsiveness to PD-1 inhibition. Unstimulated CD4 T cells expressed negligible levels of PD-1 and PD-L1, however, the expression of Pd-1 and PD-L1 was strongly upregulated when CD4 T cells were stimulated through their TCR by anti-CD3/anti-CD28 (Figure 5.18A). Neither PD-1 nor PD-L1 expression was altered by TGF-β or RA stimulation (Figure 5.18B), suggesting that TGF-β-induced effects on CD4 T cells were either downstream of PD-1 signalling or indirect through effects on other cells in the spleen.

5.2.12. Blocking PD-1 does not directly inhibit Treg conversion in purified CD4 T cells

Having shown that a PD-1 blocking antibody reduced the induction of Foxp3-expressing Treg cells, it was further investigated how PD-1 signalling is connected to TGF-β downstream effects. Since previous experiments were performed in a mixed spleen population, it was not clear whether blocking PD-1 directly affected TGF-β signalling within CD4 T cells. Previous studies investigated the link between PD-1 and TGF-β suggested that PD-1 signalling is required for TGF-β-induced Treg conversion [121, 122]. To answer this question, mixed spleen cells or purified CD4 T cells were stimulated with TGF-β and RA in presence or absence of anti-PD-1. Blocking PD-1 reduced the frequency of Foxp3-expressing Treg cells in a mixed spleen sample (Figure 5.19 A,B). Interestingly, this effect was lost when CD4 T cells were purified and stimulated in absence of other immune cells types. Blocking PD-1 even slightly increased the frequency of Treg cells (Figure 5.19B). There are several possible reasons for the differences observed between spleen cells and purified CD4 T cells. Firstly, the PD-1 blocking antibody could directly bind to PD-1 on CD4 T cells, however, other cells might help CD4 T cells in the spleen sample to convert to Treg cells through PD-1 engagement. Secondly, blocking PD-1 could affect cytokine secretion by other cells that inhibit Treg conversion. For example, IFN-γ, IL-6 and IL-4 have been suggested to interfere with TGF-β-induced Treg conversion [122].

To test the second hypothesis, cell culture supernatants were analysed for secreted cytokines. Blocking PD-1 increased the concentration of secreted IFN-γ and IL-6 in spleen cultures but not in purified CD4 T cells. IL-4 secretion was not significantly

altered in spleen cells and slightly enhanced in purified CD4 T cells. The secretion of IL-10 was enhanced by blocking PD-1 in both spleen and CD4 cultures (Figure 5.20). These results suggest that blocking PD-1 could inhibit the induction of Foxp3-expressing Treg cells through enhanced secretion of IFN-γ or IL-6.

5.2.13.Blocking PD-1 enhances anti-tumour cytotoxicity *ex vivo*

The main characteristics of effector CD8 T cells are cytokine secretion, proliferation and cytotoxicity. Having shown that blocking PD-1 restores CD8 proliferation and IFN-γ secretion in a suppressive microenvironment, the next experiment was designed to analyse the effects of anti-PD-1 on T cell cytotoxicity. Spleen cells from CT26 tumour-bearing mice were co-cultured with CT26 tumour cells *ex vivo* in presence or absence of anti-PD-1 and CT26 cell death analysed by flow cytometry. After 24 h of co-culture, levels of cell death increased with increasing effector:target ratios indicating the presence of tumour-specific cytotoxic T cells in spleens from tumour-bearing mice (Figure 5.21A). Notably, the frequency of dead CT26 cells was significantly increased by the addition of anti-PD-1 during the co-culture (Figure 5.21A). The same trend was observed when anti-PD-1 was injected i.p. in the course of tumour growth and spleen cells co-cultured with CT26 tumour in absence of the blocking antibody (Figure 5.21B). Reflecting the increase in cell death, blocking PD-1 *ex vivo* also increased the concentration of IFN-γ and TNF in the co-culture supernatants (Figure 5.21C). Blocking PD-1 had almost no effect on TGF-β secretion but increased IL-10 levels in the supernatants, confirming that blocking PD-1 enhances the secretion of both pro- and anti-inflammatory effector cytokines by T cells (Figure 5.21C). These results confirm that blocking PD-1, in addition to increasing cytokine secretion and proliferation, improves cytotoxic T cell function.

5.2.14.Blocking PD-1 does not improve the efficacy of tumour peptide vaccines

Having established that a PD-1 blocking antibody improves CD8 T cell function *in vitro* and *ex vivo*, its efficacy *in vivo* was investigated in combination with a tumour vaccine. It has been suggested that pre-existing T cell responses and T cell infiltration into the tumour are essential for successful immune checkpoint inhibition in cancer treatment [262]. Therefore, mice were immunized with TRP-$2_{180\text{-}188}$ and R848 5 weeks and 1 week before tumour challenge and subsequently treated therapeutically with anti-PD-1 on days 3, 10 and 17 post tumour induction. However, neither anti-PD-1 alone nor the

combination with the vaccine had an effect on tumour growth (Figure 5.22). In a second experiment, the same combination was tested therapeutically and the TRP-$2_{180-188}$ peptide, R848 and anti-PD-1 were given together i.p. on days 3, 10 and 17 post tumour induction. No benefit was observed when anti-PD-1 was given alone or in combination with the peptide vaccine (Figure 5.23). Importantly, the vaccine itself which reduced tumour growth when it was given s.c. (Figure 5.4) proved to be ineffective when given i.p. (Figure 5.23). In another experiment, the TRP-2 peptide and R848 were injected s.c. in the area of the tumour on days 3, 10 and 17 post tumour induction and anti-PD-1 was given i.p. always one day prior to the peptide vaccine. Although the vaccine delayed tumour growth and induced some protection, no benefits were seen when anti-PD-1 was added (Figure 5.24). Taken together, blocking PD-1 did not improve the efficacy of peptide vaccines in the B16 tumour model in three different set ups, albeit showing promising effects on T cell function *in vitro*.

5.2.15. Blocking PD-1 does not improve the efficacy of a therapeutic whole cell vaccine in the B16 tumour model

Poor immunogenicity of the TRP-2 peptide could be a reason why the combination therapy of the peptide vaccine with anti-PD-1 failed *in vivo*. Therefore, hs/irr B16 tumour cells were used instead of the TRP-2 peptide in another vaccine trial. Mice were immunized with hs/irr B16 cells and R848 on days 3, 10 and 17 and treated with anti-PD-1 one day prior to immunization. The combination of the hs/irr B16 cell vaccine with R848 induced very strong anti-tumour responses, prolonged survival and induced protection in 70% of mice (Figure 5.25 A,B,D). However, no additional benefit was observed when anti-PD-1 was added to the vaccine. Immunized mice that rejected the first tumour were re-challenged with B16 tumour cells, but no significant differences were observed between mice that received the vaccine with anti-PD-1 compared to those immunized with the vaccine alone (Figure 5.25C). These results demonstrate no beneficial effects of anti-PD-1 on the efficacy of cancer vaccines in the B16 model.

5.2.16. Blocking PD-1 improves the efficacy of a tumour vaccine in the CT26 tumour model and induces anti-tumour memory

Previous work on immunotherapies *in vivo* indicated that the efficacy of immune checkpoint inhibitors could also depend on the mouse models that were used. Lechner et al. studied six different tumour models and concluded that the immunogenicity of a tumour model was a defining feature of the response to immunotherapy. It was

suggested that immunogenic tumour models like CT26 would deliver better responses to immunotherapy than low immunogenic tumours like B16 [346].

Consequently, the efficacy of anti-PD-1 was investigated using the CT26 tumour model. Similar to the previous experiment, a vaccine combination was formulated using hs/irr CT26 cells with R848 and anti-PD-1. The vaccine was injected s.c. on days 3, 10 and 17 and anti-PD-1 was injected i.p. one day before the vaccine. Immunization with R848 alone or with the hs/irr cells modestly reduced tumour growth. However, pre-treatment with anti-PD-1 significantly enhanced the efficacy of the vaccine in the CT26 tumour model. Tumour growth was markedly reduced and survival prolonged (Figure 5.26 A,B). Overall, nearly 40 % of mice treated with the combination completely eradicated the tumours by day 23 (Figure 5.26C). Interestingly, several mice that initially showed tumour growth were capable of clearing the tumour (Figure 5.27). These findings confirm that blocking PD-1 can improve the efficacy of a tumour vaccine and induce the rejection of established tumours.

5.2.17. Inverse correlation between tumour-infiltrating IFN-γ and TNF-producing T cells and tumour size in immunized mice

Having demonstrated that blocking PD-1 enhanced the efficacy of a CT26 tumour vaccine, tumour infiltrating T cells were analysed in order to investigate the mechanism of protection. Mice were immunized as described above and dLN and tumours were removed on days 11 and 23 to analyse infiltrating T cells.

First, the relationship between T cell effector function and tumour size was analysed on day 23 post tumour induction. All treatment groups were combined and frequencies of CD4 and CD8 T cells were plotted against the tumour volume. Interestingly, the secretion of IFN-γ and TNF by CD4 and CD8 T cells inversely correlated with tumour size. Moreover, virtually no cytokines were detectable in CD4 and CD8 T cells from tumours greater than 500 mm^3. This effect was specific for pro-inflammatory effector cytokines, since the expression of IL-10, Foxp3 or granzyme B was independent of the tumour volume (Figure 5.28).

The percentage of IFN-γ and TNF-producing T cells in dLN and tumours was analysed next. In the dLN, no major differences in cytokines produced by CD4 T cells were observed between the different treatment groups on day 11 or day 23 post tumour induction. There was a slight upwards trend in IFN-γ and TNF production in the tumours when mice were immunised with the vaccine and treated with anti-PD-1. The

biggest differences between the treatment groups were observed on day 23. Mice immunized with the vaccine alone or in combination with anti-PD-1 displayed increased IFN-γ and TNF production by CD4 T cells compared with vehicle-treated mice. Notably, the frequency of IFN-γ^+ and TNF^+ CD4 T cells was the highest in mice that received the vaccine alone since mice that rejected the tumour in the group that received the vaccine with anti-PD-1 were not available for TIL analysis (Figure 5.29 A,B).

Similar trends were observed when CD8 T cells were analysed for pro-inflammatory cytokine production. In the dLN, mice treated with anti-PD-1 displayed the highest levels of IFN-γ^+CD8^+ cells on day 11 and day 23 post tumour induction. No differences in TNF production by CD8 T cells were visible in dLN. On day 11, tumour infiltrating CD8 T cells from mice that were treated with anti-PD-1 showed increased levels of IFN-γ and TNF production when compared with vehicle or vaccine-treated mice, indicating that blocking PD-1 improved CD8 T cell function. On day 23, tumour-infiltrating CD8 T cells treated with the vaccine alone or in combination with anti-PD-1 had a higher frequency of IFN-γ or TNF-producing cells compared with vehicle or anti-PD-1-treated mice. Again, mice immunized with the vaccine alone had a higher frequency of T cells producing pro-inflammatory cytokines than mice that were additionally treated with anti-PD-1 which is probably due to the fact that mice that received the combination and cleared the tumours were not available for TIL analysis (Figure 5.30 A,B).

5.2.18. The combination of a tumour vaccine with a PD-1 blocking antibody significantly reduces Treg conversion within the tumour

Previous *in vitro* experiments suggested a link between PD-1 and the induction of Foxp3-expressing Treg cells. It was demonstrated that blocking PD-1 reduced the frequency of Treg cells in a spleen cell population stimulated with TGF-β and RA (Figure 5.17). Moreover, the frequency of $Foxp3^+$ CD4 T cells drastically increased in the tumour microenvironment, where TGF-β and RA are present in abundance (Figure 5.11). To investigate whether blocking PD-1 affected the induction and infiltration of Treg cells *in vivo*, dLN and tumours from vaccinated mice were analysed by flow cytometry for Foxp3 expression on days 11 and 23 post tumour induction. In the dLN, mice that received the vaccine had a lower frequency of Treg cells than unvaccinated mice which was not altered by the addition of anti-PD-1. An examination of tumour-infiltrating T cells revealed that the vaccine alone induced some reduction of Treg cells. The combination of the vaccine with anti-PD-1, however, significantly reduced the

frequency of tumour-infiltrating Treg cells, which was observed on day 11 and day 23 post tumour induction (Figure 5.31 A,B). These findings suggest that Treg cells induced in the tumour microenvironment can be reduced by activating the immune system with a tumour vaccine and concurrent blockade of PD-1.

*5.2.19.*Blocking PD-1 increases T cell proliferation *in vivo*

Previous *in vitro* experiments demonstrated that blocking PD-1 efficiently restored T cell proliferation in a suppressive environment (Figure 5.16). To analyse T cell proliferation in response to treatment with the vaccine and anti-PD-1, cells from dLN and tumours were stained with the proliferation marker Ki-67. Treatment of mice with anti-PD-1 alone increased Ki-67 expression in CD4 and CD8 T cells in the dLN compared with vehicle-treated mice. T cells in dLN from mice treated with the vaccine and anti-PD-1 also expressed higher levels of the proliferation marker Ki-67 than vaccinated mice alone. This effect was observed for both CD4 and CD8 T cells on day 11 and day 23 post tumour induction. In the tumours, CD4 and CD8 T cells from mice that were treated with anti-PD-1 alone expressed more Ki-67 compared with mice treated with the vehicle. At both time points, T cells from vaccinated mice displayed the highest levels of proliferation with no significant differences when anti-PD-1 was added (Figure 5.32). Surprisingly, these findings indicate that immune checkpoint inhibition mostly restores T cell proliferation in the dLN while the vaccine is able to boost the frequency of proliferating T cell in the tumours.

5.2.20.The combination of a tumour vaccine with a PD-1 blocking antibody enhances T cell cytotoxicity and IFN-γ secretion

The analysis of TIL suggested that blocking PD-1 prior to vaccination decreased the infiltration of suppressive Treg cells and promoted the proliferation of T cells. However, the analysis of TIL was only possible in mice that had not cleared the tumour, which made it difficult to analyse immune cell infiltration in mice that showed the best responses to the therapy. In order to investigate the cytotoxic potential of all mice, spleens were removed on day 11 past tumour induction and co-cultured with CTV-labelled CT26 tumour cells at different effector:target ratios. After 24 h, tumour cell death induced by cytotoxic cells in the spleens was measured by flow cytometry. Spleen cells from mice vaccinated with hs/irr CT26 and R848 had greater cytotoxic activity against tumour cells than spleen cells from vehicle-treated mice. Spleen cells from mice treated with anti-PD-1 only had lower cytotoxic activity than spleen cell from

mice treated with vehicle only. Consistent with the findings on tumour growth *in vivo*, spleen cells from mice that received the combination of the tumour vaccine with anti-PD-1 had the highest cytotoxic activity against tumour cells *in vitro* (Figure 5.33A).

Cytokines secreted by spleen cells co-cultured with tumour cells were measured by ELISA. Spleen cells from mice treated with the PD-1 blocking antibody or the vaccine alone secreted slightly more IFN-γ compared with spleen cells from vehicle-treated mice. Spleen cells from mice treated with the combination of the vaccine with anti-PD-1 induced the highest concentration of secreted IFN-γ and IL-10 compared with all other groups. No differences in TNF and TGF-β secretion were observed between the groups (Figure 5.33B). Furthermore, mice that were immunized with the vaccine and treated with anti-PD-1 were split into two groups, according to whether they rejected the tumour or not. Notably, spleen cells from mice that rejected the tumours secreted significantly higher levels of IFN-γ after co-culture with CT26 tumour cells compared with spleen cells from mice that did not reject the tumours or vehicle-treated mice. No significant differences in IL-10 secretion by spleen cells from tumour-bearing mice compared with mice that rejected the tumour were observed (Figure 5.33C).

Taken together, these findings demonstrate that mice treated with an anti-PD-1 antibody in combination with two immunizations of hs/irr CT26 and R848 had improved anti-tumour responses which were reflected by an increase of tumour cell killing and IFN-γ secretion. The correlation between cytotoxic responses *ex vivo* with anti-tumour responses *in vivo* was especially evident in mice that rejected tumours compared with those that failed to reject tumours.

5.2.21. Mice that rejected CT26 tumours upon treatment with a tumour vaccine and anti-PD-1 reject tumours following re-challenge

Having shown that a significant proportion of mice immunized with hs/irr CT26 and R848 in combination with anti-PD-1 were able to reject established tumours, it was then investigated whether anti-tumour memory was induced in these mice. Two mice that had previously rejected the tumours were re-challenged with CT26 tumour cells and naïve mice given a primary tumour challenge were used as controls. In control mice, palpable tumours were visible from day 10 post tumour induction and grew very quickly, reaching an average volume of 700 mm^3 by day 17. In contrast, no tumours were detectable in re-challenged mice by day 17, providing evidence that the vaccine and anti-PD-1 combination had induced tumour-specific immunological memory (Figure

5.34). Furthermore, spleen cells from mice that rejected tumours for the second time induced higher levels of cell death when co-cultured with CT26 cells *ex vivo* compared to spleen cells from control mice (Figure 5.35), confirming the existence of tumour-specific cytotoxic T cells.

These findings demonstrate that the combination of a tumour vaccine with anti-PD-1 induces tumour protection and potent anti-tumour immunological memory capable of quickly recognising and clearing tumour cells.

5.3. Discussion

Cancer vaccines are designed to drive anti-tumour immune responses and work by expanding the pool of tumour-specific T cells. A cancer vaccine usually consists of a source of tumour antigens and adjuvants to allow effective DC activation and antigen presentation to take place. Cancer vaccines have been investigated in pre-clinical cancer models and some have made it into clinical trials in humans. Many of the trials failed due to improper choice of tumour antigens, adjuvants or delivery modes [199]. However, the biggest problem was the failure in overcoming immune subversion by the tumour. Studies of the tumour microenvironment revealed a plethora of immune suppressive mechanisms. These include the secretion of anti-inflammatory cytokines and molecules, the recruitment and modulation of regulatory immune cells, the downregulation of antigen presentation and the inactivation of immune cells through co-inhibitory receptors [73]. As a consequence, even if a cancer vaccine is successful in expanding tumour-specific T cells, the tumour microenvironment is still capable of shutting down effector functions once T cells reach their target. Therefore, a combination of a cancer vaccine with an immune checkpoint inhibitor was evaluated for its efficacy in mouse models for cancer.

The results in this chapter gave an insight into how the tumour microenvironment suppresses the immune system and which combinations are capable of outmanoeuvring immune subversion. It was demonstrated that the TLR7/8 agonist R848 is a potent adjuvant for cancer vaccines. R848 induced strong pro-inflammatory cytokine secretion and expression of maturation markers by DCs. Moreover, immunization with a tumour peptide and R848 enhanced the induction of tumour-specific CD8 T cells. Treatment of mice with the combination of TRP-$2_{180\text{-}188}$ or hs/irr tumour cells and R848 was more efficient in reducing tumour growth than either treatment alone. The vaccine increased the infiltration of IFN-γ-producing T cells which expressed high levels of the co-inhibitory receptor PD-1. *In vitro* studies revealed that blocking PD-1 stabilised CD8 T cell proliferation, cytokine secretion and cytotoxicity while reducing the induction of Foxp3-expressing Treg cells. Moreover, *in vivo* studies demonstrated that a combination of a vaccine consisting of hs/irr CT26 tumour cells and the TLR agonist R848 with a PD-1 blocking antibody significantly reduced tumour growth, prolonged survival and improved effector T cell function.

The present study demonstrated that the choice of antigen is pivotal for the efficacy of a vaccine. Two different sources of antigens were investigated *in vivo*. When comparing their efficacy, hs/irr B16 tumour cells proved to be a more immunogenic

source than TRP-2 peptides. While treatment of mice with TRP-$2_{180-188}$ and R848 induced tumour protection in 2/7 of mice, hs/irr B16 cells with R848 induced protection in 5/7 of mice. Moreover, the TRP-2-R848 combination induced no memory while mice immunized with hs/irr B16 and R848 had delayed tumour growth when re-challenged. These findings suggest that using a vaccine with a variety of antigens is better than just one antigen for the induction of anti-tumour immunity by activating a broader pool of T cells. It has been suggested that the expression of neoantigens, which arise as a consequence of mutations, may be a useful source of tumour antigens for active immunotherapies [270, 348]. The present study argues for the use of hs/irr tumour cells as antigen source since they naturally contain neoantigens.

As expected, immunization of mice with tumour peptides or hs/irr tumour cells did not induce tumour regression when used without an adjuvant. In contrast, treatment of mice with R848 alone induced some reduction in tumour growth in the B16 model. This suggests that potent antigen presentation, rather than a source of tumour antigens, is impaired in B16 tumour-bearing mice. Proper activation and maturation of DCs is crucial for T cell activation. Many cancers promote immature or regulatory DCs which are not capable of activating T cells and instead promote Treg cells or T cell suppression [349]. Shurin et al. demonstrated that DCs stimulated with tumour-conditioned medium suppressed T cell proliferation [350]. The present study shows that the TLR agonist R848 promoted an inflammatory DC phenotype and the induction of effector T cells. Indeed, an increase in tumour-specific T cells was observed when mice were immunized with a combination of antigens with R848, confirming the strong adjuvant effects of R848.

One of the key findings and the basis for the chosen cancer therapy combination was the observation of an inverse correlation between effector function and co-inhibitory receptor expression on T cells from tumour-bearing mice. High levels of pro-inflammatory cytokine secreting T cells were observed in the dLN of CT26 tumour-bearing mice. The ability of T cells to secrete effector cytokines was almost completely abrogated once they entered the tumour bed. At the same time, the expression of PD-1 and LAG-3 on T cells was vastly elevated in the tumour. *In vitro* experiments demonstrated that T cells had stronger proliferating, cytokine secreting and cytotoxic activity when PD-1 was blocked. This suggests that PD-1 signalling directly interferes with T cell effector function. Indeed, it has been shown that PD-1 signalling recruits the phosphatase SHP-2 which interferes with TCR signalling and dephosphorylates important downstream effector molecules [343].

The results of the present study showed that a proportion of PD-1 expressing T cells were still proliferating and producing IFN-γ *in vivo* depending on PD-1 expression levels. In the LN, CD4 and CD8 T cells that expressed intermediate levels of PD-1 produced significantly higher levels of IFN-γ than T cells that were PD-1 high. Moreover, more than 80 % of T cells in the tumour were PD-1 high and produced almost no IFN-γ. This suggests that PD-1 expression alone is not a definite marker for dysfunctional T cells. These findings suggest that additional factors are required to abrogate T cell function, such as the expression of other co-inhibitory molecules and their cognate ligands. Moreover, most of the *in vitro* work on Treg induction involved stimulation with TGF-β and RA which could also contribute to T cell dysfunction. It is most likely that expression of multiple co-inhibitory receptors and the presence of anti-inflammatory molecules and Treg cells together contribute to T cell exhaustion, however, their respective contributions are still not clear [93]. There is strong evidence from chronic viral infection models that co-expression of inhibitory receptors promotes T cell exhaustion. A study by Blackburn et al. has demonstrated that exhausted T cells co-expressed multiple inhibitory receptors which correlated with the severity of dysfunction [92]. This is reflected in cancer models, where combinations of checkpoint inhibitors have been more effective than single inhibitors alone [124, 246]. Clinical trials demonstrated that a combination of anti-CTLA-4 and anti-PD-1 was more effective for the treatment of melanoma than either immune checkpoint inhibitor alone [252]. Taken together, the present study adds to the view that the tumour microenvironment promotes high expression levels of multiple co-inhibitory receptors on T cells which, together with a lack of cytokine secretion, are an indication for impaired T cell responses.

PD-1 blockade has been associated with improved pro-inflammatory effector responses. Surprisingly, the present study revealed that blocking PD-1 also enhanced the secretion of IL-10 by T cells. IL-10 is considered anti-inflammatory and can suppress effector T cells which could potentially counteract beneficial effects of blocking PD-1. PD-1 knockout mice showed elevated levels of T cell derived IL-10 in their serum and were more susceptible to *T. gondii* infection [351]. Conversely, an increase of IL-10 production by triggering PD-1 has previously been described in monocytes [352]. The link between PD-1 and IL-10 secretion remains unclear but seems to be differentially regulated in different cell types. Due to its suppressive properties, IL-10 induced by PD-1 blockade could have implications for the efficacy of anti-PD-1 therapy. This aspect of PD-1 therapy would be interesting for future studies.

For example, combined therapy using anti-PD-1 and anti-IL-10 could be tested *in vivo* in murine tumour models.

Another key finding of the present study was the observation that blocking PD-1 reduced the conversion of TGF-β induced Treg cells *in vitro*. The effect of blocking PD-1 was indirect, since it did not affect Treg conversion when isolated CD4 T cells were stimulated with TGF-β. There are several possible mechanisms whereby anti-PD-1 could have affected Treg conversion in the spleen cell culture. Firstly, PD-L1-expressing non-CD4 T cells might be the dominant cells binding to PD-1 on CD4 T cells and thereby promoting Treg conversion. Wang et al. showed that PD-L1 expressing DCs promote TGF-β-induced Treg conversion [122]. More evidence that effects can be different depending on which cell expresses PD-L1 comes from a study by Iraolagoitia et al., showing that PD-L1 expressing NK cells impair maturation of PD-1^+ DCs but have no effect on PD-1^+ CD8 T cell function [134]. Secondly, blocking PD-1 strongly elevated the concentration of IFN-γ and IL-6 in the spleen cell culture but not in cultures of purified CD4 T cells. IFN-γ and IL-6 were shown to interfere with Treg induction [59, 353]. Lastly, blocking PD-1 on other cells in the spleen culture could have impaired Treg conversion, for example by promoting the proliferation and function of IFN-γ-secreting CD8 T cells. The link between TGF-β-induced Treg conversion and PD-1 signalling has been investigated in the past with contradictory conclusions. Wang et al. observed that DCs failed to promote the induction of Treg cells when PD-L1 was knocked out [122]. Moreover, a study by Francisco et al. demonstrated that PD-L1-coated beads significantly increased the frequency of TGF-β induced Treg cells [121]. Both studies suggested that PD-1 signalling on CD4 T cells triggered by PD-L1 promotes the induction of Treg cells. However, PD-L1 can also bind to CD80, therefore, it cannot be concluded that PD-L1 exerted its effects directly through PD-1 on CD4 T cells. Indeed, a study using PD-1 deficient hematopoietic stem cells found that PD-1 deficiency did not impair the generation of peripherally-induced Foxp3^+ Treg cells *in vivo* [354]. In agreement with this, our findings also suggest that blocking PD-1 indirectly impairs the induction of Treg cells.

In the present study, a vaccine consisting of hs/irr tumour cells and R848 administered with anti-PD-1 has been shown to reduce Treg cells *in vivo*. Mice treated with anti-PD-1 in combination with the vaccine had significantly reduced levels of Treg cells infiltrating the tumours. However, this reduction was not seen when anti-PD-1 was given alone. These findings indicate that the reduction of Treg cells might be promoted by anti-PD-1 when pro-inflammatory stimuli are present, such as IFN-γ-secreting T cells. A reduction

of Treg cells has also been reported by Duraiswamy et al. using a GM-CSF-CT26 cell vaccine in combination with anti-PD-1 and anti-CTLA-4 [124]. It was demonstrated that blocking PD-1 does not induce apoptosis but interfered with Treg suppressive function [124]. Future studies should investigate the mechanism of anti-PD-1 interference with Treg induction.

Most importantly, this study confirmed the hypothesis that blocking PD-1 improves the efficacy of a tumour vaccine. Treatment with anti-PD-1 significantly reduced tumour growth and prolonged survival in mice immunized with hs/irr CT26 tumour cells and R848. This combination was able to induce clearance of established tumours and protection against a second tumour challenge. The reduction of tumour growth was associated with increased tumour-infiltrating IFN-γ-secreting CD8 T cells and reduced infiltration of Treg cells which correlates with a better prognosis in the clinic [54]. While the vaccine immune checkpoint inhibitor combination was successful using the CT26 tumour model, no beneficial effects were observed in the B16 model. There are several possibilities why the therapy worked in the CT26 but not in the B16 model. CT26 tumour cells are more immunogenic and express higher levels of MHC I and thus have greater capability to induce T cell responses. An extensive study comparing six different transplantable tumour models found that the immunogenicity of a murine model defined the response to immunotherapy [346]. This is in line with other pre-clinical studies that showed that the B16 model responded poorly to immunotherapies that were protective in other models [87, 124, 346]. These findings indicate that tumour immunogenicity could potentially be a predictive marker for the response to vaccine immune checkpoint inhibitor combinations. Moreover, the expression of PD-L1 is currently investigated as a biomarker for anti-PD-1 therapy [7]. However, the present study found no differences in PD-L1 expression between B16 and CT26 tumour cells, suggesting that PD-L1 expression was not the determinant for the responsiveness towards the therapy investigated here. Both tumours expressed PD-L1 *in vivo* and upregulated expression of PD-L1 in response to IFN-γ stimulation and therefore could potentially downregulate T cell responses. Another reason why blocking PD-1 did not improve the cell vaccine in the B16 model could be that the vaccine itself was already very effective and that the expression of inhibitory receptors was not limiting the vaccine efficacy.

Notably, treatment of mice with anti-PD-1 alone did not confer protection in the B16 or CT26 tumour model. While this is in line with publications on the B16 model, other studies reported moderate beneficial effects of blocking PD-1 alone in the CT26 model

[124, 259]. Experimental design and differences in animal facilities could be a reason for the observed differences compared with previous publications. Recent studies have highlighted the impact of the microbiota on immune responses to infections and autoimmunity [271]. Differences in the microbiota have also been shown to influence the response to immunotherapy [272]. Moreover, different injection timings and frequencies were used between the present and published studies. Duraiswamy et al. reported protection in some mice treated with anti-PD-1 alone, however, it was given more frequently than in the present study [124]. Nevertheless, most studies agree that combination therapies were more effective than single immune checkpoint inhibitors alone.

Very interestingly, blocking PD-1 significantly enhanced T cell proliferation in the dLN of tumour-bearing mice. So far, it has been assumed that PD-1 blockade exerts its effects in the periphery where T cells show the highest expression levels of PD-1 and make contact with PD-L1 or PD-L2-expressing cells. While general expression of PD-1 and PD-L1 was much lower on cells in the dLN than in the tumours, PD-1:PD-L1 interaction could still limit the pool of T cells that is generated in the LN. Moreover, TLR stimulation upregulates the expression of PD-L1 and PD-L2 on DCs, which could reduce T cell proliferation in the dLN. These results reveal the novel finding that PD-1 expression is also important at the priming stage where it can limit the proliferation of T cells and thus reduce the pool of T cells.

In order to investigate the effect of the vaccine and immune checkpoint inhibitor combination therapy on effector T cell function, spleen cells were taken from immunized mice and co-cultured with CT26 tumour cells in a cytotoxicity assay. With this method it was possible to analyse cytotoxic T cells in mice that showed the best response to the therapy and cleared the tumour. Mice that were immunized with the tumour vaccine and anti-PD-1 displayed the highest levels of tumour lysis and IFN-γ secretion, confirming that the therapy elevated potent effector responses. These findings agree with the view that immune checkpoint inhibitors take the break off tumour-specific T cells which in turn are able to induce tumour cell lysis.

In line with recent developments in the cancer immunotherapy field, the present study highlights the great potential of using combinations of different immunotherapies, especially together with immune checkpoint inhibitors. Since Ipilimumab (anti-CTLA-4) became the first big breakthrough of immune checkpoint inhibition in the clinic, many immune checkpoint inhibitor combinations have been tested in pre-clinical models. Studies successfully investigated the combinations with other immune checkpoint

inhibitors [124, 246, 247, 257, 355], radiotherapy [258, 259], chemotherapeutic agents [356] and targeted therapies [357]. Recent studies have also investigated the combination of immune checkpoint inhibitors with cancer vaccines. An early study by Van Elsas et al. showed a remarkable efficacy using a GM-CSF secreting vaccine in combination with anti-CTLA-4 which led to complete rejection in 100 % of mice after a single dose [243]. Others followed showing effective vaccine immune checkpoint combinations in various tumour models including B16, CT26 and ID8 [124-126, 358].

At the moment, the long term efficacy of combinations using cancer vaccines and immune checkpoint inhibitors are yet to be tested in clinical trials. The present study adds to the emerging view that combinations of immunotherapies are more effective than single therapy regimens alone.

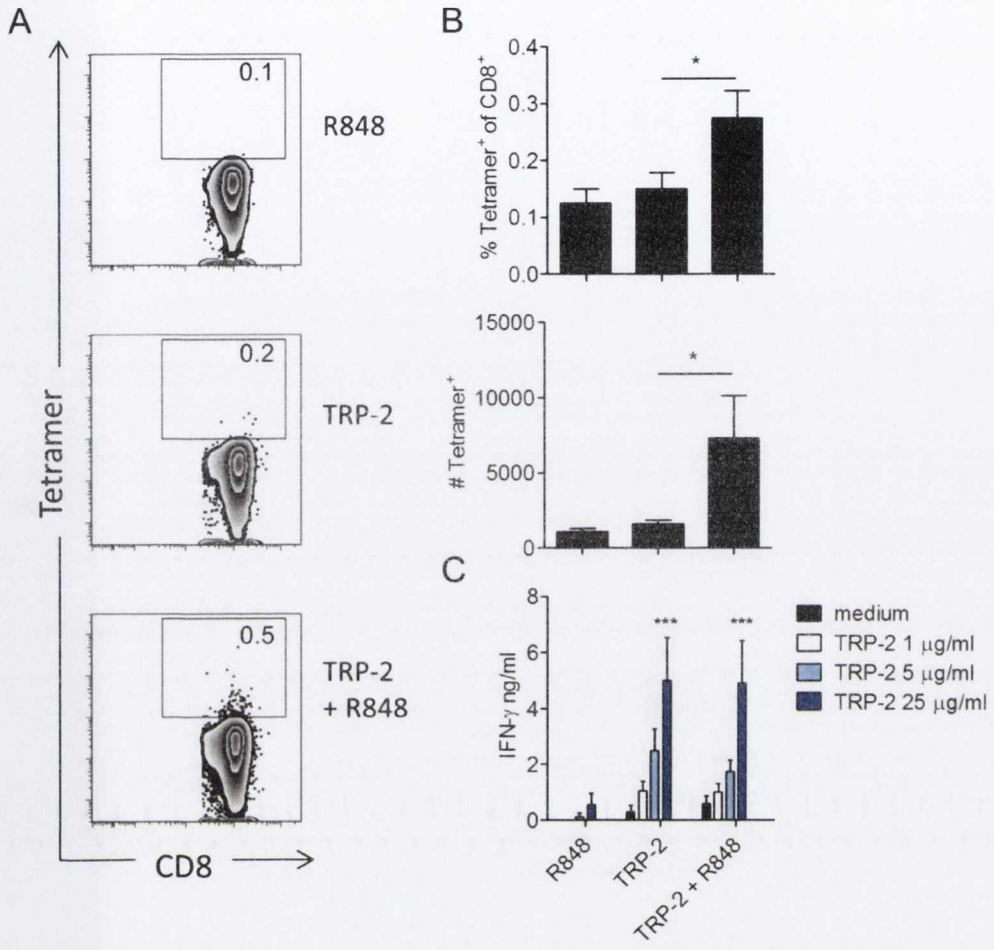


Figure 5.1
R848 enhances the frequency and total number of TRP-$2_{180-188}$ peptide-specific CD8 T cells

Naïve C57BL/6 mice were immunized with TRP-$2_{180-188}$ (100 µg/mouse), R848 (40 µg/mouse) or both into the footpads (n=4 mice/group). After 7 d, popliteal LN were isolated and single cell suspensions prepared. LN cells were stained with a TRP-$2_{180-188}$-tetramer, gating on $CD3^+CD8^+$ T cells. Results are shown as representative FACS plots (A) or mean values ± SEM (B). LN cells were re-stimulated with the indicated concentrations of TRP-$2_{180-188}$ peptide. After 5 d, supernatants were collected and IFN-γ secretion quantified by ELISA (C). *p<0.05 by 1-way ANOVA, ***p<0.001 by 2-way ANOVA versus medium. Data are representative of 3 experiments.

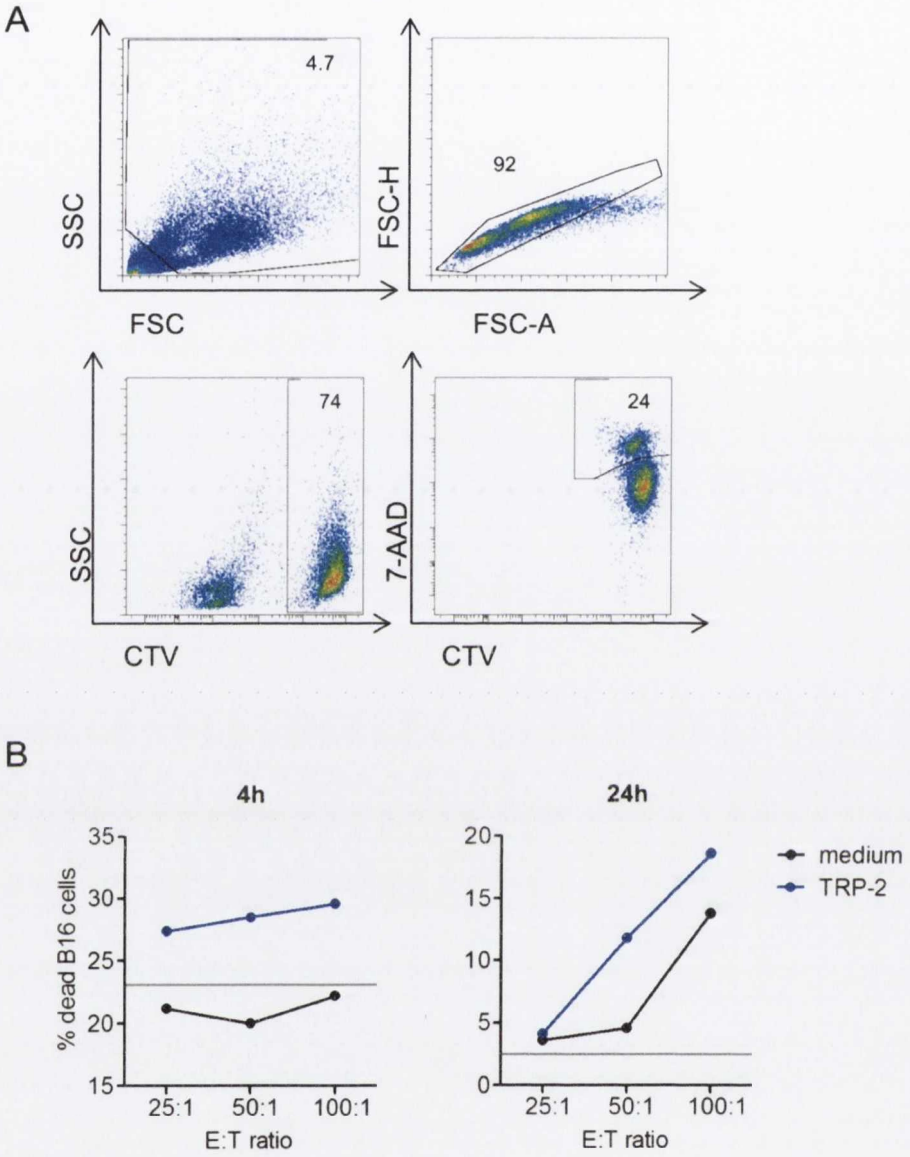


Figure 5.2
TRP-$2_{180-188}$-specific T cells induce tumour cell killing *in vitro*

B16 tumour cells were injected into 3 naïve C57BL/6 mice. After 14 d, spleen cells were isolated, pooled and re-stimulated with TRP-$2_{180-188}$ (20 µg/ml). After 5 d, cells were co-cultured with CTV-labelled B16 tumour cells at indicated ratios for 4 h or 24 h. Cell death was measured by flow cytometry using 7-AAD staining. Results are shown as example plots of FACS gating (A) and graphs of % cell death (B). The grey line represents the percentage of B16 cell death in culture alone (base line). Data are representative of 2 experiments.

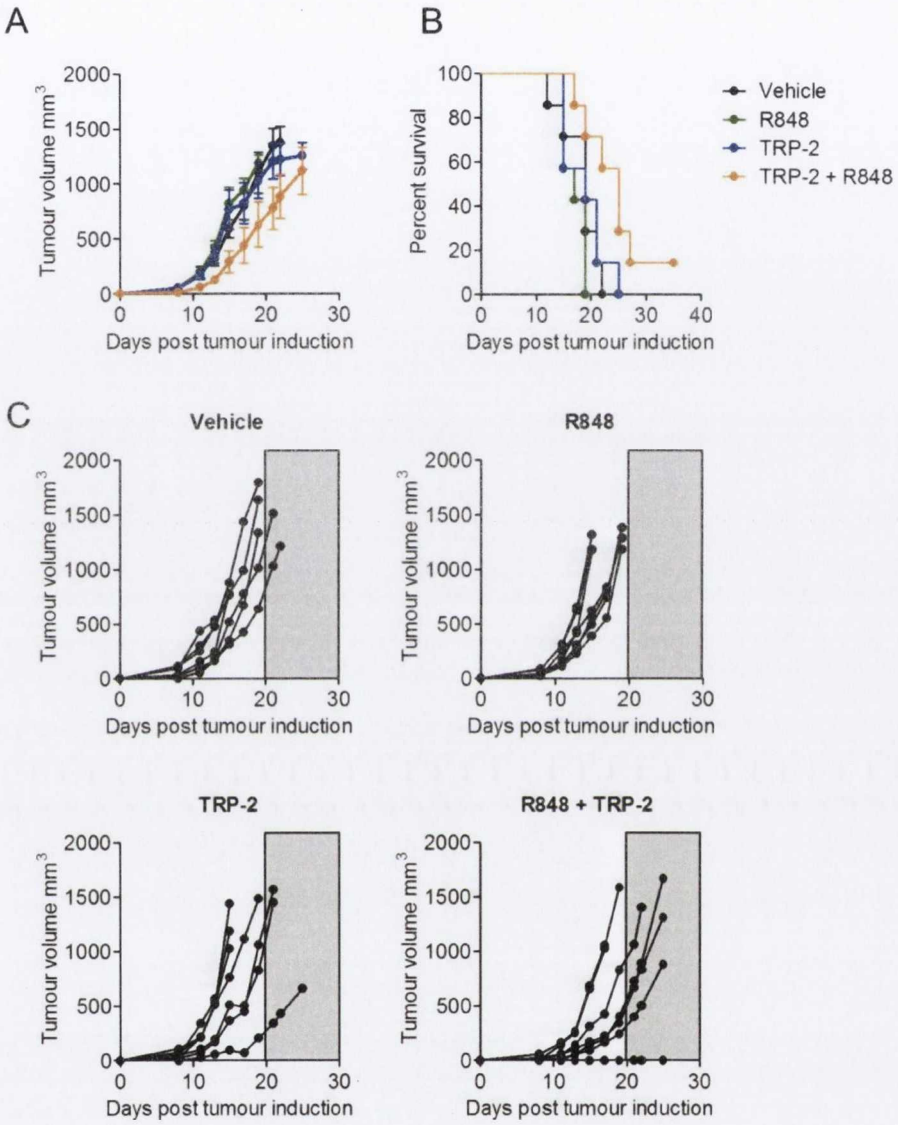


Figure 5.3
Prophylactic immunization with a tumour vaccine consisting of a TRP-2 peptide and R848 induces moderate protection in the B16 tumour model

C57BL/6 mice were immunized s.c. with TRP-$2_{180-188}$ (100 µg/mouse), R848 (40 µg/mouse) or both 5 weeks and 1 week prior to tumour induction with B16 tumour cells ($2x10^5$ cells/mouse). Results are shown as mean growth ± SEM (A), survival graphs (B) or tumour growth curves for individual mice (C). (n= 7 mice per group)

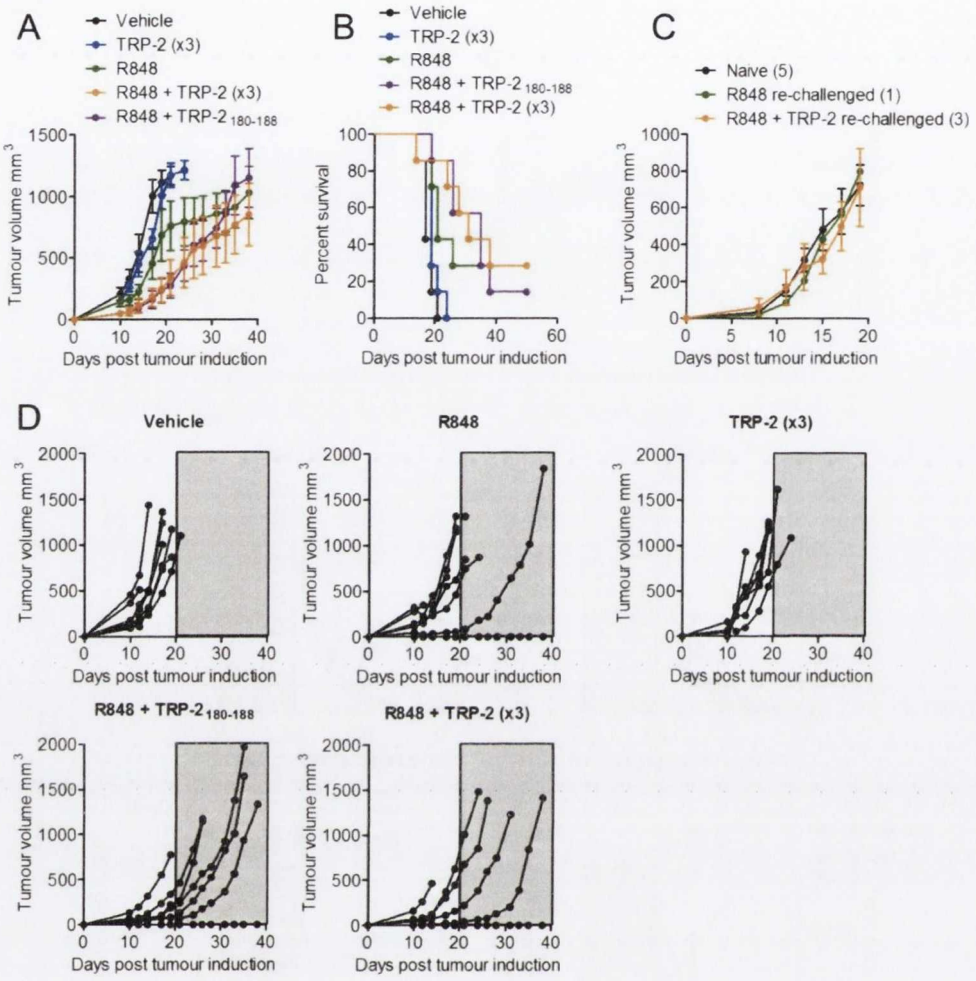


Figure 5.4

Therapeutic immunization with a tumour vaccine consisting of TRP-2 peptides and R848 induces protection in the B16 tumour model

C57BL/6 mice were injected with B16 tumour cells ($2x10^5$ cells/mouse) and immunized s.c. with TRP-2 peptides (100 µg/mouse; $TRP\text{-}2_{180\text{-}188}$ alone or $TRP\text{-}2_{180\text{-}188}$ + $TRP\text{-}2_{148\text{-}165}$ + $TRP\text{-}2_{58\text{-}78}$ (3x)), R848 (40 µg/mouse) or both on d 3, 10 and 17 post tumour induction. Results are shown as mean growth ± SEM (A), survival graphs (B) or tumour growth curves for individual mice (D). (n= 7 mice per group). Mice from (A) that rejected the tumours were re-challenged with B16 tumour cells. Number in brackets indicates number of mice per group. R848 + TRP-2 re-challenged group: combined mice from R848 + $TRP\text{-}2_{180\text{-}188}$ and R848 + TRP-2 (3x) (C).

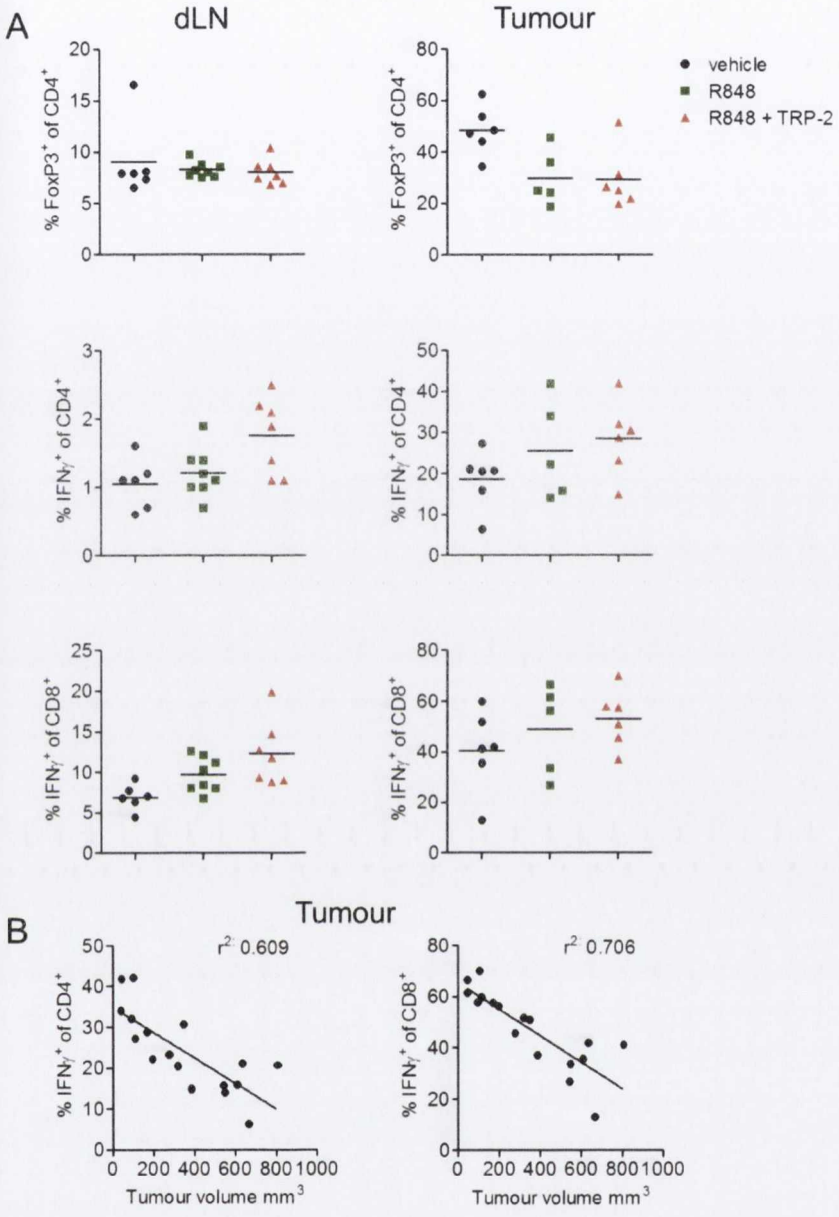


Figure 5.5
Immunization with a tumour vaccine increases infiltration of IFN-γ- secreting CD4 and CD8 T cells and decreases infiltration of Treg cells

C57BL/6 mice were injected with B16 tumour cells ($2x10^5$ cells/mouse) and immunized s.c. peritumourally with R848 (40 µg/mouse) alone or together with TRP-$2_{180-188}$ peptides (100 µg/mouse) on d 3 and 10 post tumour induction. On d 17, tumours and dLN were dissected and expression of IFN-γ and Foxp3 was analysed by flow cytometry, gating on $CD3^+CD4^+$ or $CD3^+CD8^+$ cells as indicated. Results are shown as scatter plots + mean values for dLN and tumours (A) or linear regression for tumour values pooled from groups in (A) versus tumour volume (B).

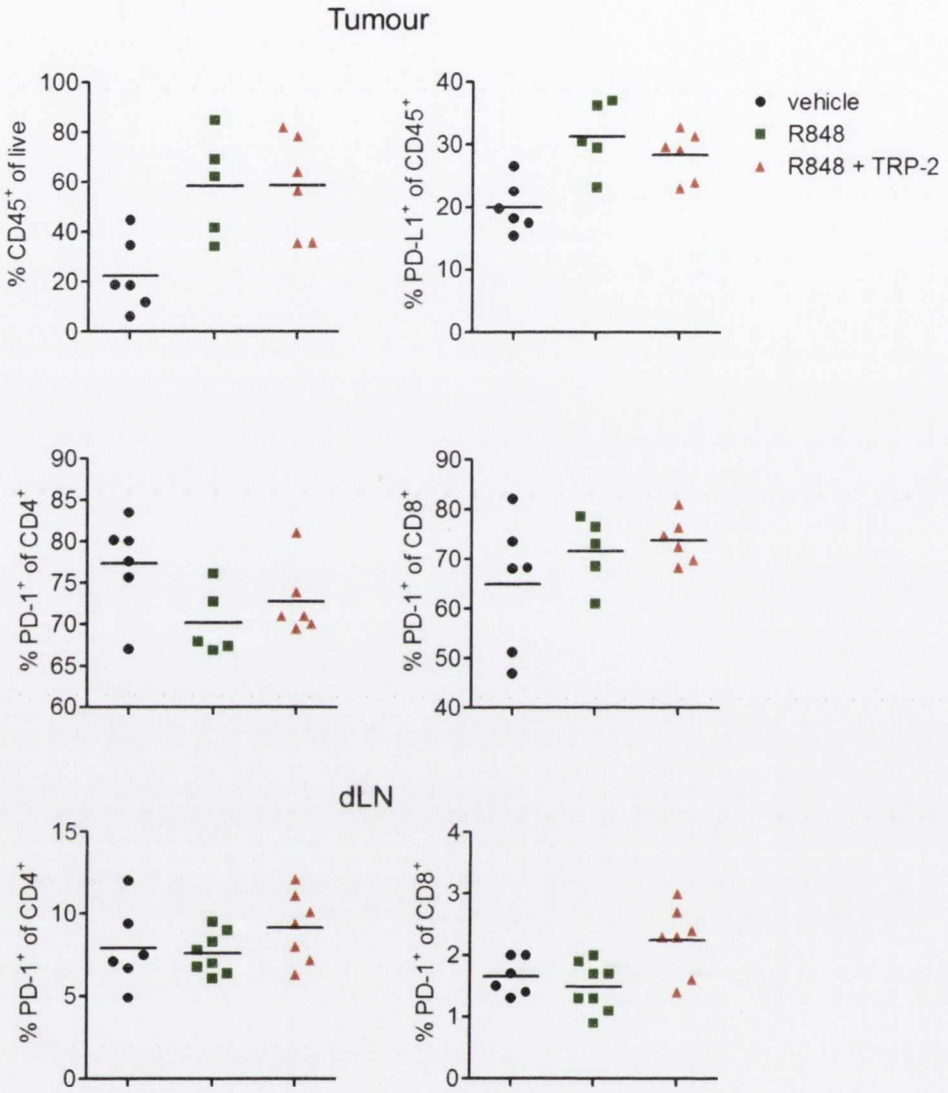


Figure 5.6
High expression of PD-1 and PD-L1 on tumour-infiltrating immune cells

C57BL/6 mice were injected with B16 tumour cells ($2x10^5$ cells/mouse) and immunized s.c. peritumourally with R848 (40 µg/mouse) alone or together with TRP-$2_{180-188}$ (100 µg/mouse) on d 3 and 10 post tumour induction. On d 17, tumours and dLN were dissected and expression of PD-1 and PD-L1 was analysed by flow cytometry, gating on $CD45^+CD3^+CD4^+$ or $CD45^+CD3^+CD8^+$ as indicated. Results are shown as scatter plots + mean values for tumours and dLN.

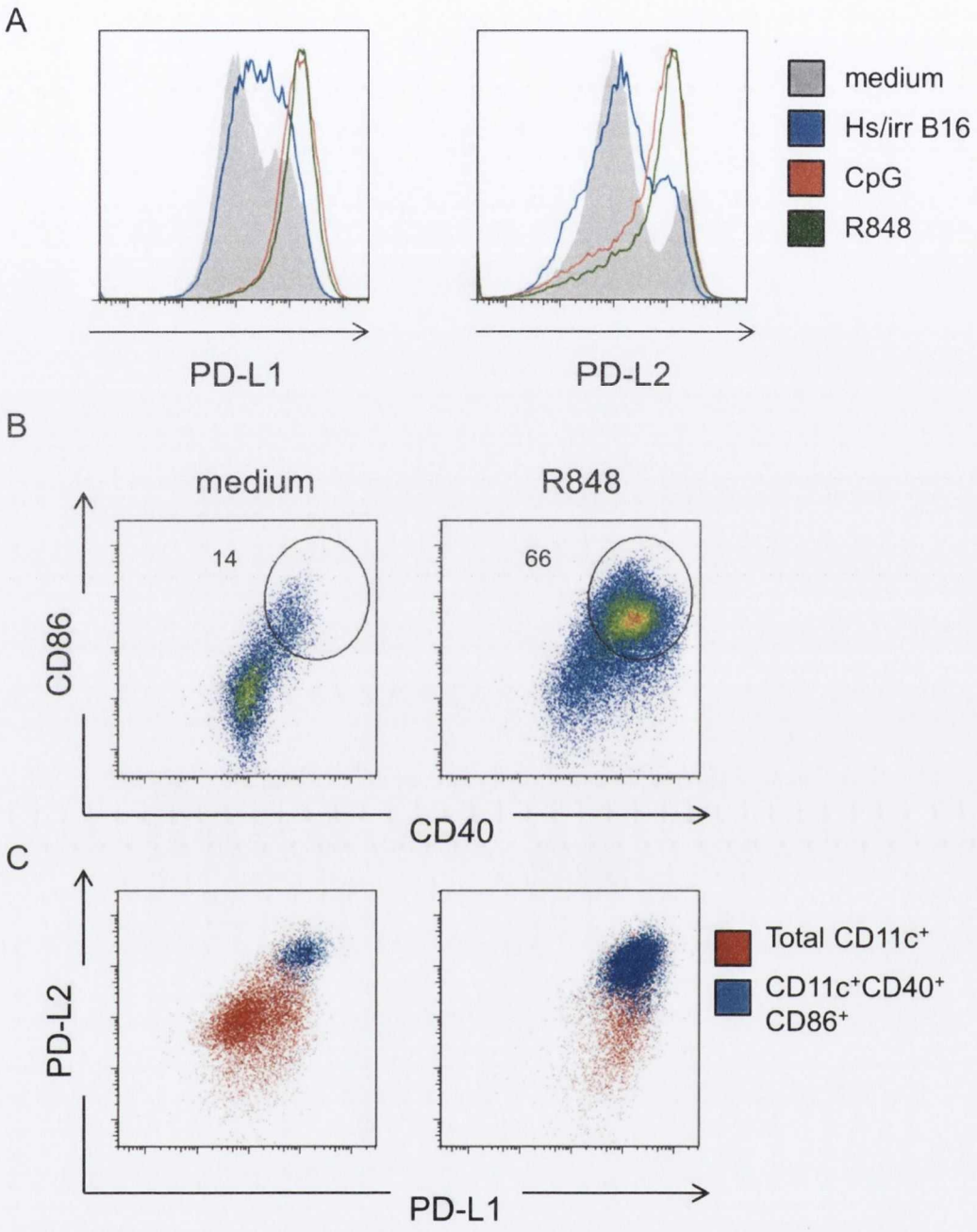


Figure 5.7
TLR agonists upregulate PD-L1 and PD-L2 expression on BMDCs

BMDCs from C57BL/6 mice were stimulated with hs/irr B16 cells (1:1), CpG (5 µg/ml) or R848 (100 ng/ml) for 24 h and expression of CD40, CD86, PD-L1 and PD-L2 analysed by flow cytometry. Results are shown as histograms of PD-L1 and PD-L2 expression after gating on $CD11c^+$ cells (A), dot plots of CD86 and CD40 after gating on $CD11c^+$ cells (B) or dot plot overlays of total $CD11c^+$ (red) and $CD11c^+CD40^+CD86^+$ cells (blue) (C).

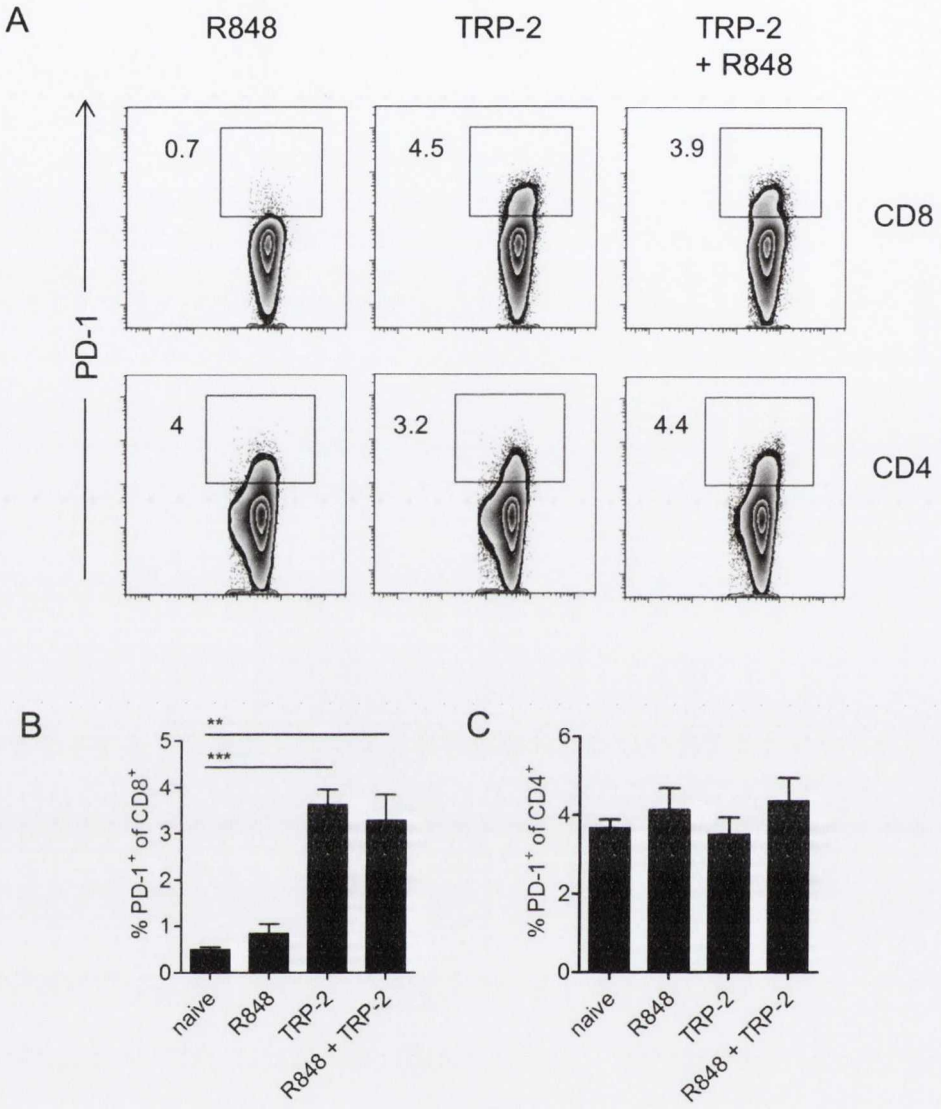


Figure 5.8
Immunization with TRP-$2_{180-188}$ stimulates PD-1 expression on CD8 T cells

Naïve C57BL/6 mice were immunized with TRP-$2_{180-188}$ (100 µg/mouse), R848 (40 µg/mouse) or both into the footpads (n=4 mice/group). After 7 d, popliteal LN were isolated and single cell suspensions prepared. LN cells were stained and analysed for expression of PD-1 by flow cytometry, gating on $CD3^+CD8^+$ or $CD3^+CD4^+$ cells as indicated. Results are shown as representative FACS plots (A) or mean values ± SEM (B). **p<0.01, ***p<0.001 by 1-way ANOVA.

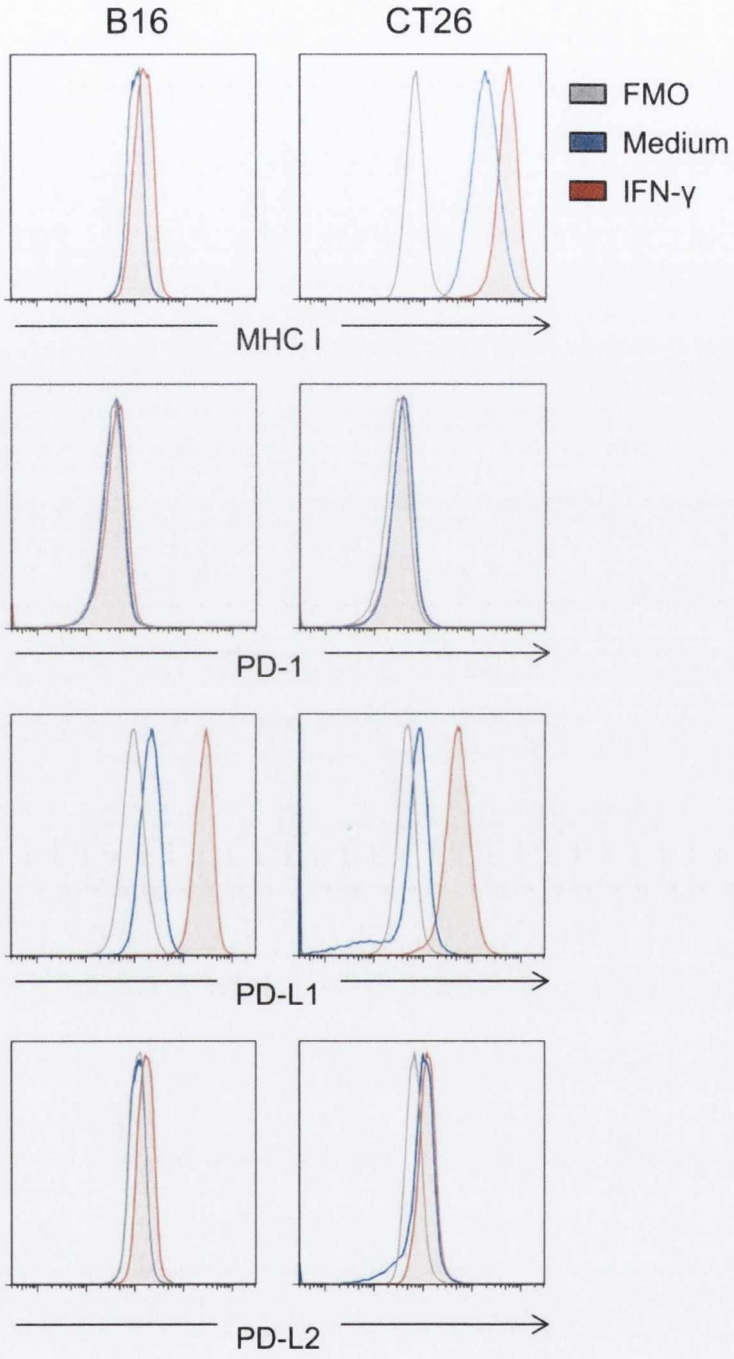


Figure 5.9
IFN-γ augments the expression of MHC I and PD-L1 but not PD-L2 or PD-1 on tumour cells

Cultured B16 and CT26 tumour cells were stimulated with IFN-γ (500 ng/ml) for 24 h. Subsequently, expression of MHC I, PD-1, PD-L1 and PD-L2 was analysed by surface staining and flow cytometry.

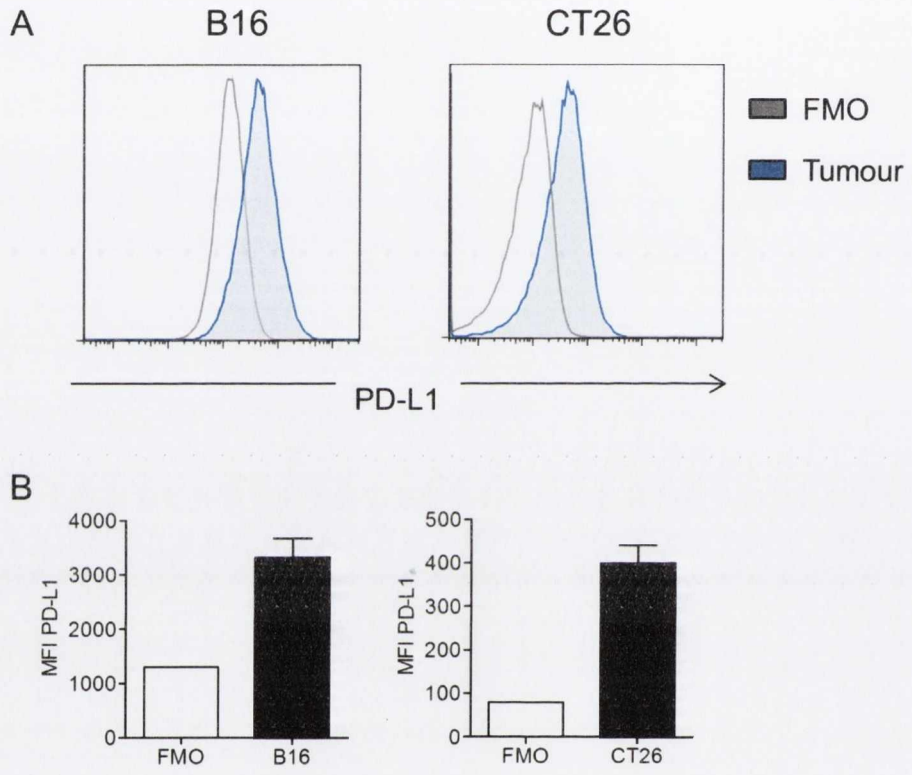


Figure 5.10
PD-L1 is expressed on B16 and CT26 tumour cells *ex vivo*

C57BL/6 or BALB/c mice were injected s.c. with B16 cell ($2x10^5$ cells/mouse) or CT26 cell ($3x10^5$ cells/mouse), respectively (n=7 mice/group). Once tumours reached a diameter of 15 mm, tumours were dissected and PD-L1 expression was analysed by flow cytometry gating on $CD45^-$ cells. Results are shown as representative overlay histograms (A) or median PD-L1 fluorescence intensites (B).

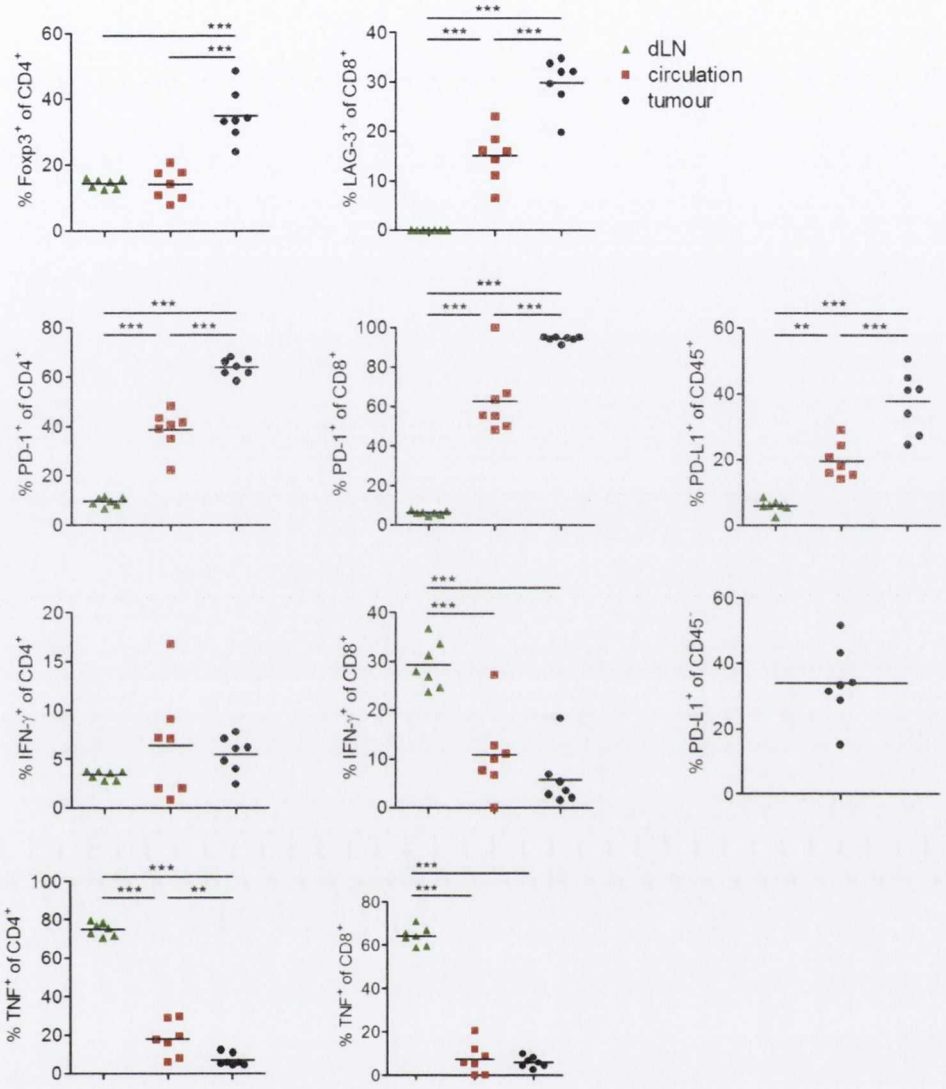


Figure 5.11
Inverse correlation between expression of pro-inflammatory cytokines and inhibitory receptors on tumour infiltrating lymphocytes

BALB/c mice were injected with CT26 tumour cells ($3x10^5$ cells/mouse). On d 23, CD45.2-PE was injected into the tail vains and mice sacrificed after 10 min. Tumours and dLN were isolated and expression of Foxp3, LAG-3, PD-1, IFN-γ, TNF and PD-L1 was analysed by flow cytometry after gating on $CD3^+CD4^+$, $CD3^+CD8^+$, $CD45^+$ or $CD45^-$ cells as indicated. Results show frequencies of cells in dLN, circulating immune cells ($CD45.2^+$ cells in tumour sample) and tumours ($CD45.2^-$ cells in tumour sample). **p<0.01, ***p<0.001 by 1-way ANOVA.

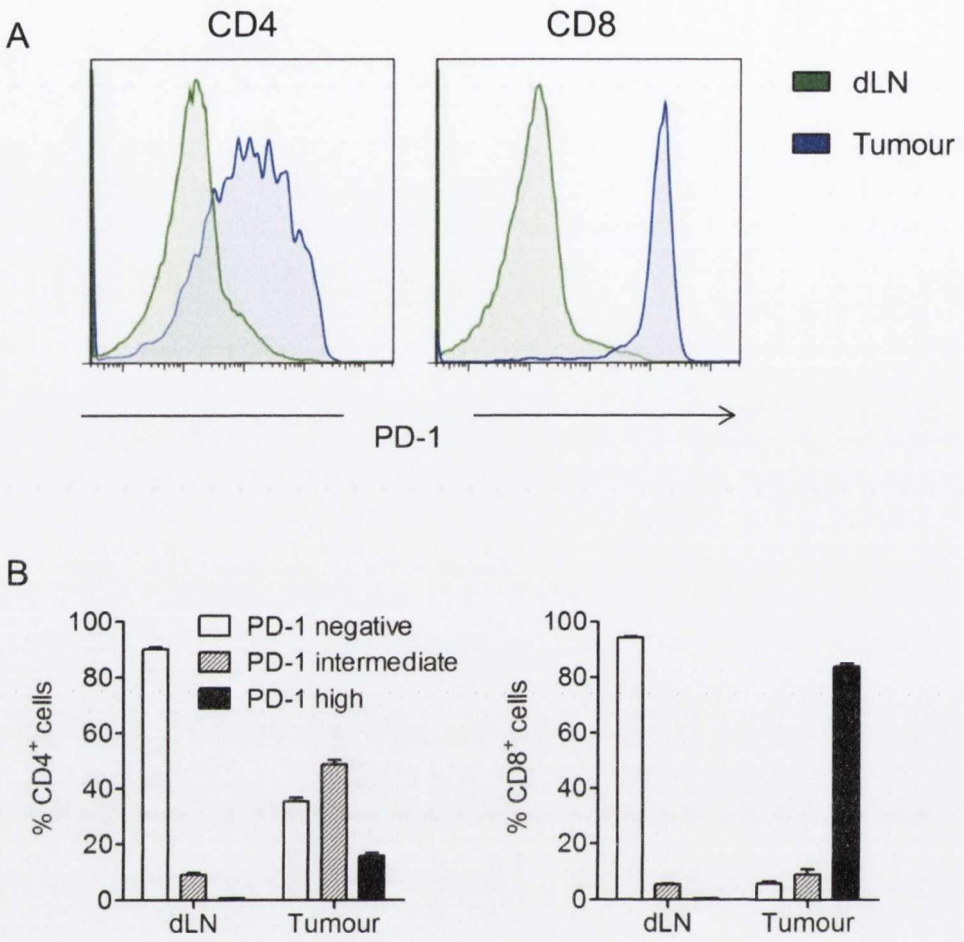


Figure 5.12
High PD-1 expression on CD4 and CD8 T cells in the tumour microenvironment

BALB/c mice were injected with CT26 tumour cells ($3x10^5$ cells/mouse). Tumours and dLN were dissected from CT26 tumour-bearing mice on d 23 and expression of PD-1 was analysed by flow cytometry, gating on $CD3^+CD4^+$ or $CD3^+CD8^+$ cells as indicated. Results are shown as representative histograms (A) or mean values + SEM (B). (n= 7 mice)

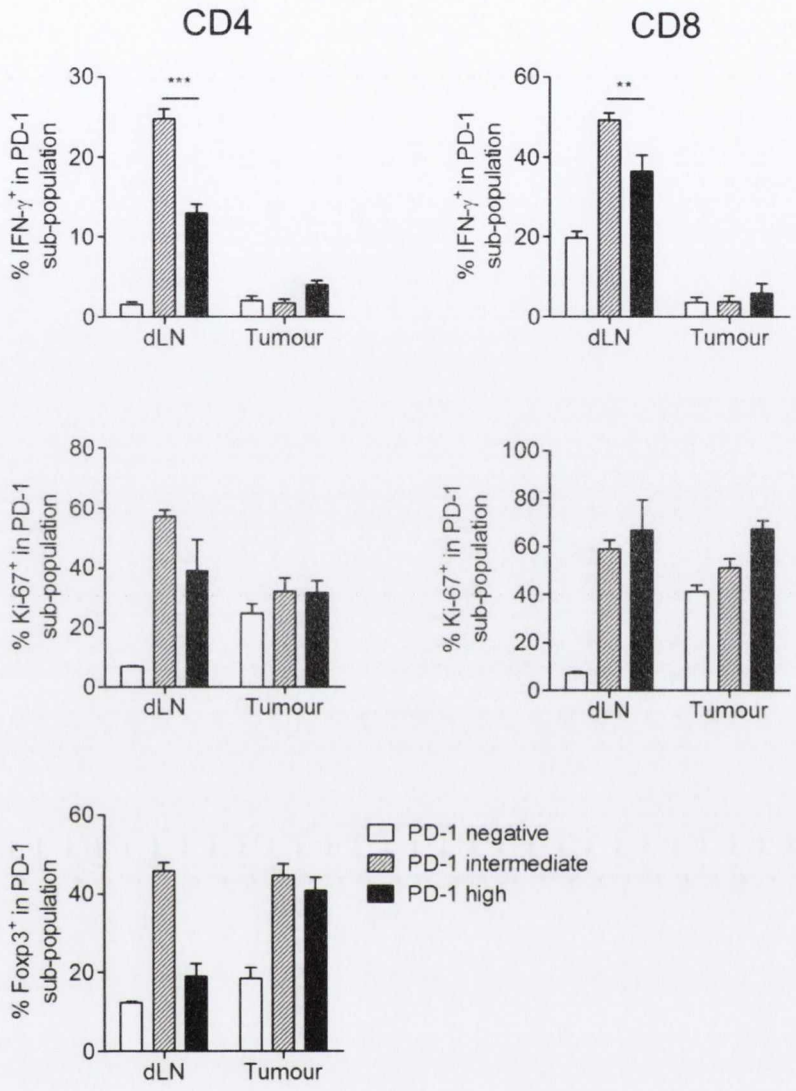


Figure 5.13
A proportion of PD-1 expressing T cells are proliferating and produce IFN-γ

BALB/c mice were injected with CT26 tumour cells ($3x10^5$ cells/mouse). Tumours and dLN were dissected from CT26 tumour-bearing mice on d 23 and expression of IFN-γ, Ki-67 and Foxp3 was analysed by flow cytometry, gating on PD-1 sub-populations of $CD3^+CD4^+$ or $CD3^+CD8^+$ cells as indicated. (n= 7 mice). **p<0.01, ***p<0.001 by 1-way ANOVA.

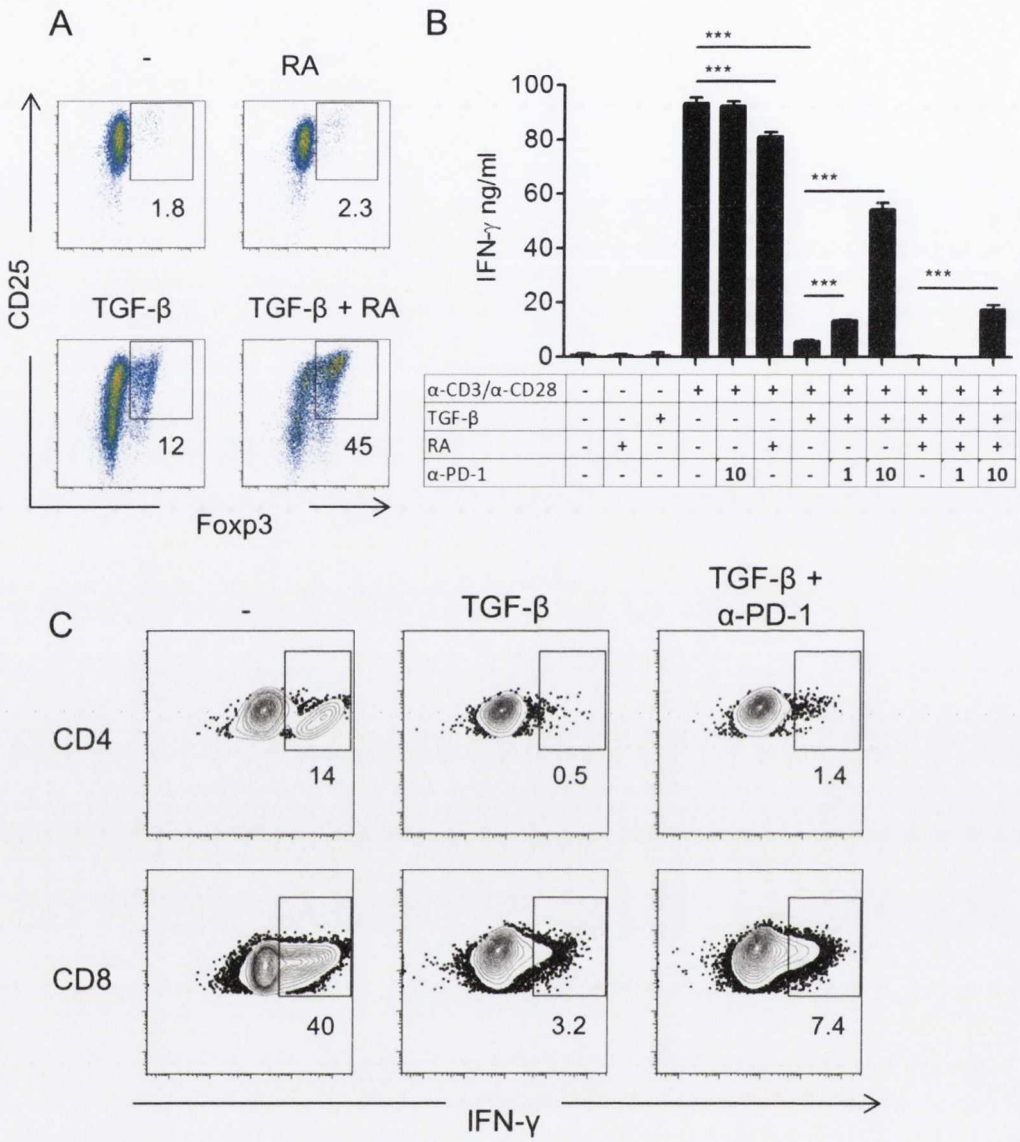


Figure 5.14
Blocking PD-1 augments IFN-γ secretion by TGF-β-stimulated spleen cells

Naïve spleen cells were cultured with the indicated concentrations of plate-bound anti-CD3 (1 µg/ml) and anti-CD28 (3 µg/ml), TGF-β (5 ng/ml), RA (100 nM) or anti-PD-1 (1-10 µg/ml) for 3 d. The expression of Foxp3 was analysed by intranuclear staining and flow cytometry, gating on $CD3^+CD4^+$ cells. Results are shown as representative FACS plots (A). IFN-γ concentrations in culture supernatants were quantified by ELISA. Results are shown as mean ± SD (n=3) (B). After 3 d, cells were stimulated with PMA/Ionomycin/BFA and intracellular expression of IFN-γ analysed by flow cytometry, gating on $CD3^+CD4^+$ or $CD3^+CD8^+$ cells. Results are shown as representative FACS plots from 2 independent experiments (C). ***$p<0.001$ by 1-way ANOVA.

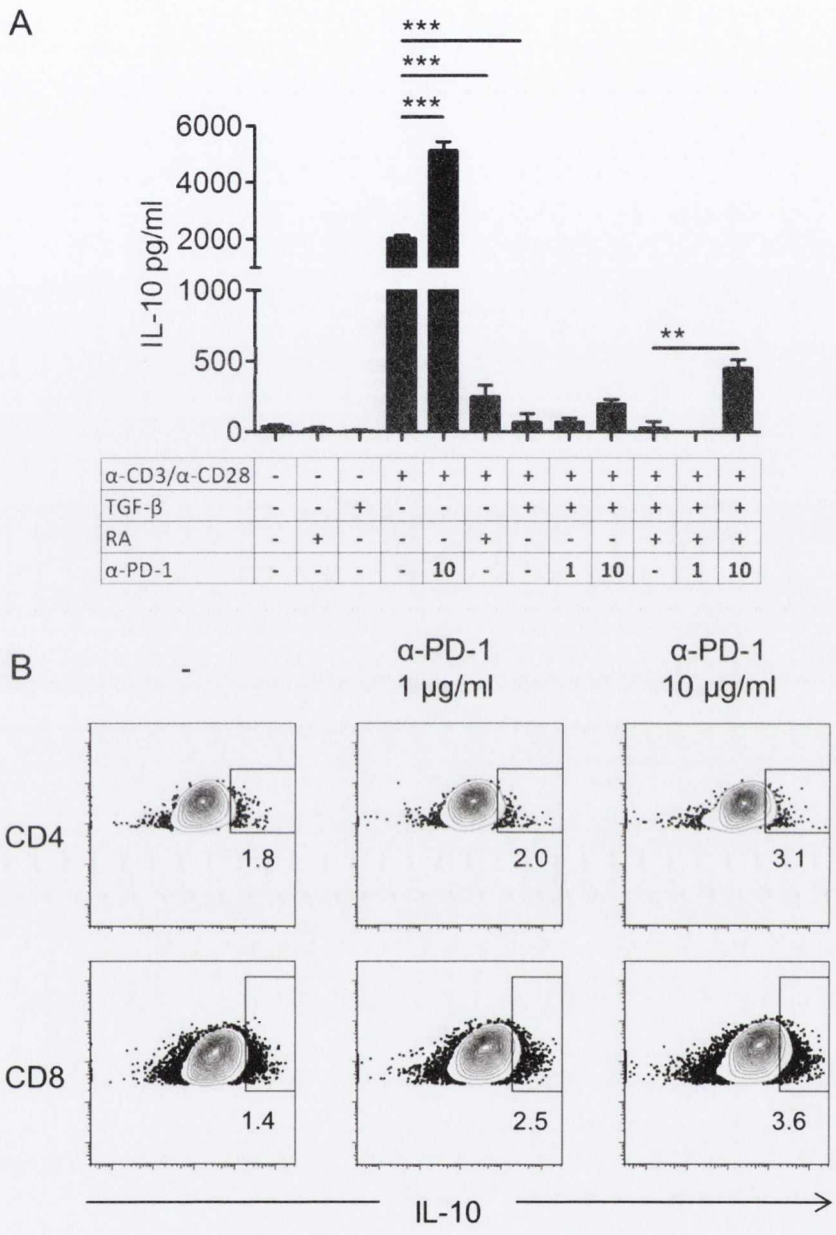


Figure 5.15
Blocking PD-1 increases IL-10 production by T cells in spleen cell cultures

Naïve spleen cells were cultured with the indicated concentrations of plate-bound anti-CD3 (1 µg/ml) and anti-CD28 (3 µg/ml), TGF-β (5 ng/ml), RA (100 nM) or anti-PD-1 (1-10 µg/ml) for 3 d. IL-10 concentrations in the culture supernatants were quantified by ELISA. Results are shown as mean ± SD (n=3) (A). After 3 d, cells were stimulated with PMA/Ionomycin/BFA and intracellular expression of IL-10 analysed by flow cytometry, gating on $CD3^+CD4^+$ or $CD3^+CD8^+$ cells. Results are shown as representative FACS plots from 2 independent experiments (B). **p<0.01, ***p<0.001 by 1-way ANOVA.

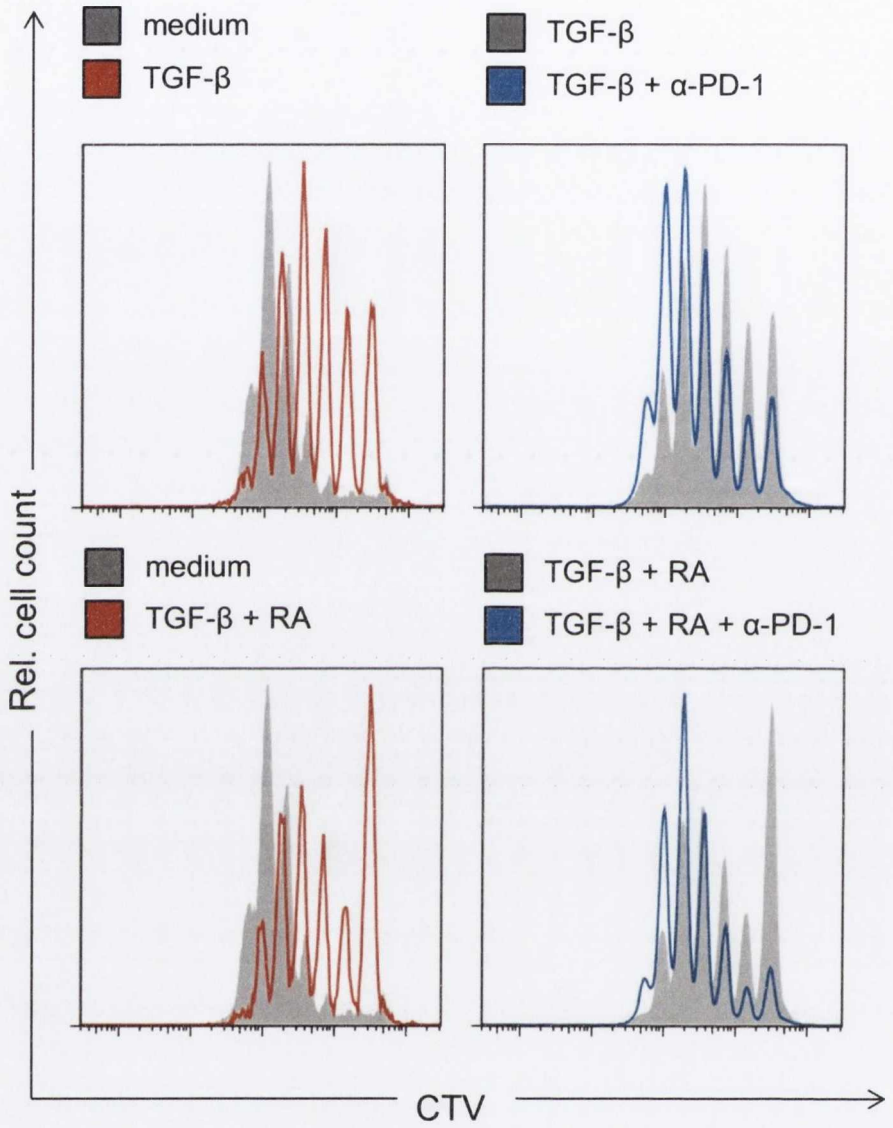


Figure 5.16
Blocking PD-1 reverses TGF-β and RA-induced inhibition of CD8 T cell proliferation

Naïve spleen cells were stained with CTV and cultured with plate-bound anti-CD3 (1 µg/ml) and anti-CD28 (3 µg/ml), TGF-β (5 ng/ml), RA (100 nM) or anti-PD-1 (10 µg/ml) for 3 d. Cell proliferation was measured by CTV fluorescence intensity and analysed by flow cytometry, gating on $CD3^+CD8^+$ cells. Results are shown as representative histograms from 2 independent experiments.

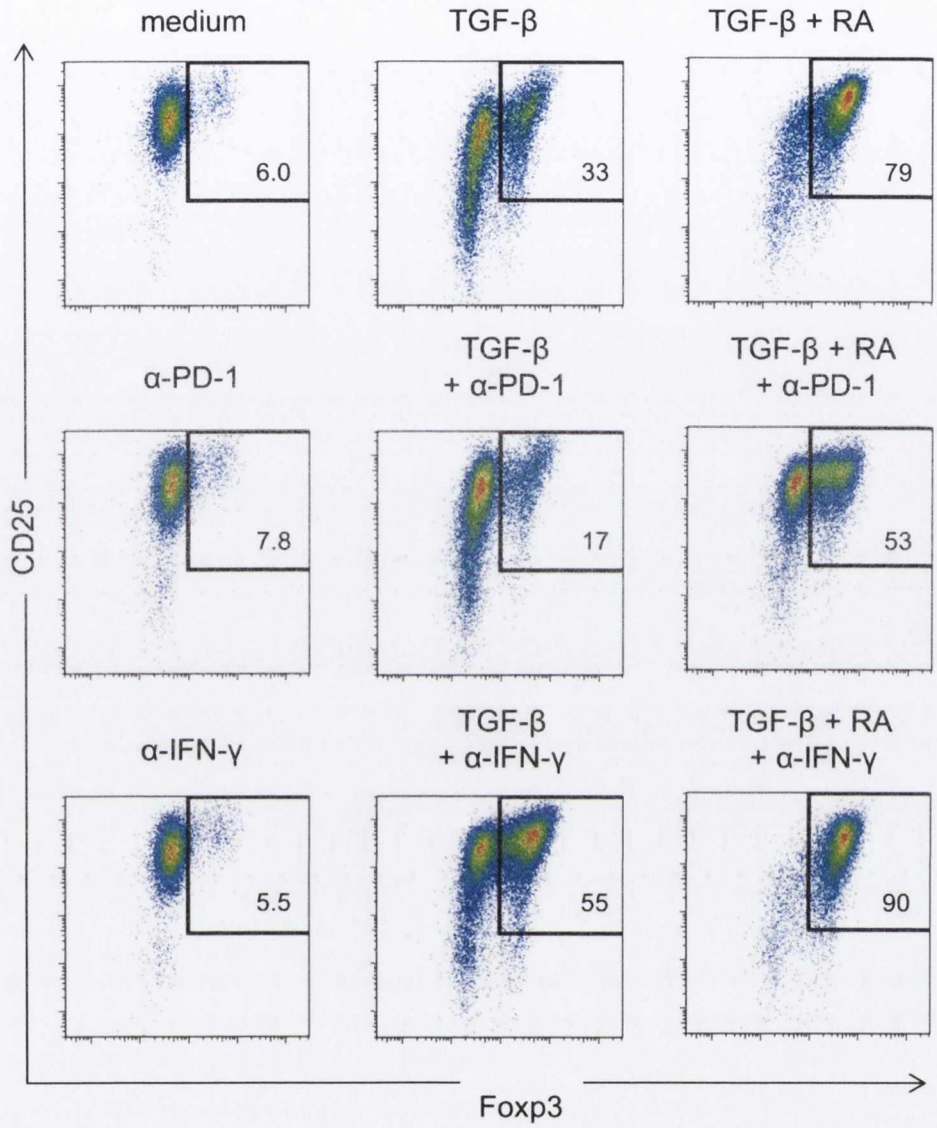


Figure 5.17
Blocking PD-1 reduces Treg conversion in TGF-β stimulated spleen cell cultures

Naïve spleen cells were cultured in presence of plate-bound anti-CD3 (1 µg/ml), anti-CD28 (3 µg/ml), TGF-β (5 ng/ml), RA (100 nM), anti-PD-1 (10 µg/ml) and anti-IFN-γ (10 µg/ml) as indicated for 3 d. Foxp3 expression was analysed by flow cytometry, gating on $CD3^+CD4^+$ cells. Results are shown as representative FACS plots from 3 independent experiments.

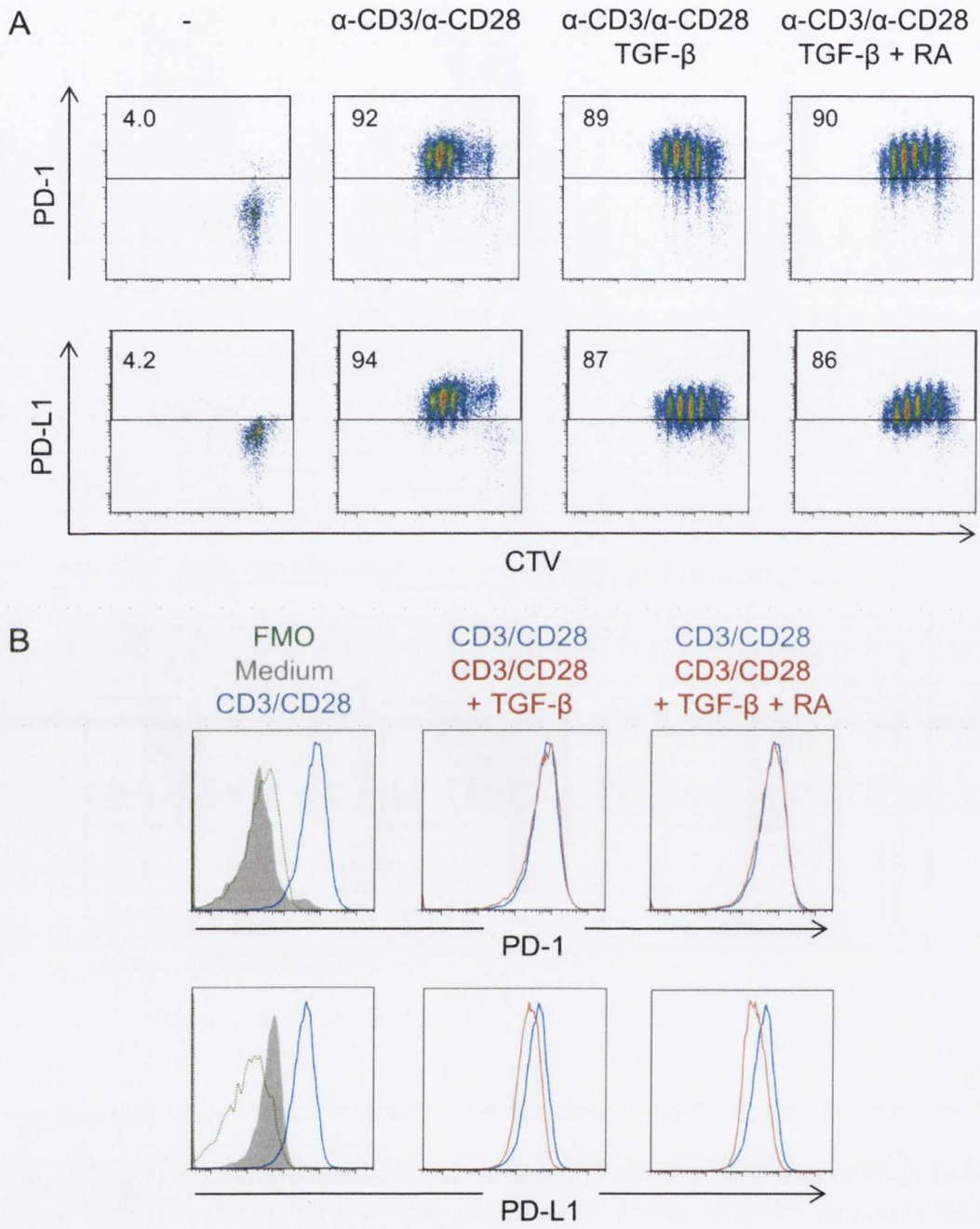


Figure 5.18
TGF-β does not alter the expression of PD-1 or PD-L1 on proliferating CD4 T cells

Naïve spleen cells were cultured with the indicated concentrations of plate-bound anti-CD3 (1 µg/ml) and anti-CD28 (3 µg/ml), TGF-β (5 ng/ml) and RA (100 nM) for 3 d. Surface expression of PD-1 and PD-L1 was analysed by flow cytometry, gating on $CD3^+CD4^+$ cells. Results are shown as representative FACS plots (A) and overlay histograms (B) from 3 independent experiments.

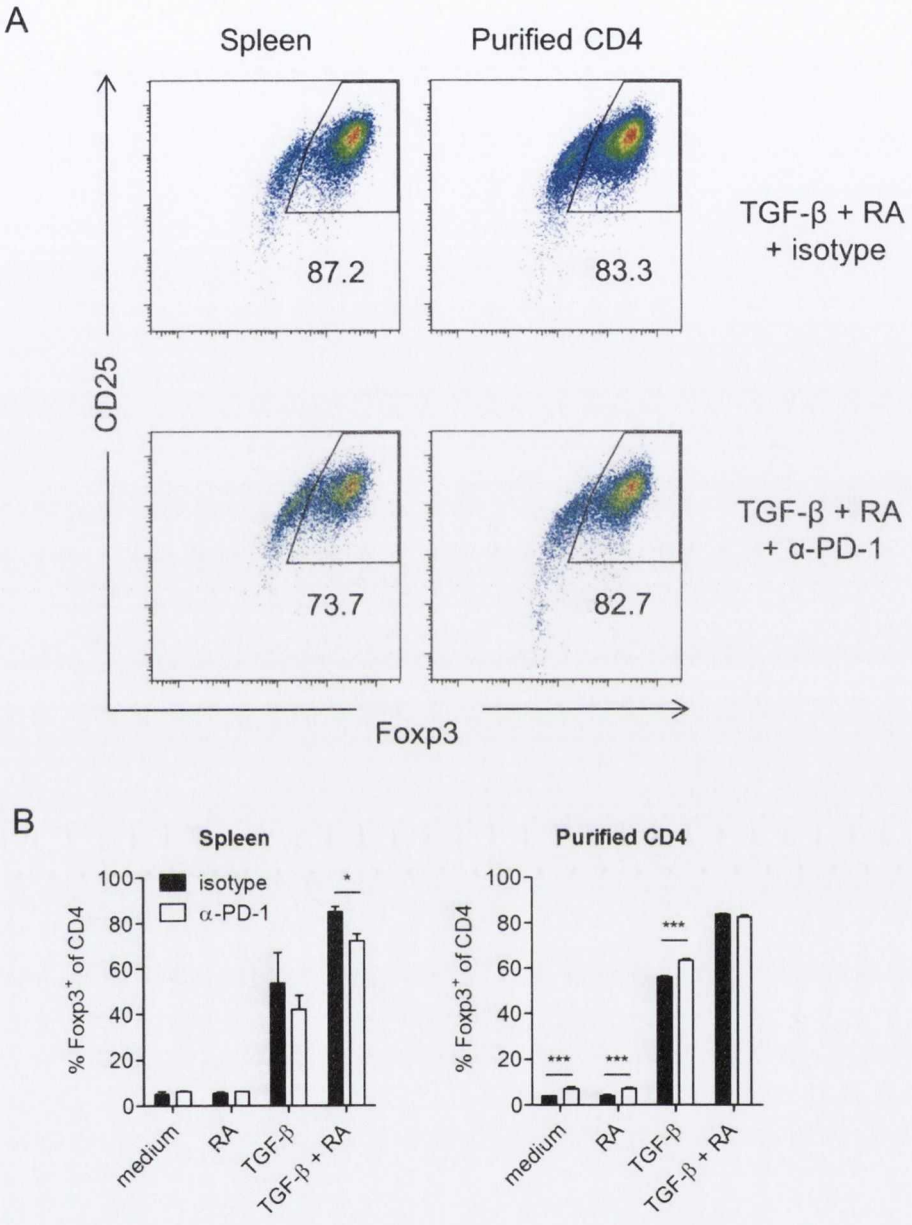


Figure 5.19
Blocking PD-1 does not directly inhibit Treg conversion in purified CD4 T cells

Naïve spleen cells or MACS-purified CD4 T cells were cultured with the indicated concentrations of plate-bound anti-CD3 (1 µg/ml), anti-CD28 (3 µg/ml), TGF-β (5 ng/ml), RA (100 nM) and anti-PD-1 (10 µg/ml) or isotype control (10 µg/ml) as indicated for 3 d. Foxp3 expression was analysed by flow cytometry, gating on $CD3^+CD4^+$ cells. Results are shown as representative FACS plots (A) or mean values + SD from triplicate samples (B). *p<0.05, ***p<0.001 by 2-way ANOVA

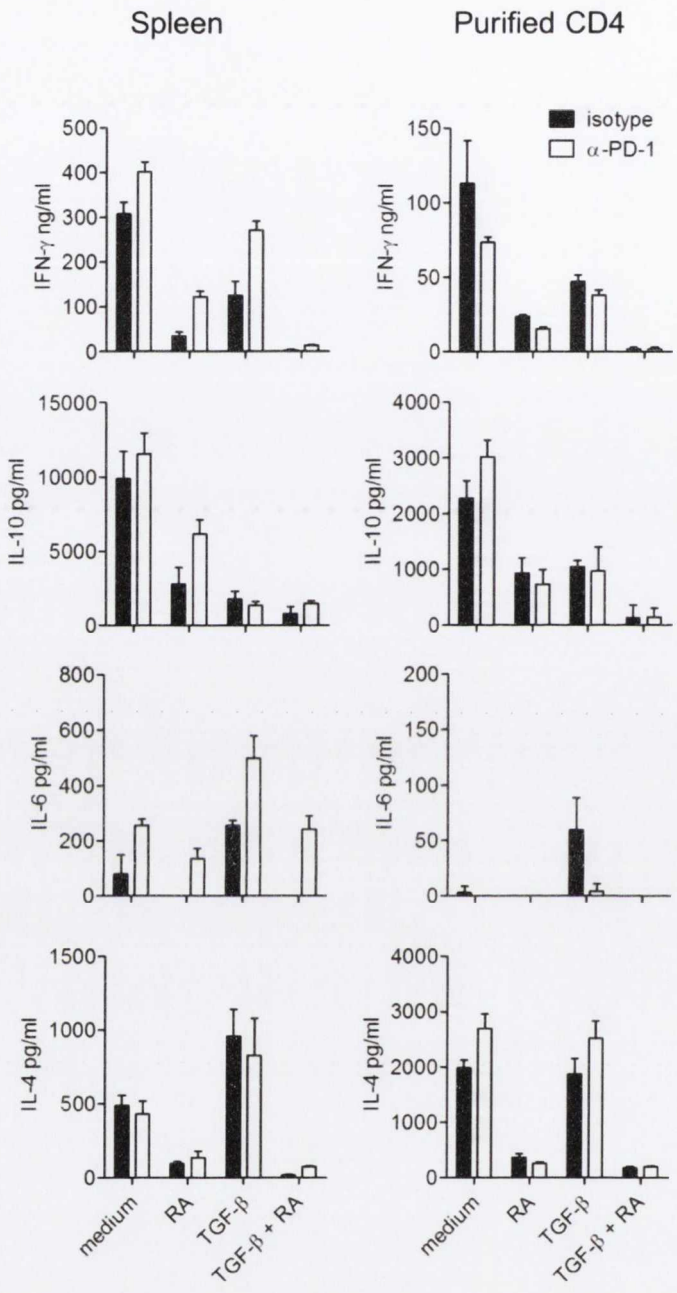


Figure 5.20
Blocking PD-1 enhances IFN-γ, IL-10 and IL-6 secretion by spleen cells but not purified CD4 T cells

Naïve spleen cells or MACS-purified CD4 T cells were cultured with the indicated concentrations of plate-bound anti-CD3 (1 µg/ml), anti-CD28 (3 µg/ml), TGF-β (5 ng/ml), RA (100 nM) and anti-PD-1 (10 µg/ml) or isotype control (10 µg/ml) as indicated for 3 d. Supernatants were collected and secreted cytokines quantified by ELISA. Results are shown as mean values + SD from triplicate samples.

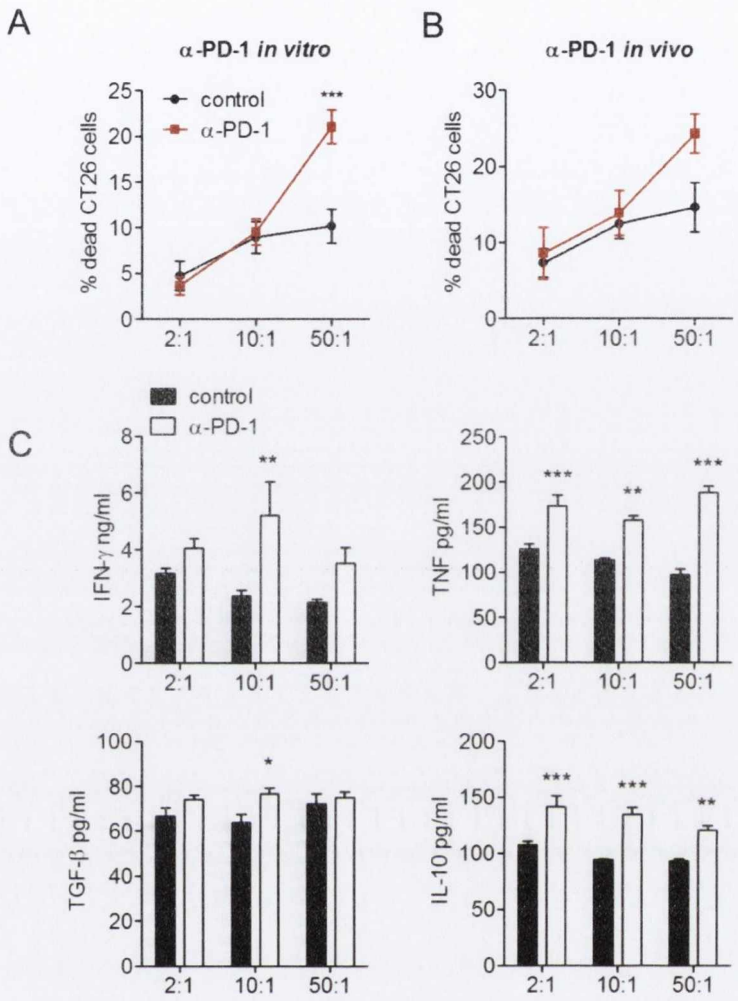


Figure 5.21
Blocking PD-1 enhances anti-tumour cytotoxicity *ex vivo*

Spleen cells from CT26 tumour-bearing mice were co-cultured with CTV labelled CT26 tumour cells at the indicated E:T ratios for 24 h. Anti-PD-1 (10 µg/ml) was added to the co-cultures *in vitro* as indicated (A,C). CT26 tumour-bearing mice were injected i.p. with anti-PD-1 (200 µg/mouse) on d 3, 10 and 17 post tumour induction (B). Cell death was measured by 7-AAD staining and flow cytometry (A,B). Cytokines in the co-culture supernatants from (A) were quantified by ELISA (C).*p<0.05, **p<0.01, ***p<0.001 by 2-way ANOVA (n= 4-7 mice per group). Data are representative of 2 experiments.

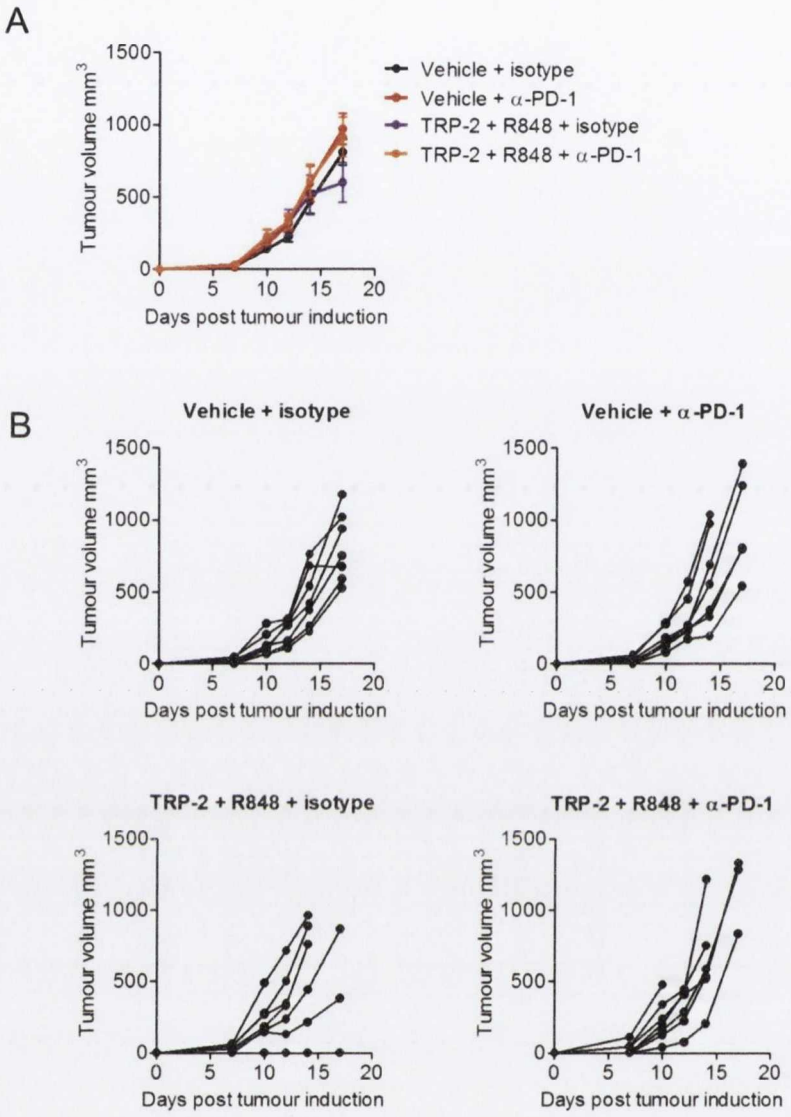


Figure 5.22
Blocking PD-1 does not improve the anti-tumour efficacy of a prophylactic peptide vaccine in the B16 tumour model

C57BL/6 mice were immunized s.c. with vehicle or TRP-$2_{180\text{-}188}$ (100 µg/mouse) and R848 (40 µg/mouse) 5 weeks and 1 week prior to tumour induction with B16 tumour cells ($2x10^5$ cells/mouse). Isotype control or anti-PD-1 (200 µg/mouse) were injected i.p. on d 3, 10 and 17 post tumour induction. Results are shown as mean tumour growth ± SEM (A) or tumour growth curves of individual mice (B). n=7 mice per group

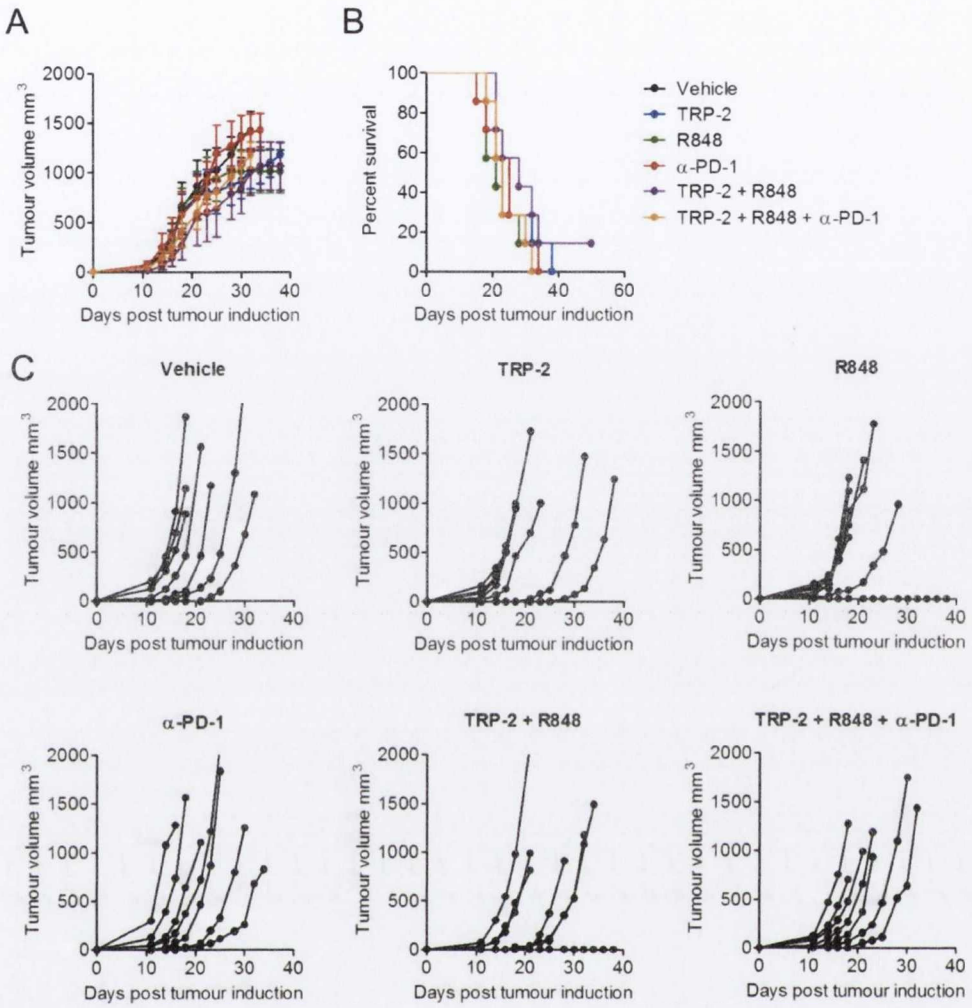


Figure 5.23
Blocking PD-1 does not improve the anti-tumour efficacy of a therapeutic peptide vaccine given i.p. in the B16 tumour model

C57BL/6 mice were injected with B16 tumour cells ($2x10^5$ cells/mouse) and immunized i.p. with vehicle or TRP-$2_{180-188}$ (100 µg/mouse), R848 (40 µg/mouse) or both on d 3, 10 and 17 post tumour induction. Anti-PD-1 (200 µg/mouse, i.p.) was injected one day prior to immunization. Results are shown as mean tumour growth ± SEM (A) survival graphs (B) or tumour growth curves of individual mice (C). n=7 mice per group

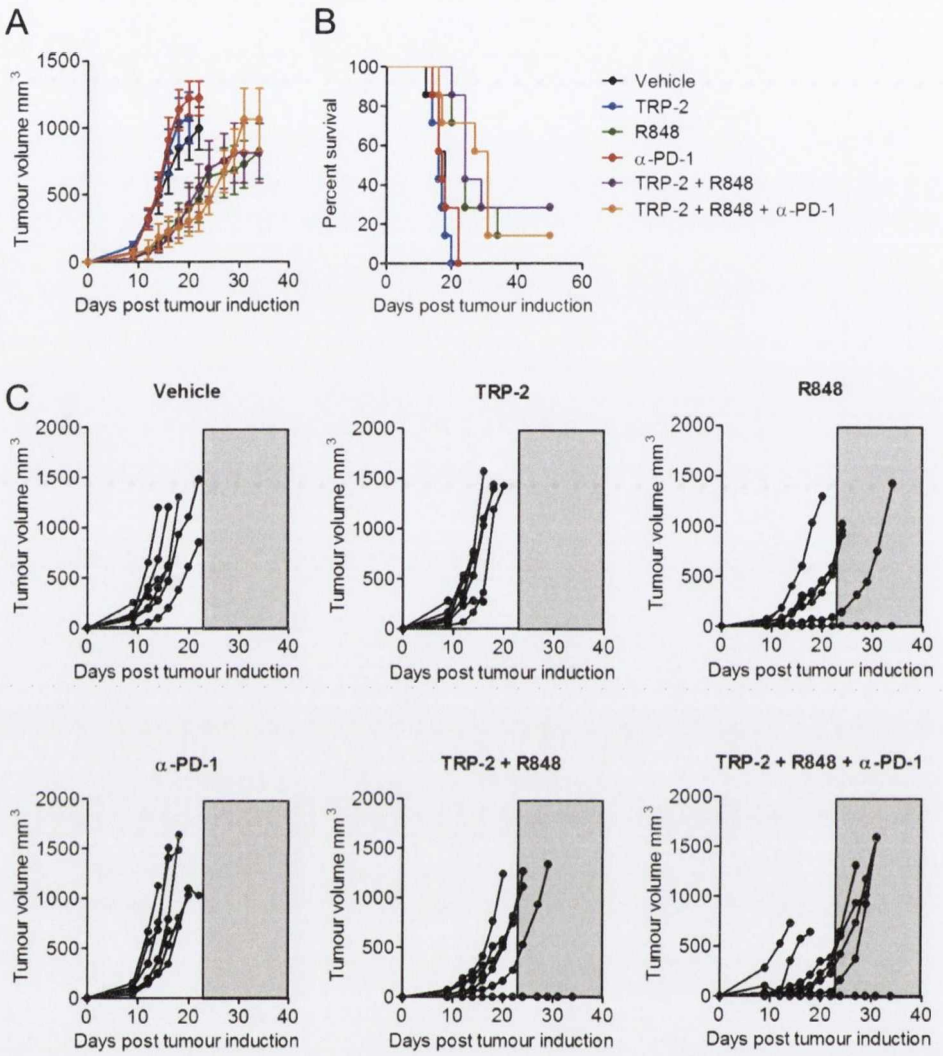


Figure 5.24
Blocking PD-1 does not improve the anti-tumour efficacy of a therapeutic peptide vaccine given s.c. in the B16 tumour model

C57BL/6 mice were injected with B16 tumour cells ($2x10^5$ cells/mouse) and immunized s.c. with vehicle or TRP-$2_{180-188}$ (100 µg/mouse), R848 (40 µg/mouse) or both on d 3, 10 and 17 post tumour induction. Anti-PD-1 (200 µg/mouse, i.p.) was injected one day prior to immunization. Results are shown as mean tumour growth ± SEM (A) survival graphs (B) or tumour growth curves of individual mice (C). n=7 mice per group

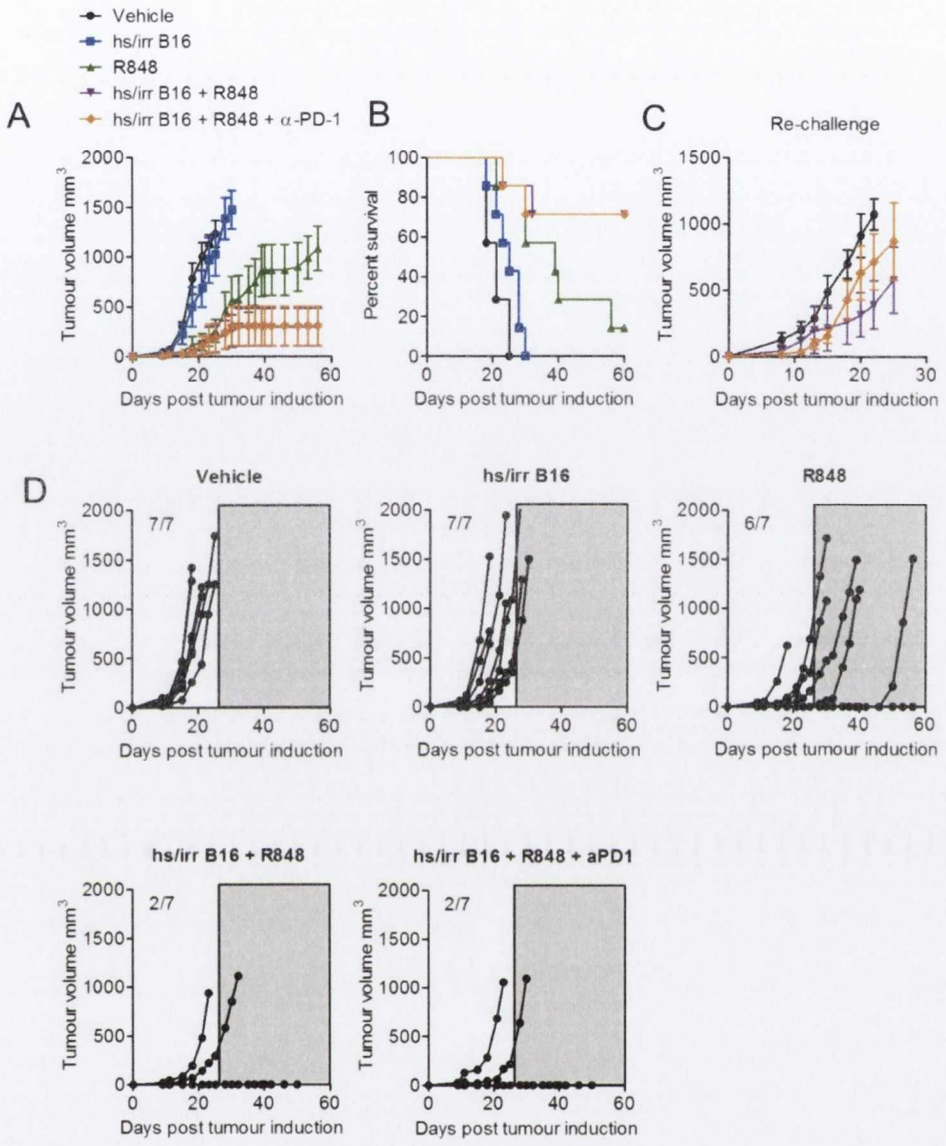


Figure 5.25
Blocking PD-1 does not improve the efficacy of a therapeutic whole cell vaccine in the B16 tumour model

C57BL/6 mice were injected with B16 tumour cells ($2x10^5$ cells/mouse) and immunized s.c. with hs/irr B16 tumour cells ($1x10^6$ cells/mouse), R848 (40 µg/mouse, s.c.) or both on d 3, 10 and 17 post tumour induction. Anti-PD-1 (200 µg/mouse, i.p.) was injected one day prior to immunization. Results are shown as mean tumour growth ± SEM (A) survival graphs (B) or tumour growth curves of individual mice (C). n=7 mice per group

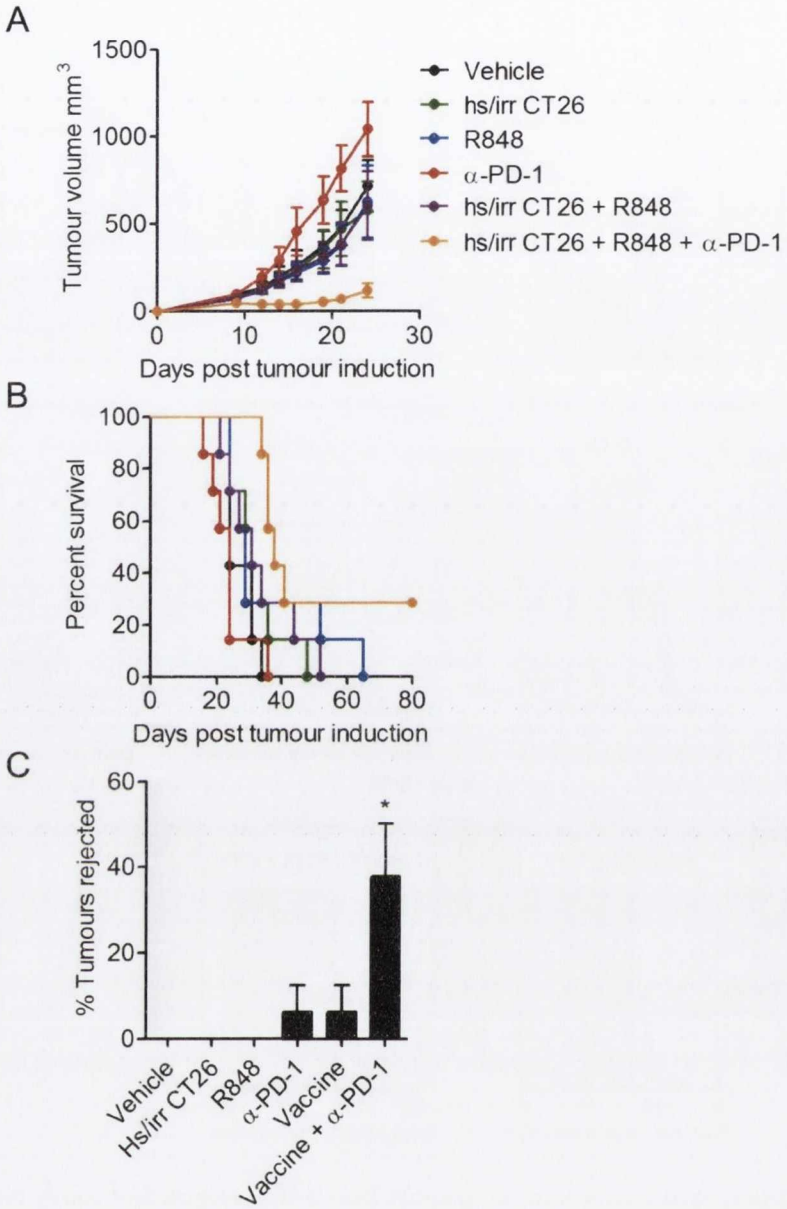


Figure 5.26
Blocking PD-1 improves the anti-tumour efficacy of a therapeutic hs/irr cell vaccine in the CT26 tumour model

BALB/c mice were injected with CT26 tumour cells ($3x10^5$ cells/mouse) and immunized s.c. with hs/irr CT26 tumour cells ($1x10^6$ cells/mouse), R848 (40 µg/mouse, s.c.) or both on d 3, 10 and 17 post tumour induction. Anti-PD-1 (200 µg/mouse, i.p.) was injected one day prior to immunization. Results are shown as mean tumour growth ± SEM (A), survival graphs (C) and percentage of mice that rejected tumours by d 23 (C). *$p<0.05$ by 1-way ANOVA

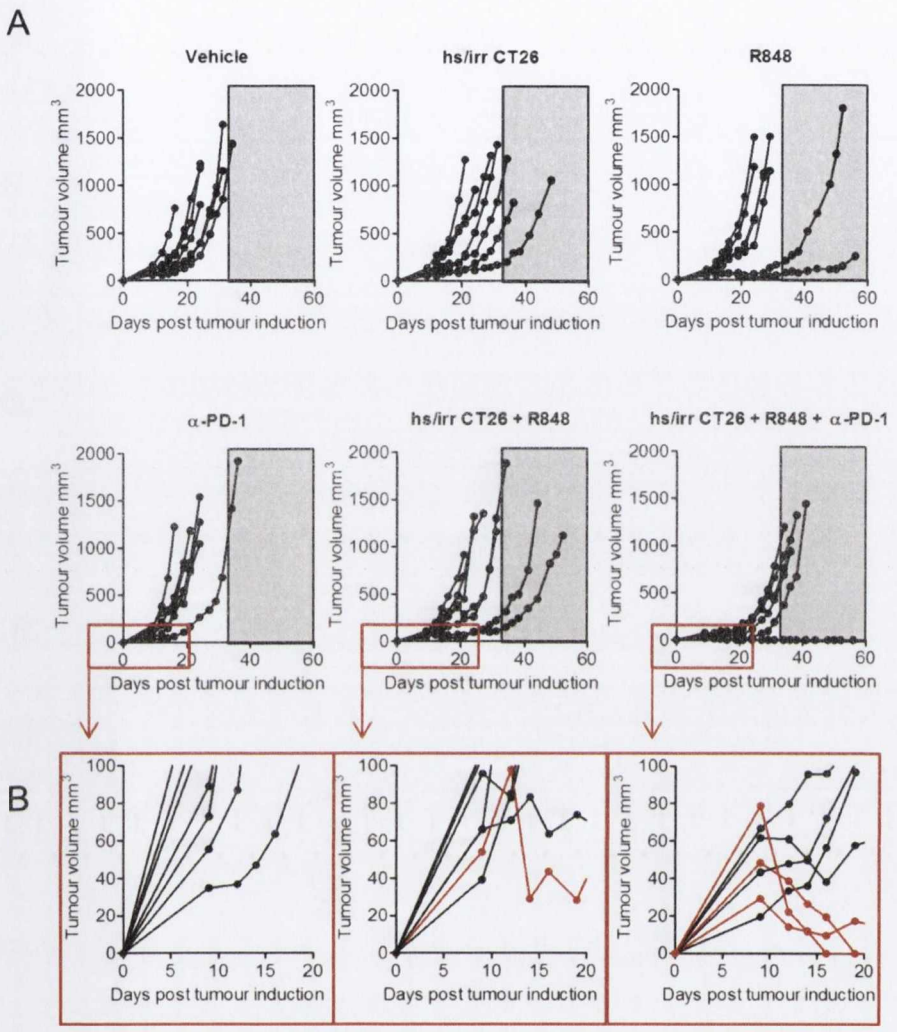


Figure 5.27
Therapeutic immunization with a tumour cell vaccine and anti-PD-1 induces clearance of established tumours

BALB/c mice were injected with CT26 tumour cells ($3x10^5$ cells/mouse) and immunized s.c. with hs/irr CT26 tumour cells ($1x10^6$ cells/mouse), R848 (40 µg/mouse, s.c.) or both on d 3, 10 and 17 post tumour induction. Anti-PD-1 (200 µg/mouse, i.p.) was injected one day prior to immunization. Results are shown as tumour growth curves of individual mice (A) and zoom into early time points of tumour growth (red lines indicate mice that showed a marked reduction in tumour size) (B).

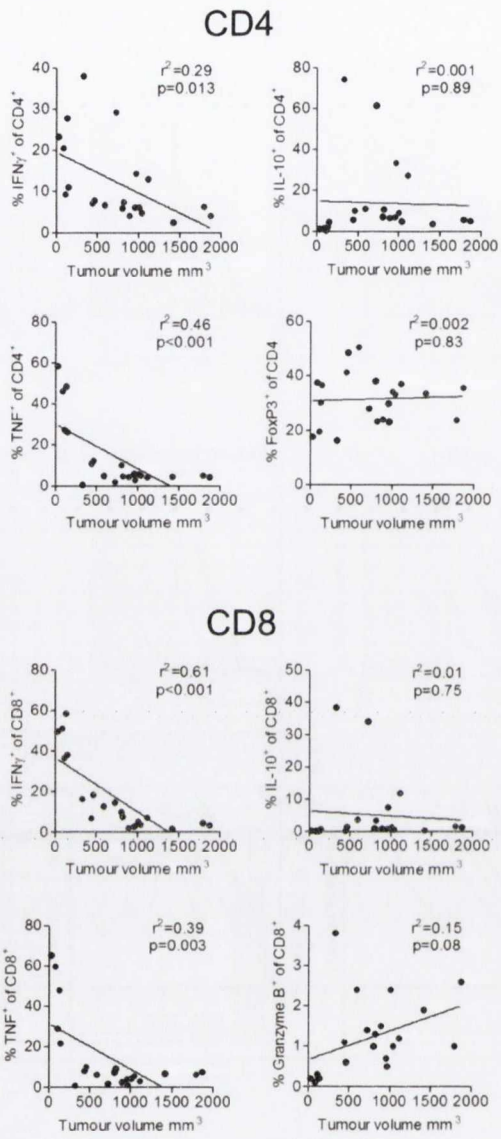


Figure 5.28
Inverse correlation between tumour infiltrating IFN-γ and TNF-producing T cells and tumour size in immunized mice

BALB/c mice were injected with CT26 tumour cells ($3x10^5$ cells/mouse) and injected with hs/irr CT26 tumour cells ($1x10^6$ cells/mouse, s.c.), R848 (40 µg/mouse, s.c.) and anti-PD-1 (200 µg/mouse, i.p.) as indicated on d 3, 10 and 17 post tumour induction. Tumours were isolated on d 23 post tumour induction and IFN-γ, TNF, IL-10 and Foxp3 expression analysed by flow cytometry. Results are shown as scatter dot plots of all groups combined + linear regression trend lines.

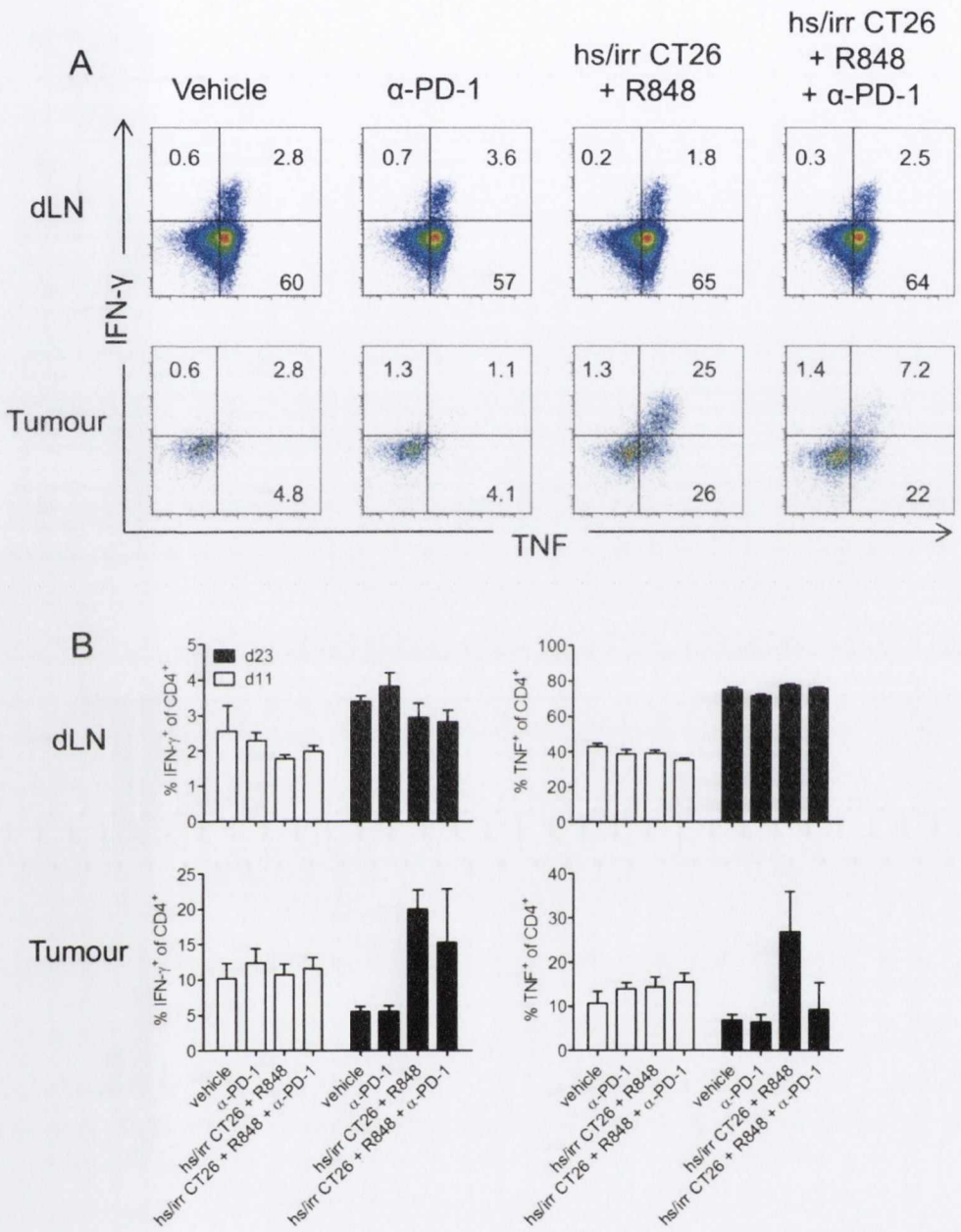


Figure 5.29
The combination of a tumour vaccine with a PD-1 blocking antibody enhances the secretion of IFN-γ and TNF by CD4 T cells

BALB/c mice were injected with CT26 tumour cells ($3x10^5$ cells/mouse) and injected with hs/irr CT26 tumour cells ($1x10^6$ cells/mouse, s.c.), R848 (40 µg/mouse, s.c.) and anti-PD-1 (200 µg/mouse, i.p.) as indicated on d 3, 10 and 17 post tumour induction. Tumours and dLN were isolated on d 11 and d 23 post tumour induction and IFN-γ and TNF expression was analysed by intracellular cytokine staining and flow cytometry, gating on $CD45^+CD3^+CD4^+$ cells. Results are shown as representative FACS plots from d 23 (A) or mean values ± SEM (B). n=4-7 mice per group.

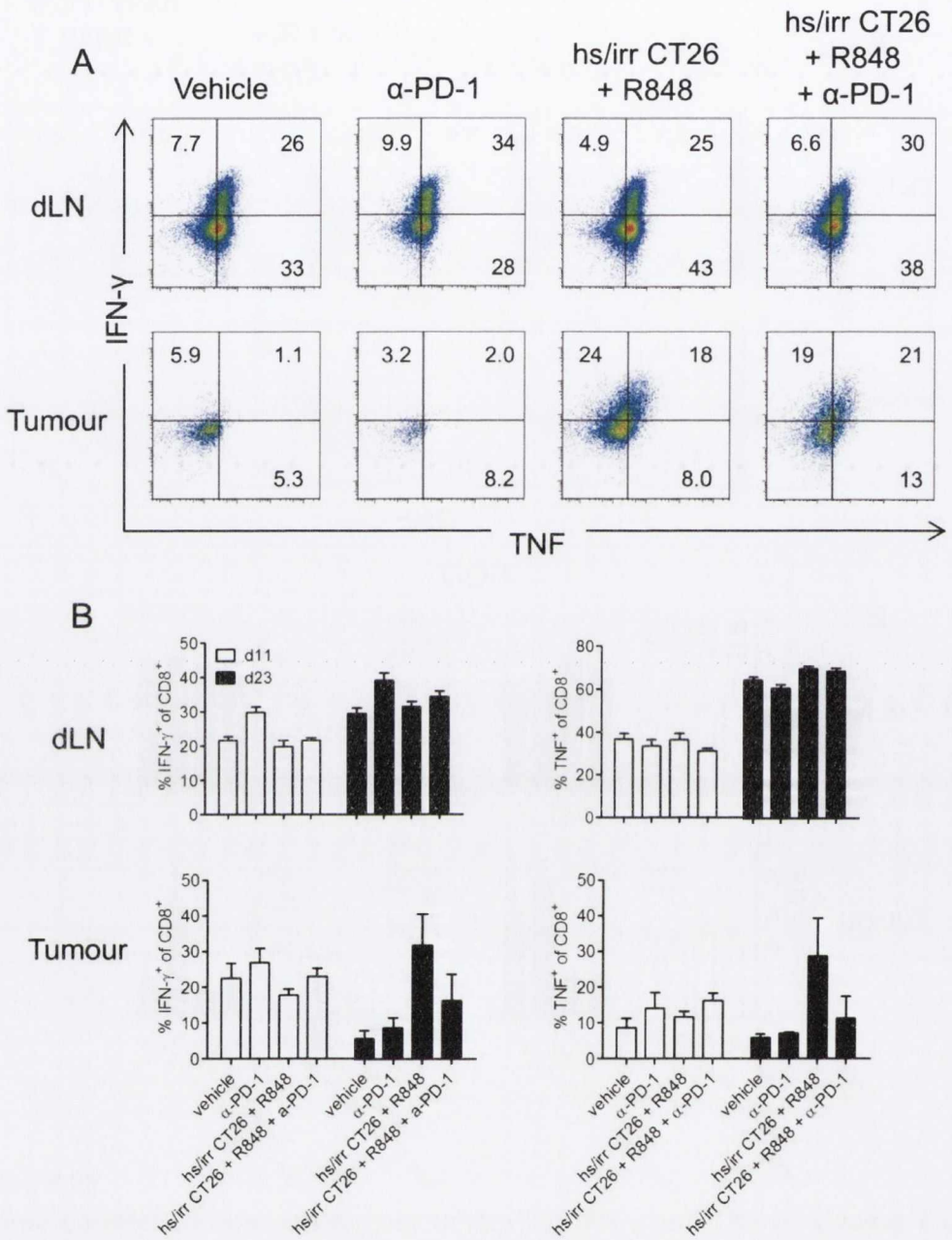


Figure 5.30
The combination of a tumour vaccine with a PD-1 blocking antibody enhances the secretion of IFN-γ and TNF by CD8 T cells

BALB/c mice were injected with CT26 tumour cells ($3x10^5$ cells/mouse) and injected with hs/irr CT26 tumour cells ($1x10^6$ cells/mouse, s.c.), R848 (40 µg/mouse, s.c.) and anti-PD-1 (200 µg/mouse, i.p.) as indicated on d 3, 10 and 17 post tumour induction. Tumours and dLN were isolated on d 11 and d 23 post tumour induction and IFN-γ and TNF expression was analysed by intracellular cytokine staining and flow cytometry, gating on $CD45^+CD3^+CD8^+$ cells. Results are shown as representative FACS plots from d 23 (A) or mean values ± SEM (B). n=4-7 mice per group.

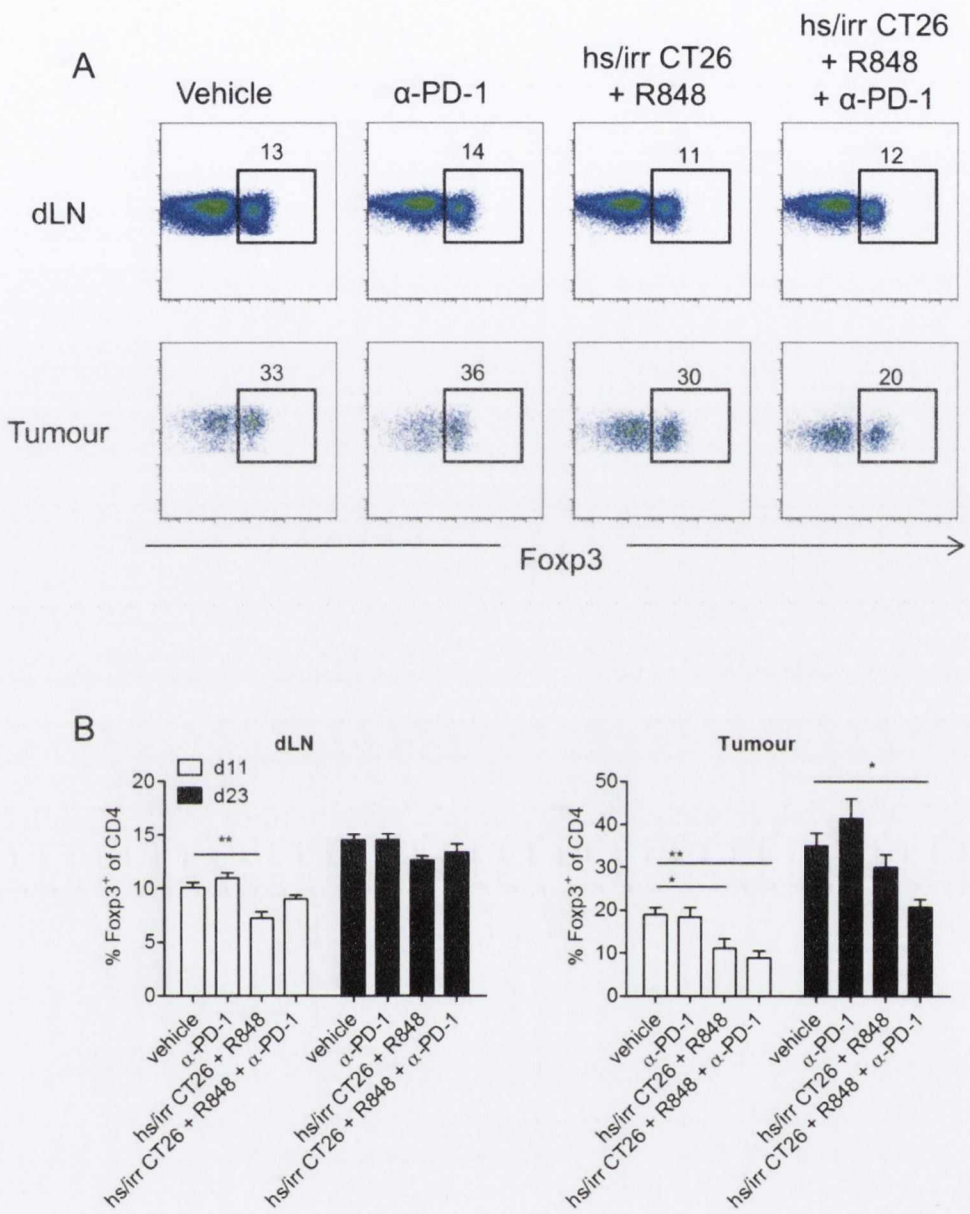


Figure 5.31
The combination of a tumour vaccine with a PD-1 blocking antibody significantly reduces Treg cells within the tumour

BALB/c mice were injected with CT26 tumour cells ($3x10^5$ cells/mouse) and injected with hs/irr CT26 tumour cells ($1x10^6$ cells/mouse, s.c.), R848 (40 µg/mouse, s.c.) and anti-PD-1 (200 µg/mouse, i.p.) as indicated on d 3, 10 and 17 post tumour induction. Tumours and dLN were isolated on d 11 and d 23 post tumour induction and Foxp3 expression analysed by intranuclear staining and flow cytometry, gating on $CD45^+CD3^+CD4^+$ cells. Results are shown as representative FACS plots from d 23 (A) and mean values + SEM (B). n=3-8 mice per group. *p<0.05, **p<0.01, ***p<0.001 by 1-way ANOVA.

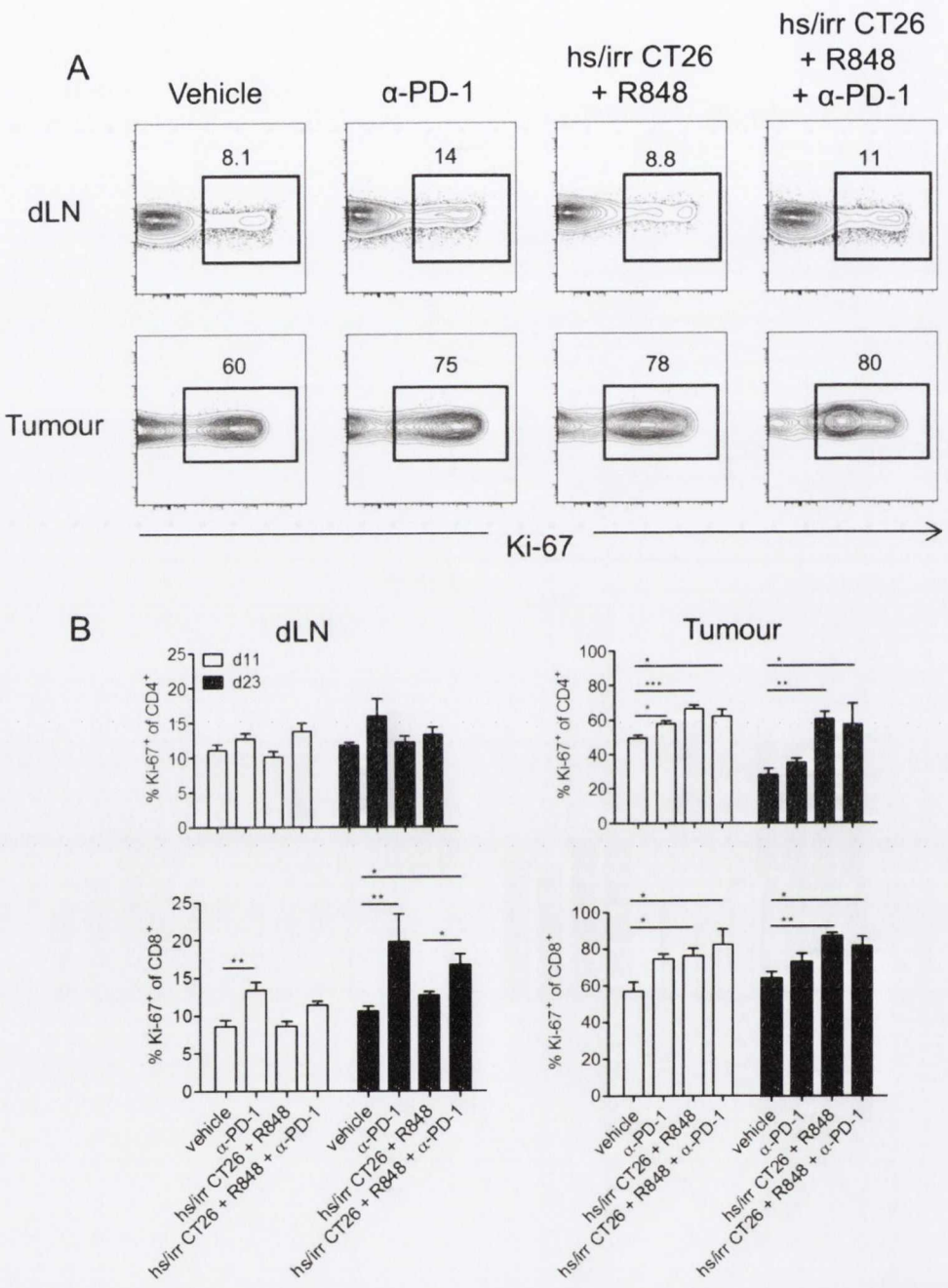


Figure 5.32
The combination of a tumour vaccine with a PD-1 blocking antibody enhances T cell proliferation *in vivo*

BALB/c mice were injected with CT26 tumour cells ($3x10^5$ cells/mouse) and injected with hs/irr CT26 tumour cells ($1x10^6$ cells/mouse, s.c.), R848 (40 µg/mouse, s.c.) and anti-PD-1 (200 µg/mouse, i.p.) as indicated on d 3, 10 and 17 post tumour induction. Tumours and dLN were isolated on d 11 and d 23 post tumour induction and Ki-67 expression analysed by flow cytometry, gating on $CD3^+CD4^+$ or $CD3^+CD8^+$ cells. Results are shown as representative FACS plots for CD8 T cells on d 23 (A) and mean values + SEM (B). n=3-8 mice per group. *p<0.05, **p<0.01, ***p<0.001 by 1-way ANOVA.

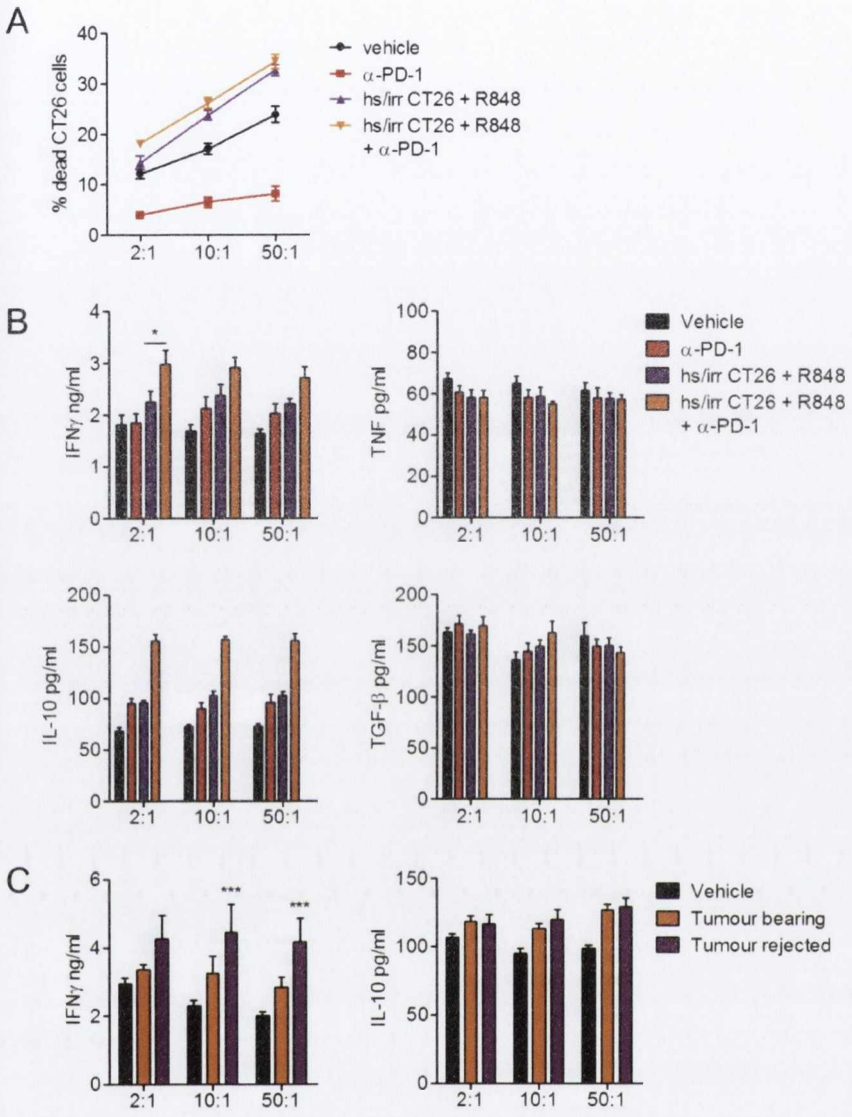


Figure 5.33
The combination of a tumour vaccine with a PD-1 blocking antibody enhances T cell cytotoxicity and IFN-γ secretion

BALB/c mice were injected with CT26 tumour cells ($3x10^5$ cells/mouse) and injected with hs/irr CT26 tumour cells ($1x10^6$ cells/mouse, s.c.), R848 (40 µg/mouse, s.c.) and anti-PD-1 (200 µg/mouse, i.p.) as indicated on d 3, 10 and 17 post tumour induction.(A,B) Spleens were isolated on d 11 post tumour induction and co-cultured with CTV-stained CT26 cells at the indicated ratios. After 24 h, cell death was measured by flow cytometry (A) and cytokine concentrations in co-culture supernatants quantified by ELISA (B). Spleens from mice injected with vehicle or hs/irr CT26, R848 and anti-PD-1 (tumour bearing + tumour rejected) were isolated on d 23 and co-cultured with CT26 cells at the indicated ratios. After 24 h, supernatants were collected and cytokine concentrations quantified by ELISA (C).

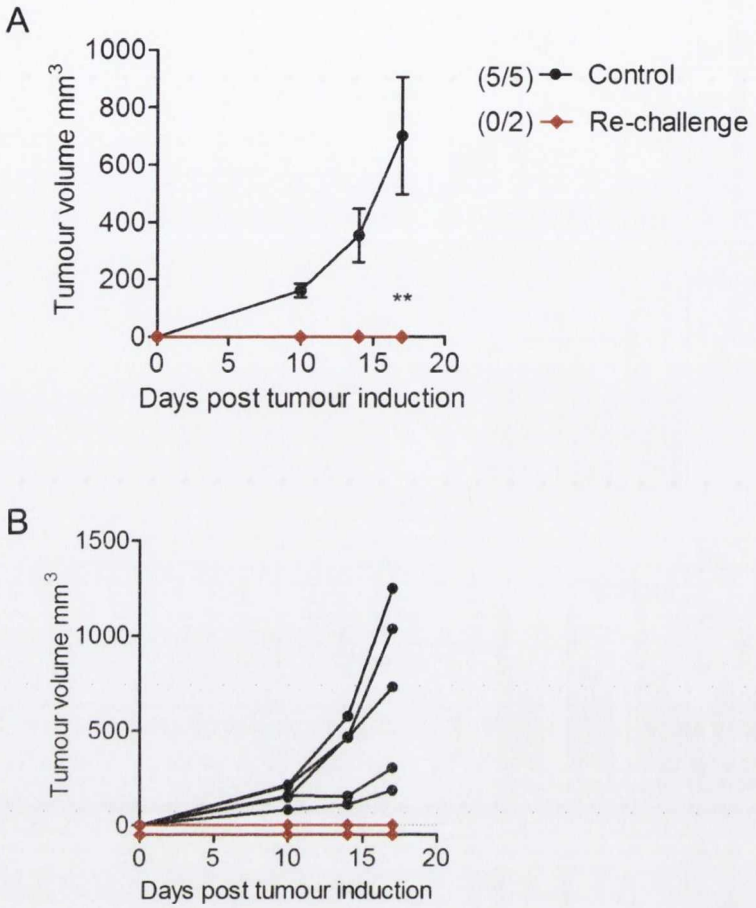


Figure 5.34
Mice that rejected CT26 tumours following treatment with a tumour vaccine and a PD-1 blocking antibody reject tumours following re-challenge

BALB/c mice were injected with CT26 tumour cells ($3x10^5$ cells/mouse) and injected with hs/irr CT26 tumour cells ($1x10^6$ cells/mouse, s.c.), R848 (40 µg/mouse, s.c.) and anti-PD-1 (200 µg/mouse, i.p.). Mice that received hs/irr CT26 + R848 + anti-PD-1 and rejected the tumours were subsequently re-challenged with CT26 tumour cells and compared to naïve mice that received the first tumour challenge. Results are shown as mean tumour growth (A) or growth curves for individual mice (B). Numbers in brackets indicate frequency of tumour-bearing mice. n=2-5 mice per group. **$p<0.01$ by 2-way ANOVA.

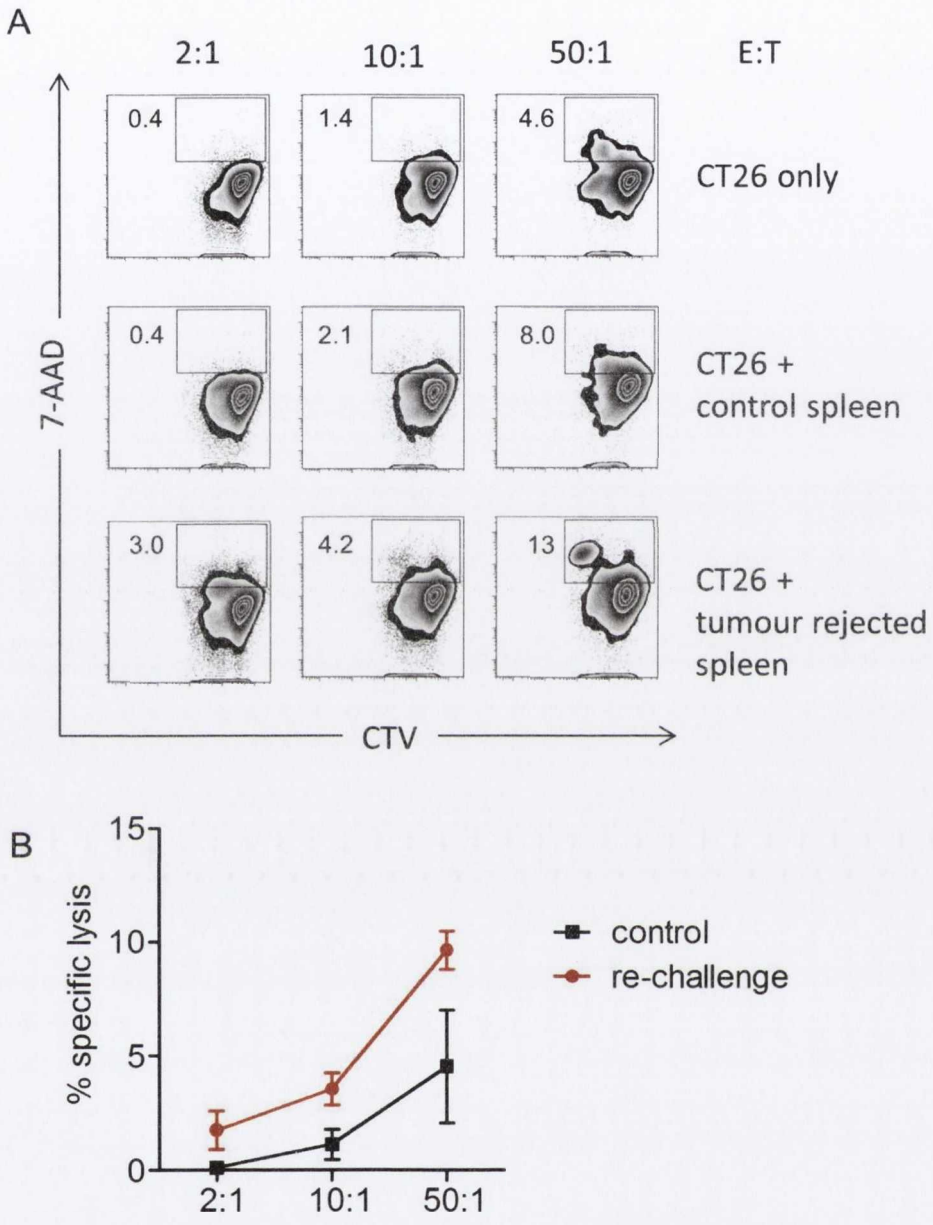


Figure 5.35
T cells from mice that rejected CT26 tumour upon treatment with a tumour vaccine and anti-PD-1 show increased cytotoxicity *ex vivo*

BALB/c mice were injected with CT26 tumour cells ($3x10^5$ cells/mouse) and injected with hs/irr CT26 tumour cells ($1x10^6$ cells/mouse, s.c.), R848 (40 µg/mouse, s.c.) and anti-PD-1 (200 µg/mouse, i.p.). Mice that received hs/irr CT26 + R848 + anti-PD-1 and rejected the tumours (=re-challenge) were subsequently re-challenged with CT26 tumour cells and compared to naïve mice that received the first tumour challenge (=control). On d 17 post re-challenge, spleens were isolated and co-cultured with CTV-labelled CT26 tumour cells at the indicated ratios. After 24 h, cell death was measured by 7-AAD staining and flow cytometry. Results are shown as representative FACS plots (A) and mean values ± SEM (B).

Chapter 6:

General discussion

6. General discussion

There have been significant advances in the development of immunotherapeutics for cancer. Until recently, researchers had struggled to induce effective anti-tumour immune responses that were comparable to standard therapies in cancer patients. Many anti-cancer immunotherapies were effective in pre-clinical models but did not induce the desired effects in cancer patients. Failure to induce tumour regression was often associated with failure to induce adequate tumour infiltration of effector T cells. The immunosuppressive tumour microenvironment was a major obstacle to successful cancer immunotherapies. Hence, strategies that managed to induce tumour-specific T cells still struggled to overcome subsequent suppression of effector T cell function.

Cancer immunotherapy has now come of age and improved considerably following a better understanding of immunosuppressive networks in the tumour microenvironment. The tumour microenvironment promotes immunosuppression by several mechanisms, including suppressive cytokines, suppressive immune cells and immune checkpoints. Strategies that targeted suppressive cytokines or immune cells induced only moderate anti-tumour effects [74, 151]. Targeting of immune checkpoints, however, turned out to be a game-changer. The big difference of immune checkpoint blockade compared to previous effective immunotherapies is that immune checkpoint blockade was effective in solid tumours while previous approaches were mainly effective in hematologic malignancies [199]. Notably, immune checkpoint blockade was much more efficient than the standard therapy, replacing Dacarbazine as first line therapy for melanoma [359]. Nevertheless, a significant proportion of patients did not respond to immune checkpoint blockade, highlighting a need for a better understanding of cancer-immune system interactions.

BAMLET is a cytotoxic PFA complex that has been under investigation for the use in cancer therapy. The present study confirmed that BAMLET is a potent inducer of tumour cell death, but also revealed the novel finding that BAMLET has immunostimulatory properties. BAMLET in combination with the TRL7/8 agonist R848 suppressed tumour growth *in vivo* and this was associated with an increase of IFN-γ-secreting T cells in lymphoid organs. These findings demonstrate that cell death-inducing drugs and immune activators are potent therapeutic combinations. The multi-potency of BAMLET also suggests that drugs that simultaneously kill tumour cells and stimulate immune responses might be more effective than drugs that only kill tumour cells. This is in line with recent reports showing that some chemotherapeutic drugs

induce immunogenic cell death which activates potent anti-tumour immune responses [360-362]. For example, Pfirschke et al. demonstrated that chemotherapy with drugs that induce immunogenic cell death, such as a combination of oxaliplatin and cyclophosphamide, sensitised tumours to checkpoint blockade and improved anti-tumour responses in several murine tumour models [361]. The efficacy of the therapy was dependent on its ability to stimulate TLR4 and CD8 T cell responses [361]. These findings could lead the way for the development of next-generation chemotherapy-immunotherapy combinations.

The present study demonstrated a novel function of BAMLET in modulating DCs, macrophages and T cells which contributed to the induction of anti-tumour immune responses. However, stimulation of immune cells with BAMLET *in vitro* induced both pro- as well as anti-inflammatory responses. While pro-inflammatory immune responses have the potential to improve cancer therapy, dichotomous immune responses induced by BAMLET could pose challenges. Anti-inflammatory responses induced by BAMLET could limit anti-tumour immunity. Conversely, excessive immune responses induced by BAMLET could potentially lead to autoimmunity. Future work needs to dissect the mechanisms of BAMLET-induced immune activation and to investigate potential adverse effects.

Early clinical trials with HAMLET indicated that PFA complexes are safe for the use in humans [284]. However, BAMLET-induced cell death is not tumour-specific and BAMLET and related PFAs have also been shown to kill healthy cells, particularly immune cells [280]. This has the potential risk of causing severe side effects. In order to improve the specificity of BAMLET and to identify effective combinations, more clarity is needed on the mechanism of BAMLET-induced cell death. Studies by Delgado et al. reported some advances in the development of PFA complexes. They found that OA was the crucial cell death-inducing component of BAMLET [281]. Moreover, they demonstrated that OA can be complexed with other proteins, such as cytochrome c [295]. This suggests that by exchanging the protein part of the complex, novel PFA complexes could be designed that enable specific targeting of tumour cells to avoid cell death of healthy cells. Moreover, PFA complexes could be designed that selectively stimulate immune cells. Future studies should investigate the balance between induction of cell death and immune-stimulation by BAMLET and related PFA complexes. Although BAMLET and PFA complexes have potential as anti-cancer drugs, more insight into the mechanisms of action is required to clarify whether they are suitable compounds for cancer therapy in patients.

In addition to activating tumour-specific T cell responses, current immunotherapy approaches target the immunosuppressive tumour microenvironment. Treg cells and anti-inflammatory molecules have been implicated in the induction of immunosuppression. The present study demonstrated that RA enhanced TGF-β-induced Treg conversion which was blocked with a RARα-antagonist. Treatment of tumour-bearing mice with RA or RARi had no effect on tumour growth. However, blocking RARα promoted a pro-inflammatory DC phenotype and enhanced the efficacy of a DC vaccine.

The approval of the DC vaccine Sipuleucel-T for patients with prostate cancer demonstrated that DC vaccines are feasible for the use in humans [224]. Early clinical trials comparing different DCs phenotypes found that mature DCs induced better anti-tumour immune responses in patients than immature DCs [36, 38]. TLR agonists and GM-CSF are commonly used as adjuvants in DC vaccines, however, TLR agonists also induce the secretion of anti-inflammatory cytokines. The present study and previous studies in our group have demonstrated that targeting suppressive signalling pathways in DCs can significantly improve DC vaccine efficacy [86, 87]. These findings could be useful for the design of DC vaccines for clinical trials, potentially improving the therapeutic efficacy of DC vaccines in humans.

An important finding of this study is the confirmation that TLR agonists induce the expression of PD-L1 and PD-L2 on DCs and monocytes, which suggests that TLR-activated DCs could dampen T cell responses as early as at the T cell priming stage [129, 130, 363, 364]. These findings strongly argue for a combination of TLR-activated DC vaccines with immune checkpoint blockade. This hypothesis is supported by pre-clinical data, showing that blockade of PD-L1 during DC vaccination improved anti-tumour effects and prolonged survival in a murine breast cancer model [365]. The DC vaccine was effective in PD-L1-positive and negative tumours, suggesting that PD-L1 expression on non-tumour cells contributes to T cell suppression [365]. Moreover, blockade of PD-1 enhanced the efficacy of a lysate-DC vaccine in a poorly immunogenic glioma mouse model [366]. There are 3 clinical trials currently recruiting patients with glioma, solid tumours and haematological malignancies which will test the hypothesis whether PD-1:PD-L1 blockade can improve the therapeutic effects of DC vaccines (NCT02529072, NCT02775292, NCT02528682).

The present study confirmed previous reports that RA synergises with TGF-β to enhance conversion of naïve T cells into Treg cells [154, 326]. Although RA signalling was investigated in the context of anti-tumour immune responses, these findings may

also be relevant in other immune-related diseases. For example, RA could be used to generate Treg cells *ex vivo* for the treatment of autoimmune diseases or in the context of transplantation to prevent the rejection of graft tissues.

The present study revealed the significant new findings that PD-1 expression on tumour-infiltrating T cells is associated with enhanced Treg cell and substantially reduced effector T cell responses. Furthermore, transient inhibition of PD-1 in the context of immune activation with a cancer vaccine released the block on cytotoxicity and cytokine secretion by tumour-infiltrating CD8 T cells. These findings suggest that PD-1 expression on T cells is, at least in part, responsible for an exhausted CD8 T cell phenotype and the induction of Treg cells in the tumour. Blocking PD-1 *in vitro* restored CD8 T cell proliferation and IFN-γ secretion in the presence of TGF-β. These findings are in line with recent reports that PD-1 interferes with TCR signalling by dephosphorylating downstream effector molecules [343].

Interestingly, the present study demonstrated that TGF-β-induced Treg conversion was reduced in presence of anti-PD-1. This is consistent with previous reports showing that PD-L1-coated beads and PD-L1-expressing DCs enhanced TGF-β-induced Treg conversion [121, 122]. Moreover, these findings provide the first evidence of a link between PD-1 and TGF-β signalling in T cells. A recent study by Baas et al. reported TGF-β-induced upregulation of PD-1 and PD-L1 on CD8 T cells [367]. However, this effect was not observed in the present study, suggesting a distinct mechanism that is downstream of TGF-β and PD-1 signalling. Identification of a possible link between PD-1 and TGF-β downstream signalling events could reveal a novel mechanism of PD-1-mediated T cell suppression. Identifying this link could also address the question whether PD-1 ligation on CD4 T cells has direct effects on TGF-β-induced conversion of naïve T cells to Treg cells.

There is little doubt that immune checkpoint blockade will constitute the backbone of standard cancer therapy for many malignancies in the future. Immune checkpoint blockade has been approved for melanoma, NSCLC, renal cancer, and bladder cancer and clinical trials have revealed promising results for a range of other cancers [7]. Even though immune checkpoint blockade showed outstanding success in the therapy of certain solid cancers, the majority of patients did not respond long-term to this therapy [252]. Moreover, some types of cancer such as melanoma and NSCLC were more responsive to immune checkpoint blockade than less immunogenic cancers, such as prostate or colorectal cancer [269]. Studies have reported that pre-existing T cell infiltration is required for successful therapy with anti-PD-1 [263, 265]. In the present

study, treatment with anti-PD-1 alone had no effect on tumour growth in the B16 or CT26 tumour model. However, the combination of anti-PD-1 with a vaccine significantly improved anti-tumour responses and led to regression of established tumours in the CT26 tumour model. These findings suggest that the combination of a cancer vaccine with immune checkpoint blockade could be a promising approach for patients that lack pre-existing T cell infiltration. This also suggests that vaccine and immune checkpoint blockade combinations might be a better approach than combinations of several immune checkpoint inhibitors, since vaccines boost the generation of tumour-specific T cells. A recent study by Antonios et al. using the low immunogenic GL261 tumour model supports this hypothesis [366]. Treatment of tumour-bearing mice with a DC vaccine or anti-PD-1 alone had little effect on survival, however, the combination of both significantly prolonged survival and led to complete eradication of tumours in some mice [366].

The present study demonstrated that a vaccine consisting of tumour lysates induces better anti-tumour immune responses than peptide vaccines. This was probably due to the presence of neoantigens in the tumour lysates. It has been shown that neoantigens are recognised by the immune system as non-self and therefore induce stronger T cell responses than self-antigens [270]. These findings suggest that vaccines containing neoantigens such as tumour lysates or selected neoantigens could induce better responses than peptide vaccines in patients. Moreover, identification of common neoantigens could be a strategy to improve the efficacy of cancer vaccines.

Immune related adverse effects (irAEs) are common side effects of immunotherapy. irAEs reported with immune checkpoint blockade include dermatitis, colitis, and hepatitis. irAEs after therapy with Ipilimumab and/or Nivolumab range from mild to severe, however, severe irAEs are rare and more commonly observed after Ipilimumab treatment than Nivolumab [7]. Management approaches have now been developed for immune checkpoint blockade-induced irAEs which have reduced life-threatening complications in patients [368]. Identification of cancer immunotherapies that only have to be given once or twice rather than chronically could reduce the risk of side effects. The present study demonstrated that 2-3 injections of a vaccine and immune checkpoint blockade combination were sufficient to induce strong anti-tumour immune responses. irAEs are an important aspect that has to be considered when deciding which therapy combination to use in patients.

Future work on immunotherapy and particularly on Immune checkpoint blockade will have to focus on two important questions. Firstly, which patients are most likely to

respond? The identification of biomarkers is crucial to improve the therapy and to decide which therapy is best for which patient. Secondly, which combinations of therapies yield the best results? Immune checkpoint inhibitors are most likely to represent the backbone of cancer therapy in many cases in the future and it is of major interest now to identify the best combination. Again, the identification of biomarkers will be important for the development of combinations.

Research on immune checkpoint blockade is advancing and alternative ways of inhibiting immune checkpoint interactions are currently under investigation. Su et al. demonstrated that it is possible to disrupt PD-1 on human T cells by CRISPR technology which could be used to improve CAR T cell therapy [369]. Moreover, Liu et al designed CAR T cells carrying a switch that transforms PD-1 ligation into an activating signal [239]. Future clinical trials should reveal whether these advanced technologies are as effective as mAb immune checkpoint blockade.

High costs may preclude universal use of immune checkpoint inhibitors as the first line therapy for different cancers. Biologics, such as monoclonal antibodies, are much more expensive than chemotherapeutic drugs or small molecule inhibitors (SMIs). Several companies and research groups are now looking into cheaper alternatives, such as SMIs that block the interaction between PD-1 and PD-L1. In 2015, the molecular details of human PD-1 and PD-L1 interaction have been identified by x-ray crystallography [370]. Consequently, several SMIs have recently been identified that bind to interaction hotspots and disrupt PD-1:PD-L1 interaction [371]. In addition to being cheaper to manufacture, SMIs have better tissue and tumour penetration than mAbs and could therefore have a higher efficacy. Moreover, SMIs have a shorter half-life which could reduce the incidence and severity of irAEs.

The generation of drugs that counteract immunosuppression in the tumour mark a paradigm shift in cancer therapy. Immune checkpoints and Treg cells are important contributors to immunosuppression and caused many past cancer immunotherapeutics to fail. In this context, the present study provides further insight into mechanisms of tumour-specific T cell activation and immunosuppressive networks in the tumour microenvironment and may contribute to the development of improved immunotherapy combinations.

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