Regulated Antagonism of Immune Suppressive Molecules in Tumours

Aliaa Amr Alamoudi

Wolfson College

University of Oxford

A thesis submitted for the degree of Doctor of Philosophy

to the Department of Oncology, University of Oxford

Hilary Term 2014

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Abstract

Despite expressing antigens that can induce immune surveillance and immune eradication, tumours demonstrate the capacity to evade anti-tumour immunity. Recently, this has been attributed to the ability of tumours to induce a local immunosuppressed micro-environment, which is a major obstacle to successful natural and vaccine induced anti-tumour immunity. Soluble factors such as transforming growth factor beta (TGFβ), and interleukin 10 (IL-10), released by cancer and stromal cells, are thought to play a significant role in this local immunosuppression.

In order to assess the influence of antagonising these soluble factors locally on tumour biology and tumour immunity, a murine CT26 colorectal carcinoma model that can express cytokine antagonists under Doxycycline (Dox) control was engineered. Two stable CT26 cell lines expressing Dox-inducible soluble extracellular domain of TGFβ receptor II (STGFβRII) or soluble extracellular domain of IL-10 receptor (SIL-10R), were established. Expression of STGFβRII *in vitro* and *in vivo* was only evident after Dox treatment. When Dox was administered directly following subcutaneous (s.c.) inoculation of STGFβRII-expressing CT26 cells into Balb/c mice, tumour growth was significantly suppressed. Interestingly, inducing STGFβRII in well-established tumours showed less suppression of tumour growth. To assess the effect of expressing STGFβRII on tumour immunity the RNA expression of 22 common cytokines was measured in the tumours of mice receiving Dox and control mice. The levels of some of these cytokines were modulated by STGFβRII expression (e.g TGFβ,Tnfsf9, IL-2). We also tested for any additive effect between expressing STGFβRII, and the administration of anti-CTLA-4 antibody on tumour growth, and tumour immunity.
The model described here could help address various limitations seen previously in studies of TGFβ blockade. It provides means of effective local antagonism, and it addresses immunological endpoints which have been limited in previous studies. Because of its tight regulation, the model also allows identification of the best timing of TGFβ blockade alone or in combination with other immunotherapeutics.

Declaration of Authentication

All the work presented in this thesis was performed by me, and in no way forms part of another thesis in this or in any other university. Contributions made by others have been acknowledged in this thesis. This work was performed under the supervision of Professor Leonard Seymour, Department of Oncology, Division of Medical Sciences University of Oxford, and Dr Robert Carlisle, Department of Engineering Science, Division of Mathematical, Physical and Life Sciences.

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List of abbreviations

/	Per
5' and 3'	5 prime and 3 prime end of RNA and DNA
293T	Human embryonic kidney cells 293 T
alk	Activin receptor like kinase
ANOVA	analysis of variance
APC	antigen presenting cells
Arg1	arginase 1
ASO	anti-sense oligonucleotides
BCS	Bicinchoninic acid
BMP	Bone morphogenetic protein
BSA	Bovine Serum Albumin
bp	Base pairs
^{o}C	Celsius
CAF	cancer associated fibroblast
CCL22	Chemokine (C-C motif) ligand 22
CIAP	Calf intestinal alkaline phosphatase
CMV	Cytomegalovirus
ConA	Concanavalin A
CT26	Mouse colon carcinoma cell line
CT26TetR	CT26 cells expressing TetR under CMV promoter
CT26PLUC	CT26TetR cells expressing luciferase under the Ptight promoter
CT26STGFβRII	CT26TetR cells expressing STGFβRII under Ptight promoter
CT26SIL-10R	CT26TetR cells expressing SIL-10R under Ptight promoter
CTL	Cytotoxic T lymphocytes
DCs	Dendritic cells
dH2O	deionised water
DMEM	Dulbecco's Modified Eagle's Mediumdimethyl sulphoxide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
Dox	Doxycycline
ECM	Extracellular matrix
EGF	epidermal growth factor
EMT	epithelial mesenchymal transition
FasL	Fas ligand
FCS	fetal calf serum
Foxp3	forkhead box P3
GDF	growth and differentiation factor
GM-CSF	Granulocyte macrophages colony stimulating factor
HER-2	human epidermal growth factor receptor 2
HGF	hepatocyte growth factor
HMG-B1	High mobility group protein B1
hrs	Hours
IDO	Indoleamine 2,3 dioxygenase
IFNγ	Interferon gamma
IL-10	Interleukin 10
IL-10R1	IL-10 receptor 1
IL-10R2	IL-10 receptor 2
i.p.	Intraperitoneal
i.v.	Intravenous
kDa	kilodalton
l	litre
LAP	latency associated peptide
LB	Luria Bertani
LTB	Lymphotoxin B
mAb	Monoclonal antibody
MDSC	Myeloid derived suppressor cells
mg	milligram

MHCI	major histocompatibility complex class I
MHCII	major histocompatibility complex class II
min	minutes
ml	milliliter
MMP	matrix metaloproteinase
Mv1Lu	mink lung epithelial cells
NEB	New England Biolabs
NK	Natural killer cells
NKT	Natural killer T cells
NOS	Nitric oxide synthase
OD	optical density
PBS	phosphate buffer saline
Pcmv	CMV promoter
Pcmv-Luc	Luciferase under the CMV promoter
PD-1	programmed cell death protein 1
PD-L1	programmed death ligand 1
PD-L2	programmed death ligand 2
PGE2	Prostaglandin E2
P-Luc	Luciferase plasmid vector (under Ptight promoter
pLUC-V	Luciferase lenti-vector (under Ptight promoter)
P/S	Penicillin and streptomycin
P-Smad2	Phosphorylated Smad2
rcf	relative centrifugal force
RLU	relative light units
ROS	reactive oxygen species
RNS	reactive nitrogen species
rpm	revolutions per minute
RPMI-1640	Roswell Park Memorial Institute
RT	room temperature
RT-qPCR	reverse transcriptase quantitative PCR
s.c.	subcutaneous
SDF-1	stromal cell derived factor 1
SDS	sodium dodecyl sulfate
SIL-10R	Soluble IL-10 receptor
STAT3	Signal transduce and activator of transcription 3
STGFβRII	Soluble TGFβ receptor II
TAA	Tumour associated antigen
TAM	Tumour associated macrophages
TEMED	Tetramethylethylenediamine
Tet system	Tetracycline controlled transcriptional activation system
tetO	Tetracycline operon sequence
TetR	Tetracycline repressor protein
TGFβ	Transformation growth factor beta
TGFβRI	TGFβ receptor I
TGFβRII	TGFβ receptor II
TH1	T helper 1
Th3	T helper 3 cells
TH17	T helper 17
TIL	Tumour infiltrating lymphocytes
TLR	Toll like receptors
Tr1	Type 1 regulatory T cells
TFBI	Transformation buffer I
TFBII	Transformation buffer II
Tnf	Tumour necrosis factor
Tnfsf	Tnf superfamily
TRAIL	Tnf-related apoptosis inducing ligand
TRE	Tet responsive elements
Tregs	Regulatory T cells
VEGF	Vascular endothelial growth factor

1 Introduction

1.1 Cancer biology and tumour microenvironment

1.1.1 Cancer biology

It is well established that the development of tumours is a multistep process in which a normal cell transforms into a cancerous phenotype by acquiring essential abilities described as the hallmarks of cancer (Hanahan and Weinberg 2011). These include the ability to sustain proliferative signals, resist growth suppression, resist cell death, induce an angiogenic switch, and the ability to invade and metastasise. Recently, two additional hallmarks have been added, the ability of cancer to evade the immune system, and reprogramming cell metabolism (Hanahan and Weinberg 2011), which both had great implications on the progress of cancer therapeutic

Although the dogmatic perception is that the ability of normal cells to acquire these functional hallmarks is largely due to mutations and genomic instability that a tumour cell evolves to acquire , it is now clear that other cells within the tumour microenvironment largely orchestrate tumour hallmarks (Hanahan and Coussens 2012).

This observation highlighted the fact that in order to understand cancer biology and cancer pathogenesis, and ultimately achieve therapeutic targets, cancer must be thought of as a complex tissue or organ made of a heterogeneous population of cells that interact with one another in a very dynamic manner.

1.1.2 Tumour microenvironment

In common with other tissue or organs, tumour development and survival requires food, oxygen, and structure. It is therefore not surprising that tumour growth is characterised by the co-development of neoplastic cells as well as other non-transformed stromal cells that can support tumour growth. Many stromal cells contribute to the tumour environment, however, they lie within three main groups; tumour vasculature cells (mainly endothelial cells), infiltrating immune cells of myeloid and lymphocytic origin, and fibroblasts which can establish and maintain an extracellular matrix for tumour structure (Hanahan and Coussens 2012, Junttila and de Sauvage 2013). The normal function of stromal cells in tissues is to maintain tissue homeostasis, and they may therefore be expected to hamper tumour formation. However, in a tumour setting

stromal cells evolve within the tumour to orchestrate changes that allow cancer progression. This can be attributed to the fact that some tumour associated stromal cells acquire different characteristics than their normal counterparts (Hanahan and Weinberg 2011, Hanahan and Coussens 2012, Junttila and de Sauvage 2013). For example the interaction of fibroblasts with carcinoma cells within the tumour may result in epigenetic changes triggering the activation of transforming growth factor beta (TGFβ) and stromal cell derived factor (SDF-1) signals which result in the activation of fibroblasts with a tumour-promoting phenotype known as cancer associated fibroblast (CAF). CAF can in turn promote tumour progression through expressing various factors and cytokines such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), TGFβ, SDF-1, and extracellular matrix proteases (Polanska and Orimo 2013).

Another example is dendritic cells (DCs), which have a function in normal tissue generating antigen specific T cell responses. However, it has been shown in certain tumour models that with advanced tumours functional changes to DCs take place. Notable, DCs derived from such tumours and their associated draining lymph nodes express lower levels of major histocompatibility complex class II (MHCII), and CD40, which are essential molecules for antigen presentation. Furthermore, these DCs suppress tumour-specific T cell proliferation more than DCs extracted from early tumours lesions, thus exhibiting an immunosuppressive phenotype induced by the tumour (Scarlett, Rutkowski et al. 2012).

Stromal cells can play a role in almost all of tumour hallmarks. Infiltrating immune cells can supply tumours with growth factors such as epidermal growth factor (EGF), for a sustained mitogenic tumour growth, or extracellular proteases for reforming the extracellular matrix. While CAF, can produce TGFβ, which plays a role in invasion and metastasis through epithelial to mesenchymal transition (EMT) processes (Hanahan and Coussens 2012).

1.1.3 Clinical implications

The improved understanding of the stromal impact on tumour growth has not only provided a better understanding of tumour biology, but has also widely influenced therapeutic approaches. This can clearly be seen in the progression from an exclusive reliance on chemotherapeutic drugs that directly target transformed epithelial cells, to the use of various other therapeutics

such as immune and anti-angiogenic therapies that aim to destroy tumours indirectly by manipulating the tumour microenvironment. It has also made it clear that identifying, understanding, and targeting crucial factors such as TGFβ, which is produced by various cells within the tumour microenvironment and is clearly a key factor in many of the hallmarks of cancer, would be an attractive therapeutic approach.

Another realization that originated from an improved understanding of the tumour microenvironment is the appreciation of the complexity and heterogeneity of cancer. Unlike a normal tissue in which different cell-types work in harmony, tumours function in a more chaotic manner. Tumour vasculature for instance is unevenly matured, poorly developed in areas, and more leaky in others (Hanahan and Weinberg 2011). This in turn has many consequences, for example on the recruitment of immune cells, on drug delivery, and on the increasing selective pressure in poorly vascularised lesions due to hypoxia and acidity (Junttila and de Sauvage 2013) . Another example of the diversity within the tumour are CAF, which are plastic in nature and can alter their phenotype and functional activity based on signals within the tumour microenvironment (Polanska and Orimo 2013).

This diversity accounts for regional differences within the tumour, which in turn accounts for the selective pressure in which the tumour microenvironment applies to actively shape tumour development. Such selection not only influences tumour progression but also drug responses and drug resistance (Aktipis, Boddy et al. 2013, Junttila and de Sauvage 2013). It is therefore crucial that the complexity of the tumour micro-environment is always considered when studying or treating cancer. Indeed, a better understanding of these complex interactions through the development of more relevant models is a crucial step. Only by answering better defined, key questions to provide an incremental framework for understanding this remarkable complexity can the current gold standards treatment be optimized and new ways to treat human cancer can be identified.

1.2 Tumour Immunity

As described previously, one of the hallmarks of cancer is the ability to evade the immune system (Hanahan and Weinberg 2011). This originates from a concept which was first postulated more than 100 years ago, that the immune system has the ability to detect tumour

cells and control their growth. This concept has recently become widely accepted and has firmly established cancer immunotherapy as a new therapeutic modality. To describe the advances in cancer immunotherapy it is important to first outline the historical background of tumour immunity and the noticeable progress that has been made in understanding the concept.

1.2.1 Cancer immunosurveillance

Although the concept that the immune system can detect tumour cells as foreign, and thus control tumour growth was established in the mid 20-th century, it was not until the 1990s with the development of better mouse models with specific engineered immune deficiencies that the concept gained wide acceptance (Dunn, Old et al. 2004, Schreiber, Old et al. 2011).

These models have shown that mice with innate and/or adaptive immune deficiencies were more prone to tumour development following exposure to carcinogens. This increased susceptibility was also evident with less immunogenic tumours that arise spontaneously. These extensive experiments proved that tumours must express tumour antigens that can be detected by the immune system, which in turn can detect and eradicate these cells (Dunn, Old et al. 2004, Schreiber, Old et al. 2011).

The concept of cancer immunosurveillance was further supported when novel *in vivo* approaches were able to demonstrate a range of tumour antigens, which disproved what was previously thought; that tumours are only composed of self-antigen and unable to elicit an immune response. The tumour antigens detected could be categorised into: differentiation antigens such as melanocyte differentiation antigens in melanoma, overexpressed or aberrant cellular antigen such as human epidermal growth factor receptor 2 (HER-2) in breast cancer, mutational antigens, viral antigens such as those associated with human papilloma virus in cervical cancer, and cancer/testis antigens which are normally only expressed in germ cells of testis and ovaries (Dunn, Old et al. 2004).

The final line of evidence for cancer immunosurveillance perhaps came from a large number of studies that showed positive correlation between the presence of tumour infiltrating lymphocytes (TIL) and improved survival. This was seen in a variety of cancer types, including metastatic melanoma (Clark, Elder et al. 1989), while more specific correlation was seen with increased

CD3+ cells in advanced ovarian carcinoma (Zhang, Conejo-Garcia et al. 2003), and increased CD8+ cells in colon carcinoma (Naito, Saito et al. 1998).

1.2.2 Cancer immunoediting

It was not long after the introduction of the concept of cancer immunosurveillance that a new hypothesis emerged to refine the role of the immune system not only in controlling tumour growth but in shaping tumour immunogenicity. This has been described as the cancer immunoediting hypothesis, which describes a process in which tumour cells co-evolving with tumour immunity must pass through three phases known as the 3Es ; elimination, equilibrium, and escape (Dunn, Old et al. 2004, Schreiber, Old et al. 2011).

Elimination

The elimination phase describes, in essence, cancer immunosurveillance, in which the innate and adaptive immune systems function together to recognise and eliminate tumour cells. This is thought to happen initially through damage/stress associated molecular patterns that are induced during early tumour development. Such signals can originate from molecules that are released directly from dying tumours (e.g. high mobility group protein B1 (HMGB1), deoxyribonucleic acid (DNA)), stress ligands that are presented on the surface of tumour cells (e.g. heat shock protein 60 (HSP60)) which can bind to receptors on the innate immune cells, and/or can be pro-inflammatory molecules and cytokines that are produced by stromal cells during stromal remodeling. The end result being an activated innate immune system, and the recruitment of various innate cells such as natural killer cells (NK), natural killer T cells (NKT), DCs, and macrophages (Dunn, Old et al. 2004, Schreiber, Old et al. 2011).

As a result of innate immune cell recruitment, and recognition of tumour cells, a type I interferon signal is generated and the production of interferon gamma (IFNγ), and other pro-inflammatory cytokines results in further recruitment of innate immune cells. The cytokines produced by the innate immune system can have a profound effect on eliminating tumour cells first by amplifying the scale of innate response, for example IFNγ produced from NK stimulates IL-12 secretion from recruited macrophages, which in turn can induce further IFNγ production from NK cells. Secondly, by the activation of a number of IFN dependent processes such anti-proliferation, pro-apoptosis, and angiostasis, all of which results in a significant killing of tumour cells. However,

the ultimate result for successful immunosurveillance is the activation of the adaptive immune response. Tumour destruction by the innate immune system results in the release of tumour antigens from dead cells, which are taken up by antigen presenting cells (APC) such as DCs (Figure 1.1). Once activated, DCs can successively migrate to draining lymph nodes to activate tumour specific T helper 1 (Th1) CD4+ T cell, which in turn facilitate the activation of tumour specific CD8+ (Cytotoxic T lymphocyte (CTL) via cross presentation of antigenic tumour derived peptides. The homing of tumour specific CD8+ into the tumour then results in killing of tumour through direct and/or indirect mechanisms. The generation of this tumour specific adaptive immune response therefore provides the long term capacity to eliminate any tumour cells and hamper tumour development (Dunn, Old et al. 2004, Schreiber, Old et al. 2011).

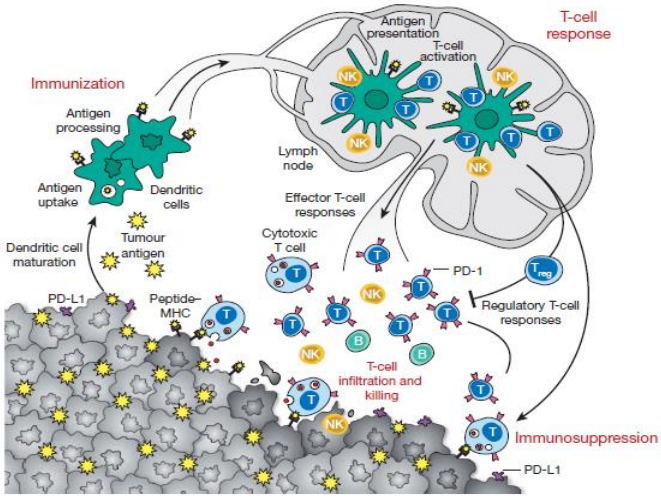

Figure 1.1 Generating anti-tumour immune responses

Tumour cells express antigens that can be captured by APC such as DCs. DCs process these antigen and present them on MHCII or MHCI (for cross presentation), migrating then to the lymph node for antigen presentation. Depending on the cytokine milieu in the microenvironment and the presence or lack of immunogenic maturation stimulus, DCs can generate an effector T cells response or instead induce a tolerogenic response in the form of T cell anergy or the induction of Tregs. Effector T cells together with NK and B cells are the recruited to the tumour site and infiltrate the tumour to mediate tumour cells killing. Used with permission from (Mellman, Coukos et al. 2011)

Equilibrium

In this phase tumour variants that have survived elimination enter together with lymphocytes into a dynamic equilibrium. The adaptive immune system can prevent any tumour outgrowth for years and places a selection pressure on escaping variants, thus shaping their immunogenicity. These cells although able to establish a tumour bed remain dormant. It is thus thought that the

equilibrium stage is the longest of the 3 phases, where many solid tumours are clinically silent despite the existence of tumour cells (Dunn, Old et al. 2004, Schreiber, Old et al. 2011).

Escape

In this phase, tumour cells that have escaped elimination and have been selected for in the equilibrium phase have now acquired the ability to circumvent immune recognition and immune destruction through acquired genetic and epigenetic changes. Escaping immune responses might be due to the impact of immune system on the immunogenicity of tumour cells, resulting in the selection of poorly immunogenic tumour cell variants, or through an active process by which cancer can induce the suppression of immune cells (Dunn, Old et al. 2004). Many mechanisms have been introduced to explain how tumour cells escape the immune system. Due to the relevance of this phase to the studies performed in this thesis it is described in more details in the next section.

1.2.3 Tumour acquired immunoprivilege and immunosuppression

To become refractory to natural and vaccine induced immunity, tumours must acquire immune regulatory mechanisms which involve dynamic interactions between tumour cells and the supporting stroma and recruited immune cells. A variety of immunosuppression mechanisms that can be acquired by tumours have emerged (Zou 2005, Stewart and Smyth 2011, Ostrand-Rosenberg, Sinha et al. 2012, Motz and Coukos 2013). One can think of the various immunosuppressive mechanisms as hurdles and/or immune-checkpoints at each step of the multistep process required to generate a successful anti-tumour immune response.

Generating a strong immune response

The first step for generating an effective immune response is successful antigen presentation.

Due to selective pressure, tumours cells may develop mechanisms (via genetic and epigenetic changes) to down regulate MHC class I (MHCI) molecules on their surface and manipulate other genes encoding the antigen processing pathway (Motz and Coukos 2013), thus diminishing tumour antigen processing and presentation. The tumour microenvironment also has a profound effect on the function and maturation of APC. For example, tumour myeloid DCs display an immature phenotype with a decrease in the expression of MHCII, and co-stimulatory

molecules, and an increase in the expression of co-inhibitory molecules and immunosuppressive cytokines. Unlike mature DCs which present antigens to generate an antigen dependent immune response, immature DCs present antigen in a tolerogenic manner inducing either anergy in T effector cells, or helping in the expansion of T regulatory cells (Tregs) with inhibitory functions (Zou 2005).

One mechanism by which the tumour environment can induce immature and thus tolergenic DCs is believed to be the expression of various immunosuppressive factors, of which VEGF is the most studied. One hypothesis is that VEGF can induce DCs suppression through the induction of the expression of the immunoinhibitory molecule programmed cell death 1 ligand (PD-L1) on these cells. Other tumour secreted factors that could also play a role in DCs poor maturation are TGFβ, IL-10, and IL-6. (Zou 2005, Motz and Coukos 2013)

Migration of immune cells and reaching tumour site

If tumour reactive T cells are successfully generated, they are faced with another hurdle of needing to be recruited to the tumour site. One mechanism by which tumours manipulate this is by altering the function of chemokines which regulate the trafficking of immune cells. Another major obstacle for immune cells to reach and penetrate the tumour is tumour vasculature. The mechanisms behind this have yet to be fully defined, but it is evident that endothelial secreted VEGF decreases vasculature adhesion molecules which are necessary for T cells trafficking. Tumour vasculature endothelial cells may also play a more active role by secreting immunosuppressive factors such as Fas ligand (FasL), which can kill T cells or suppress their function (Motz and Coukos 2013)

Local tumour environment

Even upon arrival within the tumour T cells remain faced with a wide range of immunosuppressive mechanisms and immune-checkpoints within the local tumour microenvironment (Figure 1.2). For example tumour cells express a range of surface molecules, which can induce killing of T cells such as FasL and tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), or induce T cells suppression such as PD-L1 and PD-L2. In addition to membrane surface presented molecules, there is a range of soluble factors that are secreted by tumour cells and which play a major role in inhibiting T cell function. These include

TGFβ, IL-10, prostaglandin e2 (PGE2), hydrogen peroxide, and adenosine, the first two will be discussed in more details later in this chapter.

Tumour reactive T cells do not only interact with tumour cells, but also face a range of immune suppressive cells including Tregs, myeloid derived suppressor cells (MDSC), and tumour associated macrophages (TAM) which are actively recruited to the tumour microenvironment via signals and cytokines expressed by tumour cells and stromal cells (Dunn, Old et al. 2004, Schreiber, Old et al. 2011).

Tregs

Tregs are a subgroup of CD4+ T cells that function to maintain immune homeostasis by hampering effector T cells. They can be divided into the naturally occurring Tregs (nTregs) which develop in the thymus, or induced Tregs (iTregs) which are induced in the periphery in response to antigen stimulation and a certain cytokine milieu (Nishikawa and Sakaguchi 2010). Example of iTregs are the IL-10 secreting type 1 regulatory cells (Tr1) and the TGFβ secreting T helper 3 cells (Th3). Both human and mice Tregs are characterised by the expression of the CD25 receptor and the transcription factor forkhead box p3 (Foxp3) which gives Tregs their immune suppressive phenotype (Nishikawa and Sakaguchi 2010). One of the main factors that induces Foxp3 expression in Tregs is TGFβ (Bird 2010). These cells can mediate their immunosuppressive effects on a variety of immune cells mainly CD8+, CD4+, NK, and B cells. Although activated in an antigen specific manner, Tregs can mediate their immunosuppressive effect in a non-specific way. Several mechanisms have been elucidated on the way Tregs mediate their suppression these include the secretion of immunosuppressive factors such as TGFβ, IL-10, and IL-35, or contact dependent methods such as the expression granzyme B for effector T cells killing (Sakaguchi, Miyara et al. 2010).

Tregs have been strongly associated with cancer. They have been isolated in the blood and tumours of cancer patient (Audia, Nicolas et al. 2007). In addition their existence has been correlated with poor prognosis in a number of tumours (Curiel, Coukos et al. 2004, Nishikawa and Sakaguchi 2010). Therefore the ability of tumours to recruit Tregs via the expression of the chemokine CCL22, or their ability to drive the in situ induction of a Treg population with the help of its particular cytokine milieu, is thought to be one of the important tumour induced immunosuppressive mechanism.

MDSC

MDSC are a heterogeneous population of myeloid origin that consists of immature DCs, macrophages, monocytes, and granulocyte (Ostrand-Rosenberg, Sinha et al. 2012). In mice they are characterized by the expression of surface markers CD11B+ GR1+ (Khaled, Ammori et al. 2013). As their name implies, MDSC are thought to play an essential role in suppressing various innate and adaptive immune cells within the tumour. They are recruited to the tumour site by various factors such as VEGF, TGFβ, IL-10, and granulocyte-macrophage colony stimulating factor (GM-CSF), and have been found in the tumour, blood, and lymphoid organs of cancer patients (Khaled, Ammori et al. 2013). Similar to Tregs their existence is correlated with progressive disease and poor prognosis (Khaled, Ammori et al. 2013). They promote tumour progression through non-immunological mechanisms, for example up-regulation of angiogenesis, as well as a range of immunological mechanisms such as the expression of enzymes arginase I (Arg1) and nitric oxide synthase (NOS). These enzymes metabolise arginine into nitric oxide and hydroxide and the resulting reactive oxygen and nitrogen species (ROS and RNS) can in turn induce T cell apoptosis and suppress T cell activation. In addition, MDSC secrete IL-10, which in turn can manipulate DCs into presenting antigens in a tolerogenic manner. In addition to IL-10, MDSC can also secrete TGFβ, which together with IL-10 they can induce Tregs generation/ recruitment (Stewart and Smyth 2011, Ostrand-Rosenberg, Sinha et al. 2012, Khaled, Ammori et al. 2013).

TAM

As part of the innate immune system, macrophages play an essential role in eliminating infectious agents, but they also regulate the adaptive immune system and promote wound healing. Although very plastic and diverse in their functions, macrophages are phenotypically described as existing on a spectrum from ‘M1’ to ‘M2’.M1 are activated macrophages that are induced by IFNγ, and express high levels of IL-12 and low levels of IL-10, therefore promoting a Type 1 T cell response which is associated with anti-tumour immunity. M2 on the other hand are induced my IL-4, IL-13, and IL-10, and conversely are IL-10^{high} and IL-12^{low}. This immunosuppressive phenotype supports tumour development by inducing angiogenesis, supporting tumour proliferation, and suppressing tumour immunity (Ruffell, Affara et al. 2012).

Tumours are believed to polarize macrophages into an M2 profile therefore utilising their immunosuppressive ability to promote tumour growth (Ostrand-Rosenberg, Sinha et al. 2012) .

It is therefore clear that the tumour develops a wide range of immunosuppressive strategies that can overcome effective anti-tumour immunity, and instead favour tumour development and progression. As discussed in the next section the understanding of tumour immunosuppressive mechanisms has led to drastic changes in the way tumour immunotherapy is approached, and this has been recently reflected in the clinics. This demonstrates the importance of these mechanisms and how improved understanding can lead to advances in immunotherapy for cancer.

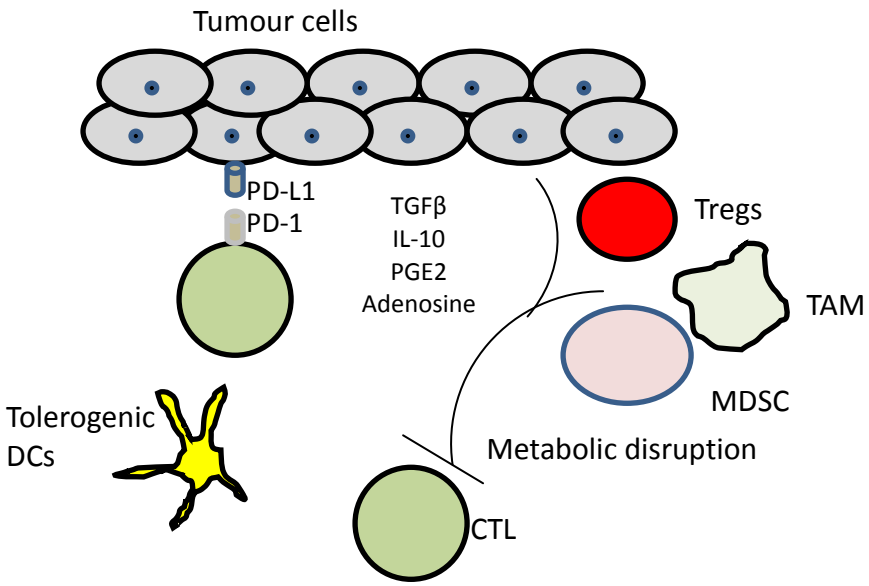

Figure 1.2 Local immunosuppression in tumours

Several immunosuppression factors face effector T cells upon arrival to tumour site. Tumour cells secrete various soluble factors such as TGFβ, IL-10,PGE2 and Adenosine which can limit CTL function. In addition CTL are faced with immunosuppressive cells such as Tregs, MDSC, and TAM, which can inhibit CTL function through secreting similar soluble factors and/or through metabolic disruption. In addition to soluble factors tumour cells can express immunosuppressive molecules on their surface. An example is PD-L1 which can interact with PD-1 receptor on T cells to terminate T cell activation. The immunosuppressive milieu in the tumour microenvironment also helps in generating tolerogenic DCs which can induce T cell anergy and generate Tregs. Figure modified from (Motz and Coukos 2013)

1.3 Cancer immunotherapy

1.3.1 Introduction

As it became widely accepted that the immune system could recognise and, occasionally, eradicate tumour cells, it is not surprising that the concept of immunotherapy became a very desirable approach. In simple terms, cancer immunotherapy is an approach that aims to boost various components of the immune system to prevent cancer development/progression. Unlike conventional cancer therapeutics such as some chemotherapies, or radiotherapy, immunotherapy has the great advantage of being tumour specific. In addition, the ability of the immune system to develop a memory towards tumour antigens makes it an ideal approach to overcome two of the main obstacles to successful cancer therapy; metastasis to other organs and recurrence (Stewart and Smyth 2011) .

Similar to our progress in understanding tumour immunity, cancer immunotherapy development has followed similar steps. Initially, cancer immunotherapy aimed to boost the patient's immune system to induce an effective anti-tumour response, however, there has been a paradigm shift towards approaches that aim to eliminate tumour induced immunosuppression and block immune-checkpoints (Mellman, Coukos et al. 2011, Stewart and Smyth 2011).

1.3.2 History and challenges

Passive immunity

Immunotherapy in cancer can be divided into two main strategies: passive immunity and active immunity. Passive immunity aims to simply provide patients with either monoclonal antibodies (mAb) or T cells that can target tumour associated antigens (TAA). Tumour specific mAb can target oncogenic pathways that are essential for tumour growth and survival. However, tumour specific mAb are also thought to be able to induce tumour cell killing by opsonizing tumour cells (complement dependent cell cytotoxicity CDC) which can also render them susceptible to phagocytosis by macrophages or antibody dependent cell mediated cytotoxicity(ADCC) by NK cells (Scott, Wolchok et al. 2012). A range of mAb have been FDA approved for solid and hematological malignancies e.g. is Trastuzumab which targets HER-2 molecules in breast cancer (Hudis 2007), and Rituximab which targets CD20 in B cell lymophoma (Maloney, Grillo-

Lopez et al. 1997). Allogeneic bone marrow transplant has also been successful in treating hematological malignancies (Mellman, Coukos et al. 2011). The approach utilises the ability of donor lymphocytes to attack tumour cells in the recipient due to a graft versus leukemia response which mimics the graft versus host response. Using the same principles of allogeneic bone marrow transfer, adoptive transfer of tumour-specific T cells that are expanded *ex vivo* and re-infused into the patients has also been introduced as an approach for passive immunity. Adoptive T cell therapy has also shown greater efficacy with the generation of more tumour specific T cells through genetically engineered chimeric antigen receptors (CARs) or T cell receptors (TCRs) (Lipowska-Bhalla, Gilham et al. 2012).

However there are obvious drawbacks that can be seen with passive immunity. The anti-tumour effect seen with mAb would be of short duration due to the short half-life associated with these antibodies which would require multiple, costly re-administrations (Mellman, Coukos et al. 2011). While adoptive T cell therapy is time and labour consuming due to difficulties associated with the isolation and expansion of antigen specific T cells. This may ultimately make it hard to apply across large patient populations especially in low resource settings.

Active immunity

The second form of vaccination is active immunisation, which utilises the fact that TAA can generate durable memory CD4+ and CD8+. The history of active immunization in cancer comes first from the work of William Coley, who demonstrated that intratumoural injection of pathogens was able to stimulate immune responses which can destroy tumour cells (Wiemann and Starnes 1994) . The approach showed some significant anti-tumour response in some cases, however, the responses were not always reproducible and the approach was not scientifically sound enough to be adopted.

However the idea of active immunisation has recently been re-visited. First attempts started using short TAA peptides not associated with immune adjuvant (Mellman, Coukos et al. 2011). Unfortunately several trials were doomed to failure with this approach. Freely administrated short peptides had very poor pharmacokinetics and with no adjuvant they proved to be poor in generating mature DCs activated responses resulting in very poor clinical efficacy. However, the further development of tumour vaccines was finally able to show some signs of clinical

activity, particularly with the introduction of immunostimulants such as IL-2. Indeed, the combination of the gp100 melanoma peptide with IL-2 in advanced melanoma showed slight augmentation of clinical responses and progression free survival compared to IL-2 alone (Schwartzentruber, Lawson et al. 2011). Also full length antigenic proteins or cell based vaccines have been explored in attempts to broaden the profile of epitope exposed to DCs (Mellman, Coukos et al. 2011). An example of this was the MAGE-A3 (cancer testis antigen) which was combined with toll like receptor (TLR) agonists in non-small cell lung carcinoma (NSCL) (Vansteenkiste, Zielinski et al. 2013). However the treatment failed to show clear survival benefits in the treated group. Independent analysis of phase III trial of MAGE-A3 in melanoma has demonstrated that it failed to meet its primary end points (GSK). Other platforms that have been used for vaccination include the use of viruses expressing TAA alone or together with cytokines (Cawood, Hills et al. 2012).The aim in this approach is to utilise the strong immunogenicity of viruses to generate an immune response which could augment tumour specific immune response. An example is the modified vaccinia virus used to express 5T4 and oncofetal antigen expressed in colon cancer and other malignancies. In phase I/II trials the vector (TROVAX) has shown to stabilise the disease in 5 out of 17 patients of colorectal carcinoma. Although clear anti 5T4 humoral responses were detected, T cells responses were limited (Harrop, Connolly et al. 2006) .

Another approach for tumour vaccination is DCs based cell vaccination. In this approach DCs from patients are isolated and loaded with tumour antigen *ex vivo* to allow activation, and then re-infused in the patient (Mellman, Coukos et al. 2011). The approach has shown some clinical efficacy, however, it has not been adopted by pharmaceutical companies probably due to the complexities associated with generating it. An exception to the rule is the FDA approved DCs cell based vaccine Provenge (Sipuleucel –T) for prostate cancer (Higano, Small et al. 2010). The vaccine was the first clinically validated treatment using active immunotherapy as an approach for treating cancer. The vaccine is comprised of a mixture of PBMCs isolated from patients, which is supplemented with cytokines, and a tumour derived antigen (fusion protein of prostatic acid phosphatase and GM-CSF) before being re-infused into patients. However, despite its FDA approval Provenge has shown limited clinical benefits. There was no evidence

of tumour shrinkage or any delay in disease progression. However there was a 4.1 months improvement in median survival, which itself was not associated with T cell responses. This has made it hard to fully determine the mechanism of action by which the treatment has mediated improvement of survival (Kantoff, Higano et al. 2010)

These examples have demonstrated that although many strategies using active immunotherapy have shown a hint of activity they completely failed to meet to expectations and provide dramatic clinical responses. It is of note that clinical trial design and endpoints determination has been a great obstacle in the face of immunotherapy (Lesterhuis, Haanen et al. 2011), however, several reasons can account for the limited clinical responses seen. For instance there is still a lack of clear understanding of what constitutes an optimal antigen and an optimal adjuvant in a tumour setting. But perhaps the more important explanation lies in the various multiple immunosuppression mechanisms that tumour acquire, which has been explained previously.

The importance of this oversight has been emphasised by reports that show that vaccine-induced immune attack is abruptly attenuated and instead initiates the induction of various immunosuppressive factors (McGray, Hallett et al. 2014). In this study vaccination in mouse melanoma bearing mice using a replication-defective adenoviral vector expressing the TAA dopachrome tautomerase resulted in a significant expansion of antigen-specific CD8+ T cells in the periphery and in tumours. However, this did not correlate with the modest tumour growth delay seen. When studying TIL, a progressive loss of their function (indicated by reduced IFNγ, TNF-α expression and degranulation) was observed when comparing TIL at 5 or 10 days post immunisation. Furthermore, TIL showed a reduction in function when compared to CD8+ in the periphery. In addition, vaccination with the antigen expressing vector in tumour bearing mice rapidly induced the expression of a range of immunosuppressive factors including PD-L1, PD-L2, Arg1, and TGFβ1.

From this we can conclude that while vaccination can induce strong antigen specific CD8+ T cells, these cells progressively lose their function in the tumour, indicating the importance of the local tumour environment in suppressing tumour immunity.

Reversing tumour immunosuppression:

The modest and slightly disappointing clinical results seen with early cancer immunotherapeutics and the ever-improving understanding of tumour immunity being attained have made it clear that trying to boost the immune system while the 'brakes' of immunosuppression are still active is likely to be ineffective. This realisation has made a great shift in cancer immunotherapy approaches, and introduced a wide range of therapeutics that aim to reverse tumour induced immunosuppression (Figure 1.3). The majority of these therapies fall into four main categories that target various immune-checkpoints in tumours and include; those that target regulatory cells, therapies that inhibit T cells immune-checkpoints, therapies that inhibit immunosuppressive soluble factors, and those that can alter tumour metabolism.

Recently this change in strategy has started to show benefits and generate attention in cancer immunotherapy as evidenced by its nomination by Science journal as the 'breakthrough of the year' for 2013. This has largely been attributed to the success recently seen in clinical trials with two therapies which aim to reverse tumour immunosuppression and inhibit immune-checkpoints; cytotoxic T lymphocyte antigen 4 (CTLA-4), and programmed cell death 1 (PD-1).

Anti CTLA-4

CTLA-4 is a negative regulatory molecule that is expressed on activated T cells (Vanneman and Dranoff 2012). While CD28 is a co-stimulatory molecule which binds to ligands B7-1 and B7-2 on APC to induce T cell proliferation and activation following stimulation of the TCR, CTLA-4 acts as an inhibitory signal that competes with CD28 for the B7-1 and B7-2 ligands. The receptor shows homology with the CD28 receptor and binds to its ligands with higher affinity, therefore small amounts of CTLA-4 recruited to the immune synapse can out-compete CD28 co-stimulatory signals, resulting in an attenuated T cell response. The molecule is also expressed on Tregs and is associated with the ability of these cells to express immunosuppressive cytokines (Grosso and Jure-Kunkel 2013). The breakthrough in targeting CTLA-4 was reported in 1996 by Allison and colleagues who demonstrated that treatment with anti-CTLA-4 antibodies resulted in the rejection of pre-established tumours in mice (Leach, Krummel et al. 1996). Since then, CTLA-4 blockade has shown success in a variety of tumour models both as a monotherapy and in combination with other agents and has proved able to recover T cell function and increase tumour specific T cell responses (Grosso and Jure-Kunkel

2013) However, the real validation of the importance of targeting immunosuppressive mechanisms came with the clinical success of Ipilimumab a fully humainsed mAb that blocks CTLA-4. In a phase 3 clinical trial, Ipilimumab increased overall survival in advanced metastatic melanoma, which was the first demonstration of an experimental therapy extending survival in metastatic melanoma and was the basis for Ipilimumab's FDA approval (Hodi, O'Day et al. 2010). It is of-note, however, that despite the durable clinical responses seen with Ipilimumab only a small fraction of patients have gained benefit (Mellman, Coukos et al. 2011). In addition side effect related to autoimmunity is an area that requires further optimisation . The success of Ipilimumab has clearly validated the importance of targeting immune-checkpoints in the tumour in order to induce a strong anti-tumour immune response.

Another approach that has been studied in attempts to reverse tumour induced immunosuppression is targeting regulatory cells. Tregs infiltration, for instance, has a negative effect on potentially tumouricidal T cells and blocking their function would be desirable. The limiting factor, however, remains finding specific inhibitory agents since most of Tregs surface markers are shared with other T lymphocytes. However, anti-CD25 antibody therapies, despite not being fully specific to Tregs cells, have been shown to increase the efficacy of tumour immunization (Nishikawa and Sakaguchi 2014).

Similarly, the attempts to reverse the function of soluble immunosuppressive factors are promising. For example, VEGF blockade has resulted in an increase in T cells homing and infiltration in tumours and improvements in immunotherapy efficacy in various preclinical studies (Stewart and Smyth 2011).

The influence of tumour cells and stromal cells on the metabolism of nutrients that are essential in T cell function, also presents an interesting target for therapy. In particular, inhibitors of immunosuppressive enzymes secreted or expressed in the tumours have been developed. Examples include inhibitors designed to prevent indoleamine-pyrrole 2,3-dioxygenase (IDO) degrading L-tryptophan and Arg1 degrading arginine. These agents are showing promising enhancement of the efficacy of immunotherapy in preclinical studies. (Stewart and Smyth 2011)

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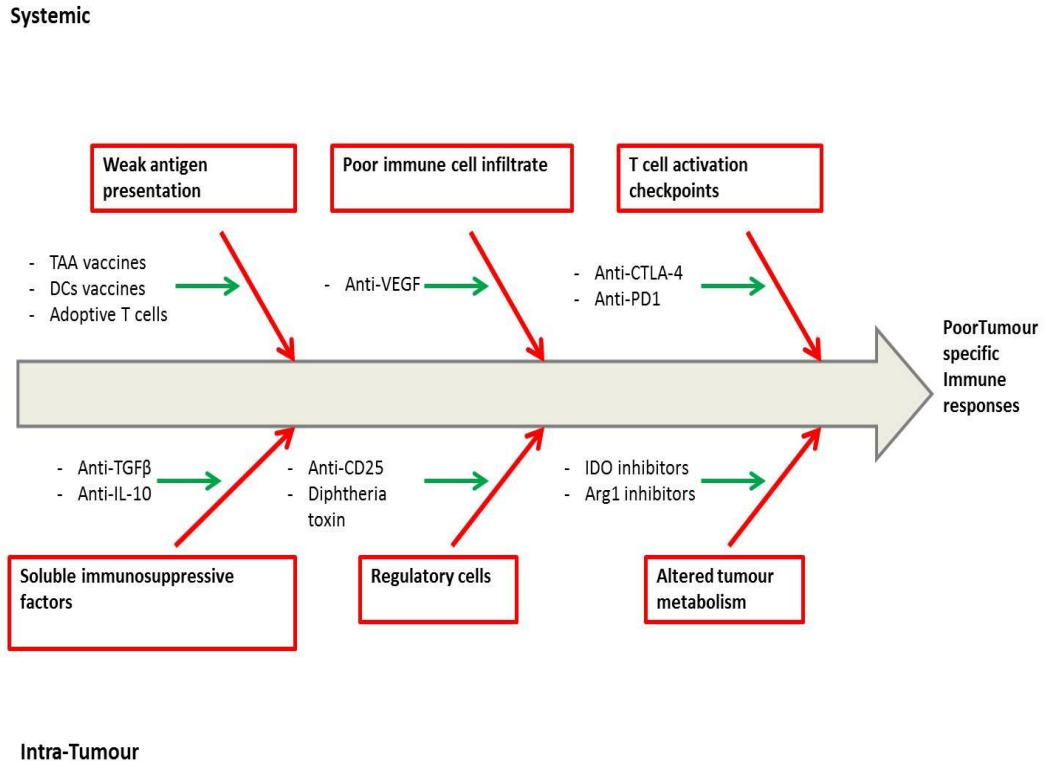


Figure 1.3 Summary of anti-tumour immunity checkpoints and immunotherapeutics

A summary of the systemic and intra-tumour hurdles/checkpoints that face the generation of successful tumour specific immune responses, and examples of therapeutics that address them. Red boxes show systemic or intra-tumour hurdles/checkpoints against anti-tumour immunity, while green arrows show examples of immunotherapeutic that target them

1.4 Soluble immunosuppressive factors

As described previously, tumour and stromal cells can secrete immunosuppressive factors within the local tumour microenvironment and these factors are thought to be key mediators in reversing tumour induced immunity. Two of the most important and well-studied factors are TGFβ and IL-10 which are known to have strong immunosuppressive effects on both the innate and the adaptive immune systems (Mosser and Zhang 2008). Due to the pleiotropic effects of these factors, it is crucial to understand their function within the local tumour environment and their effect on tumour immunity. However, attempts to obtain such an understanding and thereby completely define the effect of blocking these factors on cancer development has been stymied by the lack of model systems which allow precise enough control over the levels, timing and distribution of TGFβ and IL-10 .

1.4.1 TGFβ

1.4.1.1 Introduction

TGFβ is a pleiotropic cytokine that has a dual role in the progression of cancer (Massague 2008). The cytokine can act as a tumour suppressor through the regulation of cell proliferation and apoptosis, it can also act as a tumour promoter by mediating invasion, metastasis, angiogenesis, and immune evasion (Massague 2008, Akhurst and Hata 2012). Combined genetic and epigenetic changes in tumour cells, together with signals from the tumour microenvironment, can alter the biological responses of TGFβ giving it its characteristic plasticity in a context dependent manner (Massague 2008). TGFβ is a potent immunosuppressive cytokine which is secreted by both tumour cells and stromal cells, and can alter the maturation, activation, and differentiation of innate and adaptive immune cells and it is thought to be one of the crucial factors in tumour induced immunosuppression (Flavell, Sanjabi et al. 2010, Yang, Pang et al. 2010).

In addition, reports have shown high levels of plasma TGFβ in a number of tumours, and association with disease progression in colon carcinoma (Tsushima, Kawata et al. 1996), and prostate cancer (Adler, McCurdy et al. 1999) . Furthermore, the cytokine is correlated with metastasis in a number of tumours (Ikushima and Miyazono 2010). Therefore, the blockade of TGFβ signaling in tumours to stop its various tumour promoting activities is an attractive strategy (Akhurst and Hata 2012).

1.4.1.2 TGFβ and TGFβ Signaling

TGFβ belongs to the TGFβ superfamily of proteins which comprises more than 30 members (Massague 2008). The family includes TGFβ, activins, inhibins, Nodal, bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), and anti-Mullerian hormone (Massague 2008, Akhurst and Hata 2012). Their expression is tissue and time dependent, and they exert a wide range of functional properties (Massague 2008, Akhurst and Hata 2012).

TGFβ exists in three highly homologous variant forms (TGFβ1, 2, 3). Although they share the same signaling pathway their expression varies from different cell types and they function differently. TGFβ1 has been the most intensively studied isoform, and it is the predominant

variant expressed in hematopoietic cells (Rubtsov and Rudensky 2007). TGFβ1 has been linked to both tumourigenesis and immunosuppression (Laverty, Wakefield et al. 2009). In contrast, the effect of TGFβ3 on cancer development is less known, its role in fibrosis is more clear (Laverty, Wakefield et al. 2009).

TGFβ is secreted as an inactive complex composed of TGFβ bound non-covalently to the latency associated protein (LAP) and latent TGFβ binding protein, the resulting larger complex is known as the large latent complex (Massague 2008, Akhurst and Hata 2012). In order to bind to its receptors TGFβ requires activation. This is achieved by proteolysis of LAP in order to release TGFβ. The exact factors that can activate TGFβ are not yet full identified, however, matrix metalloproteinases 2, and 9 (MMP2, MM9), thrombospandin 1, and low pH have all been associated with the ability to release TGFβ into its active form (Annes, Munger et al. 2003). The TGFβ superfamily signals via binding to transmembrane type I and type II serine/threonine kinase receptors (Massague 2008, Akhurst and Hata 2012). Active TGFβ homodimers bind to the transmembrane type II TGFβ receptor (TGFβRII) which then, as a result of conformational changes, forms a heterodimerised complex with the type 1 TGFβ receptor (TGFβRI) also known as activin receptor-like kinase 5 (alk5) . This conformational change facilitates the cytoplasmic serine/threonine kinase domain of the TGFβRII to phosphorylate and thus activate TGFβRI (Massague 2008). Once activated, TGFβRI can activate two independent signaling pathways; the canonical Smad-dependent pathway (Figure 1.4), and the non-Smad dependent pathway. In the Smad pathway, only Smad2 and Smad3 can be phosphorylated by TGF mediated signaling via TGFβRI. Other Smads 1,5,8 can be phosphorylated by signals mediated by the BMP and AMH family members (via the alk1 receptor). Before activation, Smad2 and Smad3 are recruited to the plasma membrane by interaction with an anchor protein (Massague 2008). Once phosphorylated by TGFβRI, Smad2 and Smad3 form a complex with Smad4 which is then shuttled to the nucleus (Akhurst and Hata 2012). The complex further recruits DNA-interacting proteins (transcriptional factors) which in turn can further recruit additional co-activators and co-repressors, allowing TGFβ to activate or repress a great number of genes at one time. It is also thought that activated Smad2 and Smad3 play a role in post-transcriptional regulation through microRNA, which could be a further mechanism on how TGFβ regulates gene expression. In addition to the Smad pathway, TGFβ activated receptor complex can

further activate other non-Smad pathways such as the extracellular signal regulated kinase (ERK), TNF receptor associated factor 4 and 6, phosphoinositide 3 kinase JUN N terminal Kinase P38 mitogen activated protein kinase and nuclear factor κβ (NF-κβ) (Massague 2008, Akhurst and Hata 2012). In addition to the activating Smads, two inhibitory Smads, 6 and 7 exist, and can antagonize TGFβ superfamily signaling. While Smad6 acts specifically on BMPs, Smad 7 antagonises the signaling of all members of TGFβ superfamily (Ikushima and Miyazono 2010, Akhurst and Hata 2012).

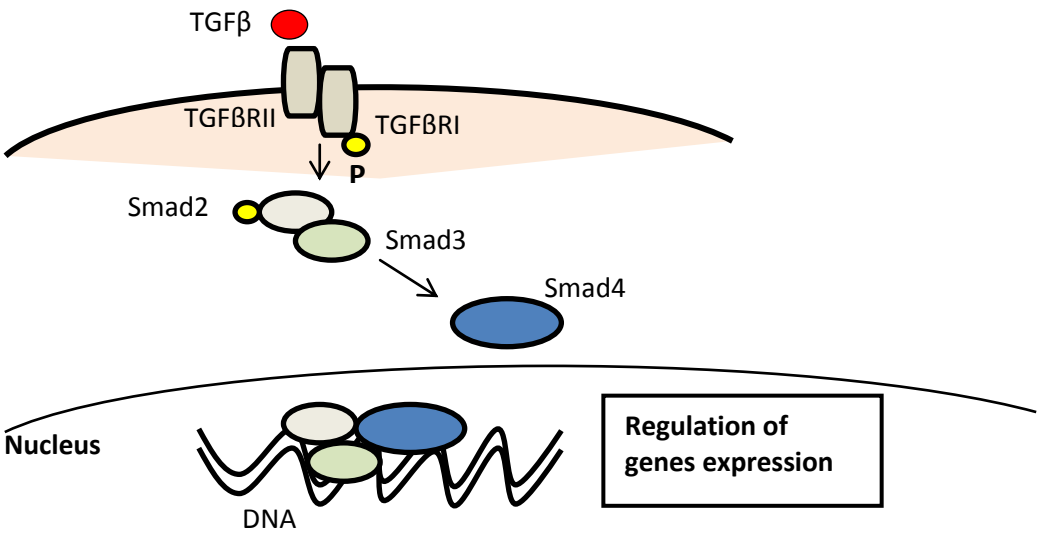


Figure 1.4 Smad dependent TGFβ signaling

The active form of TGFβ binds to TGFβRII which can then recruit TGβRI and phosphorylate it. The activated TGFβRI can then phosphorylate Smad2 and Smad3, which together can bind to Smad4 forming a complex that is shuttled to the nucleus. The complex can bind to Smad binding sites on DNA and allow the binding of various proteins that can ultimately regulate the transcription of a vast number of genes.

1.4.1.3 Biological activity of TGFβ

TGFβ is a pleiotropic cytokine that plays various roles in embryonic and adult tissue homeostasis (Massague 2008) (Akhurst and Hata 2012). One of its main functions is inhibiting the cell proliferation of various cell types including epithelial, endothelial, and immune cells. This is due to its ability to regulate the expression of various factors that control cell cycle arrest, cell differentiation, and/or induce apoptosis. The cytokine can also act as a chemotactic factor for cells such as macrophages, lymphocytes and fibroblasts. TGFβ plays an essential role in

stimulating extracellular matrix (ECM) production by mesenchymal cells. It is therefore thought to regulate the overall process of wound healing, starting by the recruitment of inflammatory cells such as macrophages, to inducing angiogenesis and stimulating ECM production by fibroblasts, to finally ensuring proper tissue remodeling (Faler 2006).

However, one of the main characteristics of TGFβ is its ability to ensure immune homeostasis. As discussed later the cytokine acts as a potent immune suppressor which alters the function of both the innate and adaptive immune system. It's role in immune homeostasis had become more clear with studying the biology of TGFβ in knockout mice and transgenic models (Bottinger, Letterio et al. 1997). The predominant phenotype of the TGFβ1 knockout mice confirmed the major role TGFβ plays in immune regulation. The knockout model is characterised with an extensive inflammatory response and an infiltrate of leukocyte in various organs. This multifocal inflammatory disease results in various cardiopulmonary complications resulting in rapid wasting and death by ~ the 4^{th} week of life (Shull, Ormsby et al. 1992, Kulkarni, Huh et al. 1993, Bottinger, Letterio et al. 1997). The model is also characterised by circulating anti-ds DNA and anti-ssDNA , an increase in the number of activated cells in lymphoid organs, and an increase in MHCI and MHCII antigen expression, suggesting an autoimmune phenotype (Bottinger, Letterio et al. 1997, Prud'homme and Piccirillo 2000).

Thus studies from the knockout TGFβ model have demonstrated the critical role for TGFβ in autoimmunity and immune tolerance.

1.4.1.4 TGFβ in cancer

Despite the tumour suppressive function TGFβ can play by inhibiting cell proliferation, the pleiotropic cytokine can have a pro-tumorigenic effect, and tumours can evade the tumour suppressing effect of TGFβ. This can be achieved by inactivating components of the TGFβ-Smad pathway, for instance TGFβRII is mutated (inactivated) in most of the human gastrointestinal tumours with microsatellite instability. Alternatively, TGFβ can be pro-tumourigenic by disabling the tumour suppression arm of the signalling pathway. If tumour cells lose the autocrine anti-proliferative effect of TGFβ, they continue to make use of its paracrine effect and its ability to alter various stromal cells to induce angiogenesis, metastasis, invasion, and immunosuppression. Tumour cells can specifically disable the suppressive arm of TGFβ signalling, but they maintain autocrine signalling to induce the expression of protumourgenic factors, mitogenic factors, and to acquire invasive abilities (Massague 2008, Ikushima and Miyazono 2010, Akhurst and Hata 2012).

Another strong link between TGFβ and cancer lies in its ability to promote invasion and metastasis, primarily due to TGFβ's strong induction of EMT and a myofibroblastic phenotype. Such a phenotype results in the loss of the cell-cell contact normally seen in epithelial cells, and the generation of a fibroblast-like cell that is able to migrate more freely. The EMT induced by TGFβ is thought to occur as a result of the ability of TGFβ to induce SNAIL and SLUG transcription factors which strongly suppress E-cadherin, an essential protein for epithelial cells adhesion and cell to cell contact (Massague 2008, Akhurst and Hata 2012).

TGFβ is also a strong regulator of ECM production. It is thought to increase collagen production and collagen deposition by fibroblasts and it regulates the expression of various genes that are responsible for ECM protein production. It is therefore not surprising that vast number of TGFβ targeting therapeutics have been used for the treatment of fibrotic diseases that are characterised by excessive ECM production and deposition (Massague 2008, Akhurst and Hata 2012).

Perhaps one of the most important tumour-promoting effects of TGFβ results from its ability to suppress various innate and adaptive immune cells, which is discussed in more details in the next section. Based on the pleiotropic and plastic feature of TGFβ, it is clear that defining the

tumour-promoting effect at different stages of the tumour development and how this might differ between cancer cells of different tissue origin is essential for determining when therapeutic targeting of TGFβ would be beneficial and what selection criteria is required for patients.

1.4.1.5 TGFβ a potent immunosuppressor of the innate and adaptive immunity

TGFβ is a potent immunosuppressive cytokine (Flavell, Sanjabi et al. 2010, Yang, Pang et al. 2010). Secreted by both tumour cells and immune cells, it can alter the maturation, activation and differentiation of innate and adaptive immune cells (Figure 1.5). In regards to innate immune cells, TGFβ was shown to inhibit NK cells proliferation and function and attenuate IFNγ production by those cells directly through Smad3 (Flavell, Sanjabi et al. 2010) or indirectly through Tregs activation (Yang, Pang et al. 2010). TGFβ inhibits NKP30 and NKG2D expression on NK which represent NK activating receptors ((Yang, Pang et al. 2010) and neutralizing TGFβ was shown to restore NK anti-tumour effects. TGFβ has also been implicated in the ability to polarise innate immune cells, including macrophages and neutrophils, toward the immunosuppressive phenotypes M2 and N2 respectively, both of which can promote tumourigenesis through mediating angiogenesis and metastasis (Yang, Pang et al. 2010). TGFβ is also known to be an important factor for the recruitment of MDSCs to tumour sites, which in turn have an immunosuppressive effect on the tumour (Bierie and Moses 2010). The cytokine has also shown a pivotal role in DCs function, affecting its mobilization to the lymph nodes for antigen presentation. TGFβ can also alter the maturation of DCs as DCs exposed to TGFβ show a decrease in their expression of MHCII expression, co-stimulatory molecules CD40, CD80, CD86, and immunostimulatory cytokines such as IL-2 and IFNγ (Flavell, Sanjabi et al. 2010).

Furthermore TGFβ is thought also to suppress the adaptive immune system. It can stop CD8+ cells proliferation and reduce the transcription of perforins and granzymes secreted by these cells (Bierie and Moses 2010, Flavell, Sanjabi et al. 2010, Yang, Pang et al. 2010). TGFβ is also known to be a strong inducer of Foxp3 expression and consequently the generation of Tregs. In the presence of IL-6, TGFβ can also induce Th17 cells from CD4+ which are known to express IL-17 and have a controversial role in tumours regarding whether they can instigate a chronic inflammatory response mediating tumour formation (Yang, Pang et al. 2010).

Overall, TGFβ has a strong ability to polarise various immune cells, which can ultimately dampen anti-tumour responses. With the strong immunosuppressive functions of TGFβ it is clear that inhibiting TGFβ signaling is a desirable approach in cancer.

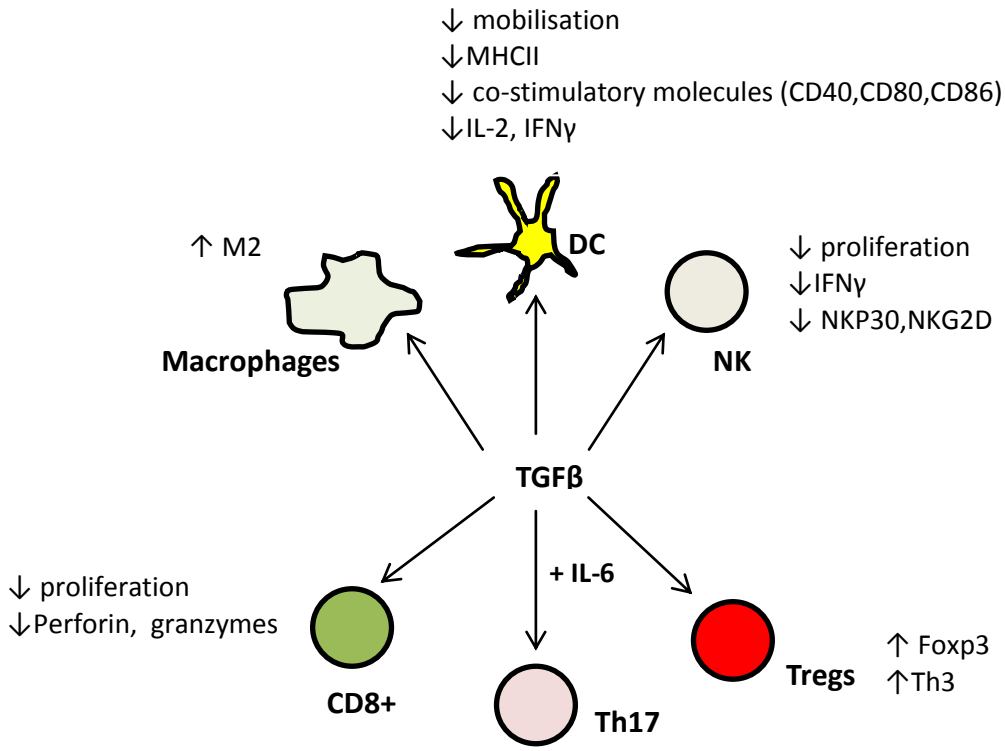


Figure 1.5 The effect of TGFβ on the innate and adaptive immune system

TGFβ exerts suppressive effects on the innate and adaptive immune systems. It decreases DCs function and maturation by decreasing the expression of MHCII, co-stimulatory molecules, and immunostimulatory cytokines. It induces an M2 phenotype in macrophages. It decreases NK proliferation and the expression of NK stimulatory molecules. In the adaptive immune system, it can induce Foxp3 expression and induce Th3 cells (a Tregs subtype).In the presence of IL-6, it induces TH17 differentiation and it can decrease CD8+ function by suppressing proliferation and decreasing the expression of cytotoxic proteins such as perforins and granzymes.

1.4.1.6 Targeting TGFβ signaling

1.4.1.6.1 TGFβ blockade strategies

The importance of TGFβ in cancer progression as described in the section 1.4.1.3 has led to the investigation of several strategies that aim to target TGFβ signaling in neoplastic diseases over the past decade. These strategies have been studied extensively in preclinical models and some have reached the clinics for treatment of neoplastic and non-neoplastic disorders (Akhurst and Hata 2012).

The strategies for antagonising TGFβ signaling can be divided into four categories, which are: anti-ligand antisense oligonucleotides, antibodies that target TGFβ ligands or receptors, small molecule inhibitors targeting the signaling pathway, or ligand traps that competitively inhibit binding of ligand to receptors. Each of these strategies has advantages, and limitations, which will be briefly summarized below.

Anti-TGFβ antisense oligonucleotides

Anti-sense oligonucleotides (ASO) have been developed to target TGFβ mRNA translation and thus ligand synthesis. The main focus has been on TGFβ2 in glioblastomas and pancreatic carcinoma, and has now reached phase II clinical trials (Akhurst and Hata 2012, Buijs, Stayrook et al. 2012). The main challenge with this strategy remains to be achieving effective local delivery methods to avoid loss of dose and off targets effects that result from systemic delivery. Another strategy employing ASO has been in the context of a tumour vaccine. NSLC cells genetically engineered to express an anti-TGFβ2 anti-sense constructs have been used as a tumour vaccine and have shown superior activity as a vaccine and enhanced survival in clinical trial (Nemunaitis, Dillman et al. 2006).This approach overcomes problems of delivery, which together with its promising clinical effects in early-stage trials have allowed it to progress to phase III in clinical trials.

Anti-ligand and anti-receptors antibodies

A range of mAb against TGFβ ligands or TGFβ receptors have developed (Akhurst and Hata 2012). This approach has the advantage of blocking extracellular ligands, which is important in the context of cancer where several cell types secrete the ligand. The agent most advanced in clinical trials is the pan-ligand humanised antibody; fresolimumab, which is currently in clinical trials for non-neoplastic disorders (Trachtman, Fervenza et al. 2011). An antagonising anti TGFβRII antibody has also entered clinical trials for breast and colon carcinoma (Akhurst and Hata 2012).

Small molecules inhibitors

A group of small molecules inhibitors exist, mainly against TGFβRI. The advantage of these drugs is their availability for oral administration and chemical stability. However, they remain to be sub-optimal due to their non-specificity. The drugs have shown preclinical success in metastatic carcinoma and have entered clinical trials.(Akhurst and Hata 2012)

Ligand traps

Ligand traps that competitively inhibit ligand binding to receptors are also an attractive approach for blocking TGFβ signaling. An example is the STGFβRII which is extracellular domain of the TGFβRII. Due to the relevance of this protein in this thesis, it will be discussed in more details in the next section. Another form of ligand trap is a peptide that mimics TGFβRIII and has been tested in clinical trials for fibrotic diseases such as scleroderma and skin fibrosis (Akhurst and Hata 2012).

Other approaches that have also been proven successful in targeting TGFβ signaling including the expression of antagonising signaling molecules such as Smad7. However, a very interesting approach, which also acted as proof of principle of the inhibitory effect TGFβ has on CTL, was generating a TGFβ unresponsive CTL that expressed a dominant negative TGFβRII. The genetically engineered cell line was shown to exceed unmodified CTL in clearing Epstein-Barr virus positive lymphoma, and is currently in a Phase I clinical trial (Foster, Dotti et al. 2008).

1.4.1.6.2 The STGFβRII

Human and Mouse TGFβRII is comprised of a TGFβ binding extracellular domain, a transmembrane domain, and a cytoplasmic domain which initiates the downstream signalling cascade (Sean Lawler and Derynck 1994). The STGFβRII can bind to TGFβ acting as truncated receptor, and thereby competitively preventing TGFβ signaling through the wild type receptor (Tsang, Zhou et al. 1995). The molecule is known to have strong affinity to TGFβ and has been used in several preclinical studies for targeting TGFβ signaling in tumour models (Zhao, Kobayashi et al. 2002, Hu, Gupta et al. 2012, Zhang, Hu et al. 2012) . The molecule has several advantages as a strategy for blocking TGFβ. It allows the same specificity seen with mAb yet holds a further advantage of being a small protein that can be distributed more efficiently if expressed in tumours.

Several preclinical studies have shown an anti-tumour activity associated with the expression of STGFβRII. For example, rat hepatoma cells (Zhao, Kobayashi et al. 2002) and mouse pancreatic carcinoma (Rowland-Goldsmith, Maruyama et al. 2002) cell lines engineered to express the STGFβRII have shown significant reduction in tumour growth and an increase in animal survival. The influence of another form of the STGFβRII protein comprised of the STGFβRII fused to the mouse or human IgG1 Fc portion has also been tested . The chimeric

protein has showed similar properties to the original receptor in terms of binding affinity for TGFβ (Sylviane Komesli 1998). The rationale of Fc fusion proteins comes from the ability of Fc to enhance the stability of proteins, allowing an increase in serum half-life of proteins *in vivo* (Huang 2009). Systemic administration of oncolytic adenoviruses expressing STGFβRII-Fc has shown the ability to reduce bone metastasis in both xenografts (Rowland-Goldsmith, Maruyama et al. 2002), and syngeneic breast cancer models (Zhang, Hu et al. 2012).

Together these studies show STGFβRII to be a successful method for blocking TGFβ signaling.

1.4.1.6.3 Challenges with Targeting TGFβ

Despite the various preclinical and the few clinical trials evaluating TGFβ blockade as means of anti-tumour therapy, no clear clinical benefits have been seen (Akhurst and Hata 2012). One of the main problems of these studies is the limited immunological endpoints, which makes it hard to interpret the minor clinical response seen (Flavell, Sanjabi et al. 2010, Akhurst and Hata 2012). Given the pleiotropic effect of TGFβ it is important to be able to identify the main mechanisms by which TGFβ blockade results in anti-tumor responses. From an immunotherapy perspective, categorising when is the best time to block TGFβ in established tumours in coordination with a tumour vaccine or other immunotherapeutic would also assist in gaining the best results from reversing TGFβ induced immunosuppression .

Furthermore blocking TGFβ locally would still be the preferred approach. This would allow avoidance of potential toxicities such as autoimmunity in addition to other toxicities that can be anticipated from blocking such a crucial factor in tissue homeostasis. Designing better methods to deliver specific TGFβ inhibitors locally within the tumour remains an important challenge (Flavell, Sanjabi et al. 2010, Akhurst and Hata 2012).

1.4.2 IL-10

One of the other important soluble immunosuppressive factor that is thought to play a role in the tumour local environment is IL-10 (Motz and Coukos 2013). IL-10 has been identified as an important anti-inflammatory cytokine that is secreted by a number of immune and non-immune cells. IL-10 belongs to the type II cytokine family which includes other cytokines that share similar structural components and signals through activating the signal transducer and activator of transcription 3 (STAT3) system (Mosser and Zhang 2008). Human IL-10 is found as a non-

covalent homodimer and has 73% homology with the mouse form (Sato, Terai et al. 2011). The cytokine signals through the IL-10 receptor complex, which is comprised of heterodimers of the IL-10 receptor 1 (IL-10R1), and IL-10 receptor 2 (IL-10R2). IL-10R1 binds to IL-10 with strong affinity (50-200 pM) (Mosser and Zhang 2008) and is expressed on immune and non-immune cells in low quantities which can be up-regulated upon stimulation. The binding results in conformational changes in the receptor allowing it to dimerise with IL-10R2. The formation of the heterodimer complex allows downstream signal transduction to occur. The complex can activate Janus Tyrosine kinases and Tyrosine kinase 2 proteins which can then result in the recruitment of STAT3 to IL-10R1, and activation of STAT3. Once STAT3 is homodimerised it can be released from IL-10R1, translocate into the nucleus and regulate the transcription of various genes by binding to STAT binding sites on gene promoters (Mosser and Zhang 2008, Sato, Terai et al. 2011)

In common with TGFβ, IL-10 plays a significant role in immune regulation (Mosser and Zhang 2008, Saraiva and O'Garra 2010, Sato, Terai et al. 2011) (Figure 1.6). It plays an important role in regulating inflammation by decreasing the production of pro-inflammatory cytokines from macrophages and DCs such as IL-1, IL-6, IL-12, TNFα, and GM-CSF. IL-10 can inhibit successful antigen presentation and thus T cell function and activation; it decreases the expression of MHCII antigens and co-stimulatory molecules on DCs, and can decrease the differentiation and maturation of DCs. It therefore ultimately results in a decrease in Th1 cytokine production (IL-2, TNFα, and IFN-γ). IL-10 is secreted by Tregs and is one of the main mechanisms by which Tregs induce their immunosuppressive functions. It can also induce the generation of an inducible Tregs population Tr1 which in turn secrete more IL-10 (Saraiva and O'Garra 2010).

However, the data from several models studying the role of IL-10 in tumours has been controversial showing various diverse effects depending on the experimental model used (Mocellin, Marincola et al. 2005). Some reports have shown that tumour infiltrating DCs were incapable of being activated *in vitro*, however, in the presence of anti-IL-10 antibody and immunostimulatory CpG this tumour induced DCs suppression was reversed. The combination also showed significant anti-tumour effect when administered *in vivo* (Vicari, Chiodoni et al. 2002*)* . While other reports have shown that IL-10 was essential for immunosurveillance and

immune eradication of endogenous skin tumours, and appeared to increase granzymes and IFNγ expression from tumours specific CD8+ T cells (Mumm, Emmerich et al. 2011) (This was also associated with an increase in tumour incidence in IL-10 $^{-/-}$ mice. Thus supporting the argument that IL-10 can augment anti tumour immunity through various mechanisms (Mumm, Emmerich et al. 2011).

Due to its varying biological functions further understanding and investigation of the effect of IL-10 within the local tumour environment is required. Models that can address the influence of IL-10 during different stages of tumour development can address some of this complexity. For example, one could hypothesise that the pro-tumour immunity effect of IL-10 is linked to the early effect on the recruitment and function of innate immune cells For instance IL-10 can decrease macrophage recruitment which might favor a decrease in the number of TAM within the tumour and thus stop tumour progression. Conversely, the tumour-promoting effects can be linked to suppressing T cell function within the tumour by IL-10 secreting Tregs.

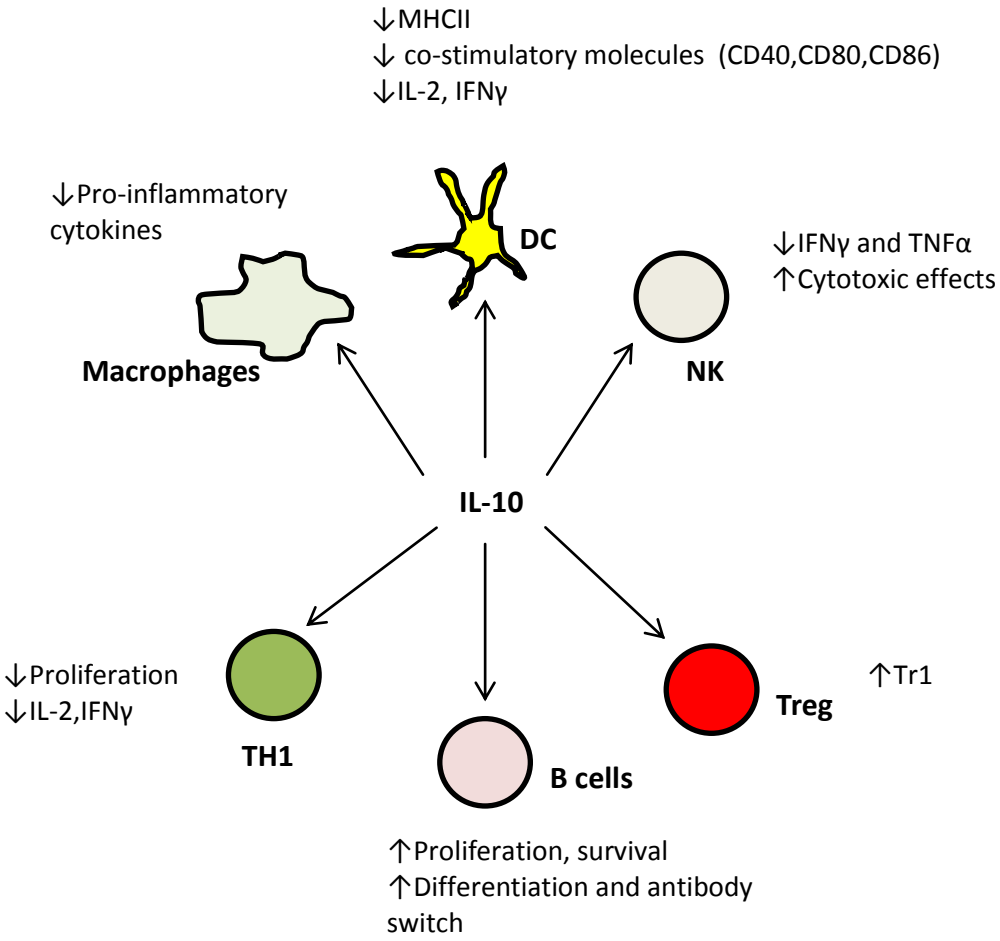


Figure 1.6 The effect of IL-10 on the innate and adaptive immune system

IL-10 decreases DCs function and maturation by decreasing the expression of MHCII, co-stimulatory molecules, and immunostimulatory cytokines. It is secreted my macrophages and can suppress the secretion of pro-inflammatory cytokines from these cells. Despite *invitro* studies showing the ability of IL-10 to decrease IFNγ and TNFα secretion from NK cells, the cytokine can activate NK and enhance their cytotoxic ability *invivo*. In the adaptive immune system, it can induce Tr1 cells (a Tregs subtype).It increases B cells proliferation, survival, differentiation and antibody switching. Finally, the cytokine is thought to inhibit a TH1 phenotype and decrease IL-2 and IFNγ production by these cells.

1.5 The Tetracycline controlled transcriptional regulation system

1.5.1 Introduction

Gene therapy is the delivery of genetic material into host cells to achieve therapeutic benefit. A wide range of vectors have been used to deliver genetic material in preclinical and clinical settings, the majority are adenoviruses, retroviruses, or naked DNA. To overcome the limitations of studying gene regulation and function with models using constitutive gene expression, there has been much interest in developing models where gene expression is regulated. The most commonly used inducible gene expression models is the Tetracycline controlled transcriptional regulation system (Tet system) (Goverdhana, Puntel et al. 2005) which allows tight regulation of gene expression under Dox control. If used in the context of immunotherapy and the targeting of local soluble immunosuppressive factors, it would allow the expression of soluble local antagonists only on demand, for example only after tumours have been established in mice. Such tumours can be given the chance to grow and establish their stroma and immune cell infiltrate, more closely resembling the clinical situation in humans, before studying the effect of immune modulating factors on them. An interesting model is the Lenti-X Tet-on system which uses the highly efficient lentivirus based vector to deliver tightly regulated genes of interest under the Dox control (Clontech). In this section the rationale for using an inducible gene expression model and the use of the Tet system in particular is explained. In addition, a description of the Tet system mechanism of action and its use in the context of a lentivirus based gene delivery is also included.

1.5.2 Inducible gene expression

Gene transfer has been a useful approach for studying gene and protein function, and for providing effective strategies to treat and manage a wide range of diseases (Guo, Li et al. 2008). However, it is becoming increasingly apparent that systems which are reliant on constitutive expression of transgenes will provide an inappropriate model for many heterogeneous, dynamic, and complex human diseases. Indeed, modeling the role of a few

selected proteins in the interplay between a tumour and the immune system represents a particularly difficult challenge. It was therefore necessary to establish regulated gene expression systems to allow refined temporal and spatial control of genes expression (Guo, Li et al. 2008). The ability to tightly control the time of gene expression permitted the production of a wider array of proteins, including those with a low therapeutic index, that otherwise would be lethal and/or toxic if expressed continuously (Goverdhana, Puntel et al. 2005). This system can also address questions regarding the reversibility of a transgene's biological effects to be addressed. Furthermore, as many immunomodulatory proteins have different effects at different stages of tumour development, the ability to switch expression on and off may help characterise what role a protein may have during a particular stage of disease development, which could have important implications for clinical therapy. Regulated gene expression could also avoid the generation of compensatory responses to the effects of a transgene product, a problem otherwise seen with continuous expression of genes. For all these reasons, great progress in generating a variety of regulated expression systems has been made and despite their limited implementation in human patients (Guo, Li et al. 2008) they have proved successful in many applications in vitro and in pre-clinical models (Stieger, Belbellaa et al. 2009).

In the context of this thesis, an inducible system allows in situ expression of immunomodulatory proteins of interest only after normal physiological development and establishment of the tumour with surrounding stroma and immune cells and also enables expression to be switched on and off on demand. This facilitates characterisation of the effect of local antagonism of soluble immunosuppressive molecules on tumour-induced immune tolerance at different stages of tumour growth, and would allow prediction of the biological activity of protein-based therapeutics that may be expressed using gene therapy approaches.

1.5.3 Tet system

1.5.3.1 Advantages of the Tet system

There are several inducible systems currently available, however, the Tet system remains the most frequently used, and has been proven to be successful in various models. The system has several useful features, including tightly regulated gene expression with very low basal level of

expression in the ‘off’ state but with high levels of expression in the ‘on’ state, giving it a wide dynamic range of expression. Unlike other systems like the Cre-recombinase inducible system, it is reversible once the inducer is removed and the levels of the induction can be adjusted by varying the dose of the inducer of expression Dox (a Tetracycline derivative) (Zhu, Zheng et al. 2002, Goverdhana, Puntel et al. 2005). Another advantage of the Tet system is that Dox is widely available, can be administrated orally, is relatively cheap, and shows no undesirable side effects. It also has good bioavailability and has a reasonable half-life (Zhu, Zheng et al. 2002, Goverdhana, Puntel et al. 2005). For these reasons, the Tet system is an ideal strategy to enable regulated induction of the expression of antagonists of soluble immunosuppressive mediators *in vivo*.

1.5.3.2 Mechanism of action of the Tet system

The Tet system is based on the E coli Tn10 Tetracycline resistance operon. In the absence of Tetracycline, the Tet repressor protein (TetR) in E coli maintains the off-state of tetracycline resistance genes on the Tn10 transposon, through binding to the tet operator (tetO) sequence. However, in the presence of tetracycline, TetR protein undergoes conformational changes causing its dissociation from the tetO sequences, and therefore allowing the transcription of tetracycline resistance genes.

Similarly, the Tet system consists of the same three elements: a regulatory protein, a responsive element linked to the genes of interest, and an inducer. There are two variants of the Tet system, the Tet-off and the Tet-on system. In the Tet-off system the regulatory element, referred to here as TetR, is a fusion of the DNA-binding domain TetR with the transcription activator domain of virion protein 16 of herpes simplex (VP16). The responsive element is mainly the gene of interest under the control of the Ptight promoter which is the minimal promoter sequence of the human cytomegalovirus promoter (Pcmv) combined with the tetO sequence. In the absence of Dox, the TetR binds to the tetO sequences and activates the Ptight promoter which in turn induces the expression of the target gene. If Dox is added the TetR undergoes a conformational change and is released from the tetO sequence and transcription is

stopped. Thus the addition of Dox switches the system off and stops the transcription of genes of interest.

However, some of the drawbacks of the Tet-off system led to the development of the Tet-on variant. In contrast to the Tet-off system the Tet-on variant is a mutated form of the TetR protein that can only bind to the tetO sequence in the presence of Dox, therefore, the system is switched on in the presences of Dox. The Tet-on system therefore avoids any undesirable effects that can result from long exposure of Dox to maintain a switched off state. In addition, the induction of gene expression with the Tet-on system responds rapidly to the addition of Dox, rather than depending on the clearance of the drug from the circulation as is required with the Tet-off system.(Zhu, Zheng et al. 2002, Goverdhana, Puntel et al. 2005, Guo, Li et al. 2008, Stieger, Belbellaa et al. 2009)

1.5.4 Lentivirus vectors

Lentivirus vectors have been used as effective delivery vehicle for gene therapy. Their ability to infect both dividing and non-dividing cells and their ability to accommodate large transgenes have made them one of the vectors of choice in biological research (Matrai, Chuah et al. 2010). Lentiviruses are RNA retroviral based vectors (HIV-1), which similar to retroviruses have the ability of integrating their genome into the host genome, thus allowing stable long term gene expression. In addition to retroviruses basic components, lentiviruses have additional regulatory and accessory genes that regulate their virus life cycle and infectivity (Lever 2006).

Lentiviruses have proteins and enzymes enabling them to incorporate their genome into the infected cell genome. They express the reverse transcriptase enzyme which can reverse their RNA genome into double stranded DNA, which is then referred to as provirus. They also express the integrase enzyme which helps in translocating the provirus into the nucleus and integrating it into the host genome. The provirus DNA is composed of identical long terminal repeats with a promoter activity at the 5’ end and a polyadenylation signal at the 3’ end and three main ORF that comprise the gag, pol and envelope genes. The Gag gene is responsible for the production of core structural proteins, while the pol gene encodes all the important enzymatic proteins such as reverse transcriptase and integrase. Finally, the env gene encodes the envelope glycoproteins of the virus which are required for binding to the cellular receptor

(Lever 2006, Cockrell and Kafri 2007). Substitution of the HIV-1 native glycoprotein with other glycoporteins to enhance tropism have led to the development of pseudotyped lentivriuses vectors, the most common strategy being to pseudotype with the vesicular stomatitis virus G protein. In addition to those major components, lentiviruses have a number of accessory regulatory genes; Tat, Rev, Nef, Vpr, Vpu, Vif which play a role in the infectivity of wild type lentiviruses (Lever 2006, Cockrell and Kafri 2007, Matrai, Chuah et al. 2010).

Lentivirus vectors have developed through several generations to ensure their biosafety and to eliminate the possibilities of generating replication competent lentivriuses. This has been achieved mainly by generating packaging cassettes that have the gag/pol/ rev genes placed on two separate expression cassettes. This together with a separate envelope cassette has resulted in increasing the minimal numbers of recombination events required to generate a RCL (Matrai, Chuah et al. 2010).

To generate a replication incompetent lentivirus, the envelope and the packaging expression cassettes are transfected with the vector expression cassette into a packaging cell line. A vector is then produced that can be used to infect cells of interest only once, and allow the expression of transgene in those cells.

1.5.5 Lenti-X-Tet-on advanced system

The Lenti-Tet-on is an inducible gene expression system. It utilises the Tet system in highly efficient lentivirus vectors. It can therefore allow long term gene expression of genes of interests under a Dox controlled inducible system (Clontech). The system is explained in more details in Chapter 3.

1.6 Thesis Objectives

1.6.1 Introduction

Limitations to successful anti-tumour immunity arises from the local tumour induced immunosuppressive mechanisms and immune-checkpoints that hamper natural and vaccine induced tumour immune responses. Validation of immunosuppressive therapeutic targets within the tumour microenvironment is currently an area of interest. One of the hurdles is the limitation of tumour models that would allow studying the effects of antagonising various soluble immunosuppressive factors locally both in early, and in well-established tumours representative of the clinical situation.

1.6.2 Hypotheses

A strategy that allows inducible expression of immune-checkpoint inhibitors from genetically engineered cells within tumours under the Tet-on system and Dox control would give precise answers to questions related to tumour induced immunosuppression, and may allow validation of new immunosuppressive therapeutic targets within the tumour microenvironment

We hypothesise that this model can validate whether antagonising the soluble immunosuppressive cytokine TGFβ locally in the tumour can reverse acquired immune tolerance and restore tumour immunity. We also hypothesise that this would suppress tumour growth in mice and prolong mice survival.

1.6.3 Aims

- Establish and assess the Lenti-X-Tet-on system *in vitro* and *in vivo*
- Establish and characterize a CT26 cell line expressing antagonists of immunosuppressive molecules under the Lent-X-Tet-on system
- Characterise the effects of TGFβ blockade (by the inducible STGFβRII) on tumour growth and tumour immunity
- Charactersise the effects of TGFβ blockade in combination with CTLA-4 blockade.

2 Materials and Methods

This chapter describes in detail the development of methods used for the experimental studies in this thesis.

2.1 List of solutions

2.1.1 Cell lines and tissue culture solutions

Dulbecco's Modified Eagle's Medium (DMEM, PAA-Laboratories): 4 mM L-glutamine, 4500 mg/l glucose, 1 mM sodium pyruvate, 1500 mg/l sodium bicarbonate, Roswell Park Memorial Institute-1640 (RPMI-1640, PAA-Laboratories): Contains 300 mg/l L-glutamine and Eagle's Minimum Essential Medium (ATCC): Earle's Balanced Salt Solution, non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, and 1500 mg/l sodium bicarbonate

2.1.2 Cloning solutions

LB medium: Luria-Bertani broth base (LB, 20 g/l, Sigma-Aldrich) in deionized water (dH2O) was autoclaved and stored at 4°C. LB agar: LB broth agar (32 g/l, Sigma-Aldrich) in dH2O was autoclaved and stored at 4°C

2xYT broth medium:

Element	volume
Bacto-tryptone	16 g
Bacto-yeast extract	10 g
NACL	5 g
dH2O	800 ml
pH 7 with 5 M NaOH up to 1 L with dH2O Autoclaved and stored at 4°C	

Transformation buffer I (TFBI):

Element	volume
Potassium acetate (30mM)	589 g
Rubidium chloride (100mM)	2.418 g
Calcium chloride dihydrate (10mM)	0.294 g
Manganese chloride tetrahydrate (50mM)	1.939 g
Glycerol (15% v/v)	30 ml
pH 5.8 with 0.2 M acetic acid up to 200 ml with dH2O Autoclaved and stored at 4 °C	

Transformation buffer II (TFBII):

Element	volume
MOPS (10mm)	0.419 g
Rubidium chloride (10mM)	0.242 g
Calcium chloride dehydrate (10mM)	2.2.05 g
Glycerol (15% v/v)	30 ml
pH 6.6 with 5 M NaOH made up to 200 ml with dH2O	
Autoclaved and stored at 4 °C	

TAE buffer (100x):

Element	volume
Tris base	4.84 g
Glacial acetic acid	1.09 ml
EDTA	0.29 g
Made up to 1L in dH2O, diluted 1x with dH2O before use	

2.1.3 Sodium dodecyl sulfate (SDS) page and western blotting solutions

1.5 M Tris HCl pH 8.8: 27.23 g Tris hydroxyl methylamine (Tris base, Fisher Chemical), 80 ml dH2O. Adjusted pH with 6M HCl, and made up to 150 ml with dH2O.
1 M Tris HCl pH 6.8: 12 g Tris base, 60 ml dH2O, adjusted pH with 6M HCl and made up to 100 ml with dH2O
10%SDS: 10 g sodium dodecyl sulfate (SDS, Sigma Life Science), 90 ml dH2O, and made up to 100 ml of dH2O
10% Ammonium persulphate: 100 mg Ammonium Persulphate (Sigma-Aldrich), 1 ml of dH2O. Made fresh for every time experiment

Sample buffer (3x)

Element	volume
1 M Tris HCL 6.8	1.5 ml
Glycerol (Sigma-Life Science)	2.4 ml
10% SDS	4.8 ml
B-mercaptoethanol	1.2 ml
Bromophenol blue	80 mg
Stored at 4°C	

Running buffer (5x)

Element	volume
Tris base	9.9 g
Glycine (Sigma-Life Science)	43.2 g
SDS	3.9 g
dH2O	600 ml
pH with HCL to 8.3	
Stored at 4°C, and use 1X diluted in dH2O	

Transfer buffer:

Element	volume
Tris base	3.02 g
Glycine	14.4
dH2O	600 ml
Methanol (Sigma-Aldrich)	200 ml
pH with HCL to 8.3	
Stored at 4°C, and use 1x diluted in dH2O	

Antibody stripping buffer: 1,504 g Glycine, 1% SDS, in 100 ml of dH2O, pH to 2.5 with HCL

Phospho-proteins lysing buffer:

Element	volume
1% NP40 (1ml NP40 (Sigma-Aldrich),5 ml 1M Tris HCL, ph 7.4, 3 ml 5M NaCl, 200µl of 0.5M stock)	10 ml
1 mM NaVO4 (activated by heating 100°C,5 min)	50 µl
1mM NaF	50 µl
1 Tablet protease inhibitor	
Stored at 4°C	

2.2 Cell lines and tissue culture

2.2.1 Cell lines and cell passage

Cell lines were cultured under sterile conditions in a class II laminar-flow cabinet, and maintained in appropriate growth media (Table 1.1) containing 10% fetal calf serum (FCS, PAA Laboratories) or 10% Tet-system approved FCS (FCS, Clontech) and 1% penicillin (104 units/ml) and streptomycin (10 mg/ml) (Sigma-Aldrich). This full growth medium is referred to as medium in the text. Cells were cultured in a humidified incubator at 37°C and 5% CO_2. When confluent (80-90%) cells were split after washing with phosphate-buffer saline (PBS, PAA Laboratories) and incubated with 2 mls of trypsin EDTA (T/E, Sigma-Aldrich) for 5 minutes (min) at 37°C and 5% CO_2. Once cells were detached from the surface, medium (3 times the amount of trypsin) was added to deactivated T/E activity, and the cell suspension together with fresh medium was transferred to new flasks.

2.2.2 Storage of cell lines

To store cells, medium was removed and cells were washed with PBS then incubated with T/E for 5 min until cells were detached. Cell suspension was then centrifuged at 1,250 revolutions per minute (rpm),(300 relative centrifugal force (rcf) at room temperature (RT),for 5 min. Supernatant was removed and cell pellet was re-suspended in 1 ml of freezing medium (5% sterile dimethyl sulphoxide DMSO, Sigma-Aldrich, 95% medium) and transferred to cryovials which were stored overnight in Mr. Frosty container (Nalgene) at -80°C before transferred to storage boxes at -80°C. In order to thaw stored cells, frozen cells cryovials were thawed at 37°C in a water bath and quickly transferred to tubes with pre-warmed medium. The tubes were then spun at 1,250 rpm, at RT, for 5 min to remove any excess DMSO, and the cell pellet was suspended in fresh medium and transferred to a T25 flask. 24 hours (hrs) later medium was removed, cells were washed with PBS and cultured in fresh medium until ready for passage.

Table 2.1 Cell line and growth medium

Cell line	Obtained from	Description	Growth medium
293T	A gift from the Jenner institute (University of Oxford)	Human embryonic kidney cells 293 cell variant containing the SV40 large T antigen	DMEM,10%FCS,P/S
Mv1Lu	American Type Culture Collection (ATCC)	Mink lung epithelial cells	EMEM,10%FCS,P/S
RAW 264.7	A gift from Prof Ruth Muschel lab (University of Oxford)	Mouse macrophages, Abelson murine Leukemia virus transformed	DMEM,10%FCS,P/S
CT26	ATCC	Mouse colon carcinoma	RMPI-1640, 10%FCS,P/S
CT26TetR	Generated in the lab	Expresses TetR protein	RMPI-1640, 10% Tet free FCS,P/S
CT26PLUC	As above	Expresses TetR and luciferase under the PTight promoter	As above
CT26STGFβRII	As above	Expresses TetR and soluble TGFβRII under the PTight promoter	As above
CT26SIL-10R	As above	Expresses TetR and SIL-10R under the PTight promoter	As above

2.2.3 Cell plating

To seed cells for various assays, a cell suspension was first prepared as described previously in section 2.2.2 and centrifuged at 1,250 rpm,(300 rcf) at RT, for 5 min. The concentration of cells was then determined by counting cells using a haemocytometer and the final concentration required was achieved by addition of the appropriate amount of medium. Cells were then seeded into well plates appropriate to the particular assay and kept in the incubator over-night before any further treatment or analysis.

2.3 Cloning assays and strategy

2.3.1 Preparation of bacterial medium and agar

Bacterial medium and Agar plates were prepared using the LB medium and LB agar respectively (see section 2.1.2Both LB medium and LB agar were sterilised by autoclave. Once cooled, ampicillin (100 µg/ml, Sigma-Aldrich) was added for colony selection. LB agar was poured in 10 cm dishes and together with LB medium stored at 4°C.

2.3.2 Preparation of heat competent bacterial cells

XL-10 Gold Ultracompetent E.coli cells (Agilent technologies, UK) were used to prepare heat shock competent bacterial cells. Briefly, a single colony of the E.coli cells was first cultured in 3 ml of the 2xYT broth medium (see section 2.1.2) at 37°C in bacterial incubator shaker. Once optical density at a wavelength of 550 nm (OD550) reached 0.3 the 3 ml culture was transferred into 200 ml of pre warmed 2xYT and incubated at 37°C in the shaking incubator until OD550 reached ~ 0.480. Culture was kept on ice for 5 min and then spun down at 3000 rpm, at 4°C, for 20 min. Supernatant was discarded and pellet was re-suspended in 80 mls of pre-chilled transformation TFBI (see section 2.1.2) and kept on ice for 5 min. Suspension was spun again as previously described and the pellet was resuspended in 8 mls pre-chilled of TFBII, Suspension was kept on ice for 15 min before aliquoting in pre-chilled 1.5 ml microfuge tubes kept on dried ice and then transferred and stored at -80°C.

2.3.3 Heat shock bacterial transformation

For plasmid DNA amplification the heat shock competent E.coli prepared as described in section were used. E.coli cells were thawed on ice for 2-3 minutes before 2 µl DNA (10-100 ng) was added. The mix was kept on ice for an additional 20 min before being heat shocked at 37°C for 3 min. Ampicillin free LB medium was added (900 µl) and the culture was then incubated for an additional 15 min. Transformed cells were then added to LB agar containing ampicillin and incubated overnight at 37°C.

2.3.4 Plasmid DNA amplification and extraction from bacterial culture

Transformed colonies were then isolated from the LB agar plate and incubated with fresh LB medium containing ampicillin (5ml) at 37°C in a shaker incubator set at 220 rpm. Plasmid DNA was then extracted and purified from bacterial culture using the QIAprep spin Miniprep Kit (Qiagen, UK). The plasmid DNA was then verified by restriction digest and running on agarose gel electrophoresis to visualize the various DNA sizes. Once verified 200 µl of the 5 ml culture was then transferred to 200 ml of fresh LB medium with ampicillin and incubated at 37°C to further amplify the plasmid DNA. Isolation of plasmid DNA was then achieved using the EndoFree plasmid Maxi prep kit (Qiagen, UK), and the purified DNA was further verified by sequencing (Source Bioscience, UK). DNA was quantified when required using a nanodrop spectrometer (Thermo Scientific, UK).

2.3.5 DNA restriction digests

Plasmid DNA was digested using the appropriate endonuclease enzymes (New England, Biolabs, (NEB), UK) either to create sticky end for subsequent ligation of desired genetic material, or for screening and verification of desired constructs. For screening purposes a 10 µl test digest was performed using 2 µl of DNA (0.5 µg), 1 µl of the appropriate 10x NEB buffer,1 µl of 10x bovine serum albumin (BSA) (NEB), 0.3 µl of each of the appropriate endonuclease enzymes needed, and nuclease free water. For creating sticky ends for subsequent ligation the backbone of the donor vector and the recipient vector were digested in a similar manner with scaling up of the quantities to a 75 µl and a 100 µl reaction respectively. The reaction was then

incubated at 37°C for 1 hour before screening for the correct size DNA fragments by agarose gel electrophoresis.

2.3.6 DNA ligation

To construct Plasmid DNA vectors expressing genetic material of interest a gene or a fragment of DNA from a donor plasmid must be ligated into a recipient vector. For this both the donor and the recipient vectors are digested using the appropriate endonuclease enzymes as described in the section above. To prevent the recipient vector from re-ligating to itself prior to insertion of the new DNA fragment 2 µl of calf intestinal alkaline phosphatase (CIAP, Invitrogen, UK) was added for 1 hour at 37°C. The recipient vector was then cleaned up from all the enzymes using miniprep kit washing buffers. The donor plasmid underwent DNA electrophoresis and DNA bands were visualized using a dark reader trans-illuminator. Based on DNA sizes the desired fragment was then gel extracted using gel extraction kit (Qiagen, UK). Once DNA fragment and recipient vector were ready a 20 µl ligation reaction was set up using the T4 DNA ligase (NEB), ligation buffer, together with the appropriate volumes of DNA fragment and recipient vector based on their sizes. Control ligation missing the fragment or the ligase enzyme was also included, and the reaction was incubated for 1 hour at RT before being transformed on to LB agar plates. Plates were incubated overnight at 37°C and colonies were selected, grown in LB medium and screened for using a test digest as described in section 2.3.5

2.3.7 Agarose gel electrophoresis

Separation and visualization of DNA fragment based on size was achieved using agarose gel electrophoresis. Agarose gels consisted of 1% agarose (Sigma-Aldrich) and 0.5 µg/ml of ethidium bromide (Sigma-Alrdich) in TAE buffer. Gels were poured in electrophoresis gel boxes and DNA samples together with DNA hyper ladder I (Bioline) were run at 120 volts for 60 min. DNA bands were then visualized by UV transillumination (Alpha Imager, Alpha Innotech, USA).

2.3.8 Constructing the STGFβBRII, STGFβRII-Fc, and SIL-10R vectors

The cDNA sequence for the extracellular domain of mouse TGFβRII (STGFβRII) (1-184 a.a), and mouse IL-10Rα (SIL-10R) (1-238 a.a), and the Fc region of mouse IgG1 were both obtained from Mr Gene (Regebsburg, Germany). The STGFβRII was subcloned using Not1 and Xba1 into the IgG1 Fc plasmid to generate the STGFβRII-Fc fused protein. Both sequences STGFβRII alone or STGFβRII-Fc, and SIL-10R were then subcloned in the pLVX-Tight-Puromycin vector (Lenti-X-Tet-On Advanced System, Clontech) using the appropriate restriction sites.

2.4 Lenti-X-Tet-On Advanced system

2.4.1 Lenti-X-Tet-On advanced system components

The Lenti-X-Tet-On advance system (632162, (Clontech, USA)) includes the list of components and constructs described in Figure 2.1 and Table 2.2.

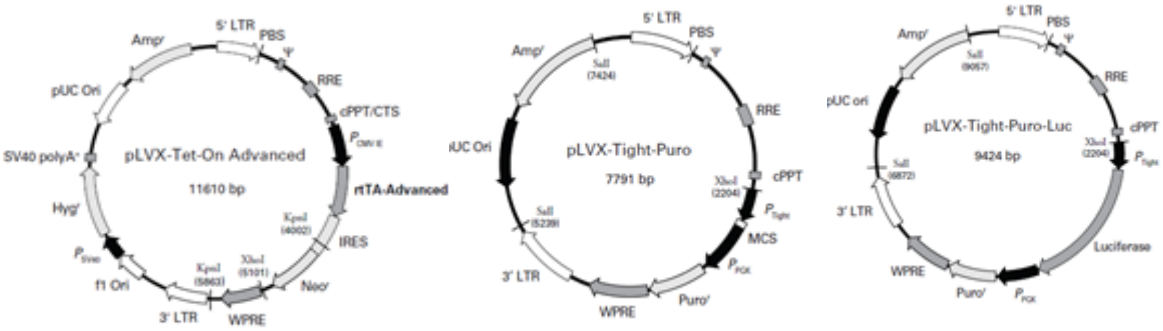


Figure 2.1 The Lenti-X-Tet-on Advanced system plasmid constructs

Maps of the 3 plasmid vectors supplied with the Lenti-X-Tet-On Advanced system. A complete vectors description is supplied in the vector information packet available from clontech.

Table 2.2 Lenti-Tet-On- Advanced system components

Components	Comment
pLVX-Tet-On advance vector	Harbours the expression of TetR under the CMV promoter, and the gene for G418 resistance
pLVX-Tight-Puro-luc	Expressed the inducible Luciferase under the Ptight promoter, and the gene for puromycin resistance (Control vector)
pLVX-Tight-Puro-vector	Expresses genes of interest under the Ptight promoter, and the gene for puromycin resistance
Lenti-X-HT Packaging mix	Contains all lentiviral packaging components including Pol, IN, Tat, Rev, and Gag lentiviral proteins & VSV-G envelope
Tet system Approved FBS	Tetracycline free FBS
Additional Materials	
G418 (Clontech)	For selection of cells transduced with pLVX-Tet-On
Puromycin (Clontech)	For selection of cells transduced with pLVX-Tight-Puro
Polybrene (Sigma-aldrich)	To facilitate the infection of cells with lenti vector particles
Doxycycline (Dox) (clontech 631311)	To induce the expression of genes in cells transduced with the tet-on system

2.4.2 Lentivirus packaging

In order to generate high titers of Lentivirus vectors, the main plasmid constructs are co-transfected with all the packaging components into a packaging cell line, in this case 293T. Briefly, 293T cells were plated $4x10^6$ /100 mm^2 plate in 10 mls of growth medium. 24 hours later cells were 80% confluent and transfected with polymer-DNA solution. To prepare the polymer-DNA solution, DNA 7µg of the Tet-On, P-Luc, Ptight STGFβRII, Ptight STGFβRII-Fc or Ptight SIL-10R plasmid constructs were mixed with 25 µg of DNA packaging mix. The DNA mix was then added to xfect polymer and incubated for 30 min. The polymer DNA solution was then added to the cells, and cells were incubated at 37°C 5% CO_2 in the incubator. 10 hrs post transfection, medium was replaced with 10 mls of fresh medium. 48 hrs post transfection cell supernatant containing the lentivirus particles was harvested and filtered through 0.45 µm filters

and stored until use in -80 °C. All steps were performed under sterile conditions in a class II laminar-flow cabinet.

2.4.3 Titrating antibiotics for selection of single and double stable cells lines

In order to select for stable cell lines infected with the lentiviruses it was important to first titrate each selection agent to determine the optimal concentration for CT26 cell line. G418 is the selection agent for the PpLVX-Tet-on virus, while puromycin is the selection agent for all the other viruses. To determine the optimal concentration for selection, CT26 were plated $1x10^4$ cells in each well of a 96 well plate containing 200 µl of medium. 24 hrs later medium was replaced with fresh medium containing G418 (0, 100, 200, 400, 800, 1000 µg/ml) or puromycin (0, 2.5, 5, 7.5, 10, 12.5 µg/ml), cells were incubated for 5 days and an MTS assay was performed to determine cell viability. Based on results shown in Figure 2.2 the lowest concentrations that resulted in complete cell death by day 5 was chosen, 400 µg/ml for G418 and 12.5 µg/ml for puromycin.

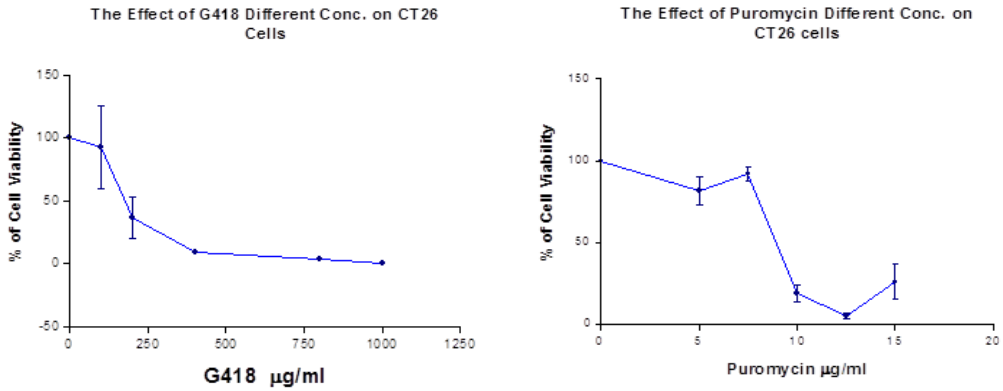


Figure 2.2 Titrating G418 and Puromycin for CT26 viability

CT26 cells were plated $1x10^4$ cells per well in a 96 well plate. 24 hrs later cells were treated with different concentrations of the selection agent. 5 days later an MTS assay was performed to determine cell viability. n =5 ,mean and SD shown.

2.4.4 Colony formation assay

In order to determine the viral titers a colony formation assay was used, providing information on the viability of the viral stock and the number of infectious particle. Colony formation also provided single and/or a double stable cell line generated from a single colony i.e. equal virus particle per cell, to use in all future experiments. To do this, CT26 cells were plated at $2x10^5$ cells per well in a 6 well plate. 24 hrs later six 10-fold serial dilutions of supernatant containing lentivirus particles were mixed together with polybrene (Sigma-Aldrich) 4 µg/ml. The mixture was then added to the cells and cells were centrifuged at 1000 g, at 29 °C, for 60 min. 22 hrs post transfection media was replaced with fresh medium containing the optimal concentrations of selection antibiotics. 2 weeks later single resistant clones could be seen and the virus titer was determined as $1x10^6$ colony forming units, which corresponded to the number of colonies generated by the most diluted virus concentration. Colonies were then selected and transferred using colony cylinders (Corning, Life sciences, USA) into a 96 well plate, and allowed to grown under antibiotic selection for 2 weeks until transfered into a confluent T75 flask.

2.5 DNA transfections

For DNA plasmid transfections, cells were seeded $4x10^5$ cells in a 24 well dish overnight. Cells were then treated with 0.5 µg of plasmid DNA (based on experiment) per well, diluted in Opti-MEM reduced serum (Invitrogen), and mixed with lipofectamine LTX and the plus reagent buffer following protocol (Invitrogen). 4 hrs later DNA-lipofectamine mix was removed and cells were washed with PBS and kept in fresh medium containing any required treatment based on experiment e.g. Dox or TGFβ as explained in more details in results section.

2.6 MTS assay

MTS assay (Promega) is a colorimetric assay that measures the ability of reductase enzymes to reduce MTS dye to Formazan dye giving a purple colour which can be determined by measuring its absorption at wavelength 490 nm. The conversion can only take place if cells are viable and express active reductase enzymes, thus the assay is used often to asses cell viability and proliferation. The MTS was thawed and added to cells in a 96 well plate at 40 µl per well,,in addition to wells with no cells (only medium) for background reading. Cells were then incubated

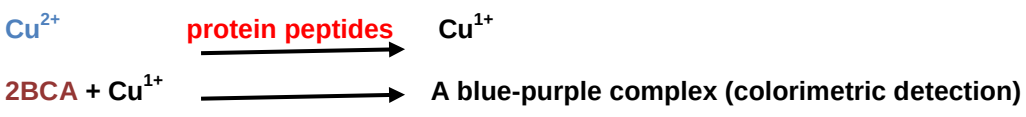
at 37°C 5%CO_2 for 30 min and the absorbance was measured in Wallac 1420 Victor^2 multilabel counter (Perkin Elmer) at wavelength 490nm. Background reading was subtracted and data was represented as % of cell viability of control untreated cells.

2.7 Luciferase assay in vitro

The lentivirusvirus (pLUC–V) expresses firefly luciferase under the Ptight promoter. The luciferase activity can be measured through a chemiluminescent assay that measures the conversion of luciferin to the luminescent product oxyluciferin in the presence of ATP and Mg^{2+} as co-factors. To measure the activity of luciferase in cells infected with pLUC–V, cells were plated $1x10^4$ cells per well in a 96 well plate, 24 hrs later cells were either left untreated or treated with different concentrations of Dox. 20 hrs later medium was removed and cells were washed before adding 100 µl per well of pre-chilled lysing buffer (Promega, UK). To ensure further lysis cells were kept for 6 hrs at -80 °C before analysis. Analysis was performed using 25 µl of cell lysate added to a luminometer tube and placed in luminometer (Lumat LB 9507, Berthold Technologies) pre-injected with 25 µl luciferin. Luminescence is measured as relative luminescence units (RLU) for 10 seconds. All values were then corrected for the amount of cells per well based on protein concentration determined by protein assay described below.

2.8 Bicinchoninic acid assay (BCA) for protein concentration

BCA assay (Sigma-Aldrich) is used to determine the protein concentration in a sample based on a standard curve of a known concentration of a known protein e.g. BSA. The assay is based on the following reaction:



Therefore the reduction of Cu^{2+} and consequently the absorbance of the end product complex is proportional to the amount of protein in a sample. To carry out the assay a standard curve of know concentrations of BSA (Sigma-Aldrich) (30 µg/ml- 2 mg/ml) diluted in the lysis buffer used

to lyse samples was first made. Protein quantification was performed in a 96 well plate by adding 50µl BCA mixture (made up according to manufacturer's protocol) to 50µl cell lysate diluted with lysis buffer, as well as the BSA standard curve. Plates were then incubated for at 37°C, for 1hr, or 60°C for 30 min. Absorbance was read at wavelength 595 nm using a Wallac 1420 Victor^2 multi-label plate reader (PerkinElmer Life). Protein quantification was done by extrapolating resulting OD values from the standard curve.

2.9 TGFβ1 expression from CT26 detected by ELISA

CT26 alone or CT26PLUC were seeded at 2x10^5 cells/ml in a 6 well plate, and cultured for 48 hrs in serum free medium to exclude any TGFβ1 originating from the FCS. Culture medium was then collected. For TGFβ1 an activation step with HCl is required to activate any latent TGFβ1 to immune reactive TGFβ1. To do this 20 µl of 1 N HCl was added to 100µl of cell culture medium and incubation for 10 min at room temperature performed. To neutralize the activated sample 20 µl of 1.2 N NaOH/0.5 M HEPES was added. TGFβ1 and IL-10 concentration was then detected by Mouse TGFβ1 ELISA kit (R& D systems.) according to manufacturer's instruction. Plates were read at 450 nm using a Wallac 1420 Victor^2 multilabel counter.

2.10 SDS page and Western-blot assay

Sample preparation

For detection of soluble secreted transgenes expressed under Dox control, cells were plated at 4x10^5 cells per well,in 2 mls in a 6 well dish. 24 hrs later cells were washed with PBS and treated with stated concentrations of Dox in serum free medium. 48 hrs later 2 mls per well of supernatant was collected and concentrated using centricon tubes 3k (Millipore, UK) spun at 4000 rcf, 20 mins, at 10 °C. The concentrated supernatant (200 µl) was then stored in -20°C until needed. For detection of unsecreted proteins cells were lysed using either the tissue protein extraction reagent (T-PER, Thermo scientific, Pierce products, USA) or Phosphoprotein lysing buffer (section 2.1.3). Cell lysate was then spun at 13,000 rcf, at 4°C, for 5 min, and the supernatant was collected and stored at -20°C. For detection of proteins from CT26 tumours extracted from Balb/c mice, tumours were extracted as described in section 2.11 and directly frozen on dry ice or in liquid nitrogen. Tumours were thawed on ice and cut into small pieces in

5 ml tubes. 2 ml of the T-PER lysing buffer was added and tumours were homogenized using the T18 basic Ultra-Turrax homogenizer (Thermofisher Scientific,IKA, UK) at full speed for 30 sec. The homogeniser was washed thoroughly with 3 dilutions of distilled water between samples. Samples were then spun and stored as previously described for cell lysate. Protein concentrations of samples were measured by BCA protein assay as described previously.

Gel preparation and SDS page

Gels (12%) were prepared as follow:

Reagent	Separating gel	Stalking gel
dH2O (ml)	4.9	4.1
1.5 M Tris HCL pH 8.8 (ml)	3.8	-
1 M Tris HCL pH 6.8 (ml)	-	0.75
10% SDS (µl)	150	60
Acrylamide/ bis acrylamide (Biorad) (ml)	6	1
10% Ammonium persulphate (Sigma) (µl)	150	60
Temed (Tetramethylethylenediamine,Sigma) (µl)	15	6

Samples were thawed and diluted to the required equivalent concentration using lysis buffer and mixed with sample buffer (section 2.1.3). Samples were heated at 95°C for 5 min to denature proteins. Samples were then loaded on the gel alongside a pre-stained protein molecular weight marker (Precision Plus Protein dual color standard (Bio-rad)). Gels were then placed in gel tank filled with 1x running buffer and run at 160 V, at RT for 50-60 min.

Nitrocellulose transfer

To transfer protein from gel to nitrocellulose membrane a gel transfer sandwich was assembled. All components were pre-soaked in transfer buffer and arranged in the following order: pad (Bio-rad) - filter paper - gel - nitrocellulose membrane (Bio-rad) - filter paper – pad. Transfer sandwich was assembled in a transfer cassette and placed in tank with running buffer and run at 30 V overnight at 4°C.

Western blot

Nitrocellulose membrane was removed from transfer cassette and washed in PBS. The membrane was then stained with Ponceau stain (protein stain) to ensure efficient transfer. Membranes were then destained by washing in PBS. Membranes were blocked in 5% skimmed milk (Sigma-Alrdich, and Bio-rad) in PBS/0.1% Tween,at RT, for 1 hour. Membranes were then washed three times in PBS/0.1 Tween, and incubated with primary antibodies diluted in 2.5% skimmed milk followed by washing and secondary antibodies incubation, described in Table 2.3. If a membrane was to be probed with another set of antibodies, membranes were incubated with antibody stripping buffe, at RT, for 10 mins. Membrane were then washed with PBS until a normalised pH of 7 was reached again, blocked with skimmed milk and re-probed with the appropriate primary and secondary again.

To detect the signal, membranes were washed 3 times after secondary incubation, and incubated with Supersignal west dura extended duration substrate (Thermoscientific) before the chemiluminescence signal was developed using X-ray film (SuperRx,Fujifilm) and an automatic developer (CP1000, Agfa). For densitometric analysis of band intensity Image J software was used.

Table 2.3 Antibodies used for westernblot

Antibody	Species	Conc.	Incubation	Company
Primary				
TetR	mouse,monoclonal	1:500	1 hr RT	Clontech
Anti mouse TGFβRII	goat, polyclonal	2 µg/ml	3 hrs RT	R&D
Mouse IL-10R alpha	goat,polyclonal	0.1µg/ml	3 hrs RT	R&D
Phospho-smad2	rabbit,monoclonal	1:500	Overnight 4°C	Cell signaling
Smad2	rabbit,monoclonal	1:500	Overnight 4°C	Cell signaling
Actin-beta	mouse,monoclonal	1:1000	1 hr RT	Sigma-Aldrich
Secondary				
anti goat IgG-HRP	mouse, polyclonal	1:1000	1 hr RT	Santacruz
anti mouse IgG2a-HRP	goat,polyclonal	1:1000	1 hr RT	Santacruz
anti rabbit IgG HRP	donkey,polyclonal	1:7000	1 hr RT	Promega

2.11 Flow-cytometry analysis

Sample preparation

CT26 Tumour sections:

Tumours extracted from mice were kept in ice cold serum free RPMI medium. Tumours were then washed three times with serum free medium in 50 ml falcon tubes. In 100 mm dishes tumours were cut into sections of approximately 3 mm x 1mm. Each section was then disrupted mechanically using autoclaved tweezers, and sterile scalpels, until a homogenised mix was produced. The mix was then transferred into 1.5 ml microfuge tubes together with a dissociation enzyme mix made of 1 in 100 Collagenase IV (8000 units) and DNase or Dispase in RPMI medium. The mix was then incubated at 37°C and 5% CO_2 in a tube rotator for 1-2 hrs. Mix was then filtered using a 70 µM cell strainer (Bectom Dickinson) and the filtered mix was centrifuged at 500 rcf, at RT, for 5 min. Supernatant was discarded and the resulting pellet was re-suspended in PBS and spun as previously. This was repeated three times. After the final wash

cells were re-suspended in 1 ml of PBS and were counted using haemocytometer and diluted in appropriate volume of PBS to make a concentration of $1x10^6$ cell/ ml.

Mice splenocytes

Mice spleens were kept in ice cold serum free RPMI. Spleens were crushed in 24 well plates with a 5 ml syringe plunger until a homogenies mix is produced. The mix was then filtered using a 70 µM cell strainer into a 50 ml falcon tube containing 10 mls of fresh serum free RPMI. Tubes were then spun at 1350 rpm, at RT, for 5 min. Supernatant was discarded and cell pellet was re-suspended in 10 mls of PBS and spun as previously. Cells were then counted using haemocytometer and diluted in appropriate volume of PBS to make a concentration of $1x10^6$ cells/ ml.

Antibody staining

Spleen and tumour cells were kept as $1x10^6$ cells/ml per tube. Tubes were spun at 500 rcf, at RT, for 5 min. Supernatant was discarded and cells were re suspended in 100 µl of PBS with purified rat anti-mouse CD16/CD32 (BD) 1µg/ million cells/100 µl, at 4°C, for 5 min. This step was performed to block FC receptors and prevent nonspecific binding of antibodies to leukocytes.Cells were then stained with various primary antibodies (BD) 1:100 described in Table 2.4, and incubated at 4°C, for 1hr. For Foxp3 staining splenocytes were kept at $3x10^6$ per tube, blocked with CD16/CD32 and stained with anti-CD4 antibody prior to fixation and permeabilisation and staining with anti-mouse Foxp3 (following manufacture instruction, Mouse Foxp3 Buffer Set kit).

Cells were then washed with PBS and spun 500 rcf, at RT, for 5 min, before re suspending the pellet in PBS and 5% formalin. Cells were kept at 4°C overnight before analysis using a FACS Caliber machine (BD, Biosciences). For data analysis FlowJo V10 was used.

Table 2.4 Antibodies used for flow-cytometry analysis

Antibody	Clone	Flurophorme
Anti-mouse CD8a	53-6.7 (Rat, IgG2ak)	AlexaFluor 488
Anti-mouse CD4	RM4-5 (Rat, IgG2ak)	PE-Cy7
Anti-mouse CD11B	M1/70 (Rat, IgG2bk)	AlexaFluor 488
Anti-mouse LY-6G,LY-6C	RB6-8C5 (Rat, IgG2bk)	PE-Cy7
Anti-mouse CD44	IM7 (Rat, IgG2bk)	PE-Cy7
Anti-mouse Foxp3	MF23 (Rat IgG2b)	AlexaFluor 488

Isotype control	A95-1 Rat IgG2bk	AlexaFluor 488
Isotype control	A95-1 Rat IgG2bk	PE-Cy7

2.12 Enzyme-linked immunosorbent spot (Elispot) for IFNγ

High protein binding PVDF 96 well plate (Millipore) were first activated with 35% ethanol 50µl/well, for a maximum of 1 min. Plates were then washed with sterile PBS and coated with 50µl/well of diluted capture antibody (IFNγ Elispot Alp kit, Mabtech, AN18 5µg/ml) and incubated overnight at 4°C. Next day plates were washed five times with sterile PBS and blocked with RMPI containing 20% FCS, at 37°C, for 2 hrs

Mouse splenocytes were prepared as described in section 2.11, however, red blood cells were first lysed using the ACK lysis buffer for 5 mins. PBS was then added and splenocytes were spun down at 1350 rpm, at RT, for 5 min. Cell pellet was resuspended in 10 mls of RMPI medium (with 20% FCS).

When splenocytes were ready, blocking medium was removed from plates and cells were plated $2.5x10^5$ -$5x10^5$ cells/ well in 50µl medium. Cells were then either treated with Concanavalin A (ConA) type VI (Sigma-Aldrich, 1mg/ml stock in 0.9% Sodium Chloride solution, 1µg/ well) as a positive control, or the recombinant AH-1 peptide (1µg/ml) in 50 µl of medium. Plates were then incubated at 37°C for ~ 20 hrs. Plates were then washed five times with sterile PBS and incubated with 50µl/well of the biotinylated detection antibody (R4-642,1µg/ml diluted in PBS), at RT, for 2 hrs, covered in tin foil. Plates were then washed five times with PBS and incubated with 50µl/well of streptavidin-ALP (1µg/ml), at RT, for 45 mins. Plates were washed five times with PBS and 50µl/well of BCIP/NBT colour developer buffer (Sigma-Aldrich) was added to each well. Plates were left at RT until spots could be seen. Plates were then washed thoroughly with tap water to stop colour development. Plates were dried thoroughly and left to dry further overnight in the dark. Plates were then read using a plate reader (Jenner Institute).

2.13 Reverse transcription quantitative polymerase chain reaction (RT-QPCR)

2.13.1 Sample preparation and RNA extraction

Tumour sections were thawed on ice and cut to smaller sections of approximately 30 mg. Sections were then homogenised in 600 µl of RLT lysis buffer (Rneasy RNA extraction kit, Qiagen, UK). Lysate was spun at 10,000 rcf, at RT, 3 min. Supernatant was then collected for total RNA extraction using the Rneasy kit and including the DNase step to eliminate any genomic DNA. RNA was then measured by nanodrop and stored at -80 °C.

2.13.2 RT^2 profiler PCR array

cDNA preparation and RT-QPCR

Preparation of cDNA from 1µg of purified RNA was achieved using the RT^2 First strand kit (Qiagen,UK) in a 20 µl reaction. Resulting cDNA was diluted 5 times in nuclease free water and stored at -20 °C. RT qPCR was carried out using RT^2 SYBR green master mix and prepared cDNA in a 25 µl reaction diluted in nuclease free water using the RT^2 profiler 96 well plate. Plates were sealed and spun down at 1000 rpm, at RT, for 30 sec, to remove any bubbles.

PCR cycling program was set using the Applied biosystem, step one plus model, as follow:

	Step 1	Step 2	Step 3
Temperature °C.	95	95	60
Time	10 min	15 sec (40 cycles)	1 min (40 cycles)

Threshold cycle (CT) values were exported and the following criteria and analysis was used according to the RT^2 profiler handbook; CT>35 were considered as a negative call. CT>35 of the control GDC (Figure 2.3) indicated that levels of contamination of genomic DNA were too low to interfere with results. To determine fold change between mice groups ΔCT was first calculated (CT (gene of interest (GOI))- CT (house-keeping gene)). This was followed by calculating the $2^{-\Delta\Delta CT}$, which represents the normalised expression of the GOI in the treated

group divided by the normalised expression of GOI in the control group using the following formula:

$$\frac{2^{-\Delta CT\ (GOI\ treatment)}}{2^{-\Delta CT\ (GOI\ control)}}$$

For statistical analysis data was presented as $2^{-\Delta CT}$ for each gene studied.

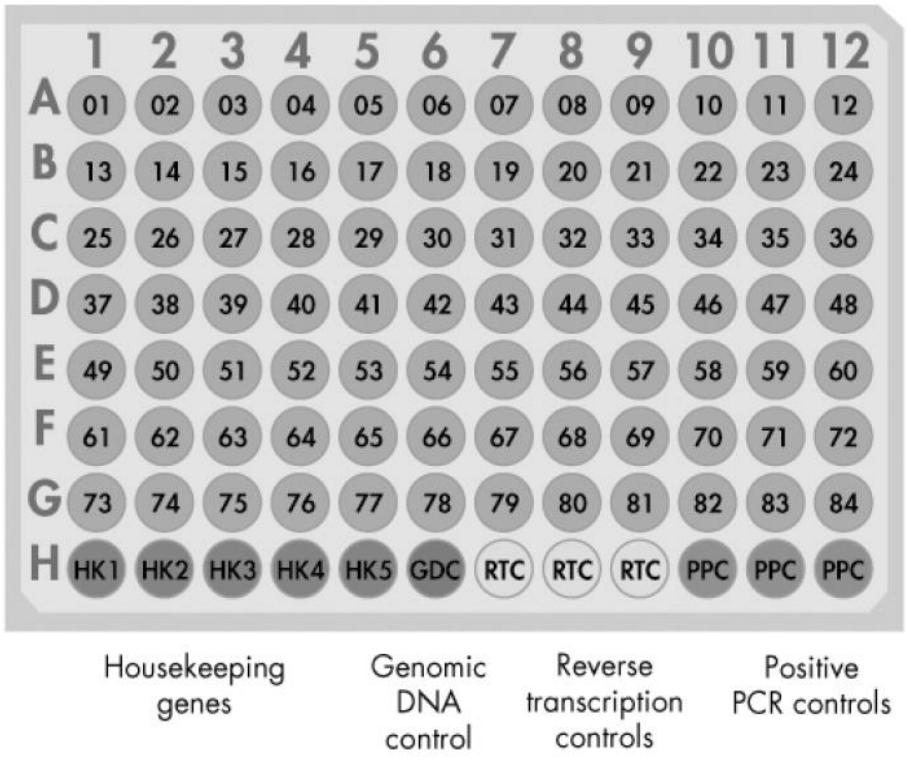


Figure 2.3 RT^2 profiler common cytokine profile 96 well PCR plate

Wells A1 to G12 represent real time PCR assay for the common cytokine profile (84 genes).Wells H1 to H5 are the house-keeping gene panel to normalise array data. Well H6 is genomic DNA control that detects non transcribed genomic DNA. Wells H7 to H9 contain the replicate reverse transcription reaction controls (RTC), testing the efficiency of the reverse transcription reaction. Wells H10 to H12 contain the positive PCR controls (PPC) assay which consists of the predispensed artificial DNA sequence and the assay to detect it to test for the efficiency of the PCR reaction. Figure acquired from RT^2 profiler PCR array handbook.

2.13.3 Animal studies

All animal experiments were performed in accordance with the terms of the UK Home Office guidelines and with the approval of the Medical Sciences Animal Ethics Committee, University of Oxford. Experiments were conducted under the Home Office Project Licence number PPL 30/2816 (2011 – 2012) protocol 19b and PPL 30/2819 (2013) protocol 2 and under the personal licence 30/9272

2.13.4 Housing conditions and diet

Mice were kept in temperature and humidity controlled conditions at the biomedical services, John Radcliff Hospital Oxford. Mice were give sterile tap water and formulated diet and housed in individually ventilated cages (IVC). For mice receiving Dox, 2mg/ml of powder Dox (Clontech) and 5% of sucrose (Sigma-Aldrich) were dissolved in water bottles. Water bottles were covered with foil to protect Dox from light. Control mice received 5% sucrose in water without Dox. Water was changed every 48 hrs to avoid any inactivation of Dox due to its instability in water.

2.13.5 General anesthesia

For all procedures except measuring tumours mice were first anesthetized, by inhaling oxygen and isoflurane (2-5%).

2.13.6 Subcutaneous CT26 tumours

To establish CT26 (our various genetically modified CT26 cells) tumours in Balb/c mice, cells were implanted at $1x10^6$ cells in 100µl/mouse. Mice underwent anesthesia and the right flank was shaved before injecting the cells subcutaneously (s.c.) using a 27 gauge (27G) needle and a 1 ml syringe. Once established, tumour length, width and depth was measured using calipers and tumour volume was calculated as 1/2x(LxWxD) and presented as mm^3.

2.13.7 Luciferase expression *in vivo*

Control CT26TetR or CT26PLUC were injected s.c. in Balb/c mice. Two weeks later tumour size reached ~ 200 mm^3 and mice received no Dox, 0.5, or 2 mg/ml of Dox with 5 % sucrose in the drinking water. To detect luminescence signal mice were first anesthetised and then injected

intra peritoneal (i.p.) with 15.8mg/ml D-lucifirin(Gold Biotechnology) 75µl on each side. Mice were kept for 4 mins under anesthetisia before undergoing non-invasive measurement using an IVIS 100 system (Xenogen) set for either 10 or 20 sec exposure.

2.13.8 Adoptive transfer of splenocytes in Balb/c mice

Splenocytes were prepared as described in section 2.11 and 2.12. However, they were resuspended in PBS ($1x10^7$ cells/ 100µl of PBS) (Saika, Kusaka et al. 2006, Naas, Ghorbani et al. 2010) for adoptive transfer. Cells were kept on ice until ready to be injected. Female Balb/c mice (5-6 weeks) with established CT26 tumours were kept in an heat box for 30 mins to dilate tail vein. Cells were then inject intravenously (i.v.) using a 29G needle.

2.13.9 Animal experimentation ethics statement

All animal experiments were performed in accordance with the terms of the UK Home Office guidelines and with the approval of the Medical Sciences Animal Ethics Committee, University of Oxford. The Home Office Project Licence number under which these experiments were conducted were PPL 30/2816 (2011 – 2012) and PPL 30/2819 (2013). Studies were carried out under the personal licence PIL 30/9272

2.14 Data processing and Statistical analysis

Data was processed using Microsoft Excel 2007 or Graphpad prism 3 and 5. Statistical analysis was done using Graphpad prism 3 and 5. The identity of the statistical test used (analysis of variance (ANOVA), or unpaired Student's T test) has been stated in the figure legends.

3 Results

3 Establishing and Characterising the Lenti-X-Tet-On Advanced System.

3.1 Introduction

This chapter describes the development of a new tumour model to allow the study of the effects of antagonising soluble immunosuppressive factors locally in well-established tumours. The concept was to genetically engineer murine CT26 colorectal carcinoma cells to stably express secreted cytokine antagonists (antagonists to TGF-β and IL-10) under an inducible system. There are several inducible systems currently available, however, Tet system remains the most frequently used, and has been proven to be successful in various models (Zhu, Zheng et al. 2002). For the reasons outlined in section 1.5.3 the Lenti-X-Tet-on advance system was chosen as a model to allow the stable expression of proteins of interest under the strong regulation of the Tet-on system. This system (represented in Figure 3.1), and the evaluation of its performance *in vitro* and *in vivo* using luciferase as a reporter gene is described in this chapter.

The Lenti-X Tet-on Advanced Inducible Expression System

The Lenti-X-Tet-on advance system (Clontech) is composed of two vectors, the regulator vector and the response vector which are based on the Tet system (section 1.5.3.2)

The Regulator Vector: pLVX-Tet-on advance, which constitutively expresses the tetracycline-controlled transactivator fusion protein, referred to as TetR. The protein expression is under the Pcmv, and unlike the previous systems from Clontech the advanced system utilises human codon preference to enhance the protein expression in mammalian cells.

The Response Vector: pLVX-Tight-puro response vector. This carries the inducible Ptight promoter for the transcription of genes of interest. The Ptight promoter consists of modified Tet-Responsive Elements (TRE) made up of seven direct repeats of altered tetO sequences combined to a modified minimal Pcmv.

When expressed in cells the two components can be used to make a doubly transfected stable cell line. Normally the transgene encoded in the Response Vector will be silenced (Figure 3.1), but in the presence of the system's inducer Dox, the TetR can bind to the Ptight promoter and allow robust transgene expression.

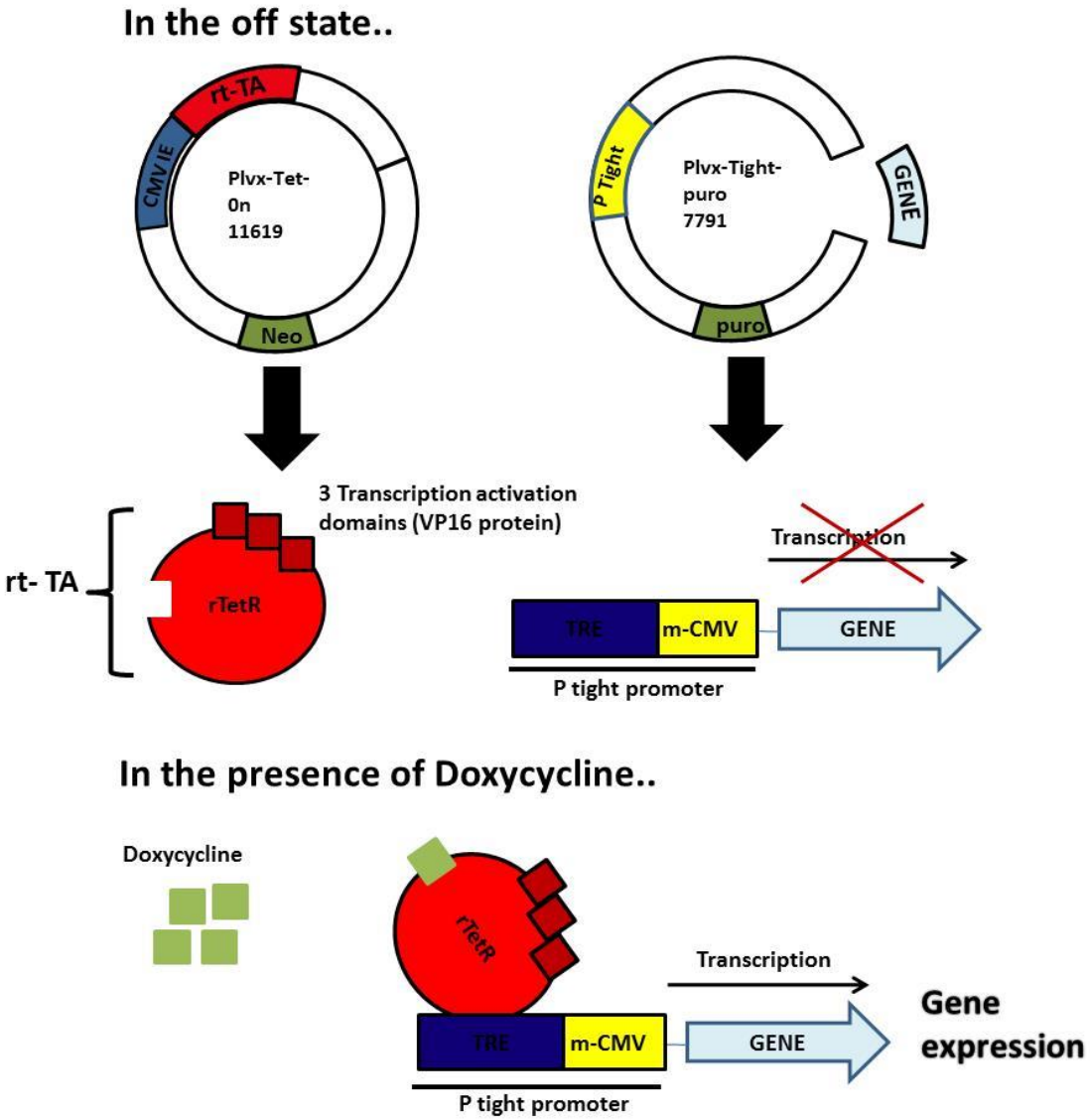


Figure 3.1 The Lenti-Tet-on system

The two components of the Lenti-x-Tet-on system, the pLVX-Tet-on plasmid expressing TetR, and the pLVX-Tight-puro plasmid which expresses genes of interest under the Ptight promoter. In the absence of Dox, transcription of genes of interest cannot be carried out, however, in the presence of Dox TetR repressor protein (red circle) together with the transactivators (red squares) bind to tetO sequence (TRE) on the Ptight promoter of the pLVX-Tight-puro allowing transcription of the genes of interest to proceed.

3.2 Chapter Objectives

1-**Establish a CT26 stable cell line expressing the TetR protein**: this stable cell line was engineered for use in all future experiments, enabling any gene of interest to be expressed under the transcriptional control of the TetR protein by the presence of Dox.

2-**Establish a double-transgenic stable cell line expressing TetR and luciferase to permit evaluation of the performance of the system**: this stable cell line would allow testing of the system *in vitro* and *in vivo* using luciferase as a reporter gene before proceeding with the immunomodulatory genes of interest.

3-**Assessing the strength and performance of the Ptight promoter**: to compare with other promoters and give an indication of levels of transgene expression that might be achieved.

3.3 Results

3.3.1 Engineering a CT26 stable cell line expressing the TetR protein

The first step in developing the Tet-on advanced system was to engineer a CT26 cell line that stably expressed the TetR protein, which could then be used in all future experiments. To engineer the cell line pLVX-Tet-on plasmid, expressing the TetR protein constitutively, was first transfected together with packaging plasmids into human embryonic kidney 293T (293T) cells to produce a non-replicating pLVX-Tet-on lentivirus vector (Figure 3.2). Supernatant from 293T cells was collected 48 hrs post transfection, and CT26 cells were then infected with 6 to 10-fold serial dilutions of pLVX-Tet-on lentivirus, to allow for the colony formation of CT26 cells expressing TetR. Transfectants were then selected with G418, the selection marker for the pLVX-Tet-on. Nine resistant cell clones were isolated, grown, and screened by western blot for expression of TetR. Using an anti-TetR monoclonal antibody against wild type TetR, a positive band at the correct molecular weight (30 kDa) could be detected from the cell lysate of all 9 transfected clones but not in the control non-infected CT26 cells (Figure 3.3A). Using

densitometry the level of TetR protein in each of the 9 clones relative to the level of the internal control β-actin (Figure 3.3B) was also measured. This allowed the stratification of the 9 clones as high, intermediate, or low TetR expressing and enabled the impact of TetR expression level on the expression of test transgenes to be characterised. A high expression of the TetR (and thus the promoter transactivator) might be expected to induce higher transgene expression, although, this enhancement, if present, might come at the cost of higher background levels in the form of higher transgene expression in the absence of Dox. Three CT26 TetR-expressing clones were chosen and designated high (colony 8), intermediate (colony 5) and low (colony 1) based on their TetR expression to allow correlation of TetR expression to the expression of genes of interest

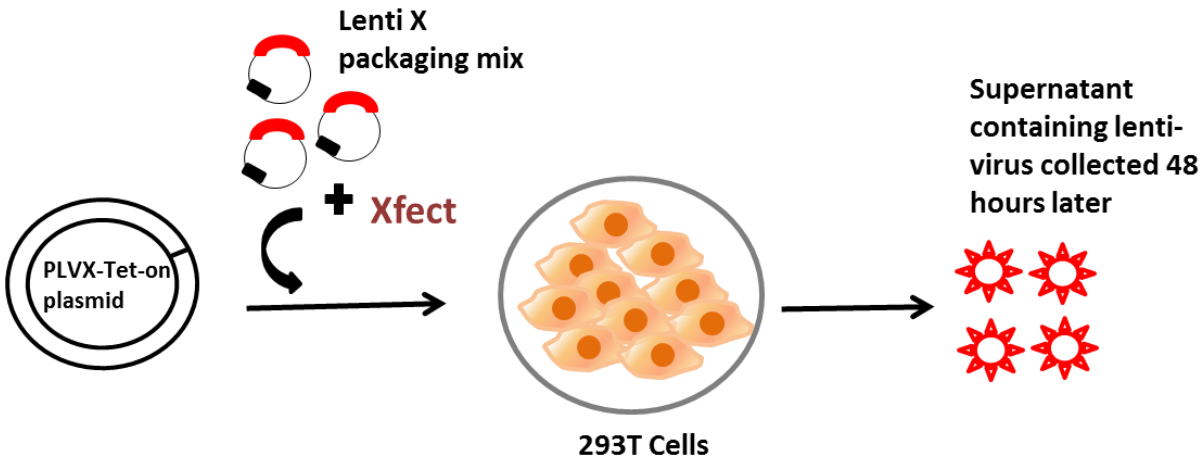


Figure 3.2 Producing Lentiviruses with the Lenti-X packaging mix

The PLVX-Tet-on plasmid was co-transfected together with the Lenti-X packaging mix and the transfection reagent Xfect in to 293T cells. 48 hrs later the supernatant containing Lentivirus was collected, filtered and stored at -80°C until usage.

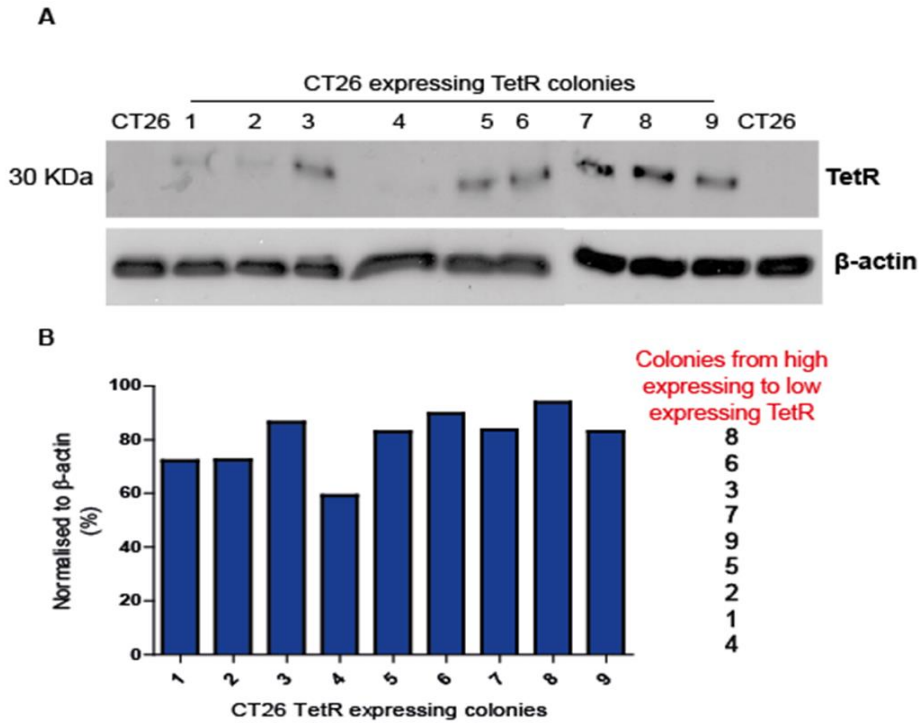


Figure 3.3 The detection and quantification of the TetR protein expression.

A Western blot analysis of cell lysate from nine G418 resistant cell clones of CT26 post pLVX-Tet-on lentivirus transfection and control non-infected CT26 cells ('CT26') for TetR expression. TetR protein detected at 30 kDa in all transfected colonies but not in the negative control cells. **B** The percentage of TetR protein relative to the loading control β-actin in all 9 colonies.

3.3.2 Characterisation of the Tet-On advanced system *in vitro*

3.3.2.1 Testing the induction of Luciferase under the Tet-On system in CT26 expressing TetR colonies

Prior to using the Tet-on system to express immunoregulatory proteins it was important to validate that regulation of transgene expression by Dox was responsive, reliable, and well controlled. This was tested using a luciferase reporter gene. Luciferase has a logarithmic dynamic range and is amenable to imaging *in vitro* and to real-time imaging *in vivo,* making it ideal for testing the system. Firstly a luciferase lenti vector (pLuc-V) was generated (as in Figure 3.2) using the pLVX-Tight-Luc plasmid, instead of the pLVX-Tet-on, which expressed luciferase under the Ptight promoter. Three CT26 TetR cell lines; high expressing (colony 8), intermediate

expressing (colony 5) and low expressing (colony 1) TetR were then transfected with serial dilutions of pLuc-V to generate double-transfected stable cell clones expressing both TetR and luciferase. Transfected cells were then selected with both puromycin, the selection marker for pLVX-Tight-Luc, and G418. The double resistant cell clones were harvested using cloning cylinders and transferred to suitable culture vessels, and allowed to grow. Once a population was established clones were then screened for luciferase expression 24 hrs after Dox treatment. Cells treated with Dox showed high luciferase expression, reaching close to $1x10^5$ RLU/µg of cell protein, in the high TetR expressing colonies (Figure 3.4). This was more than 150-fold higher than levels observed in the absence of Dox treatment ($p<0.05$), or in control CT26TetR cells not infected with pLuc-V (CT26TetR). Luciferase expression appeared to show a trend towards Dox dose dependence; however, this was not statistically significant. Cells that did not receive Dox showed very low levels of signal, which was comparable to control cells CT26TetR, indicating the tight control of gene expression by Dox treatment (Figure 3.4).

From the results we could conclude that, firstly, higher TetR levels did not correlate with higher background levels of luciferase expression, since colony 8 showed similar background levels to colonies 5 and 1 in the absence of Dox. Secondly, TetR levels correlate positively with the maximum transgene levels (luciferase) levels achieved, since colony 8 showed the highest luciferase expression post-Dox treatment (10-fold higher than colonies 5 and 1). This suggests that the higher the transactivator levels the higher the levels of expression of the transgene. Thirdly, the system is tightly regulated and controlled by Dox since the levels of luminescent signal in the absence of Dox in all colonies did not exceed the signal from control non-transfected cells. It was therefore decided to proceed with the high expressing TetR colony, colony 8, in all future studies and it is referred to as CT26TetR in the following text.

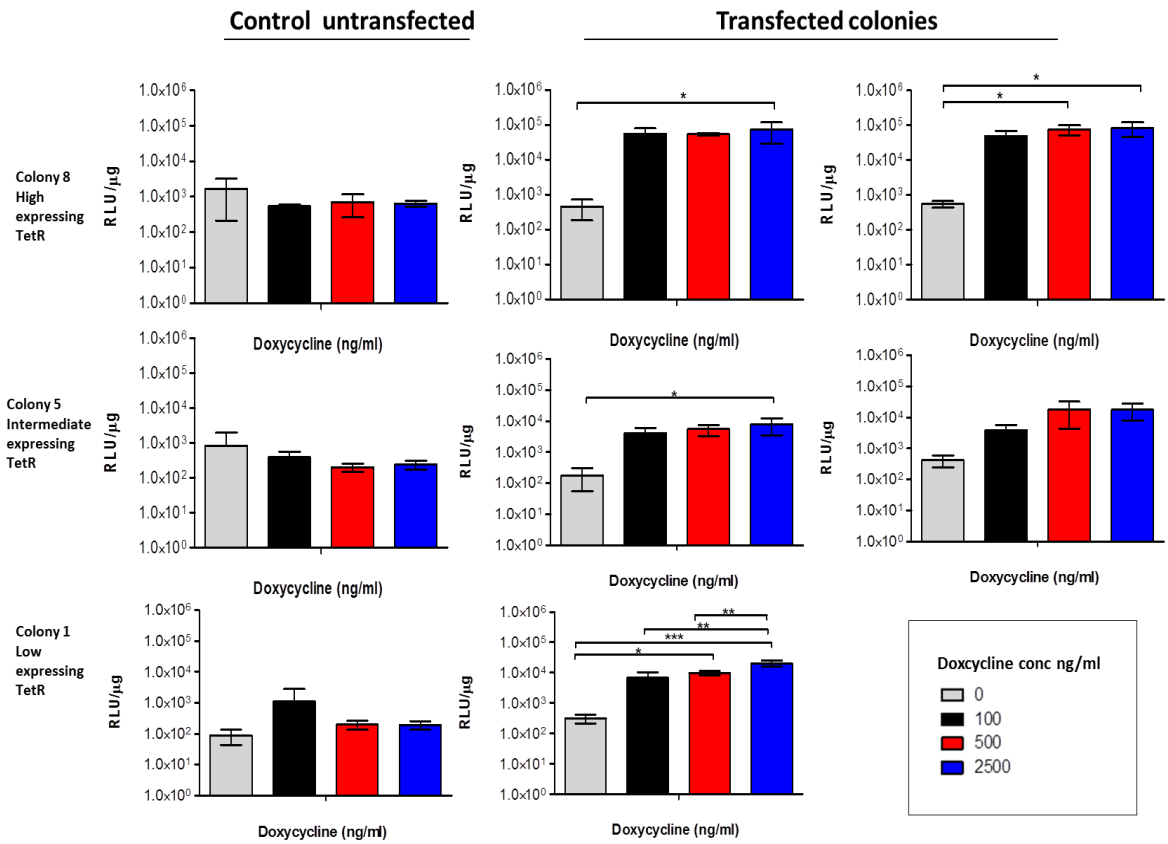


Figure 3.4 Expression of the Luciferase transgene in CT26-TetR cells in response to different Dox concentrations

Luciferase expression (RLU/µg cell protein) in CT26 high, intermediate, and low expressing TetR colonies and control after transfection with the pLVX-Tight-Luc or not, 24 hours post Dox addition. Data represents n=3, mean and standard deviation (SD) shown. Significance tested by ANOVA * = $p < 0.05$

3.3.2.2 Comparing the expression of Luciferase under the Ptight promoter and CMV promoter

Although our studies showed a strong induction of luciferase, it was important to conduct a comparison between the levels of expression mediated by the Ptight promoter and those mediated by other constitutive promoters such as Pcmv. Since the Ptight promoter consists partially of a modified minimal Pcmv it was anticipated that the levels of expression achieved would be comparable.

To study this, CT26TetR (high) cells were transiently transfected with either a plasmid expressing luciferase under the Ptight promoter (P-Luc), or one that expressed luciferase under the CMV promoter (Pcmv-Luc). A positive outcome using the CT26 TetR cells would also verify

that sufficient TetR production is achieved for a strong Ptight promoter transactivation. Cells that were transiently transfected with the P-Luc were either treated with Dox (4hrs post transfection) to induce luciferase expression or left untreated. As a control, Pcmv-Luc transfected cells were also treated with Dox, to control for direct effects of Dox on luciferase expression. At 24 hrs post-transfection cells were lysed and luciferase levels were measured. P-Luc and Pcmv-Luc transfected cells treated with Dox showed comparable levels of luciferase expression with the average being ~$2x10^4$ and $1.7x10^3$ RLU/µg respectively (Figure 3.5). Similar to results presented in Figure 3.4 P-Luc transfetced cells that did not receive Dox showed negligible levels of luminescent signal comparable to non-transfected cells, whereas cells treated with Dox showed luciferase levels approximately 140-fold higher. This confirmed that the strong induction of the Ptight promoter under the control of TetR protein was comparable to that achieved with a constitutive promoter like Pcmv.

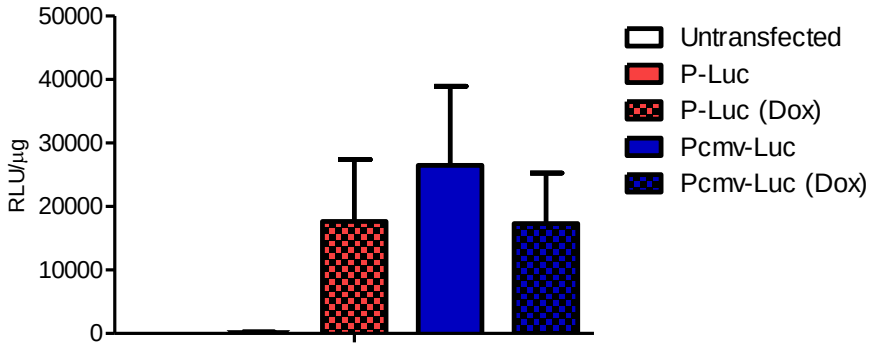


Figure 3.5 Luciferase expression under the Ptight promoter and Pcmv in CT26TetR cells

CT26 TetR cells were transiently transfected with P-Luc or Pcmv-Luc. Cells were then treated with Dox to induce the P-Luc expression or left untreated. As a control Pcmv-Luc transfected cells were also treated with Dox. 24 hrs later cells were lysed and luciferase levels were measured. Data represents n=3. Mean and SD shown, one way ANOVA analysis showed no significant difference between P-Luc Dox and Pcmv-Luc Dox.

3.3.3 Characterising the Tet-On advanced system *in vivo*

After observing the powerful inducible regulation of luciferase expression by Dox *in vitro*, it was important to assess that the Dox control mechanism was as responsive and reliable *in vivo.* This would be important to validate high bioavailability of Dox and good penetration of this inducer

chemical into tumour tissue growing s.c. It was also important to characterise the toxicity profile of the Dox in the chosen *in vivo* model.

A pilot study was therefore performed in which control CT26TetR or CT26 expressing TetR and luciferase (CT26PLUC) were injected s.c. in Balb/c mice. The CT26 cell line was originally isolated from a Balb/c mouse (Pearlstein, Ambrogio et al. 1984) and can therefore establish syngeneic tumours when injected s.c. in this strain. To estimate the tumour growth parameters that might apply in future efficacy studies tumours were allowed to reach approximately 200 mm^3 (10 days post implantation) before starting the mice on Dox in their drinking water. This was expected to permit a well-established tumour with a mature immune-milieu to develop.

Prior to Dox administration all mice were injected i.v. with luciferin and imaged to give baseline pre-Dox readings.

Tumours from mice that were injected with CT26TetR cell line (Control group) showed no signal above background levels seen in non-tumour areas in mice (30165 photons/cm^2/sec) (Figure 3.6). Mice that were injected with CT26PLUC showed low levels of expression prior to Dox administration, with the maximum readings being lower than $2x10^5$ photons/cm^2/sec. Over the next 48 hrs mice were divided into groups, and received 5% sucrose in the drinking water (to enhance its taste) with: Dox at 0,0.5 or 2 mg/ml. A level of 2 mg/ml is the recommended maximum Dox dose, however, 0.5 mg/ml was also tested to investigate dose dependent induction of luciferase, and to test if lower concentrations might actually provide better induction, as the mice on the lower dose may tolerate the bitterness of Dox and drink more readily thereby increasing overall uptake.

After 24 hrs of Dox exposure mice that received 0.5mg/ml showed the highest level of induction, reaching an average of $2.2x10^7$ photons/cm^2/sec, whereas mice which received 2 mg/ml showed an average of $1.9x10^7$ photons/cm^2/sec (Figure 3.6A). Thus 24 hrs post Dox luciferase levels increased 153-fold in the 0.5mg/ml Dox treated mice and 67-fold in the 2mg/ml group relative to pre-Dox readings (Figure 3.6B). Mice that did not receive Dox showed very low background which was only 3-fold higher than CT26TetR mice without the luciferase transgene, with levels reaching only $1.2x10^4$ photons/cm^2/sec. After 48 hrs post-Dox treatment there was a drift towards higher luciferase expression in mice receiving 2mg/ml of Dox. This might be related to the adaptation of mice to the taste of Dox in water. At this time-point mice which received 2

mg/ml showed an average of $9.1x10^7$ photons/cm^2/sec (Figure 3.6A) which was ~317-fold higher than the pre-Dox readings in this group (Figure 3.6B), whereas there was less increase in the 0.5 mg/ml group with levels reaching an average of $2.6x10^7$ photons/cm^2/sec.

To test if luciferase expression could be switched off by removal of Dox all mice were kept off Dox and then imaged again 24 hrs later (point 72 hrs). Levels of expression dropped in both groups reaching an average of $3x10^6$ photons/cm^2/sec in the 2mg/ml group and $4.8x10^6$ photons/cm^2/sec in the 0.5 mg/ml group.

Notably no differences in weight or well-being were observed between mice exposed to Dox and those not exposed to Dox.

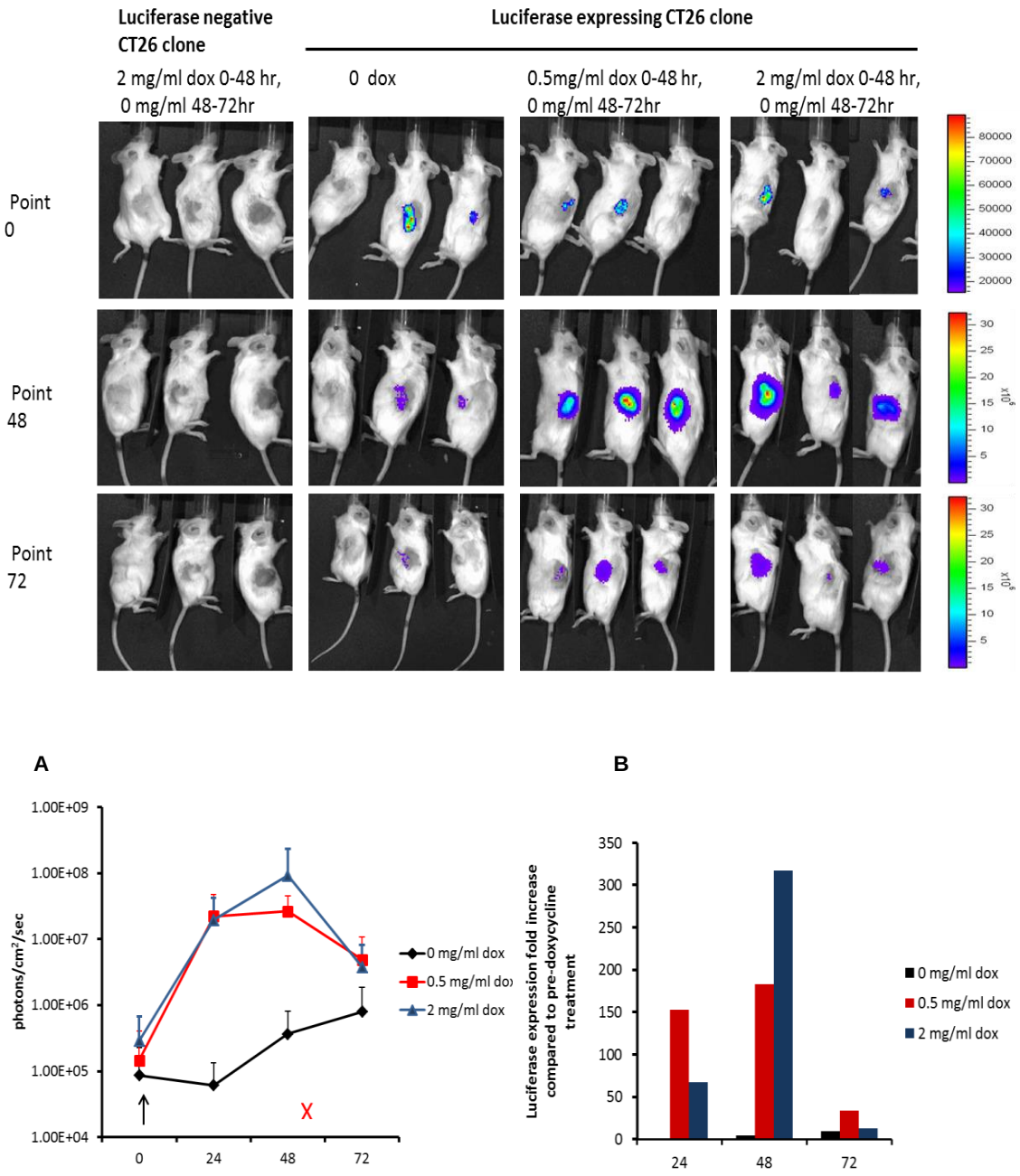


Figure 3.6 In vivo regulation of luciferase expression by addition and removal of Dox

CT26PLUC and CT26TetR were implanted in Balb/c mice s.c. and allowed to grow for 10 days. Mice were then imaged for luciferase expression before Dox addition (point 0) and at 24 and 48 hrs after its addition (point 24 and 48 on graph). At 48 hrs Dox treatment was removed and mice were imaged 24 hrs later (point 72 on graph). Black arrow on graph indicates start of Dox treatment, red cross indicates cessation of Dox treatment. In vivo bioluminescence imaging was conducted using IVIS system. Panel **A** shows raw data, panel **B** shows fold increase in mean photons/sec/cm^2 compared to levels pre-Dox addition. Data represent n=3,mean and SD. One way ANOVA analysis showed no significant difference between the groups.

3.4 Conclusion and discussion

Reversing local immunosuppressive factors is an attractive approach to enhance anti-tumour immunity. Very few *in vivo* studies have addressed whether blocking these factors locally may induce a more efficient tumour immune response that might result in tumour regression (Flavell, Sanjabi et al. 2010). A particular limitation of some of the studies that have tried to address this question is that attempts to block these immunosuppressive factors have been studied early in tumour growth (Zhao, Kobayashi et al. 2002). For example, in one study expressing STGFβRII protein from mouse thymoma cell line (EL4) showed a significant effect in lowering the overall tumour incidence in those mice ($p<0.001$) (Won, Kim et al. 1999). In addition lymphocytes from spleens of mice bearing the genetically engineered cell line showed higher cytotoxicity effect compared to mice bearing the wild type cell line (Won, Kim et al. 1999). However, such studies may not reflect the clinical condition since most of the local immunosuppressive mechanisms are thought to be acquired later on in a well-established and larger tumour (Stewart and Smyth 2011). Another drawback of these studies is the use of systemic approaches to block these factors. For example the i.p delivery of the anti-TGFβ antibody was able to supress 4T1 cells metastasis to the lung which was also associated with T cell infiltrate in both the primary and metastatic site (Nam, Terabe et al. 2008). Systemic blockage of TGFβ however would be undesirable firstly, since delivery of blocking agents into the tumour interstitium may be inefficient and secondly, because the pleiotropic and fundamental homeostatic roles of some of these factors may render their systemic blockade undesirable (Flavell, Sanjabi et al. 2010). Thus, till this moment little insight is known on the advantage of blocking immunosuppressive molecules locally in well-established tumours.

Thus the aim of this work was to engineer a cell line that can express immunosuppressive molecule antagonists under an inducible system, and study the effect of this on tumour immunity and tumour growth. Such technology should have many applications in studying the local microenvironment of cancer at different stages of development, and also in comparing possible protein therapeutics without having to address complicating issues of protein delivery.

In this chapter the performance of the Lenti-Tet-on advanced system was established and evaluated. This was achieved by the engineering of lenti vectors which make up the components of the Lenti-x-Tet-on system. These vectors were then used to establish a CT26 stable cell line expressing the TetR protein and a double transgenic stable cell line expressing TetR and luciferase. The CT26PLUC cell line was used to evaluate the response of the system, *in vitro* and *in vivo,* to its chemical inducer Dox. The system was shown to be tightly regulated by Dox since very low basal background expression was seen *in vitro* and *in vivo* its absence, whereas upon its addition luciferase levels of expression increased up to/over 150-fold *in vitro* and up to 300-fold *in vivo.* The high levels of expression were comparable to alternative constitutive promoters such as Pcmv, demonstrating the power of the system. Furthermore removal of Dox resulted in dramatically reduced levels of luciferase expression *in vivo*

One of the limitations of the first generations of Tet-on systems was the retained residual affinity of the transactivator to the tetO sequence in the absence of Dox, which was translated into high basal background levels. In addition the prokaryotic nature of the first generation of transactivators gave it less stability and weaker ability to function in mammalian cells post induction (Urlinger, Baron et al. 2000) . Indeed this was reflected in studies that showed that the expression of luciferase under the Tet-on system plasmids injected intramuscularly in Balb/c mice showed high levels of background expression before treatment with Dox (15 folds higher than control mice) which only showed a 10 fold increase after giving Dox (Perez, Plence et al. 2002). The Lenti-X- Tet-on advanced system is a modified Tet-on system that utilise a novel synthetic transactivator engineered to utilise human codons to increase protein expression and stability in mammalian cells (Urlinger, Baron et al. 2000). The sequence of novel transactivators were discovered after random and directed mutagenesis in the first generation, which yielded novel transactivators that had lower residual affinity to the tetO sequence in the absence of Dox, and were more stable *in vivo* (Urlinger, Baron et al. 2000). Indeed this might explain why we were able to see a 300 fold induction of luciferase expression 48 hrs post Dox administration, while only very low background levels (6 fold increase of control mice) was detected.

In chapter 3 it was concluded that luciferase expression under the Lenti-X-Tet-on system was comparable to luciferase expression under the strong constitutively expressed promoter Pcmv. Other reports have shown that the maximum expression levels of the Tet system (Tet-off) was

approximately 35 fold higher than that of Pcmv system (Yin, Zhu et al. 1996). However, it was noted in the same report that the selection of clones expressing the transactivators and clones expressing the transgene was an important factor, as clonal variation could be detected due to different levels of transactivator expression. In addition different cell lines reacted differently with the Tet-system.

In summary studies were successfully preformed to evaluate the Lenti-X-Tet-on system in CT26 cells and CT26 tumours. These studies showed that the system is responsive, reversible, well controlled by sub-toxic doses of Dox, and well tolerated by the animal model used. These experiments therefore validated the Tet-on system as a suitable strategy to achieve tightly controlled, inducible expression of immunomodulating proteins from within genetically engineered tumours *in vivo.*

4 Establishing and Characterizing a CT26 cell line Expressing Immunosuppressive Molecules Antagonists Under the Tet-on system.

4.1 Introduction

Two of the functionally important immunosuppressive molecules expressed locally within the tumour microenvironment are TGFβ and IL-10. As described in section 1.4.1.4 and 1.4.2 both cytokines have considerable potency in suppressing the function of a range of immune cells, suggesting they maybe key mediators. This has made them attractive targets, and approaches to antagonise their functions have long been an area of interest.

Blocking TGFβ signaling

The observation of the importance of TGFβ in cancer progression, as described in section 1.4.1.3 has led to the development of several strategies that aim to inhibit TGFβ signaling (Akhurst and Hata 2012).These strategies have developed into clinical trials for numerous neoplastic diseases and some non-neoplastic disorders and are explained in more details in section 1.4.1.5 .Two of the strategies used to inhibit TGFβ signaling are the use of neutralising mAb, or the application of ligand traps. The advantage of mAb and ligand trap strategies is their ability to sequester extracellular ligands, which in the context of TGFβ mediated immunosuppression in tumours is essential since the ligand would be secreted from various stromal and immune cells within the tumour, and acts in both an autocrine and paracrine manner (Massague 2008) In addition such strategies provide ligand-specific intervention, a level of selectivity which is a limiting factor in some of the other strategies e.g anti-ligand anti-sense oligonucleotides(Akhurst and Hata 2012). A ligand trap also has the advantage of being smaller in size than a mAb and thus better able to penetrate through the tumour (Scott, Wolchok et al. 2012).

STGFβRII

An example of the ligand trap strategy is the delivery of recombinant STGFβRII protein. As explained in more details in section 1.4.1.5.2, the STGFβRII can bind to TGFβ acting as

truncated receptor, and thereby preventing TGFβ signaling through the wild type receptor (Tsang, Zhou et al. 1995). It has shown strong affinity for TGFβ and has been used in several preclinical studies in tumour models (Zhao, Kobayashi et al. 2002, Hu, Gupta et al. 2012, Zhang, Hu et al. 2012).

STGFβRII-Fc

Another form of the STGFβRII, a chimeric recombinant protein using STGFβRII and Fc region of human immunoglobulin (STGFβRII-Fc), has also been used in preclinical studies (Zhang, Hu et al. 2012), and shown similar properties to the original receptor in terms of binding affinity to TGFβ (Komesli, Vivien et al. 1998). Several proteins (ligands, enzymes, soluble receptors) have been genetically engineered to be fused to Fc regions for therapeutic purposes. The rationale for inclusion of the Fc region comes from the ability of Fc to enhance the immobility and stability of proteins, allowing an increase in serum half-life *in vivo* (Huang 2009). One way the addition of Fc region can facilitate this is through its ability in generating homodimers, which decreases size mediated renal clearance(Huang 2009).

SIL-10

IL-10R1 is a transmembrane protein that acts as the IL-10 binding domain in the IL-10R complex. The mouse IL-10R1 is composed of the extracellular domain (225 a.a), a transmembrane domain (21 a.a), and a cytoplasmic signaling domain (313 a.a). The IL-10R1 binds with high affinity to IL-10 (70 pM) (Ho, Liu et al. 1993) and therefore the soluble extracellular domain of IL-10R1 (SIL-10R), has been has been used commercially as an IL-10 inhibitor (R&D systems). However, no preclinical or clinical studies have used the protein as a therapeutic modality to block IL-10.

Expressing STGFβRII,STGFβRII-Fc and SIL-10 under the Tet-on system

The Lenti-X-Tet-on system can allow the TGFβ1 or IL-10 sequestering receptors to be expressed in a regulated manner and secreted from CT26 cells within the tumour microenvironment, so that their capacity to act as a TGFβ1 or IL-10 antagonists can be gauged. The influence of the delivery of recombinant forms of these antagonists on tumour growth following systemic delivery would be difficult to characterise due to uncertainties surrounding pharmacokinetics, tumour deposition and distribution within the tumour. The approach described here allows the level and timing of production of these proteins to be controlled in

situ. This chapter describes the approach that was used to establish and characterise a CT26 cell line expressing these antagonists under the Tet-on system.

4.2 Chapter objectives

1- **To engineer a CT26 cell line expressing STGFβRII,STGFβRII-Fc, and a CT26 cell line expressing SIL-10 proteins under the Tet-on system**

2- **To characterise the secretion and biological activity of the STGFβRII *in vitro***

3- **To characterise the secretion and biological activity of the STGFβRII *in vivo:*** with emphasis on the timing of induction with respect to the establishment of CT26 tumours grown in Balb/c mice

4.3 Results

4.3.1 Characterising the expression of TGFβ from CT26 cell line and effect on growth of these cells

Before establishing a CT26 cell line expressing the STGFβRII *in vivo* it was important to first characterise the proliferative or anti-proliferative effect of TGFβ on this cell line. As discussed previously TGFβ is a pleiotropic molecule that can act as a promoter of tumour growth but can also act as a tumour growth inhibitor through its anti-proliferative effect on epithelial cells. It was therefore necessary to evaluate whether CT26 cells respond to the anti-proliferative effects of TGFβ1, which might indicate that TGFβ can act as a tumour suppressor in this cell line. If this was the case inhibiting TGFβ1 with STGFβRII may actually promote tumour growth. Conversely, an up-regulated expression of TGFβ by these cells might indicate that it is one of the mechanisms acquired to induce immunosuppression and thus validating the value in antagonising it. Indeed, very high expression of TGFβ by these cells might indicate that full antagonism may be difficult to achieve with STGFβRII. Therefore it was important to measure the expression of TGFβ by CT26.

4.3.1.1 TGFβ1 is secreted in the culture medium of CT26 cells

To test for TGFβ1 secretion, CT26 cells were seeded in a 6 well plate and cultured for 48 hrs in serum free medium. For comparison, two other mouse cell lines were also tested; the B16 melanoma cell line and C2C12 myoblast cell line. After 48 hrs culture medium was collected from all 3 cell lines and TGFβ1 was detected by ELISA. CT26 were shown to secrete 0.25 ng/ml of TGFβ1 (from $4x10^5$ cells/well), however, this was a significantly lower level than produced by the B16 ($p<0.05$) and C2C12 ($p<0.001$) cell lines which were tested in conjunction (~ 0.4 and 1.3 ng/ml respectively) (Figure 4.1).

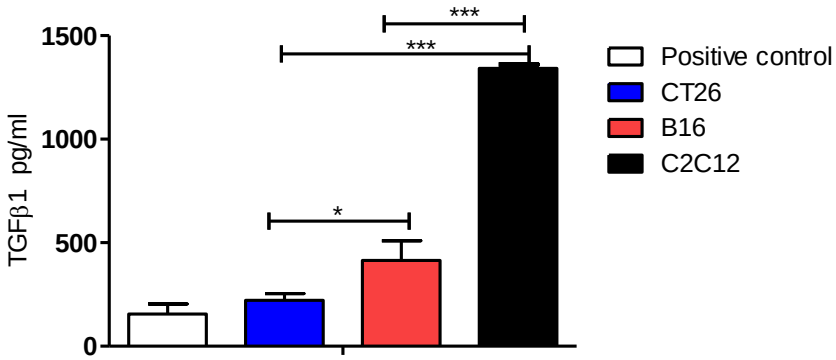


Figure 4.1 TGFβ1 expression from mouse cancer cell lines

Cells were seeded ($2x10^5$ cells /well) and cultured in serum free medium for 48 hrs. Culture medium was the collected and activated by HCl according to manufacturer's protocol section 2.9 and TGFβ1 concentration was detected by ELISA. Positive control (recombinant TGFβ1) was provided with the kit. Results represent n=3, mean and SD shown. One way ANOVA was performed for statistical analysis * = P<0.05, ***= P<0.001

4.3.1.2 CT26 cells have intact TGFβ signaling but do not respond to TGFβ1 anti-proliferative effect

TGFβ1 has an anti-proliferative effect on epithelial cells (section 1.4.1.3). However various tumours and cell lines lose their sensitivity to this effect. It was therefore important to study the effect of TGFβ on CT26 proliferation.

An *in vitro* reporter system was designed to determine whether CT26 cells have intact TGFβ signaling pathways. Cells were transfected the P-3TP-LUX plasmid, a TGFβ responsive reporter vector which expresses luciferase under a TGFβ responsive promoter that contains

three consecutive tissue plasminogen activator (TPA) response elements (TRE) and a portion of the plasminogen activator inhibitor 1 (PAI-1) promoter region, since PAI-1 expression is strongly induced by TGFβ (Figure 4.2) (Chen, Lebrun et al. 1996). Mv1Lu is known to contain intact TGFβ signaling, and responds to the anti-proliferative effect of TGFβ, and therefore were also transfected with P-3TP-LUX and used as a positive control. Post transfection with the P-3TP-LUX vector both cell lines were treated with different concentrations of recombinant TGFβ1, and luciferase expression was measured 16 hrs later.

Both Mv1Lu and CT26 showed an increase in luciferase expression (~ 5 fold) after the addition of TGFβ1 (0.1 ng/ml) (Figure 4.3A). However this expression was not linearly dose dependent at increasing levels of TGFβ1, which caused luciferase levels to plateau and then to slightly decline.

To test if CT26 would show inhibited proliferation in the presence of TGFβ, cells were plated and treated after 18 hrs with no TGFβ1 (control) or a range of increasing concentrations of TGFβ1 in serum free medium. Three days later cell viability was determined by MTS assay, Mv1Lu was used as a positive control. Mv1Lu viability dropped to approximately 50% of mock untreated cells when treated with TGFβ1 at 0.1 ng/ml, and the viability declined further to 30% of mock untreated cell with higher concentrations (Figure 4.3B). Unlike Mv1Lu, however, CT26 were resistant to the anti-proliferative effect of TGFβ1 seen in Mv1Lu, even at high concentrations (Figure 4.3B).

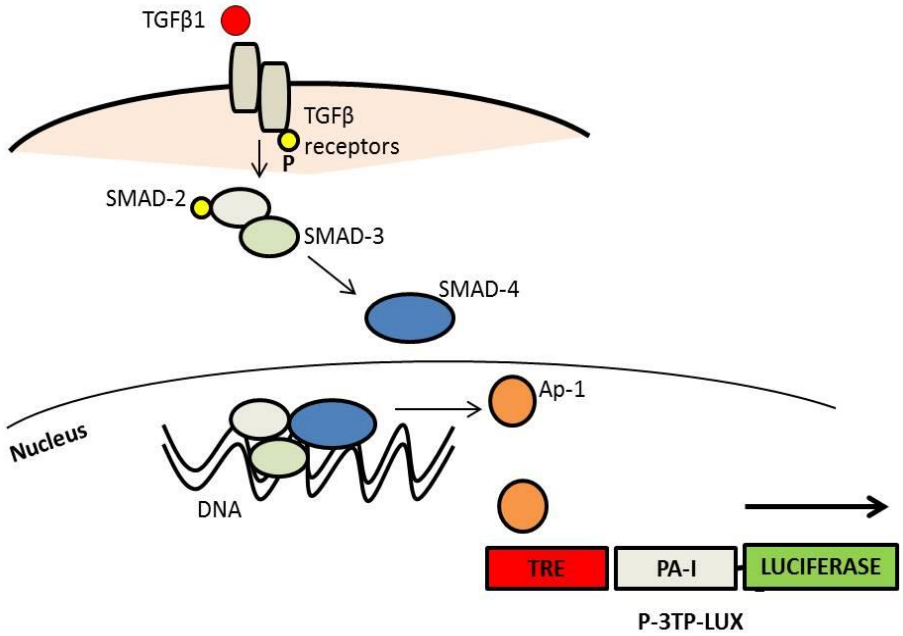


Figure 4.2 The P-3TP-LUX plasmid mechanism of action

Upon binding of TGFβ to its receptor the downstream signaling cascade is activated and results in the expression of the activator protein 1(Ap-1) transcription factor. Ap-1 can in turn bind to the TRE and thus activate the PA-I promoter to drive luciferase expression from the P-3TP-LUX plasmid.

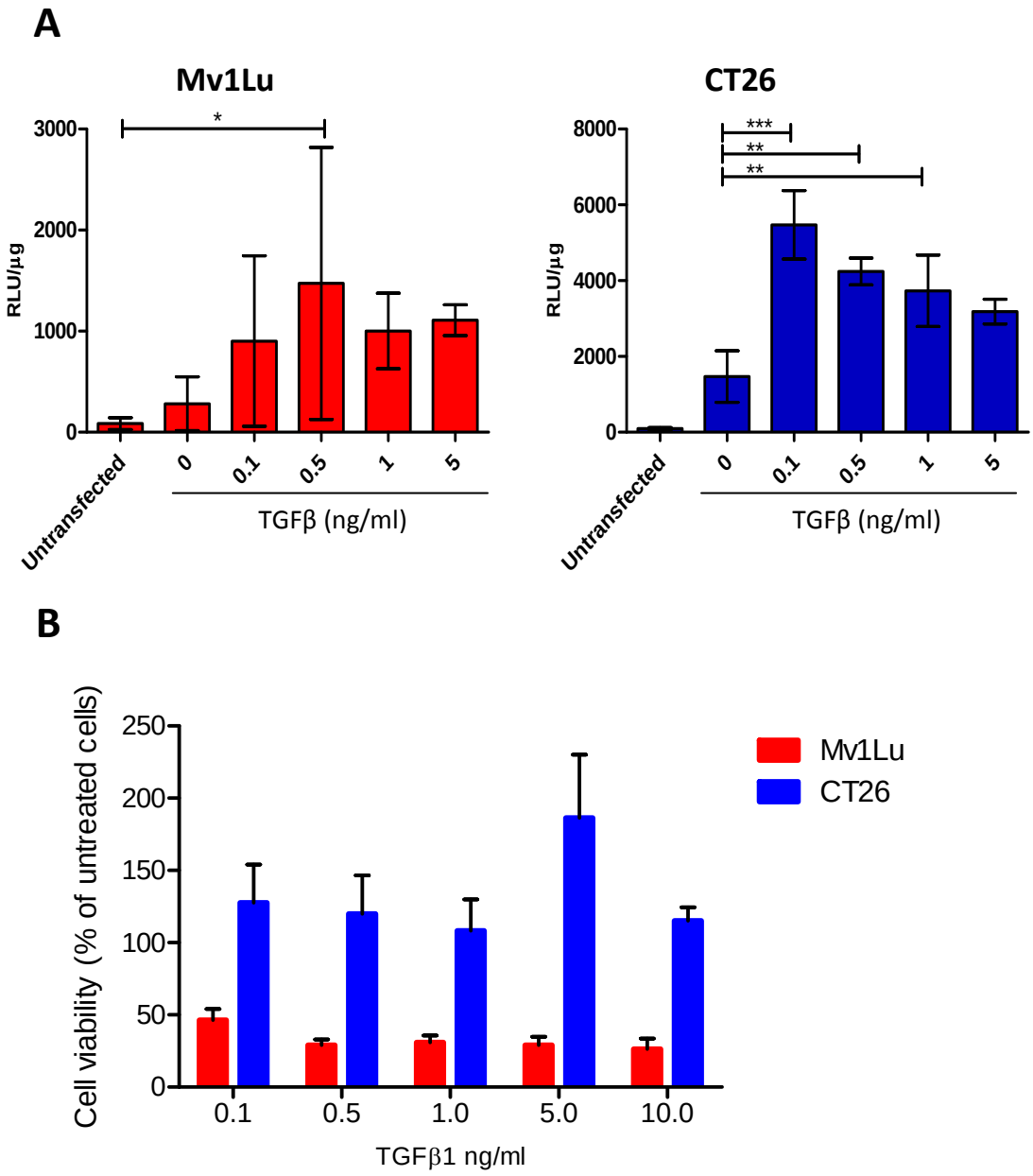


Figure 4.3 CT26 maintains intact TGFβ signaling but is not sensitive to TGFβ1 anti-proliferative function

A CT26 and Mv1Lu cells were seeded 10,000 cells/well and transfected 24 hrs later with the P-3TP-LUX plasmid. After 4 hrs post transfection, cells were treated with different concentrations of TGFβ1 in serum free medium for 16 hrs before luciferase activity was measured as RLU/µg. Data represents n=3, mean and SD shown, significance tested by one way ANOVA *** = $p < 0.001$, **= $p < 0.01$,* = $p < 0.05$. **B** CT26 and Mv1Lu cells were plated 5000 cells/well and treated 24 hrs later with different concentrations of TGFβ1. Three days later cell viability was measured by MTS assay. Data represents n=3, mean and SD shown and is presented as the % of the viability obtained when no TGF β1 was added.

4.3.2 Establishing a CT26 cell line expressing the STGFβRII and STGFβRII-Fc under a Dox inducible promoter

4.3.2.1 Cloning of the STGFβRII and STGFβRII-Fc sequence into the pLVX-Tight plasmid

The cDNA sequence for the extracellular domain (1-184 a.a) of mouse TGFβRII alone (STGFβRII) and the Fc region of mouse IgG1 (Fc) was synthesized (commissioned from Mr.gene) (Figure 4.4). The STGFβRII sequence was excised from its original plasmid with the appropriate endonuclease enzymes (Not1 and Xba1) to produce sticky ends compatible for ligation. The fragment was then gel extracted and purified and ligated into the Fc plasmid to generate the fused STGFβRII-Fc sequence. A test digest using the appropriate endonuclease enzymes was then conducted to verify the desired construct (Figure 4.5A), bands at ~ 1200 bp indicated the expected size for the STGFβRII-Fc construct and thus proved the successful ligation of the STGFβRII fragment. Both STGFβII alone and the STGFβRII-Fc were excised from their original plasmid and sub-cloned into the pLVX-Tight vector using the appropriate restriction sites and endonuclease enzymes (Not1 and Xba1 for the STGFβRII, BamH1 and EcoR1 for the STGFβRII-Fc) and a test digest was performed (Figure 4.5B). Band at ~1200 bp represented the expected size for the STGFβRII-Fc while band at ~500 bp represented the expected size for the STGFβRII fragment. The cloned pLVX-Tight-STGFβRII and the pLVX-Tight-STGFβRII-Fc plasmids were then sequenced to further ensure the correct sequence was cloned before use in further experiments.

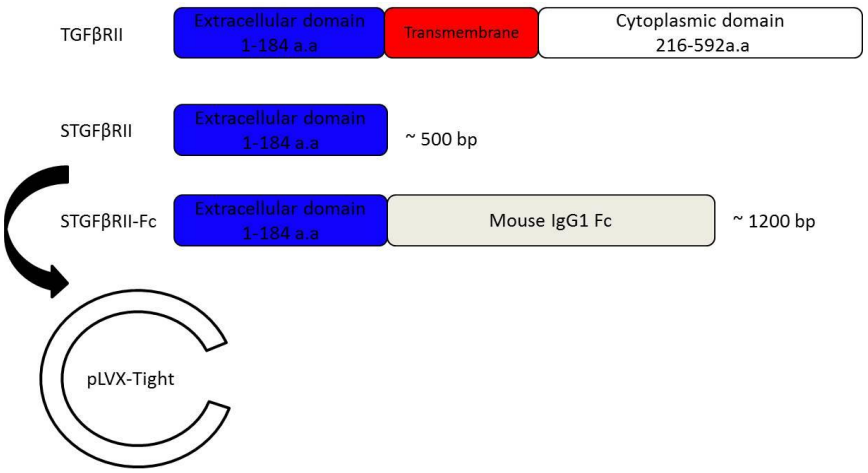


Figure 4.4 A schematic diagram of the cloning strategy of the STGFβRII and the STGFβRII-Fc sequence into the pLVX-Tight plasmid

The mouse TGFβRII consists of the ligand binding extracellular domain, a transmembrane domain and the signaling cytoplasmic domain. The cDNA sequence of the extracellular domain (184 a.a) which acts as the STGFβRII was obtained from Mr.gene and subcloned to be ligated to the mouse IgG1-Fc sequence also obtained from Mr.gene. Both fragments the STGFβRII and the STGFβRII-Fc were subcloned into the pLVX-Tight plasmid.

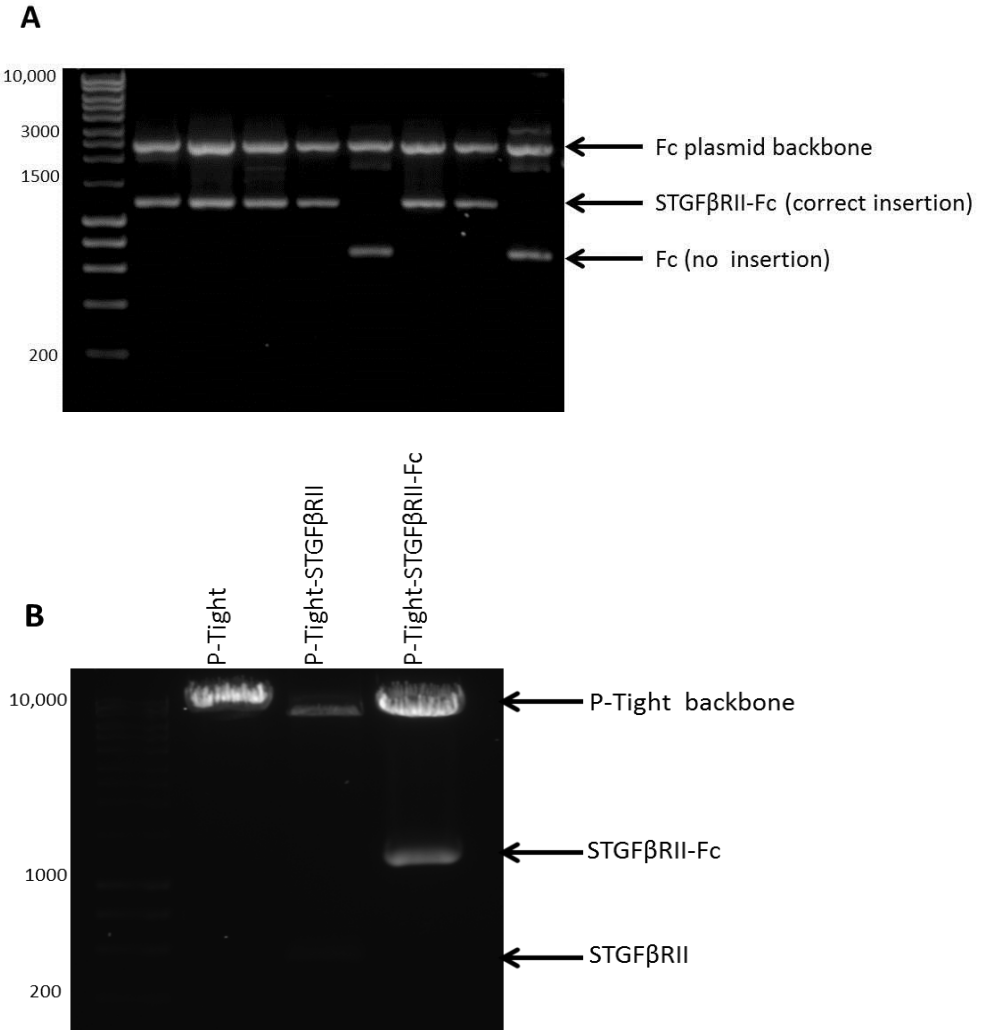


Figure 4.5 Cloning of the STGFβRII and STGFβRII-Fc into the pLVX-Tight plasmid

A The STGFβRII fragment was cut from its original plasmid using Not1 and Xba1 endonuclease enzymes, and subcloned into the mouse IgG1 Fc plasmid. Plasmid was grown and 8 colonies were picked and digested with endonuclease to screen for the correct fragment. Top arrow indicates the correct molecular weight of the mouse IgG1 Fc plasmid backbone. Middle arrow indicates the correct molecular weight for the STGFβRII-Fc ligated sequence indicating insertion of the fragment. Lowest arrow indicates molecular weight of the mouse IgG1 Fc alone indicating clones that do not have the insert. **B** Both the STGFβRII alone or the STGFβRII-Fc were subcloned from their original plasmids and ligated into the pLVX-Tight plasmid (P-Tight on graph) using (Not1 and Xba1 for STGFβRII and BamH1 and EcoR1 for STGFβRII-Fc). A test digest was conducted to compare the pLVX-Tight alone (negative control) and the pLVX-Tight expressing STGFβRII or STGFβRII-Fc. Above arrow indicates the molecular weight of the pLVX-Tight backbone, middle arrow indicates the predicted size of the STGFβRII-Fc fragment and lower arrow indicates the predicted molecular weight of the STGFβRII fragment. Weights were determined using hyper ladder I (Bioline) gel was run as described in methods 2.3.9.

4.3.2.2 Establishing CT26 cell lines expressing STGFβRII or STGFβRII-Fc

To generate stable CT26 cells lines expressing STGFβRII or STGFβRII-Fc, CT26 cells needed to be infected with Tet-on lenti virus vectors expressing those proteins. Therefore, the pLVX-

Tight-STGFβRII and pLVX-Tight-STGFβRII-Fc plasmids, constructed in section 4.3.2.1, were first transfected together with packaging plasmids in 293T cells to produce non-replicating lentivirus vectors expressing STGFβRII or STGFβRII-Fc. Supernatant from 293T cells (now containing lentivirus vectors) was then collected 48 hrs post transfection, and CT26TetR cells were infected with 6 lots of 10-fold serial dilutions of the supernatant containing lentivirus vectors, to allow for the colony formation of CT26 cells expressing STGFβRII or STGFβRII-Fc. Transfectants were then selected by using G418 and Puromycin. It was possible to isolate resistant clones, indicating a double stable cell line expressing both the TetR and the newly introduced transgenes. These clones were then allowed to grow before they were tested for transgenes expression.

4.3.2.3 Engineered CT26 cell lines show Dox regulated transgene expression *in vitro*

To assess the expression of STGFβRII and STGFβRII-Fc under Dox control, cells were seeded in a 6 well plate in serum free medium and treated with a range of concentrations of Dox (0, 100, 500 or 2500 ng/ml). The supernatant was collected and concentrated using the Amicon centricons as described in materials and methods section 2.10. Both the cell lysate and supernatant culture medium were studied for the expression of STGFβRII and STGBFβRII-Fc, 24 and 48 hrs post Dox treatment by Western blot (Figure 4.6A.B). Both cell lines were able to express the respective proteins under Dox control after 24 and 48 hrs, with protein detected in the cell lysate and concentrated supernatant. STGFβRII-Fc was detected at its predicted molecular weight ~ 70 kDa, while STGFβRII was detected (as expected) at ~ 30 kDa. The expression of both proteins was dependent on the dose of Dox. No expression was seen in cells not treated with Dox or in control non-infected CT26TetR cells Whilst tests on supernatant revealed single bands, cell lysates produced multiple bands probably reflecting the heterogeneous glycosylation of the protein which was described in other reports (Lin, Moustakas et al. 1995).

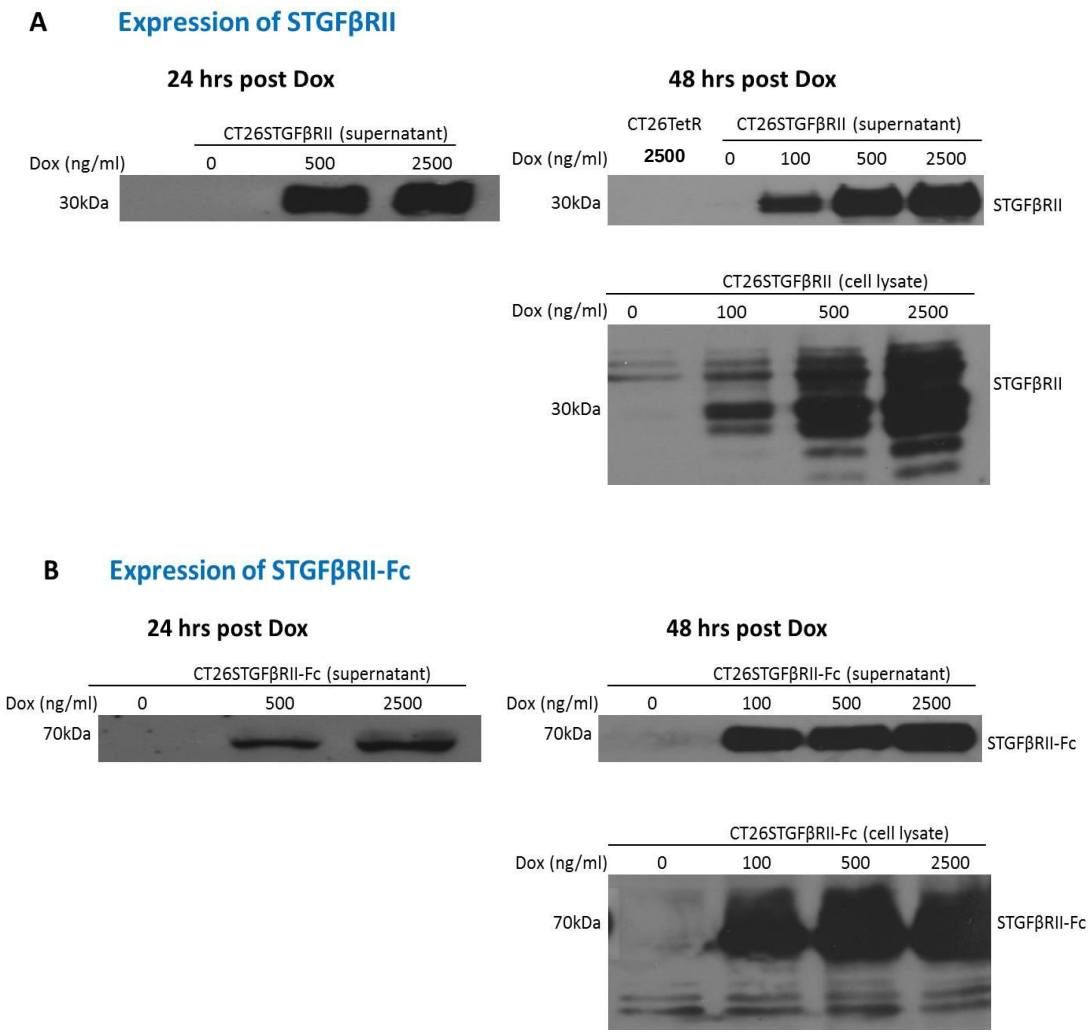


Figure 4.6 STGFβRII and STGFβRII-Fc can be expressed under Dox control and are detectable in the supernatant and cell lysate of CT26 cells

Engineered CT26 cells were plated 8x10^{5}cells/well. The following day, cells were either left untreated or treated with different concentrations of Dox in serum free medium. Expression of STGFβRII **A,** or STGFβRII-Fc **B** was studied in the cell lysate or the concentrated supernatant by Western blot either 24 hrs or 48 hrs post Dox treatment. CT26TetR cells were used as negative control. **A** a band at MW ~ 30 kDa (corresponding to STGFβRII) could be detected post Dox treatment in both the cell lysate and supernatant of CT26STGFβRII. The expression could be detected 24 hrs and 48 hrs post Dox treatment and was dependent on the dose of Dox. **B** Similarly the expression of the STGFβRII-Fc could be detected at ~ 70 kDa. The blots are representative of 5 independent experiments. For conditions and antibodies see material and methods section 2.10 and Table 2.3.

4.3.2.4 Estimation of the amounts of STGFβRII and STGFβRII-Fc being secreted

To estimate the amount of STGFβRII and STGFβRII-Fc being produced by the engineered cells, levels in the supernatant were compared with a standard curve of known concentrations of a commercial recombinant mouse STGFβRII-Fc (commercial STGFβRII-Fc). The protein is composed of the Mouse TGFβRII extracellular domain linked to mouse IgG_{2a} produced in a mouse myeloma cell line and is detected at 66 kDa in reducing conditions of SDS-PAGE (R&D systems), thus its gel migration should be similar to the engineered STGFβRII-Fc protein, and approximately double the migration of the STGFβRII protein.

To measure the concentration of STGFβRII being produced in the supernatant, CT26 expressing STGFβRII were plated at $8x10^5$cells/well. The next day, cells were treated with 2500 ng/ml of Dox in fresh serum free-medium. 48 hrs later the supernatant was collected and concentrated to 200 µL. Since at this stage the amount being produced could not be predicted, different dilutions of the supernatant (neat,1:2,1:5,1:10) and a range of concentrations of the commercial STGFβRII-Fc (25,10,5,1 ng per well) were used (Figure 4.7). Densitometric analysis of Western blots was then conducted to determine the amount of STGFβRII protein expressed in supernatant relative to the commercial protein. Roughly 10 µl of concentrated supernatant gave equivalent staining to 25 ng of commercial protein, therefore a level of 500 ng was calculated to be present in the 200 µl of concentrated supernatant. Thus in total an estimation of 250 ng/ml of supernatant (500 ng per 1 mg of total cell protein) of STGFβRII was produced. When comparing the STGFβRII-Fc from concentrated supernatant to 25 ng of commercial protein, an estimated total of 125 ng/ml (250 ng per 1 mg of total cell protein) was obtained.

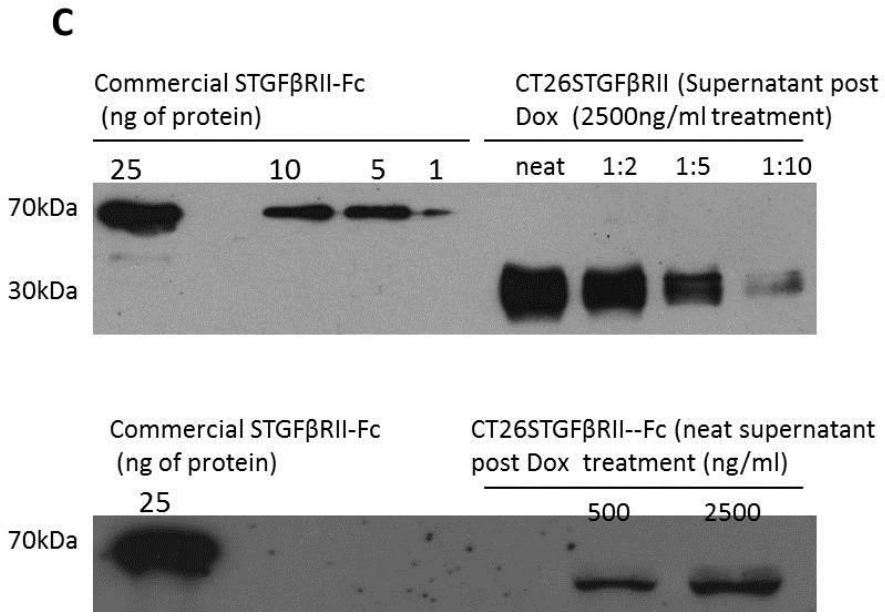


Figure 4.7 Determining the concentrations of STGFβRII and STGFβRII-Fc secreted in the supernatant of Dox treated CT26 cells

CT26 cells expressing STGFβRII or STGFβRII-Fc were plated and treated next day with Dox. 48 hrs later the supernatant was collected and concentrated. To determine the concentration of STGFβRII a Western blot running different dilutions of the concentrated supernatant against different known concentrations of the commercial STGFβRII-Fc was conducted (upper blot image). Similarly, dilutions of the concentrated supernatant from CT26STGFβRII-Fc were compared to 25 ng of the commercial STGFβRII-Fc (lower blot image). Densitometric analysis of bands was then conducted using Image J.

4.3.3 Expressed STGFβRII is functionally active and can prevent TGFβ induced phosphorylation of Smad2 *in vitro* in CT26 cells

The interaction between TGFβ and TGFβRII leads to a downstream signaling cascade with phosphorylation of Smad2 (P-Smad2) as a key outcome and indicator of intact signaling section 1.4.1.2. After confirming that STGFβRII was expressed in sufficient dose dependent amounts from transfected CT26 cells, it was necessary to validate the biological activity of STGFβRII through studying its ability to stop the TGFβ induced P-Smad2.

First, it was important to validate the ability of TGFβ to induce P-Smad2 in CT26 cells, and that this could be detected by Western blot. CT26 cells were therefore seeded at $4x10^5$ cells/well and the next day cells were treated with different concentrations of recombinant TGFβ1 in fresh medium for 30 mins. Medium was then removed and cells were lysed using phospho-protein

lysis buffer. Cell lysate was then studied for the expression of P-Smad2. TGFβ1 treatment of CT26 was able to induce the expression of P-Smad2 and signal was observed from treatment with TGFβ1 levels of 0.25 ng/ml and greater (Figure 4.8A). However, the expression seemed to plateau after 1 ng/ml of TGFβ, which is similar to previous results seen with the expression of luciferase under the 3TP promoter (Figure 4.3A)

Next the ability of the commercial STGFβRII-Fc to reverse the TGFβ induced P-Smad2 was tested to provide a positive control and allow the identification of the concentration of soluble receptor protein required to achieve this. The level identified could then be correlated to the amount of STGFβRII secreted from CT26STGFβRII cells. To achieve this CT26 cells were plated as previously described. The following day 1 ng/ml of TGFβ was mixed with different concentrations of the commercial STGFβRII-Fc for 10 min in serum free medium before addition of the mixture to the cells. 30 min later cells were washed and lysed and studied for the expression of P-Smad2. The signal of P-Smad2 was markedly reduced by adding 10 ng/ml of the commercial STGFβRII-Fc; however, higher concentrations did not appear to reduce the signal further (Figure 4.8B)

Inhibition of the cellular activity of TGFβ by mixing with commercial (pure) STGFβRII-Fc may be an over-simplistic model of the situation where STGFβRII is produced in situ, since there will be many other proteins present in the cell supernatant. These other proteins might affect the capacity of the protein to inhibit the phosphorylation signal, or might increase the baseline P-Smad2 signal expression since endogenous TGFβ is expected to be present in the mixture of proteins in the supernatant. For testing this 1 ng/ml of TGFβ was mixed with different concentrations of the commercial STGFβRII-Fc, however, this time mixing was performed in the presence of supernatant from CT26PLUC cells seeded 48 hrs previously, before adding to CT26 cells and detection of P-Smad2. Similar to the previous result, a reduction in the P-Smad2 signal could be seen after treatment with 10 ng/ml of commercial STGFβRII-Fc and the signal was not further reduced with a higher concentration (Figure 4.8B)

The key experiment was the study of the ability of the STGFβRII engineered and secreted from CT26STGFβRII cells to inhibit the TGFβ induced P-Smad2. This would indicate that the secreted protein can efficiently bind to TGFβ and prevent its binding to the wild type receptor. To do this CT26STGFβRII cells were treated with Dox for 48 hrs and the supernatant containing

STGFβRII was collected for testing. As a negative control supernatant from two control cell lines was collected in the same manner post Dox treatment. These included CT26TetR and CT26-SIL-10R (described in section 4.4) which similar to the CT26STGβRII cell line was infected with the same Tet-On lentivirus vectors, however, it expresses SIL-10R rather than STGFβRII. All concentrated supernatant was mixed with different concentration of TGFβ for 1 min before addition of the mixture to CT26 cells. The expression of P-Smad2 was studied as formerly described. As previously observed 1 ng/ml of TGFβ was able to induce a P-Smad2 signal in CT26 cells, however, only cells treated with CT26STGFβRII supernatant failed to induce P-Smad2 (Figure 4.8C) (see * on blot C) indicating the ability of the secreted STGFβRII to prevent the TGFβ induced signal.

To confirm and analyse this result in more detail a densitometry analysis was carried out. P-Smad2 bands were first normalized to the internal control Smad2 (total un-phosphorylated protein) and a relative fold up-regulation of P-Smad2 when treated with 1 ng/ml of TGFβ was compared to untreated cells. Treatment with 1ng/ml in the presence of supernatant from the two control cell lines CT26TetR and CT26-SIL-10 induced a 3 fold increase in the level of P-Smad2, however, in the presence of supernatant from CT26STGFβRII there was no induction (bar graph in Figure 4.8C)

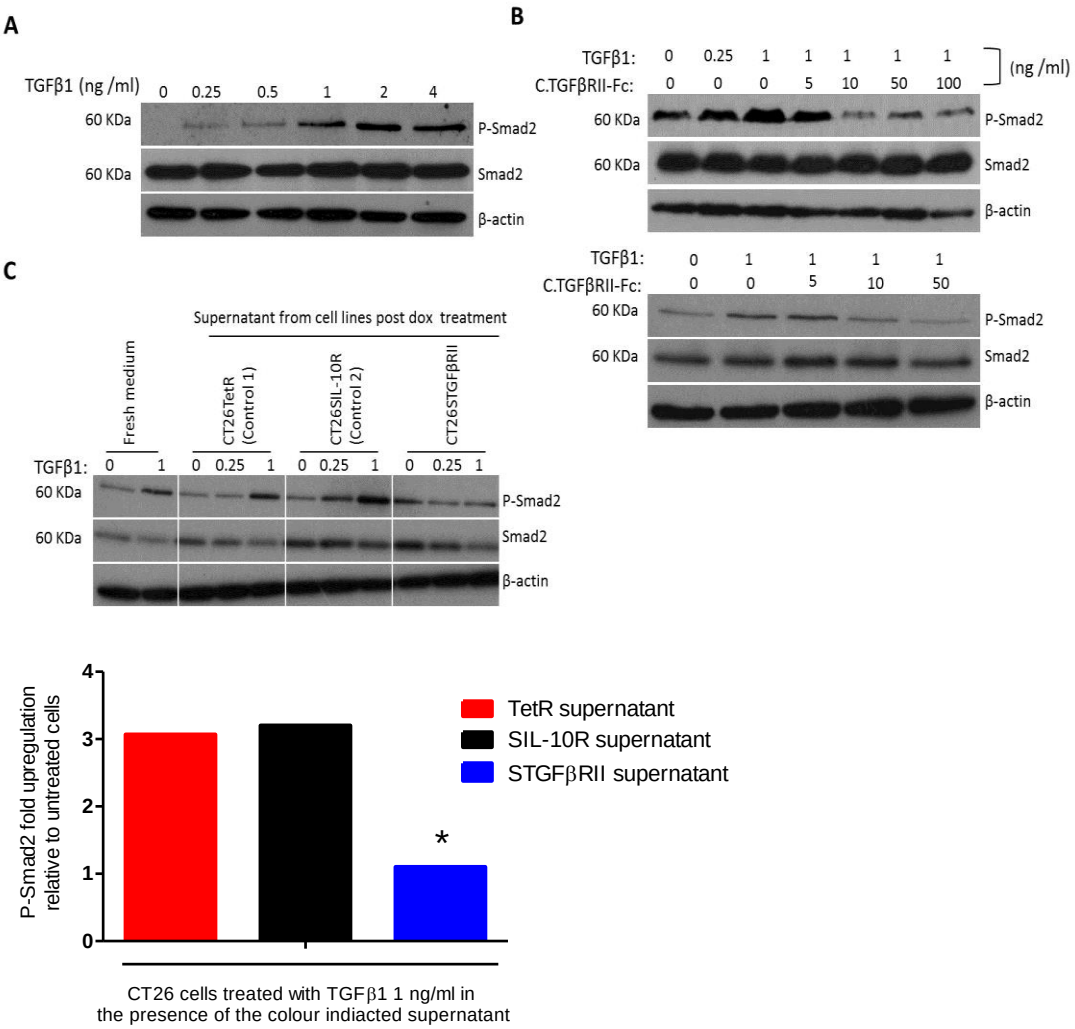


Figure 4.8 Inhibition of TGFβ induced P-Smad2 by the secreted STGFβRII in CT26 cells

A CT26 cells were seeded and treated next day with different concentration of TGFβ1 in serum free medium for 30 min. Cells were lysed and analysed for the expression of P-Smad2. **B** Different concentrations of the commercial STGFβRII-Fc (C.TGFβRII-Fc ng/ml) were mixed with TGFβ, in serum free medium (upper blot), or supernatant from seeded cells (lower blot) for 10 min before adding the mixture on seeded CT26. 30 min later cells were lysed and analysed for the expression of P-Smad2. **C** Supernatant from CT26STGFβRII cells post Dox treatment or control supernatant was mixed for 1 min with different concentrations of TGFβ before adding the mixture on seeded CT26 cells. 30 min later cells were lysed and analysed for the expression of P-Smad2. All blots were stripped and re-probed for two loading controls; the total Smad2 and β-actin. Bar graph represents P-Smad2 fold upregulation as analysed by densitometry of the blot in **C.**

4.3.4 Expression of STGFβRII or STGFβRII-Fc from CT26 cells *in vitro* does not induce cell death of CT26 cells

Successful Dox induced production of STGFβRII and confirmation of its activity as a TGβ antagonist were confirmed in section 4.3.2 and 4.3.3 respectively. However, before expressing the STGFβRII as a TGFβ antagonist *in vivo* it was necessary to confirm that expressing the protein does not in itself induce cell death of CT26 cells. Another important point to address was any anti-proliferative effect of Dox treatment on CT26 cells, since Dox has been shown to have an anti-proliferative effect on other cell lines (Fife, Rougraff et al. 1997, Cakir and Hahn 1999).

In order to test for any growth effect of these transgenes on CT26 cells, CT26PLUC, CT26STGFβRII, and CT26STGFβRII-Fc cells were plated in a 96 well plate. The next day, cells were either left untreated or treated with Dox 2500 ng/ml. To detect cell viability an MTS assay was then conducted 72 hrs later. Expressing STGFβRII or STGFβRII-Fc did not decrease cell viability; on the contrary an increase in cell viability was seen in both cell lines ($P<0.001$) (Figure 4.9). This could be related to Dox treatment as the trend for an increase in cell viability could be seen in control cell line CT26PLUC, however, it did not reach significance $p>0.05$.

It was therefore important to study if Dox treatment in itself was the cause for the induced proliferation seen, and if as shown in other reports, in high concentrations it can inhibit proliferation and/or induce apoptosis. To study this CT26 cells were plated in a 96 well plate. The next day, cells were either left untreated or treated with different concentration of Dox (0.5, 2, 10, 50, 100 µg/ml). To detect cell viability an MTS assay was then conducted 72 hrs later. In contrast to results seen with engineered CT26 expressing transgenes, there were no significant differences in cell viability between low doses of Dox (0.5 and 2 µg/ml) and Dox untreated cells (Figure 4.10). As seen in other reports higher concentration of Dox starting from 10 µg/ml had a significant deleterious effect on cell viability when compared to untreated cells ($P<0.01$) this was further exaggerated with higher concentrations of Dox (50 and 100 µg/ml, $p<0.001$) (Figure 4.10).

It was therefore concluded that low doses of Dox did not have an effect on cell viability in CT26 cells, however, with high doses starting at 10 µg/ml cell viability is compromised. The expression of STGβRII and STGFβRII-Fc from CT26 did not in induce cell death; to the contrary there was a significant increase in cell viability.

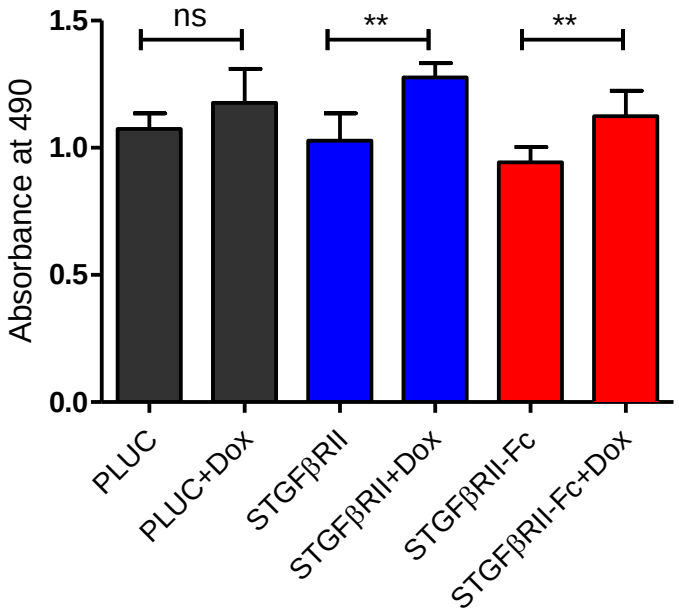


Figure 4.9 The effect of expressing STGFβRII and STGFβRII-Fc from CT26 on cell viability

CT26PLUC, CT26STGFβRII, and CT26STGFβRII-Fc cells were plated 10,000 cells/well in a 96 well plate. The next day cells were either left untreated or treated with 2500 ng/ml of Dox. Cells were incubated for 72 hrs before measuring cell viability using an MTS assay. Results represent n=5, mean and SD shown. Significance tested by unpaired Student's T test , **= $p < 0.01$

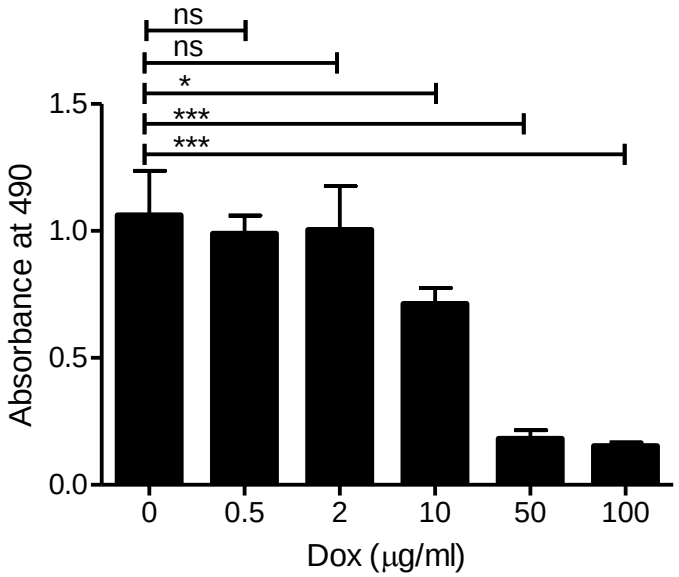


Figure 4.10 The effect of various conc of Dox on CT26 cell viability

CT26 cells were plated 10,000 cells/well in a 96 well plate. The next day cells were either left untreated or treated with different conc of Dox. Cells were incubated for 72 hrs before measuring cell viability using an MTS assay. Results represent n=3, mean and SD shown. Significance tested by one way ANOVA,*=$p<0.05$, **= $p < 0.01$, ***=$p<0.001$.

4.3.5 The STGFβRII is expressed by CT26STGFβRII tumours in Balb/c mice post Dox treatment

To examine if STGFβRII protein could be produced and secreted under Dox control *in vivo*, two small pilot studies were conducted using Balb/c mice. In the first experiment 5-6 weeks Balb/c mice were injected s.c. with CT26STGFβRII cells. When tumours reached sizes of approximately 100 mm^3, a serum sample was extracted for pre-Dox analysis and Dox treatment was then commenced. 24 hrs later a serum sample was extracted, and tumours were excised, homogenised, and lysed for Western blot analysis of STGFβRII expression. A band that corresponds to the expected molecular weight (~30kDa) of STGFβRII was detected in the mouse that received Dox while the same band was not seen in the mouse that did not receive Dox (Figure 4.11A left blot). A faint band could also be detected in the serum from the mouse receiving Dox which was not detected pre-Dox treatment or in the mouse that did not receive Dox (Figure 4.11A right blot). Another band was seen around 70 kDa in all mice, which might correspond to the endogenous TGFβRII.

To confirm that expression can be still detected after a longer duration of Dox treatment, a similar experiment was conducted, and mice were kept on Dox treatment for 7 days before tumours were excised for analysis. Similar results were seen with a band corresponding to STGFβRII detected in the Dox treated mouse while no band was detected in the control untreated mice (Figure 4.11B).

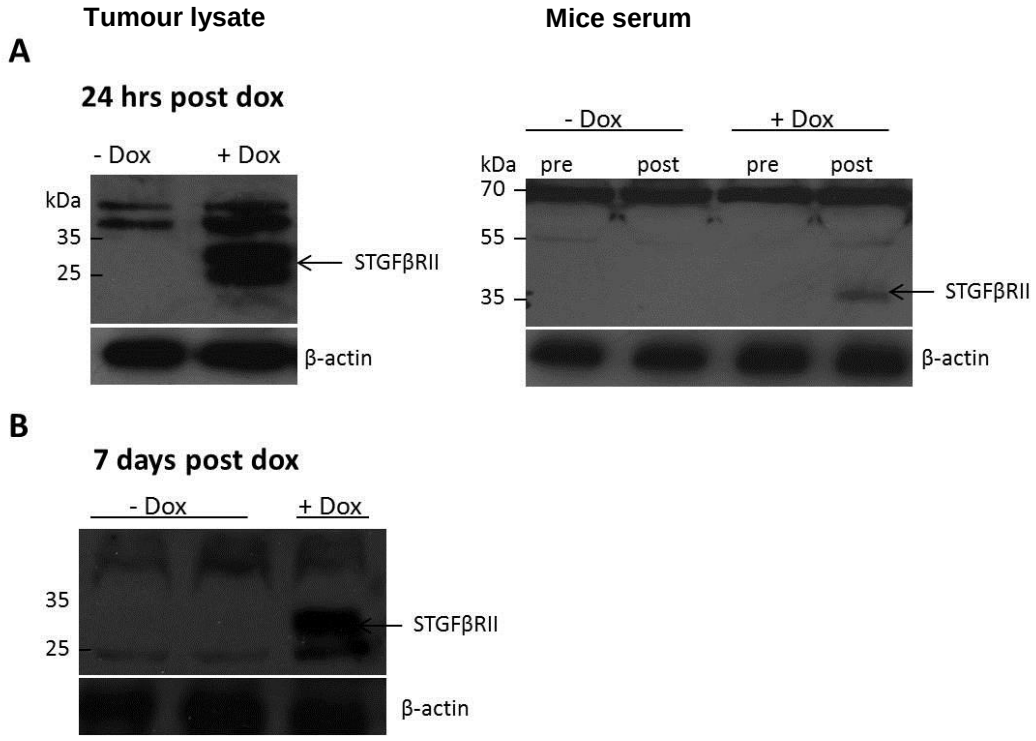


Figure 4.11 The expression of STGFβRII in CT26 tumours and its presence in mice serum post Dox treatment

A Balb/c mice were injected s.c. with CT26STGFβRII cells and treated with Dox or left untreated when tumours reached sizes of ~100 mm^3. 24 hrs later tumours (left blot) were excised and serum (right blot) was extracted for analysis of STGFβRII expression by Western blot. **B** Balb/c mice were injected s.c. with CT26STGFβRII and treated with Dox or left untreated when tumours reached sizes of ~100 mm^3. 7 days post Dox treatment tumours were excised for STGFβRII expression analysis. Arrow indicates the expected molecular weight of STGFβRII

4.4 Establishing a CT26 cell line expressing SIL-10R

4.4.1 Cloning and engineering a CT26 cell line expressing SIL-10R

The approached developed here can be viewed as a platform technology with potential applicability to the testing of a whole range of immunomodulatory proteins. Hence, after generating CT26STGFβRII and validating the ability of the protein to be induced successfully under the control of Dox *in vitro* and *in vivo,* it was interesting to test if the system could be used to express another antagonist. Having engineered a CT26TetR cell line, any other antagonist expressed under the Ptight promoter should be easily introduced in this cell line allowing the expression of the protein under the Dox control. One of the other key immunosuppressive factors to target locally is IL-10, and SIL-10R can act as an antagonist to IL-10.

Similar to section 4.3.2.1 the cDNA sequence of the extracellular domain of the IL-10R1 was ordered and subcloned in to the pLVX-Tight-Plasmid, and a lentivirus vector was generated by packaging in 293T cells. As in section 4.3.2.2 CT26TetR were infected with the SIL-10R expressing lentivirus and selected for resistance antibiotics. Resistant clones were selected and isolated and grown.

4.4.2 CT26SIL-10R cells produce SIL-10R under Dox control *in vitro*

As in section 4.3.2.1, the isolated CT26TetR cells were seeded in a 6 well plate in serum free medium and treated with a range of concentrations of Dox (0,100, 500 or 2500 ng/ml). Both the cell lysate and concentrated supernatant culture medium were studied for the expression of SIL-10R 48 hrs post Dox treatment by Western blot (Figure 4.12 A,B). Dox regulated expression was observed after 48 hours, with protein detected in the cell lysate and concentrated supernatant. This study demonstrates the wider utility of the system developed here.

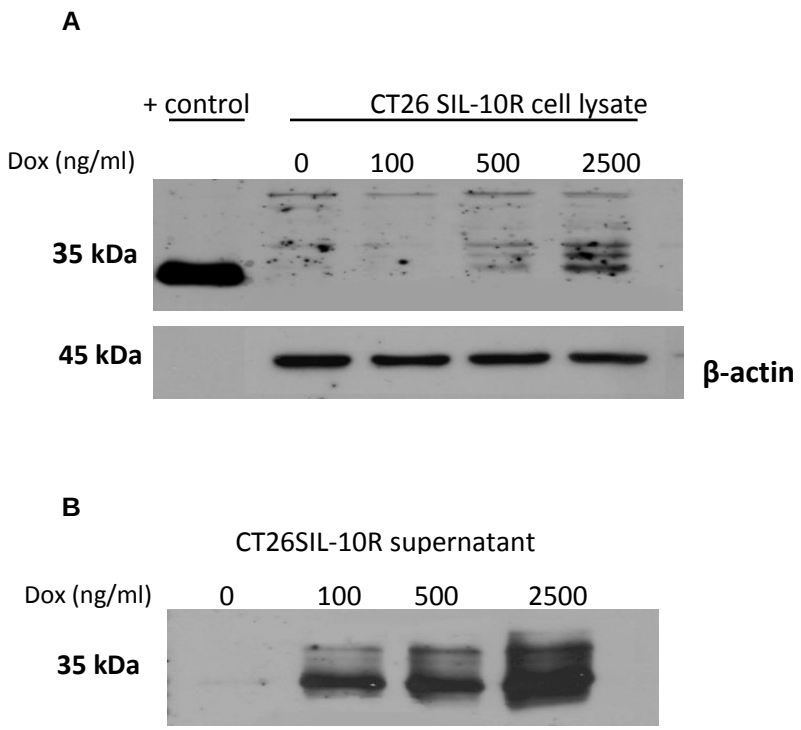


Figure 4.12 SIL-10R is expressed under Dox control in the cell lysate and supernatant of CT26 cells

Engineered CT26SIL-10R cells were plated $8x10^5$cells/well. The following day, cells were either left untreated or treated with different concentrations of Dox in serum free medium. Expression of SIL-10R in the cell lysate **A,** or supernatant **B** by western blot at MW ~ 35 kDa (corresponding toSIL-10R) could be detected post Dox treatment in both the cell lysate and supernatant of CT26SIL-10R. The expression could be detected 48 hrs post Dox treatment and was dependent on the dose of Dox.

4.5 Conclusion

The aim of this work was to develop a system to characterise the effect of reversing TGFβ locally in the tumour microenvironment. However, due to its pleiotropic and dynamic effect on epithelial cells it was first necessary to characterise the influence of TGFβ on CT26 cells before *in vivo* studies could be performed. Such *in vitro* characterisation was therefore the focus of the first half of this chapter.

There are several strategies for reversing TGFβ (Akhurst and Hata 2012), ligand traps are an attractive approach as they have the advantage of sequestering any extracellular ligands within the tumour environment (Akhurst and Hata 2012).

The STGFβRII and the STGFβRII-Fc have been used before as recombinant exogenously applied TGFβ antagonists and have been shown to competitively inhibit the binding of TGFβ to its receptor (Rowland-Goldsmith, Maruyama et al. 2002, Hu, Gupta et al. 2012) . However, in all reports there was a lack of control over the timing, level, and distribution of the presence of

these antagonists within the tumour. The aim of the second half of this chapter was therefore to establish and characterise CT26 cell lines expressing STGFβRII or STGFβRII-Fc under Dox control.

To characterise the expression of TGFβ from CT26 cells ELISA experiments measuring TGFβ concentration demonstrated that unmodified parental CT26 express TGFβ1 in the supernatant at approximately 250 pg/ml (per 1 mg of total cell protein) in 48 hrs (Figure 4.1). This was in accordance with previous reports which showed similar levels of expression from CT26 cells (Jarnicki, Lysaght et al. 2006). However, this was less than the level detected in other mouse cell lines e.g. B16 and C2C12 (Figure 4.1). This might indicate that TGFβ expression is one of the mechanisms used by CT26 cells to induce cancer progression but might not be the only mechanism. This also meant that the probability of expressing a sufficient amount of the antagonist to achieve reversal of TGFβ in this cell line is higher than in other cell lines expressing very high levels of TGFβ. CT26 cells appeared to still have intact TGFβ signaling, since they were able to express luciferase under a TGFβ responsive promoter in a manner similar to a known TGFβ sensitive cell line; Mv1Lu (Satterwhite, Aakre et al. 1994) (Figure 4.3A). It was noticed that this was not dose dependent as levels more of than 0.5 ng/ml of TGFβ did not further enhance the luciferase expression in both cell lines. This meant that small amounts of TGFβ would be required to induce a signal which then reaches a plateau and cannot be further induced by more treatment. This also might explain why the majority of TGFβ is in its inactive form physiologically, since only very low amounts of the active form are required (Annes, Munger et al. 2003). However, one may expect that within tumours a more active form is present due to the inherent low pH in tumours which is a known factor for activating TGFβ (Annes, Munger et al. 2003).

Notably, despite having an intact TGFβ signaling, CT26 were not sensitive to the anti-proliferative effect of TGFβ since incubating with a range of TGFβ concentrations did not have an effect on cell viability (Figure 4.3B). While same concentrations of TGFβ induced 50-70 % death in Mv1Lu three days post TGFβ treatment, in accordance with previous studies (Satterwhite, Aakre et al. 1994). This indicates that CT26 do not experience a direct anti-proliferative effect of TGFβ in which case reversing TGFβ in this cell line should not be counter-productive in terms of trying to achieve tumour growth retardation. The loss of the anti-

proliferative effects of TGFβ despite the intact signaling confirms the complexity of the TGFβ signaling which has yet to be fully defined (Massague 2008, Akhurst and Hata 2012). It is known that the activated Smads are shared among many binding partners, each of which activate or deactivate a subset of TGFβ responsive genes only. A downstream mutation in any of the Smads binding partners in cancer cell might therefore explain why a subset of the TGFβ responsive genes remain to be responsive to TGFβ while others don't. In addition the role of the non Smad pathways in the paradoxical outcomes of TGFβ must not be forgotten (Massague 2008).

To establish the CT26 cell lines expressing STGFβRII and STGFβRII-Fc, the correct sequence for STGFβRII and STGFβRII-Fc was subcloned in the pLVX-Tight plasmids. Although the commercial form of STGFβRII-Fc utilizes the IgG2a constant region, we chose IgG1 constant region for our fusion protein. While mouse IgG2a can induce ADCC and CDC to high extent, IgG1 does not, and thus any immunological effect that might be seen from the fusion protein should be the effect of the STGFβRII domain. Once the plasmids were generated the lentivectors were packaged as previously described in section 3.3.1. CT26TetR cells from section 3.3.1 were infected with the lentivectors, and selected for and grown. Secreted STGFβRII and STGFβRII-Fc could be detected by Western blot post Dox treatment (Figure 4.6). The expression of protein was Dox dose-dependent and tightly regulated since Dox-untreated cells did not show any expression. A rough estimate of the amount of proteins being secreted was obtained using densitometry analysis of Western blot bands and comparison to the band intensity produced by known concentrations of a commercial STGFβRII-Fc (Figure 4.7). This gave an approximation of 500 ng of STGFβRII per mg of total cell protein in the supernatant and 250 ng of STGFβRII-Fc per mg of total cell protein. Giving that the commercial STGFβRII-Fc has shown clinical activity with doses of 2mg/kg in mice (Van Aarsen, Leone et al. 2008), we predict that the engineered STGFβRII is produced in sufficient amounts to function *in vivo*. The quantification based on the commercial STGFβRII-Fc is not ideal as many variables including the exact molecular weight, the levels of glycosylation, and the formation of multimeric protein complexes might alter its binding capacity to the antibodies detecting it. However, the aim was to have a rough estimation of the levels of secreted proteins being produced, and this

was achieved. Although, an ELISA assay would be more ideal for quantifying the protein concentrations, there were 2 main limitations preventing use of such an ELISA. Firstly, the limited availability of two functioning and reliable monoclonal antibodies that can bind the STGFβRII at two different binding sites, to enable a sandwich format. Secondly, an ELISA assay would detect both the engineered secreted form of STGFβRII and the endogenous wild type receptor. In contrast, Western blotting permits both forms to be easily distinguished based on molecular weight.

The biological activity of the STGβRII was also validated. TGFβ can induce the downstream phosphorylation of Smad2 giving increased levels of P-Smad2 (Massague 2008). This could be seen in control CT26 cells treated with 1 ng/ml of TGFβ with no further increase at higher concentrations (Figure 4.8A). Notably, the P-Smad2 was reduced when cells were treated with supernatant containing STGFβRII (Figure 4.8C) or the commercial STGFβRII-Fc (Figure 4.8B). Again it was evident that levels starting at 10 ng/ml of the commercial STGFβRII-Fc could reduce the P-Smad2 signal, but the signal was not further reduced with higher concentrations. Based on these data it can be concluded that sufficient amounts of the secreted STGFβRII should be available to reverse TGFβ induced signaling. It was concluded that the secreted engineered STGFβRII can efficiently bind to TGFβ1 and prevent its binding to the wild type receptor.

Studies also demonstrated that the expression of the proteins has no direct adverse effect on cell viability (Figure 4.9). In fact, an increase in viability could be seen post Dox treatment, Although, this might be Dox related since the same trend could be seen in control CT26PLUC cells, but the same effect could not be seen when CT26 were treated with similar doses of Dox. It is of note that the MTS assay is colometric assay that depends on the availability of reductase enzymes to reduce MTS dye to Formazan, it is therefore a metabolic assay, and thus factors that increase the metabolic activity of the cell, which could be the case in cells expressing transgenes after Dox treatment, could increase cell viability measured by this assay. High conc of Dox treatment has been shown to have anti-proliferative effects in some cell lines (Fife, Rougraff et al. 1997, Cakir and Hahn 1999), when tested in CT26 cells, conc Starting 10μg/ml had a significant effect on cell viability ($p<0.05$) with a mean average of 67% cell viability of

control untreated cells (Figure 4.10). Cell viability further decreased with higher conc. of Dox (50 and 100 µg/ml) reaching ~ 15 % viability of control untreated cells.

Finally the ability of the STGFβRII to be secreted *in vivo* from CT26STGFβRII tumours grown in Balb/c mice was shown. The secretion was Dox dependent and was detected after 24 hrs and was still evident up to 7 days post Dox treatment (Figure 4.11 A,B). Levels produced by the tumour were sufficiently high to give low levels of detectable protein in the serum of mice receiving Dox. This meant it was important to consider any biological systemic effects that could be seen from expressing the proteins *in vivo.*

After generating CT26STGFβRII and the ability of the protein to be induced successfully under the control of Dox *in vitro* and *in vivo,* experiments were performed to test whether the strategy could be used to express other antagonists with immunomodulatory capacity. To test this CT26TetR cells were infected with a lentivirus vector engineered to express the SIL-10R which acts as an IL-10 antagonist. The engineered CT26SIL-10R cell line was then studied for SIL-10R expression under Dox control. As seen with STGFβRII, SIL-10R could be detected in the cell lysate and supernatant under Dox control. Thus having engineered a CT26TetR cell line, any other antagonist expressed under the Ptight promoter can now be easily introduced into this cell line allowing the expression of the protein under the Dox control.

STGFβRII and STGFβRII-Fc have been expressed in tumour models previously. This is mainly through cell lines that have been genetically engineered to constitutively express the proteins (Rowland-Goldsmith, Maruyama et al. 2002). An alternative strategy has been to deliver the gene to already established tumours using an adenovirus vector, an approach which provides little control of the distribution or level of protein production giving the limitation of adenovirus to replicating in murine cells (Zhang, Hu et al. 2012). In this chapter a new tumour model has been successfully designed that permits the expression of STGFβRII and other antagonists in early and in well-established tumours. Our concept is to engineer murine CT26 colorectal carcinoma cells to express secreted cytokine-antagonists (specifically antagonists to TGFβ or IL-10) in the presence of endogenously supplied Dox only. Hence, tumours could be grown normally, and the

addition of Dox at a chosen time should induce expression of the cytokine-antagonists. Studies could then be performed on tumour biology as well as anticancer efficacy.

This model has never been established previously and because of its regulated manner it should thus provide precise answers to questions related to the local tumour induced immunosuppression, and may allow us to validate new immunosuppressive therapeutic targets within the tumour microenvironment. This could also validate whether local antagonism of these immunosuppressive factors is sufficient for restoring tumour immunity. Thus, allowing insights into developing more effective and less toxic antagonists that can function locally rather than systemically.

5 Characterising the Effect of Regulated Antagonism of TGFβ Signaling on Tumour Growth and immunity in murine models

5.1 Introduction

TGFβ has demonstrated the capacity to enhance as well as suppress tumour growth. Although the exact mechanisms that regulate the balance of this complex role remain elusive, it is becoming increasingly clear that this may reflect the prominent role TGFβ plays in the growth, differentiation and migration of epithelial cells, coupled with its function as an important mediator of changes in the tumour stroma. For example, TGFβ signal activation has been associated with poor prognosis, linked to its direct induction of epithelial cell proliferation and EMT (Bruna, Darken et al. 2007, Mima, Hayashi et al. 2013). However, in other studies abrogation of TGFβ signaling has been associated with a more aggressive and metastatic phenotype (Lu, Herrington et al. 2006, Malkoski, Haeger et al. 2012). In some of these reports, despite the loss of TGFβ signaling in the epithelial cells, the increased aggressiveness of the tumour has been linked with greater expression of TGFβ from those cells. Thus, even though the lack of TGFβ responsiveness deprives epithelial cells of any metastatic advantage, the paracrine stromal alteration induced by the secreted TGFβ may be sufficient to govern an aggressive metastatic phenotype (Ikushima and Miyazono 2010).

It is thus clear that without a thorough understanding of the context dependence of TGFβ within the tumour microenvironment therapeutic advantages from targeting its signaling would be limited (Ikushima and Miyazono 2010). A model which regulates TGFβ signaling globally, perhaps by controlled antagonism should therefore give more insight into the factors that influence TGFβ action and the ultimate outcome of such action. Ideally this would allow control of the precise time of signaling abrogation and allow manipulation of other factors within the tumour microenvironment before or after abrogation of TGFβ signal.

As, previously described in section 1.4.1.4 TGFβ is a potent immunosuppressive cytokine that can alter the maturation, activation, and differentiation of innate and adaptive immune cells.

Indeed, in animal studies abrogation of TGFβ signaling in T cells has been shown to induce tumour-specific CTL responses and is important for resistance to tumour challenge (Gorelik and Flavell 2001) The limitation of these models is that TGFβ blockade is generally performed at an early stage of tumour growth. This thus fails to assess possible therapeutic benefit of reversing immunosuppression in a well-established tumour, i.e. the type of tumour most commonly encountered in the clinic.

The model developed in Chapter 4 was therefore used to investigate influence of the regulated expression and secretion of STGFβRII from tumour cells and help characterise the effect of local antagonism of TGFβ on tumour growth and tumour immunity.

5.2 Chapter objectives

1- **To test the effect of inducible expression of STGFβRII from tumour cells on tumour growth and animal survival**
2- **Compare the effects of early and late expression of STGFβRII on tumour growth**
3- **Characterise the effects of inducing the expression of STGFβRII in well-established tumours on local immune cells and immune cytokine profile**

5.3 Results

5.3.1 The effect of expressing STGFβRII on tumour growth and animal survival

5.3.1.1 Early expression of STGFβRII from CT26 cells *in vivo* suppresses tumour growth

A unique benefit of using the Tet-on system to regulate expression of TGFβ-antagonising STGFβRII within tumours is the possibility for defined temporal control, including the option to halt expression when required. Accordingly the first logical step was to study the biological activity of early expression of STGFβRII in CT26 tumours *in vivo*, and assess its effects on tumour 'take rate' and early growth.

To investigate this, CT26STGFβTII or control CT26PLUC cells were injected s.c. $1x10^6$ cells/mouse in Balb/c mice, which were then either immediately started on Dox or left untreated. Tumour volumes were monitored and animals were humanely sacrificed when tumours reached a predetermined size (tumour volumes of ~1200 mm^3). The experiment was pre-designed to be terminated (and all animals put down) when 50% of the mice in any of the groups reached the maximum size. This experimental design enabled us to use a multivariate analysis based on the entire data set to relate the effect of treatment to growth rate. Furthermore the area under the curve was calculated for each individual mouse from day 0 to the experimental end date, to take into account the effect of treatment on tumour growth over the entire period. A one way ANOVA or a Student's T test was then used to compare the area under the curve between the different groups.

Mice bearing CT26STGFβRII tumours, and which were started on Dox (Dox group) showed decreased rates of tumour establishment and growth, with 5 mice out of 10 showing no tumour 'take' at all (Figure 5.1). Chi squared analysis demonstrated that this number was significantly ($p<0.05$) below the figure expected if the null hypothesis, i.e. that there was no difference in the take rate between the groups, was true. The mean area under the curve in mice that had grown a tumour in the Dox group was also lower than the no Dox group (2.07-fold). To exclude any direct effects of Dox on tumour take and growth we included another control group of mice that were inoculated with CT26PLUC cells and were immediately started on Dox. All mice in this group developed a tumour, and the tumour growth was significantly increased in this group compared to CT26STGFβRII Dox group ($p<0.01$) (Figure 5.1).

It was apparent that the growth rate of CT26PLUC was markedly different *in vitro* and *in vivo* to CT26STGFβRII regardless of the presence or absence of Dox, with the CT26PLUC being faster-growing. This may be expected since these two cells lines were originally isolated from two different colonies expressing different transgenes, and creates doubts over how appropriate a control the CT26PLUC cell line is for the CT26STGFβRII. For this reason a retrospective analysis excluding the CT26PLUC group and using a Student's T test comparing the CT26STGFβRII Dox group and CT26STGFβRII no Dox group was performed and showed a

significant decrease in tumour size of the Dox group ($p<0.01$) (Figure 5.1B). These data therefore indicate that the early expression of STGFβRII had a significant effect on tumour take rate and tumour growth.

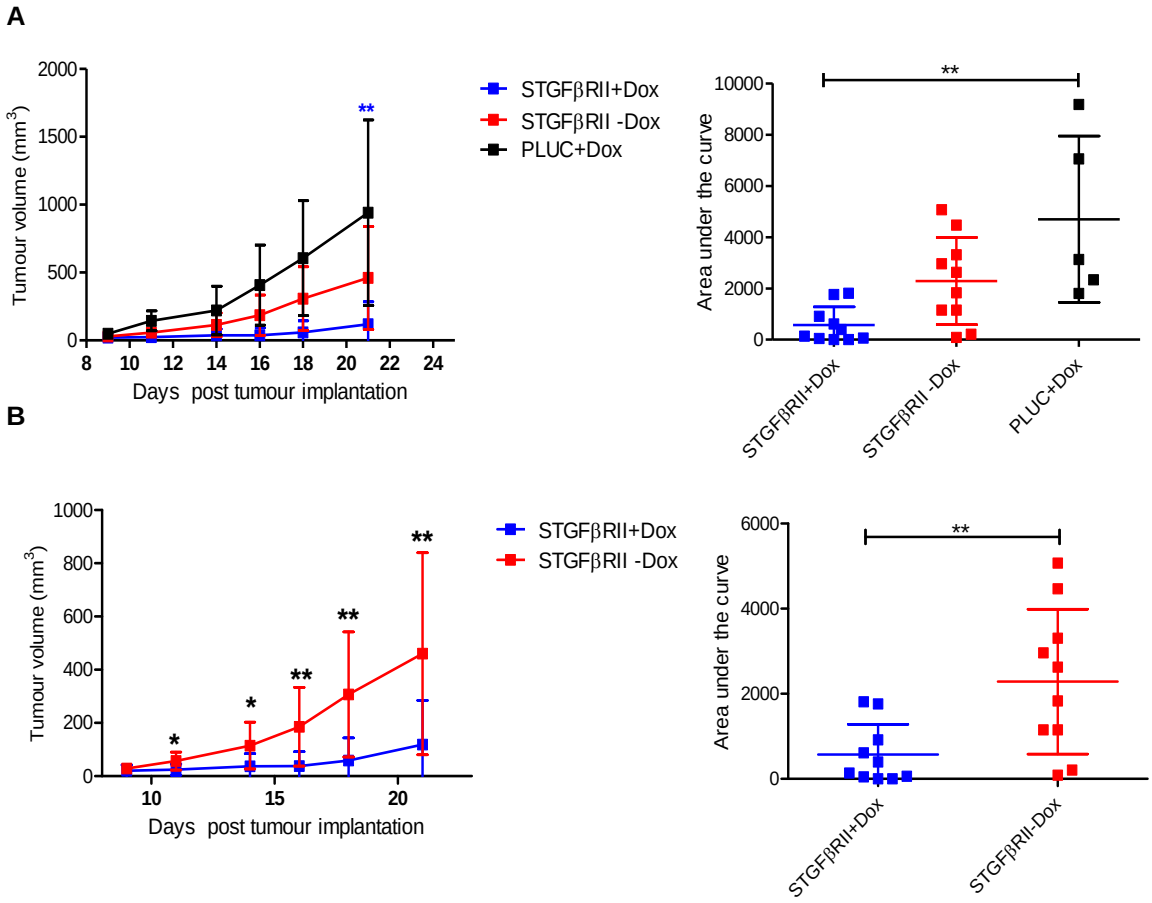


Figure 5.1 Early expression of STGFβRII suppresses tumour growth of CT26 tumours in Balb/c mice

A 1x10^6 CT26STGFβRII or CT26PLUC cells were injected s.c. in Balb/c mice. Mice were either left untreated or started on Dox (2 mg/ml in drinking water). Tumour monitoring was performed every 2-3 days and was stopped when 50% of the CT26PLUC group was put down. Graph represents n=10 or n=5 (PLUC), mean and SD shown. Graph on the right represents the area under the curve for each individual mouse in each group, significance tested using one way ANOVA for the area under the curve between groups, **= $p < 0.01$. **B** A retrospective analysis comparing CT26STGFβRII Dox and CT26STGFβRII no Dox from the same experiment was performed using an unpaired Student's T test. Mean and SD shown. *=$p<0.05$, **= $p < 0.01$. The figure on the Left compares tumour volumes between groups on each day post tumour implantation, while figure in the right compares the total area under the curve between the two groups.

5.3.1.2 The effect of expressing STGFβRII from established CT26 tumours on tumour growth rate (Late expression)

To assess the effects of expressing STGFβRII in well-established tumours, Balb/c mice were injected s.c. with CT26STGFβRII or CT26PLUC cells ($1x10^6$ cells/mouse). When tumours reached sizes of ~ 30 mm^3 and not more than 100 mm^3 (11 days post tumour implantation) the mice were divided into equal groups and either started on Dox treatment or left untreated. Tumour volumes were monitored until they reached a predetermined size for mice sacrifice (tumour volumes of ~1200 mm^3) and the same analysis as described in previous section was used.

Although the tumour growth rate in the Dox treated group was smaller, with the mean of the area under the curve being 1.65-fold less than the non-Dox treated group, this was not statistically significant in terms of tumour volume ($p=0.058$) or survival duration ($p = 0.078$) (Figure 5.2A).

It was also important to investigate whether any anti-tumour effect that might be seen was solely due to the Dox treatment. In a similar manner as previously described CT26PLUC tumour bearing mice were divided into two groups and either started on Dox or left untreated, and tumour volumes were monitored. As previously seen *in vitro,* Dox treatment showed a slight increase in tumour growth, however, this was not statistically significant ($p= 0.3$) (Figure 5.2B).

It was therefore obvious from these experiment and those reported in section 5.3.1.1, that there was a clear difference between the effects of antagonizing TGFβ activity during early and late tumour growth. This may reflect the multiple effects of TGFβ on tumour biology, making the outcome of reversing TGFβ activity dependent on the specific tumour context.

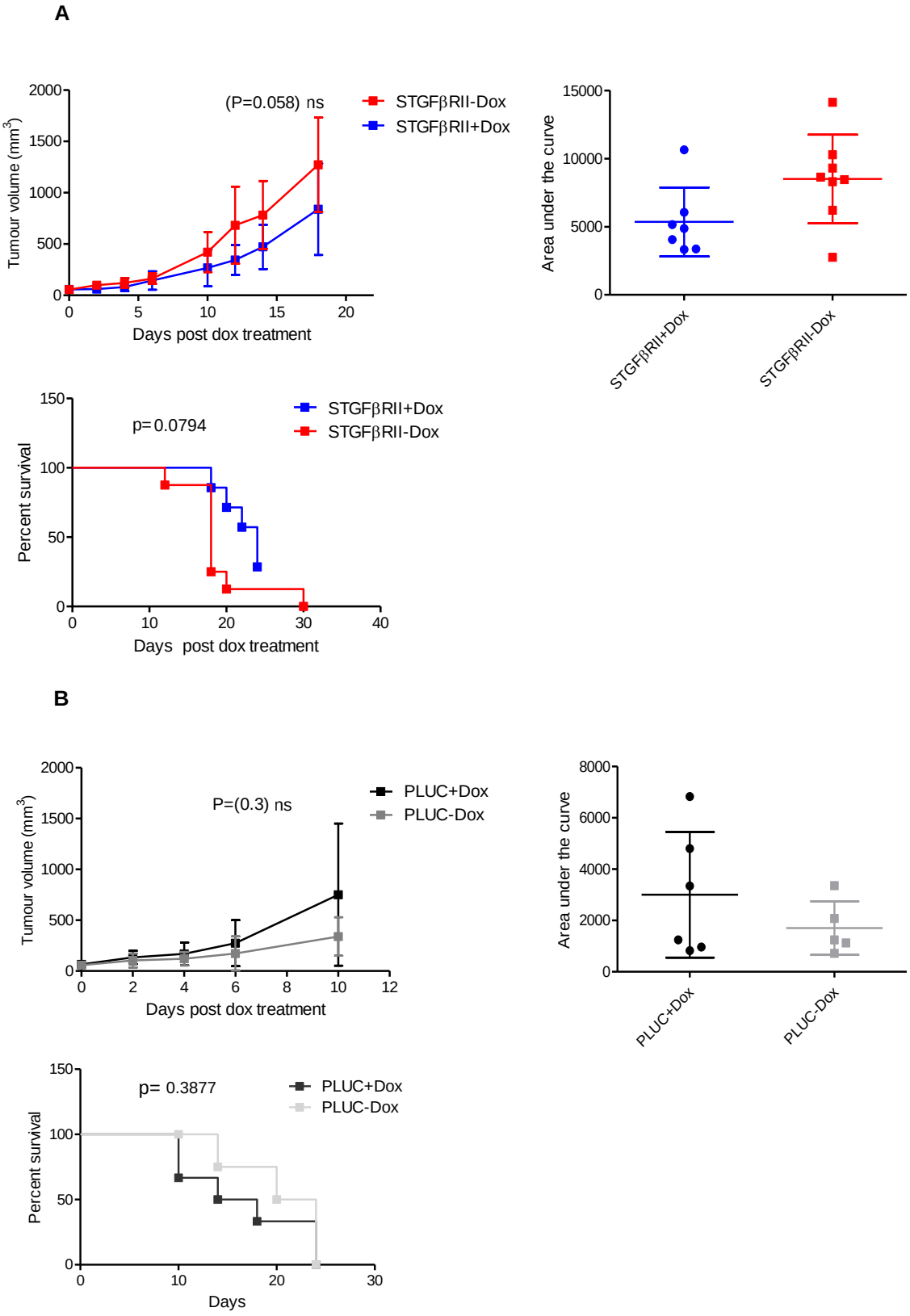


Figure 5.2 The effect of late expression of STGFβRII on established tumour growth and animal survival

The effect of late expression of STGFβRII and Dox treatment on tumour growth and survival was assessed in Balb/c mice with established CT26STGFβRII **A**, or CT26PLUC **B** tumours**.** Dox was started 11 days post tumour implantation and tumour growth was monitored. Graph represents n = (5-8), mean and SD shown. Statistical analysis was performed using Student's T test comparing the area under the curve between groups, and a log-rank test for survival curves.

To validate the results of this experiment, treatment of established tumours was repeated with a larger sample number. Balb/c mice were injected s.c. with CT26STGFβRII $1x10^6$ cells/mouse. When tumours reached sizes between 20-70 mm^3 and not more than $100mm^3$ (14 days post tumour implantation) mice were divided into equally sized groups and either started on Dox treatment or left untreated. The same analysis as described previously was used. As in the previous experiment there was a slight decrease in tumour growth in the Dox group and a slight increase in animal survival with two mice in the Dox receiving group showing no palpable tumour by the end of the experiment (Figure 5.3). However, overall, tumour growth (p=0.3) and survival duration (p=0.087) were not significantly different.

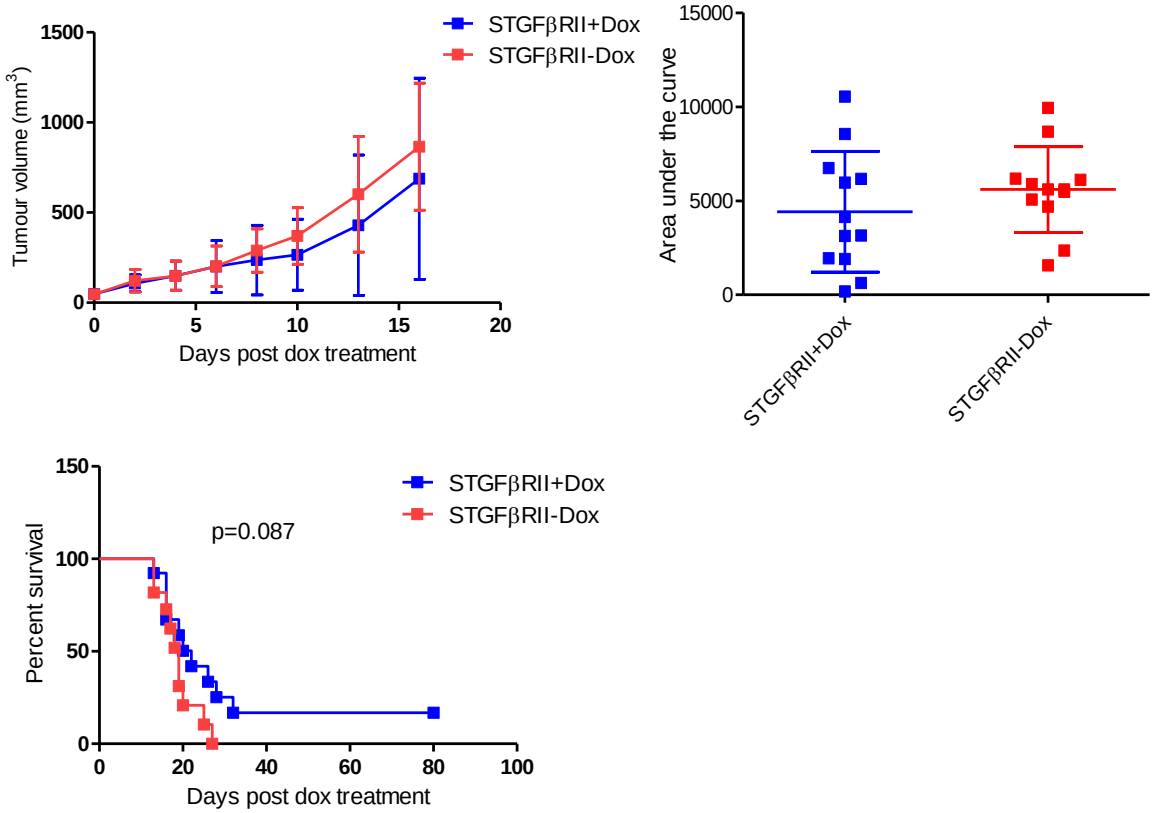


Figure 5.3 The effect of late expression of STGFβRII on tumour growth and animal survival

The effect of late expression of STGFβRII on tumour growth and survival was assessed in Balb/c mice with established CT26STGFβRII tumours**.** Dox was started 14 days post tumour implantation and tumour growth was monitored. Graph represents n=(12), mean and SD shown. Statistical analysis was performed using Student's T test comparing the area under the curve between groups, and the log-rank test for survival curves.

5.3.2 The effect of expressing STGFβRII in established tumours on the tumour immune milieu

5.3.2.1 The effect of expressing STGFβRII on tumour cytokine profile

5.3.2.1.1 Common cytokines screen analysis

One of the key objectives of this work was to assess the effect of blocking TGFβ signaling on the tumour immune milieu including immune cells, and immune cytokines within the microenvironment of well-established tumours. A shift from a more immunosuppressive profile towards an immunostimulatory one would be expected in this case. Therefore, a screening study was conducted, to determine changes in the RNA expression of a panel of common cytokines (83 genes) in a small sample using the RT^2 profiler PCR array (for details check section 2.13.2). This would allow validation of the assay system and to exclude from the next screen of cytokines that were undetectable, and thus might not be important in the CT26 tumour microenvironment. Importantly, however, the screening study allowed identification of important cytokines that would be of interest to study in a larger number of tumour samples.

For this initial screen, two equally sized CT26STGFβRII tumour samples were used. One sample obtained from one mouse receiving Dox for STGFβRII expression, and a second sample from a mouse that did not receive Dox, thus should not be expressing STGFβRII. Tumour samples were extracted 2 weeks post initiation of Dox. Total RNA was extracted as described in materials and methods and cDNA preparation and RT-QPCR was conducted using the RT^2 profile PCR common cytokine array (Hassuneh, Albini et al. 2012) This array includes a list of common cytokines that have been described previously in other reports (Parkin and Cohen 2001)

Eight cytokines were undetectable in both samples by RT-QPCR, suggesting their limited local role in development of both these CT26 tumours (see Table 5.1). Of particular interest was IL-17a which is a pro-inflammatory cytokine secreted predominantly by T helper 17 cells (TH17) an important subset of T helper cells that play an important role in promoting inflammation in autoimmune diseases . IL-17a has a controversial role in cancer development and tumour immunity (Hemdan 2013).Interestingly, this was accompanied with undetectable levels of IL-21

which is also secreted by TH17 and plays an essential role in TH17 differentiation and activation (Liu and King 2013). Other undetectable cytokines are listed (Table 5.1).

Table 5.1 Cytokines undetected in RT-QPCR screen of CT26STGFβRII tumours

Cytokines undetectable in screen	Predominant Cells secreting it	Function
Bone morphongenetic protein 1 (BMP1)	Tumour and stromal cells	Growth factor/ bone development
Growth differentiation factor (GDF) 2 (also known as BMP9) (Lamplot, Qin et al. 2013)	Tumour and stromal cells	Growth factors/osteogenic factors/ angiogensis
GDF 5 (Jin and Li 2013)	Tumour and stromal cells	Growth factors/osteogenic factors/ angiogensis
IL-17a (Hemdan 2013)	TH17	Pro-inflammatory
IL-20 (Wegenka 2010)	Monocytes, kertinocytes	Keratinocyte proliferation/ skin homeostasis
IL-21 (Liu and King 2013)	Activated $CD4^{+}$T cells (mainly TH17)	Expansion and differentiation of T cells.e.g. TH17
IL-25 (IL-17E) (Serre and Silva-Santos 2013)	TH17	Pro-inflammatory
IL-9 (Stassen, Schmitt et al. 2012)	$CD4^{+}$T cells subsets(TH9,TH2,TH17)	Proliferation and survival of immune cells/ asthma development

5.3.2.1.2 The effect of locally expressing STGFβRII on the levels of 22 cytokines in established CT26 tumours.

Of the 83 cytokines screened described in the previous section, 22 (listed in Table 5.2) were chosen to test for expression on a larger tumour sample size (n=6). Tumours from Balb/c mice were extracted one week after Dox initiation to compare the RNA expression of these cytokines between mice receiving Dox and control mice. cDNA was made from total RNA and RT-QPCR was conducted using the RT^2 profiler custom array panel. All of the 22 cytokines were detected in all of the 12 tumours anlaysed. Three of the 22 cytokines demonstrated significantly (Student's T test) decreased levels in the group receiving Dox; 2-fold decrease in Bmp4 (p=0.03), 1.44-fold decrease in Tnsf9 (p=0.02) and most importantly a 1.44-fold decrease in TGFβ1 (p=0.03). Although not significantly different, an increase in IL-2 by 4.43-fold (p=0.07), Tnsf10 also known as TRAIL by 2.06-fold (p=0.06), and IL-4 by 1.9-fold (p=0.08) was also seen in the Dox receiving group (Figure 5.4B).

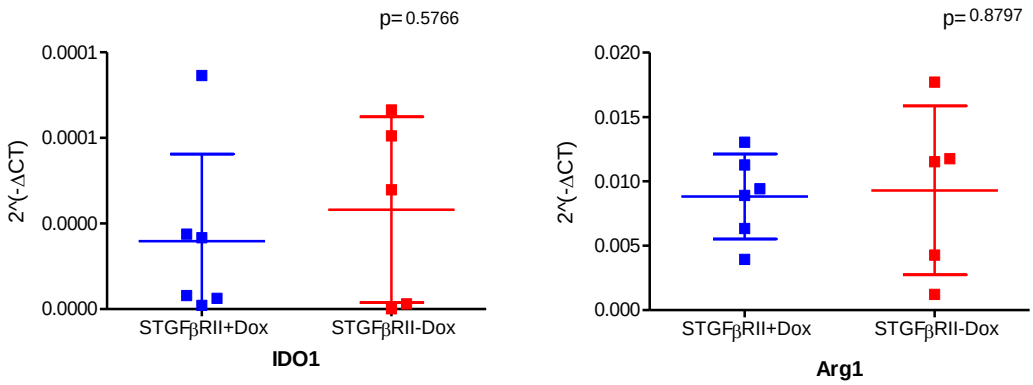
Table 5.2 List of cytokines screened in established CT26STGFβRII

Cytokine	Cells secreting it	Function
Metabolic		
Arg1 (Stewart and Smyth 2011)	Tumour and stromal cells (MDSC)	Metabolism of Arginine> inhibition of T cell function
IDO (Stewart and Smyth 2011)	Tumour and stromal cells (DCs and Macrophages)	Catabolism of Tryptophan> decrease T cell proliferation/activation
I**nterleukins**		
IL-2 (Bachmann and Oxenius 2007)	Activated T cells (CD4+)	Proliferation/activation/survival of T cells
IL-4	CD4+ T helper 2 cells	B and T cell proliferation Humoral immunity
IL-6(Rincon 2012)	Innate immune cells/ non-	Pro-inflammatory

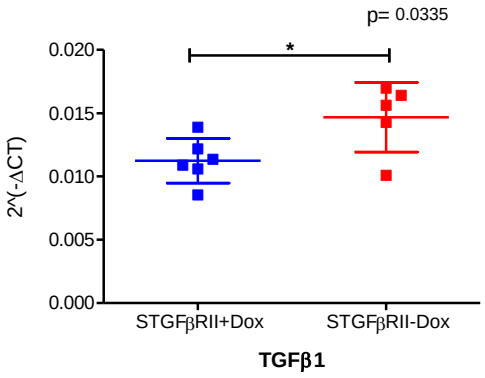
	immune cells	
IL-7(Mackall, Fry et al. 2011)	Non-haemopoietic stromal cells	Mature T cells development and homeostasis
IL-10(Mosser and Zhang 2008)	Monocytes,macrophages/ Tr1	Anti-inflammatory
IL-12b(IL-12p40)(Hamza, Barnett et al. 2010)	Monocytes,macrophages,DCs	T helper 1 cells development
IL-15(Steel, Waldmann et al. 2012)	Monocytes,macrophages	T and NK proliferation
IL-23a(Croxford, Mair et al. 2012)	Monocytes,macrophages,DCs	Pro-inflammatory
Tnf superfamily		
Tnf(Bradley 2008)	Macrophages, T lymphocytes	Pro-inflammatory
Tnf superfamily member 9 (Tnfsf9) (4-1BBL)(Vinay and Kwon 2011)	Activated APC	Binds to the co-stimulatory molecule 4-1BB to induce CD8 and T helper 1 cells expansion
Tnfsf10 (TRAIL)(Dimberg, Anderson et al. 2013)	Tumour and stromal cells	Apoptosis
Lymphotoxin B (LTB)	Activated T cells	Pro-inflammatory
Bone morphogenetic protein (Bmp) and TGFβ family		
Bmp4 (Kallioniemi 2012)	Tumour and stromal cells	Bone and cartilage development
TGFβ1(Flavell, Sanjabi et al. 2010)	Tumour and stromal cells	Innate and adaptive immune suppression
Chemokines		
CCL22 (Nishikawa and Sakaguchi 2010)	Macrophages, DCs	Treg recruitment

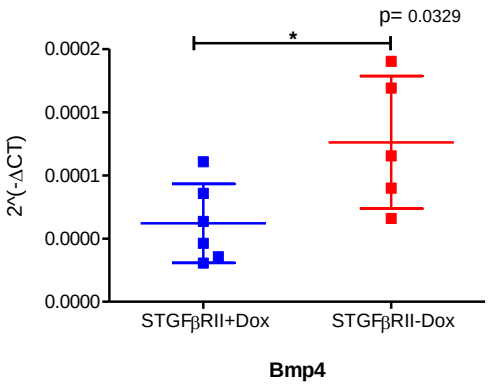
Interferons		
Ifnγ (Schroder, Hertzog et al. 2004)	APC,NK,CD8+, T helper1	Macrophage activation and immunostimulation
Others		
VEGFa (Stewart and Smyth 2011)	Endothelial cells	Angiogenesis, immunosuppresion
Fibroblast growth factor 10 (Fgf10) (Turner and Grose 2010)	Fibroblast	Growth factor
FasL	Tumour and stomal cells	Apoptosis and T cell killing
Colony stimulating factor 2 (Csf2) (Hercus, Broughton et al. 2012)	T and B cells, macrophage, mast cells	Growth factor for the production and differentiation of granulocytes and macrophages

Metabolic regulators

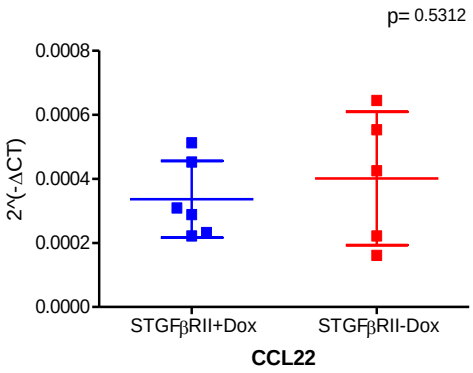


BMPS and TGFβ superfamily

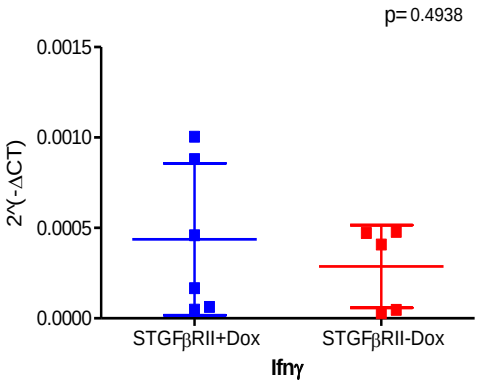




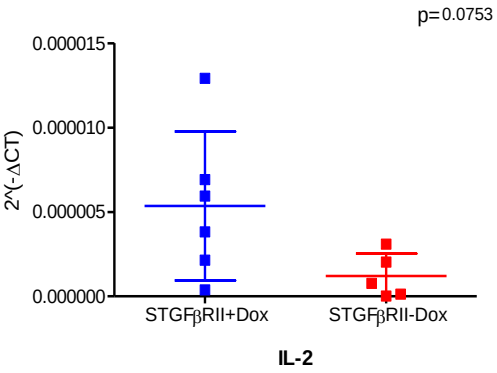
Chemokines



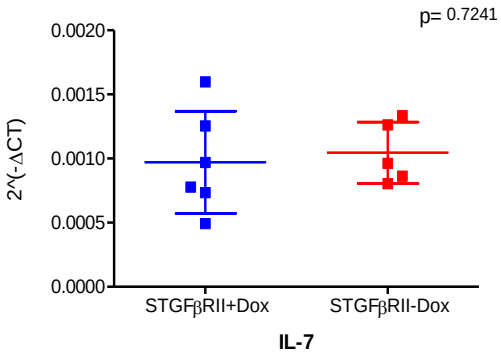
Interferons



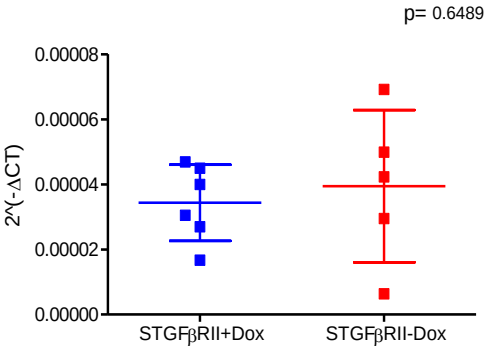
Interleukins



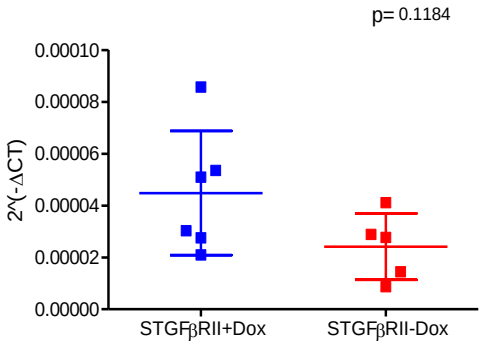
IL-2



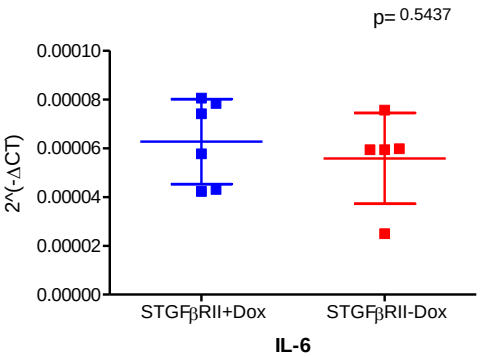
IL-7



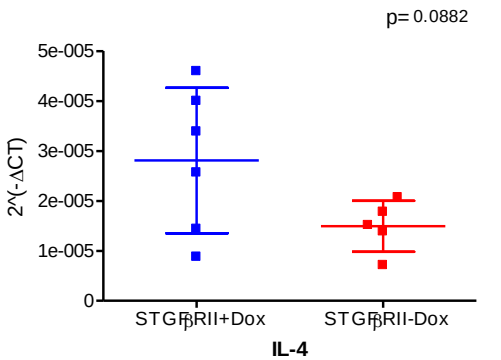
IL-23a



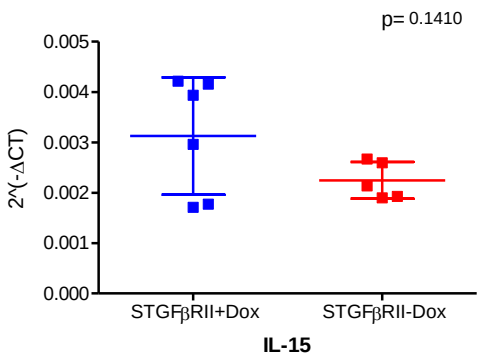
IL-10



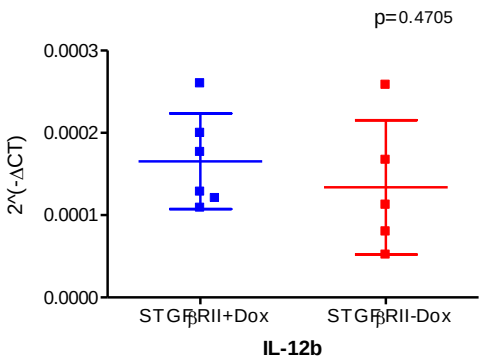
IL-6



IL-4

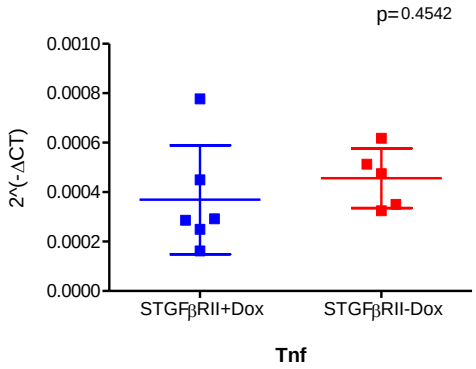


IL-15

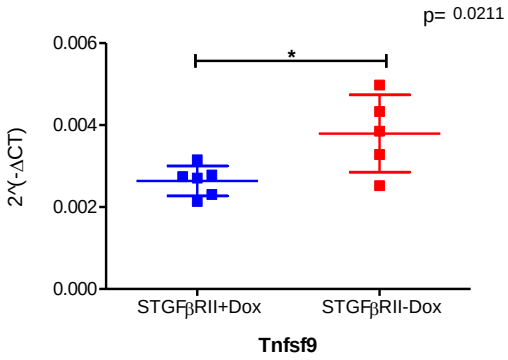


IL-12b

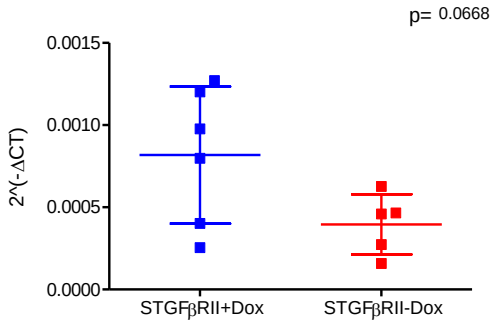
Tnf superfamily



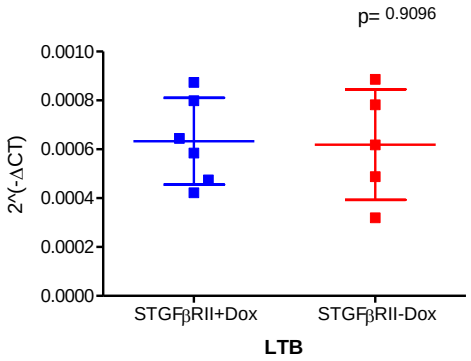
Tnf



Tnfsf9

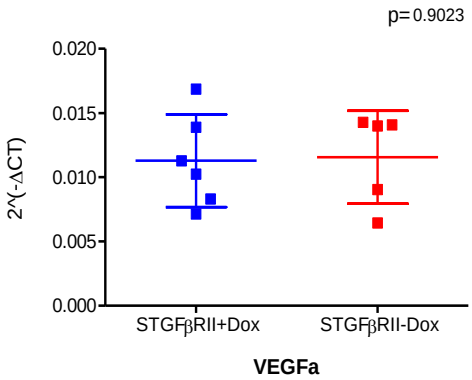


Tnfsf10

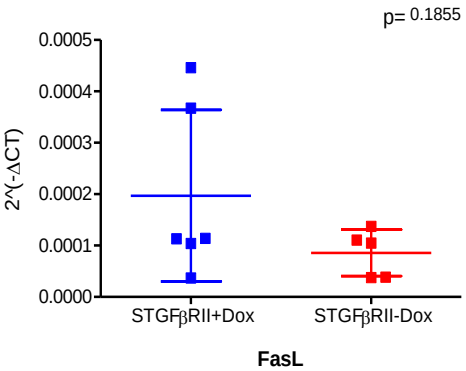


LTB

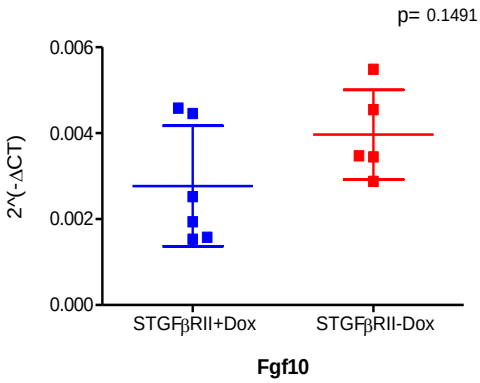
Others



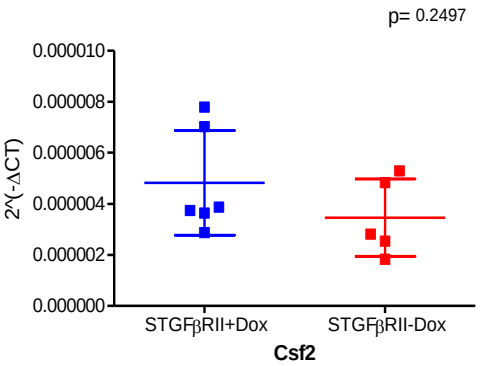
VEGFa



FasL



Fgf10



Csf2

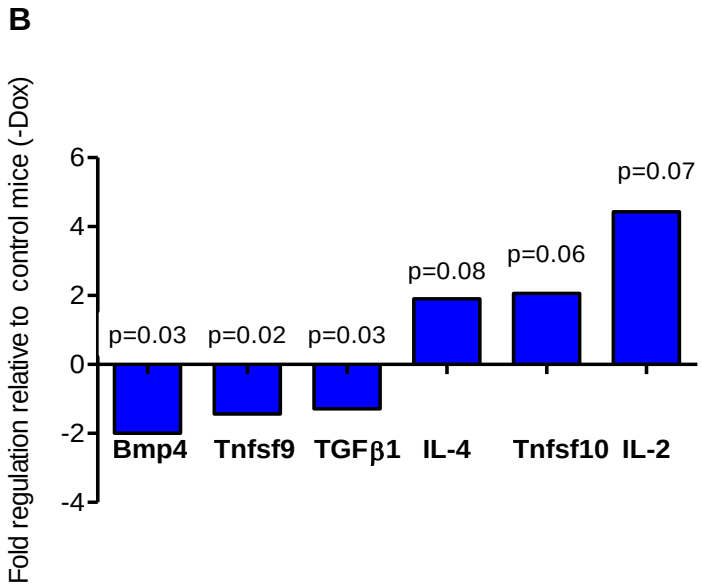


Figure 5.4 The effect of expressing STGFβRII in established CT26 tumours on cytokine profile

A Seven days post Dox treatment total RNA was extracted from CT26STGFβRII tumours of mice receiving Dox (n=6) and control mice not receiving Dox (n=5),cDNA preparation and RT-QPCR was conducted using the RT^2 profiler custom PCR array to screen for the expression of 22 immune cytokines. Data was plotted using the $2^{\wedge(-\Delta CT)}$ (gene expression normalized to housekeeping gene β-actin). Statistical analysis was performed using Student's T test, mean and SD shown, *= $p < 0.05$. **B** Graph summarises hits from screen. Fold regulation was calculated using the $2^{\wedge(-\Delta\Delta CT)}$ (normalized gene expression in Dox receiving group relative to control group no-Dox).

5.3.2.2 The effect of long term expression of STGFβRII on immune cells profile

TGFβ1 has been shown to influence the infiltration of immune cells in the tumour environment. To determine whether the expression of STGFβRII in well-established CT26 tumours would alter the infiltration of immune cells of interest, CD8+, CD4+, Tregs (CD4+Foxp3+), and MDSC (CD11B+GR1+) were stained and measured by flow-cytometry, locally within the tumour or systemically in the spleen. To achieve this tumours and spleen from section 5.3.1.2 were extracted once a tumour has reached a cutoff size of ~1200 mm^3. Tumours, and spleen were then dissociated mechanically and enzymatically before they underwent staining for various markers. Due to technical challenges it was difficult to achieve reliable staining for CD4+, and CD4+ Foxp3+ within tumours, and therefore this analysis was not included.

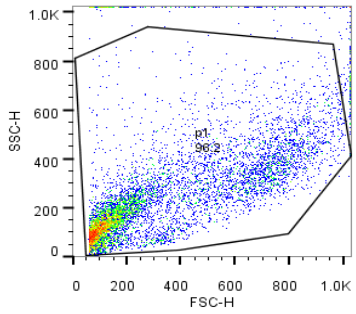
An infiltrating CD8+ population within the tumours could be detected with a mean of ~ 1% of the total tumor cell population (Figure 5.5). However, no change in infiltration was detected when comparing the Dox and the non-Dox group.

A large population of CD11B+ was detected in tumour samples suggesting a strong infiltration of monocyte/macrophages in CT26 tumours and perhaps the importance of those cells in CT26 tumour development (Figure 5.5). Of those cells, a large percentage was GR1+ which is the fundamental marker for MDSC in mice, however, no difference in the number of cells infiltrating the tumour could be seen between the Dox and non-Dox groups.

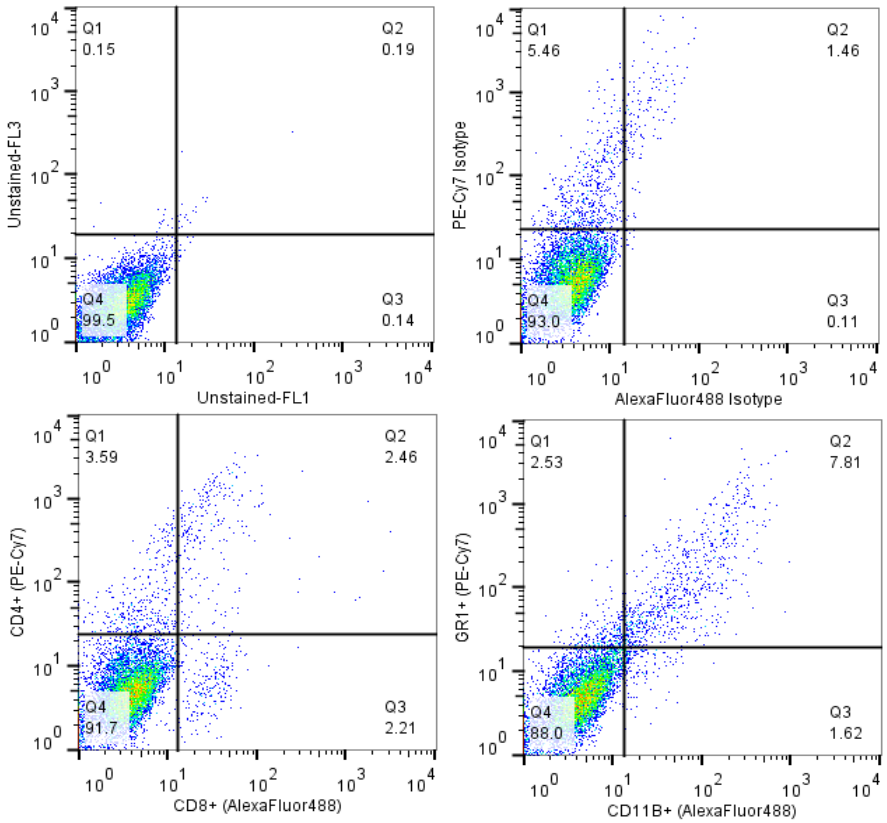
However, it was notable that there was a significant decrease ($p=0.02$) in the CD11B+ population of splenocytes from mice receiving Dox, which was also reflected in the CD11B+ GR1+ population ($p=0.06$) (Figure 5.5). A similar decrease was not seen in any of the other cell types screened for in the spleen. To exclude that this could be due to a general Dox effect rather than the expression of STGFβRII, staining of CT26PLUC tumours (control tumours) from 3 mice that received Dox and 3 mice that did not was conducted. No significant difference in any of the cell populations from these CT26PLUC mice (CD8+, CD4+, Tregs (CD4+ Foxp3+) and MDSC (CD11B+GR1+) was seen (data not shown).

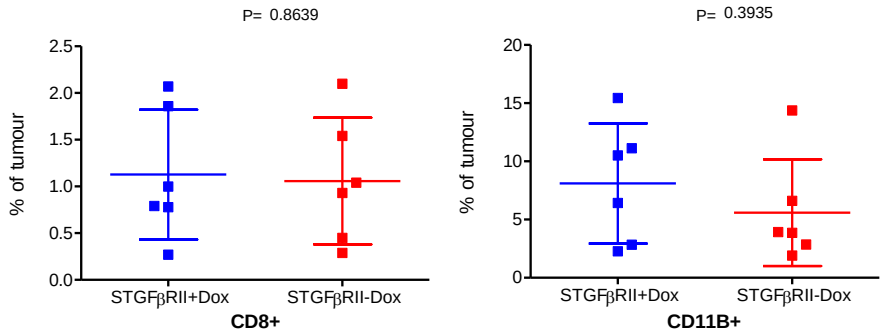
A

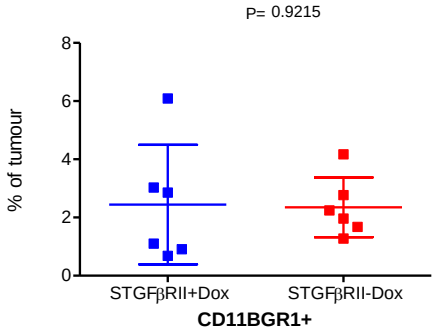
Gating of CT26 Tumours for FACS analysis



Tumour staining

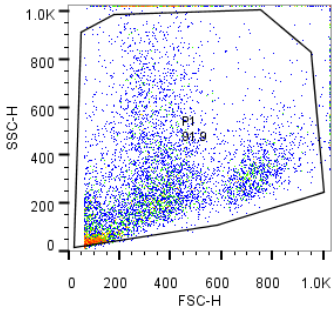




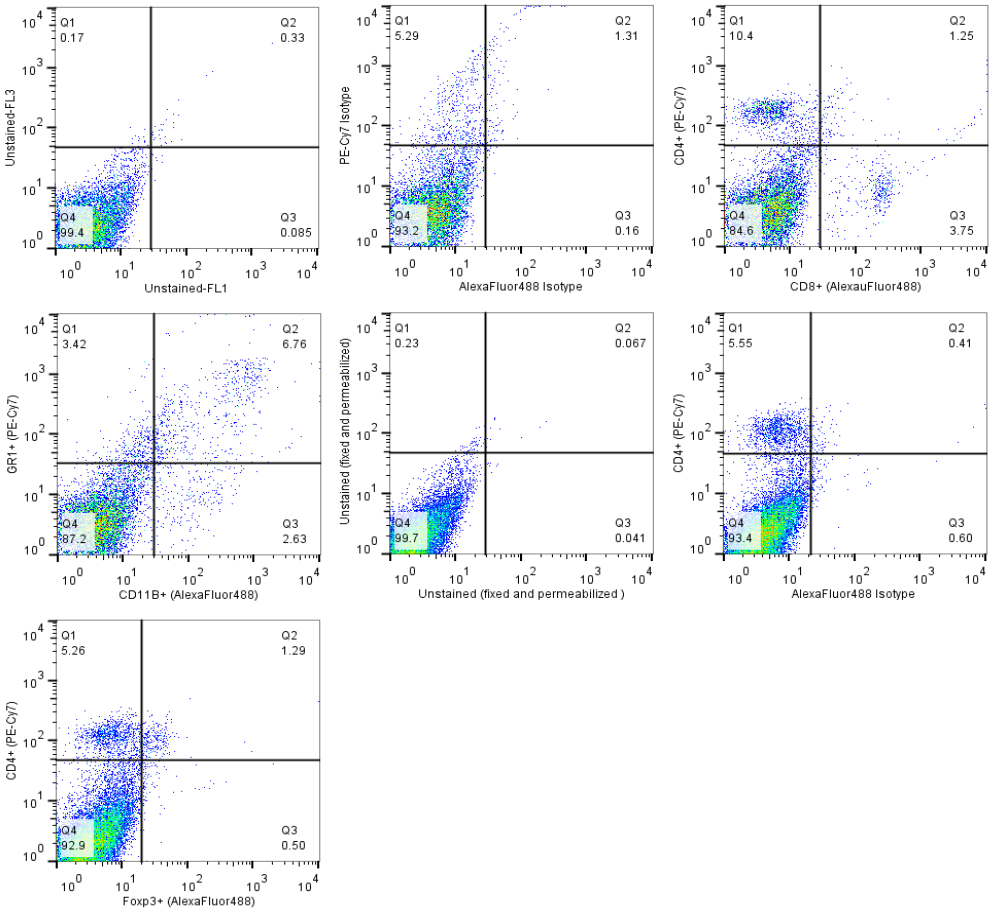


B

Gating of spleenocyte population for FACS analysis



Spleen staining



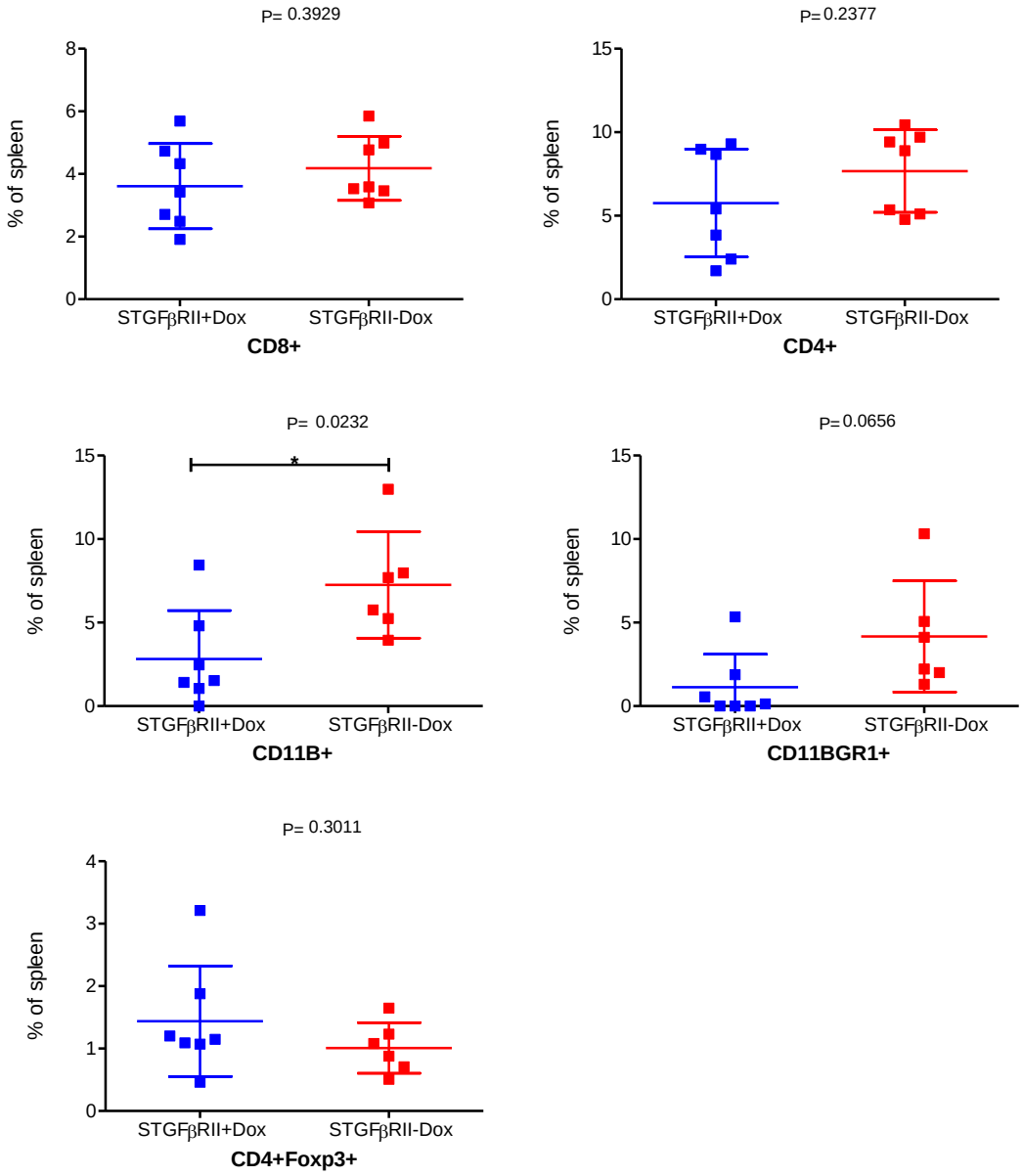


Figure 5.5 The effect of expressing STGFβRII in established CT26 tumours on immune cell profile in tumours and spleens

The effect of expressing STGFβRII in established CT26 tumours on immune cell profile was evaluated when tumours reached cutoff size of ~1200 mm^3. CT26 tumours **A**, and spleens **B,** were extracted, and mechanically and enzymatically dissociated before staining for various immune cells markers for flow-cytomteric analysis. Dot blots are representative images from one mouse treated with Dox. Statistical analysis was performed using Student's T test, mean and SD shown, *= $p < 0.05$.

Although TGFβ signaling abrogation might not have an effect on CD8+ recruitment it might play a role in the activation status of CD8+. One of the known activation markers of CD8+ is CD44+. To test if the expression of STGFβRII can induce a larger CD8+CD44+, CT26 tumours and

spleens from mice receiving Dox and control mice were stained. To avoid the potentially confounding effect of assaying all tumours when they reached the same size, this further analysis was performed on smaller and less necrotic tumours that had not reached the 1200 mm^3 cutoff limit. Thus samples were extracted approximately 15 days post Dox administration with an average size of ~ 700 mm^3 (range between 300-1200 mm^3).

In contrast to the larger tumours in Figure 5.5A, a CD8+ population could not be detected in the majority of these samples. Thus an analysis could only be conducted on splenocytes (Figure 5.6).This indicates that CD8+ infiltration in CT26 tumours could be a late event that occurs as the tumours enlarges. One hypothesis might be linked to an enhanced CD8+ recruitment associated with necrotic tissue that accompanies poor vascularization and hypoxia as the tumour enlarges.

When comparing CD8+ CD44+ percentage in spleen no difference can be seen between Dox and non-Dox groups (Figure 5.6).

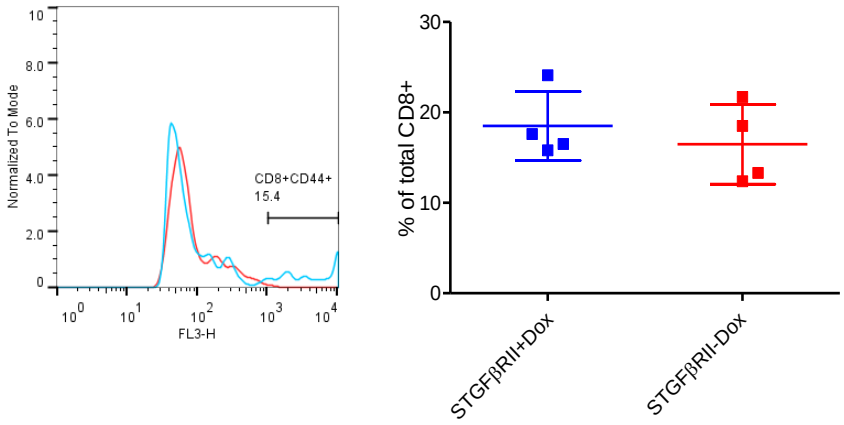


Figure 5.6 The effect of expressing STGFβRII in established CT26 tumours on CD8+ cell activation in the spleen at 15 days post Dox

The effect of expressing STGFβRII in established CT26 tumours on CD8+ CD44+ cell percentages in spleens15 days post Dox administration. Spleens were extracted, and mechanically dissociated before staining CD8 and CD44 markers for flow-cytometry analysis. Statistical analysis was performed using Student's T test, mean and SD shown. Histogram on the left is a representative of one mouse treated with Dox.

5.4 Conclusion

The aim of this chapter was to study the effect of expressing STGFβRII on tumour growth and tumour immunity. The advantage of having an inducible expression allowed the effect of early expression of STGFβRII on tumour take rate and early growth to be compared to the effect of late expression on tumour development.

To study the effect of early TGFβ abrogation on tumour growth, CT26STGFβRII cells were implanted in Balb/c mice, and mice were started immediately on Dox to allow the early expression of STGFβRII. A significant ($p<0.01$) effect on tumour take rate and tumour growth was seen in the Dox receiving group, with 50% of mice not developing tumours. The average mean growth of tumours that did develop in the Dox receiving group was 2.07-fold lower than the non-Dox group, when measured as the mean of the area under the curve.

Interestingly, this significant effect was lost with late expression of STGFβRII. In this experiment mice with established CT26STGβRII tumours were started on Dox 11 days post tumour implantation. Although the tumour volumes in the Dox treated group was smaller, with the mean being 1.65-fold less than the non-Dox treated group, this was not statistically significant in tumour volume ($p=0.058$) or animal survival ($p=0.08$).

The effect of STGFβRII expression on tumour growth has been studied in several models (Rowland-Goldsmith, Maruyama et al. 2002, Zhao, Kobayashi et al. 2002, Zhang, Hu et al. 2012). In athymic mice the constitutive expression of STGFβRII from human pancreatic carcinoma cell line yielded significantly ($p<0.001$) smaller tumours than in control mice. This was also associated with a decrease in metastasis in the orthotropic model (Rowland-Goldsmith, Maruyama et al. 2002). Similar results were also seen with another human non-metastatic pancreatic cell carcinoma cell line (Hu, Gupta et al. 2012). In a syngeneic immunocompetent model, rat hepatoma cells expressing STGFβRII showed significant delay in tumour growth when compared to cells that did not express the protein (Zhao, Kobayashi et al. 2002). In this paper there was also a significant increase in the mRNA expression of the IL-12p40 subunit in tumours expressing STGFβRII. An increase in IL-2 was also seen in splenocytes of these mice post *in vitro* stimulation. One of the plausible mechanisms for the anti-tumour effect seen in these mice as discussed in that report is the ability of STGFβRII expression to reverse the TGFβ induced suppression of IL-2 production by T cells and

tumourcidal activity of macrophages, which has been previously seen in this model (Zhao, Kobayashi et al. 2002)

To my knowledge no other reports have compared the effect of early and late expression of STGFβRII in tumours. Because of the complexity of TGFβ effects, and the multi-effects on tumour biology, the outcome of reversing TGFβ is highly contextual (Massague 2008). The decrease in tumour growth that was seen with early expression of STGFβRII may be due to reversing the effect of TGFβ on one of the early events which occur during tumour development, either directly on the cancer cell biology or in the tumour microenvironment e.g. the angiogenic switch, or innate immune cells activation. While a less significant impact on adaptive immunity might explain why no significant anti-tumour effect can be seen with the late expression of STGFβRII. This is supported by reports that showed an anti-tumour effect with STGFβRII expression in immunocompromised mice (Rowland-Goldsmith, Maruyama et al. 2002). However, another plausible explanation is the inability of STGFβRII to sequester all of the expressed TGFβ in the microenvironment as the tumour progresses. As the tumour progresses it is expected that more stromal cells are recruited, which in turn can secrete TGFβ.

One of the other objectives of this chapter was to study the effect of blocking TGFβ signaling on the tumour immune milieu. It was interesting to see whether the immune profile in tumours that express STGFβRII would attain a shift from an immunosuppressive profile towards an immunostimulatory one. A screen for mRNAs encoding a range of common cytokines was therefore conducted in established CT26STGβRII tumours. As previously described, CT26STGFβRII were allowed to develop into established tumours in Balb/c mice before they were started on Dox. One week post Dox treatment tumours were extracted for analysis. Of the 22 cytokines screened (Table 5.2) a significant decrease ($p<0.05$) of 3 cytokines; TGFβ1 , BMP4, and Tnsf9, was observed in the Dox treated group. Although not significant, a relatively high fold increase in expression was seen with IL-2,IL-4 and TRAIL.

One mechanism which could explain the decrease in the expression of TGFβ1 and BMP4 is the direct effect of STGFβRII on tumour growth. CT26 cells are known to express TGFβ1 (Figure 4.3.1.1), similarly, reports have shown that CT26 have intact BMP signaling (Jin, Yun et al. 2007), and BMP4 has been shown to be expressed in various human tumours and cell lines

(Kallioniemi 2012). BMP4 is a regulator of embryonic development and bone formation. However, it has also been associated with cancer regulation. Its role remains to be highly controversial, since despite its inhibitory effect on cancer growth it has been linked with its ability to induce metastatsis, invasion, and anginiogenesis and thus promoting tumour development (Kallioniemi 2012). By inducing an anti-tumour effect through the expression of STGFβRII, one would expect a consequential decrease in the expression of these proteins from CT26 cells. The minor changes seen in the expression of these proteins in the Dox treated group reflects the minor anti-tumour effect seen. However another mechanism might be abrogating the paracrine effect of TGFβ on the innate and adaptive cells in the tumour environment.

As described in section 1.4.1.4, TGFβ can suppress the proliferation and function of NK and T cells (Yang, Pang et al. 2010), and it has been linked to abrogation of IL-2 expression from these cells (McKarns, Schwartz et al. 2004). Reversing TGFβ signaling is therefore expected to restore IL-2 expression. IL-2 cytokines can then act in itself to induce a positive feed-back to enhance T cells proliferation and differentiation, which explains its use in various immunotherapeutic modalities to induce anti-tumour immunity (Lesterhuis, Haanen et al. 2011). Of note, however, is the observation that the cytokine can also induce Treg expansion .

In a similar mechanism, TGFβ blockade can be expected to induce IL-4 which is a CD4+ T helper 2 cytokines

Tnsf9 (4-1BBL) is a type II transmembrane glycoprotein that is mainly expressed on activated APC such as B cells, DCs and macrophages {Cheuk, 2004 #204}. The ligand binds 4-1BB, a co-stimulatory receptor expressed predominantly on activated T cells upon TCR activation, and it is thought to promote activated effector T cells expansion. It is not entirely clear how TGFβ blocking can reduce Tnsf9 expression, as to my knowledge no reports have associated TGFβ with an increased expression of Tnsf9. There is also no substantial evidence linking an increase of any of the higher expressed cytokines (IL-2,IL-4, TRAIL) with a decrease in the expression of Tnsf9. Although it is plausible to hypothesis that given that the main role of 4-1BB:4-1BBL signaling is to activate T cells and induce cytokine production by T cells, an increase in IL-2 and/or IL-4 could induce a negative feed-back that down regulates 4-1BB and 4-1BBL expression . Another hypothesis is that the high expression of TRAIL seen in the Dox treated group could be mediating APC killing (expressing Tnsf9) within the tumour.

Another point to address was whether the expression of STGFβRII can effect immune cell populations within the tumours. To do this tumours and spleens from Dox treated or non-Dox treated mice were extracted once a tumour has reached a cutoff size of ~1200 mm^3 and stained for various immune cells populations. A CD8+ population could be detected in these large tumours however there was no difference in their number between Dox and non-Dox treated group. An increase in the intratumour CD8+/Treg population can be associated with favorable clinical responses, however due to technical challenges staining for CD4+Foxp3+ could not be achieved.

Interestingly this CD8+ population could not be detected when studying smaller tumours. This indicates that CD8+ recruitment might be a late event in CT26 tumours development. This might have implications on the immunosuppressive mechanism that develop within the tumour, and might indicate that generating a T cell response in a CT26 model to start with is more crucial than reversing the immunosuppression on those T cells.

A large population of CD11B+ and CD11B+GR1+ cell could be detected in tumours. Indicating the importance of these cells in CT26 development, in accordance with their role in .immunosuppression, angiogenesis, metastasis, and tumour development. However, there was no difference between Dox and non-Dox treated groups.

It was therefore concluded that although early expression of STGFβRII showed a significant impact on tumour growth, late expression of STGFβRII showed less effect. The expression of STGFβRII in well-established tumours could be mediating minor changes in the immune cytokine profile which might account for the minor anti-tumour effect seen.

6 Studying Combined Effect of Expressing STGFβRII and CTLA-4 Blockade *in vivo*

6.1 Introduction

Cancer immunotherapy has recently seen clinical validation with the licensing of Ipilimumab in metastatic melanoma, a fully humanised mAb that blocks the immune inhibition mediated by CTLA-4 (Hodi, O'Day et al. 2010). As explained in section 1.3.2, CTLA-4 acts as a negative regulator of T cell activation through various intrinsic and extrinsic mechanisms (Vanneman and Dranoff 2012). CTLA-4 binds B71 and B72 ligands with high affinity thereby inhibiting their interaction with, and co-stimulation of CD28. Ultimately CD28 signalling is inhibited and a great attenuation of T cells expansion and activation ensues. Chronic antigen exposure is a key mediator of a sustained expression of CTLA-4, it is therefore logical that this immune-checkpoint plays an important role in cases of cancer and chronic viral infection where chronic antigen exposure is evident. This has made CTLA-4 blockade an attractive approach for reversing T cell attenuation and promoting effective anti-cancer immune responses (Grosso and Jure-Kunkel 2013) .

CTLA-4 blockade has been studied extensively in a wide range of preclinical models of solid and haematological malignancies. The aim is clearly to enhance anti-tumour immunity. Although it has shown great efficacy in numerous models as a monotherapy with complete tumour regression, it has been ineffective in others (Grosso and Jure-Kunkel 2013).

Variables and predictive markers that determine the efficacy of CTLA-4 have not yet been identified. Generally speaking, however, a factor that has been shown to predict a positive outcome is the pre-treatment immune status for e.g. TIL content, and tumour burden (Perez-Gracia, Labiano et al. 2014) (Grosso and Jure-Kunkel 2013). This might be due to the local immunosuppression associated with a larger tumour burden e.g. excessive TGFβ and IL-10 expression rendering CTLA-4 blockade unable to alter CD8 T cell tolerance in the tumour. This has consequently led to investigation of CTLA-4 blockade in combination with other therapies such as chemotherapy, radiotherapy and cancer vaccines (Grosso and Jure-Kunkel 2013). The aim is to utilise the ability of such therapies to reduce tumour bulk and therefore reduce tumour

induced anti-immune factors, to release tumour antigens, to increase immune cells recruitment to the tumour, and or to prime a tumour specific T cells response. Recently CTLA-4 has also been combined with therapies that target other immune-checkpoints such as the anti-PD-1 antibody. This combination has shown a significant ($p<0.01$) increase in tumour regression (75% of mice) in comparison to either of the therapies alone, in a CT26 model (Duraiswamy, Kaluza et al. 2013). This was also associated with an increase in the intra tumour CD8 to Treg and CD8 to MDSC ratio. This study illustrates the importance of combinational immunotherapy in overcoming the multiple mechanisms of immunosuppression in the local tumour environment, and indicated how this can result in an effective inhibition of tumour growth. Indeed the combination of anti-CTLA-4 and anti-PD-1 antibodies is now in clinical trials (Wolchok, Kluger et al. 2013).

In a CT26 model CTLA-4 antibody treatment was shown to induce protection in 90% of the mice when given simultaneously with s.c. CT26 tumour cells and a DCs based cell vaccine. However, this protection dropped to 45 % of mice when the same combination was used in mice with established tumours (Pedersen, Buus et al. 2006). This emphasises the importance of tumour burden in the response of CT26 to CTLA-4 blockade.

Seeing the impact of tumour burden on CTLA-4 blockade efficacy, it was hypothesised that the local expression of TGFβ could be an important immunosuppressive factor that limits the strong anti-tumour immune responses expected to be seen with CTLA-4 blockade. This could also explain why the clinical results with anti-CTLA-4 antibodies although promising remain suboptimal (Grosso and Jure-Kunkel 2013). It was therefore interesting to look for any additional effects that can be achieved by combining anti-CTLA-4 therapy with the expression of STGβRII in established tumours. The studies described in this chapter assess the utility of this approach.

6.2 Chapter objectives

1- **Studying the effect of combining CTLA-4 antibody therapy and STGFβRII expression on tumour growth**

2- **Studying the effect of combining CTLA-4 antibody therapy and STGFβRII expression on tumour immune status**.

6.3 Results

6.3.1 The effect of CTLA-4 antibody treatment and STGFβRII expression on tumour growth in mice with low tumour burden

CTLA-4 blockage has not been widely investigated in CT26 tumour models. In the one paper identified, CTLA-4 blockage was associated with an increase in survival; however, this was tumour burden dependent, since administering anti-CTLA-4 antibodies in mice with larger tumours showed less effect on survival (Pedersen, Buus et al. 2006). Therefore the effect of CTLA-4 blockade in a CT26 model was tested here, and the possible additive effect of combining such treatment with the in situ expression of TGFβ antagonist was studied.

Balb/c mice were injected s.c. with $1x10^6$ CT26STGFβRII cells per mouse. When tumours reached sizes of ~ 15 - 30 mm^3 (7 days post tumour implantation) they were assigned into 3 equally sized groups and were then treated with either anti-CTLA-4 antibody and Dox (CTLA-4+Dox), anti-CTLA-4 antibody and no Dox (CTLA-4-Dox), or were left untreated (-CTLA-4-Dox). The anti-CTLA-4 antibody was given i.p. as 3 consecutive doses (100, 50, 50 µg per mouse) 3 days apart. Mice that received Dox were started on Dox with the first dose of anti-CTLA-4 antibody and continued on Dox throughout the entire experiment. Tumour volumes were then monitored until a planned time for mice sacrifice (tumour volumes of ~1200 mm^3).

By day 3 post initiation of treatment a clear reduction in tumour growth was observed in both CTLA-4 receiving groups, while tumour growth progressed in the untreated group (Figure 6.1). However, there was no additive effect from receiving Dox. By day 9 almost all tumours in both groups receiving CTLA-4 had disappeared. This remained to be the case until the termination of study 18 days post treatment. This result demonstrated the strong anti-tumour effect CTLA-4 has on CT26 tumours of low burden.

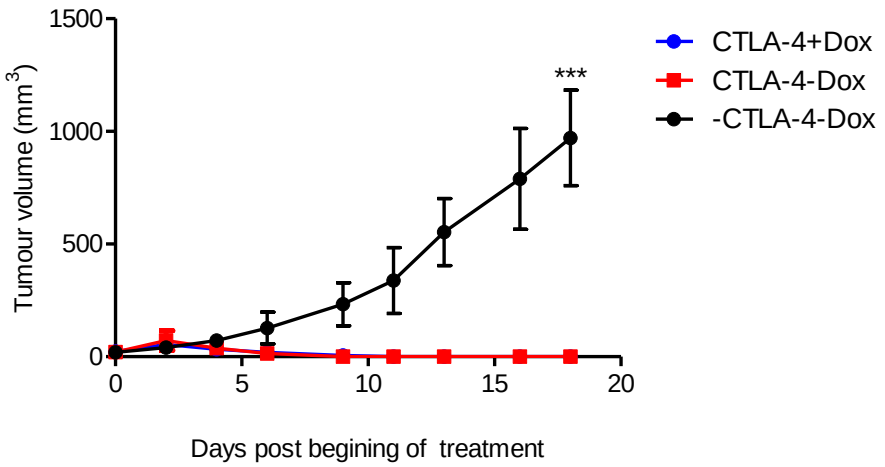


Figure 6.1 The effect of combining the expression of STGFβRII and delivery of anti-CTLA-4 antibody on tumour growth

$1x10^6$ CT26STGFβRII cells per mouse were injected s.c. in Balb/c mice. When tumours were established ~ 15-30 mm^3, mice were divided into 3 groups; and received either anti-CTLA-4 antibody and started on Dox (CTLA-4+Dox), anti-CTLA-4 antibody alone (CTLA-4-Dox), or left untreated (-CTLA-4-Dox). Tumour monitoring was performed every 2-3 days and was stopped when 50% of the -CTLA-4-Dox group was humanely sacrificed. Graph represents n=5, mean and SD shown, statistical analysis was performed using one way ANOVA comparing tumour volumes on day 18 *** = P<0.001.

6.3.2 The effect of CTLA-4 blockade and STGFβRII on inducing a tumour specific immune response.

6.3.2.1 *In vivo* CTLA-4 blockade increases the percentage of CD8+ and CD4+ in spleens of CT26 bearing tumours

The mechanism of the strong anti-tumour effect of CTLA-4 blockade on CT26 tumour growth observed in Figure 6.1 was probed by studying the immune cell status in spleens of mice post CTLA-4 blockade, (as tumours were completely eradicated by the CTLA-4 treatment, only spleens could be obtained).

To study the effect of CTLA-4 blockade on the lymphocyte population, spleens from section 6.3.1 were stained for CD8+, CD4+, and CD8CD44+. It was notable that both the percentage of CD8+ and CD4+ was significantly (P <0.001) and substantially (2-fold) higher in mice treated with anti-CTLA-4 antibody compared to controls (Figure 6.2). However there was no significant difference between groups that received anti-CTLA-4 antibody and Dox or those that received anti-CTLA-4 antibody alone.

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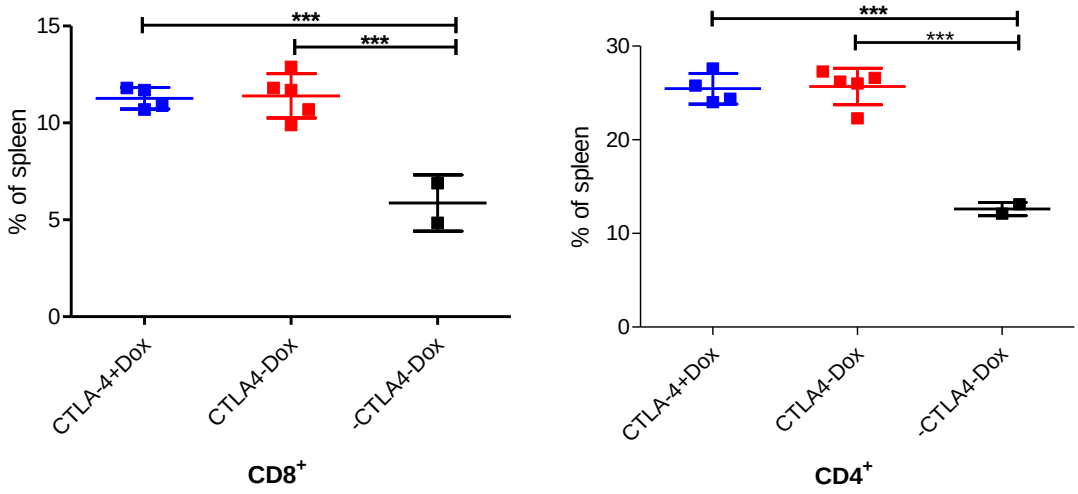


Figure 6.2 The effect of CTLA-4 blockade and STGFβRII expression on lymphocyte populations in the spleens of mice bearing poorly established CT-26 tumours

Spleens of mice from section 6.3.1 were isolated, and mechanically dissociated, and stained for CD8, CD4 for flow-cytometric analysis. Mean and SD shown, Statistical analysis was performed using one way ANOVA ***= $p < 0.001$.

6.3.2.2 *In vivo* CTLA-4 blockade increases IFNγ production by tumour antigen specific splenocytes.

Having observed the strong anti-tumour effects resulting from CTLA-4 blockade, it was interesting to investigate if such effects were associated with the induction of an antigen specific anti-tumour immune response. Two questions were probed; 1- did CTLA-4 blockade enhance tumour specific T cell response, 2 - If the answer is yes, can adoptive transfer of the generated activated tumour specific T cells induce cancer regression in mice with established CT26 tumours. Despite the strong anti-tumour effect seen with CTLA-4 blockade masking any potential additive effect of combining CTLA-4 with the expression of STGFβRII on tumour growth, it was still a possibility that an additive effect could be seen in the induction of anti-tumour immune response.

To answer the first question an Elispot assay was performed to detect IFNγ secretion from activated splenocytes. Spleens from all the mice of the 3 groups in section 6.3.1 were

mechanically disrupted and seeded in anti-IFNγ antibody coated Elispot plates. As an additional negative control, spleens from naïve, non-tumour bearing Balb/c mice (labelled as control) were also used. Splenocytes were then activated using the AH-1 peptide, a CT26 specific antigen peptide (Ahmad, Rees et al. 2005) or by ConA to induce non-specific activation as a positive control for the assay.

As expected CTLA-4 blockade showed a significant increase ($p<0.001$ for CTLA-4+Dox, $p<0.001$ for CTLA-4-Dox) in the IFNy production from activated splenocytes when compared to mice that did not receive CTLA-4 antibody, measured as SFC per 10^6 splenocytes (SFC/10^6 splenocytes) (Figure 6.3). However, there were no significant differences between groups that received Dox or did not receive Dox ($p>0.05$). Mice bearing CT26 tumours that did not receive any treatment showed a slight increase in IFNγ production (mean=77 SFC/10^6) compared to control naïve mice (mean=2 SFC/10^6), however, this was not significant. This indicates that CT26 tumours are only able to induce a week tumour specific immune response.

Notably all splenocytes from the 4 different treatment groups stimulated with ConA showed a high number of SPF/10^6 splenocytes that was not significantly different between groups (Figure 6.3). Indicating that the effect seen with CTLA-4 blockade was tumour specific.

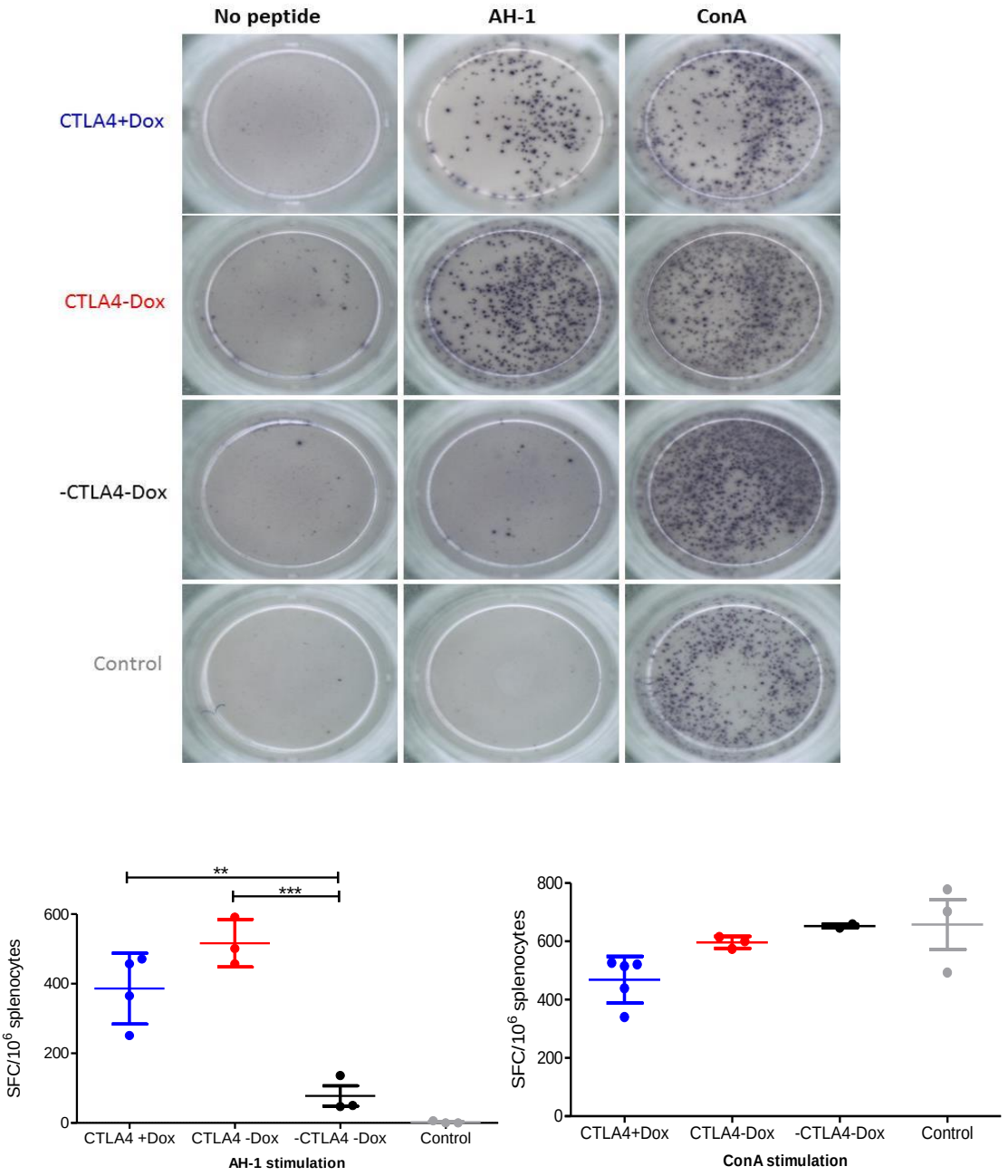


Figure 6.3 The effect of CTLA-4 blockade and STGFβRII expression *in* vivo on the activation of tumour antigen specific immune cells

Spleens from Balb/c mice bearing CT26 tumours and treated with anti-CTLA-4 antibody with or without Dox, together with control spleens from naïve control mice, were assayed by Elispot for IFNγ production after stimulation with either the CT26 antigen peptide, AH-1, or ConA. Diagram above represents one spleen from each group either left untreated (No peptide) as a negative control, treated with 1μg/ml of AH-1 peptide, or treated with ConA as a positive control. Graphs below represents 3 or 4 mice per group, mean and SD shown. Statistical analysis was performed using one way ANOVA. **= $p < 0.01$, ***= $p < 0.001$.

6.3.2.3 The effect of adoptive transfer of activated immune cells on the regression of established CT26 tumours in Balb/c mice

As seen from the Elispots in section 6.3.2.1, CTLA-4 blockade clearly induced the activation and proliferation of CT26 specific T cells. The next question to address was whether adoptive transfer of these activated splenocytes would mediate tumour regression in Balb/c mice bearing CT26 tumours.

It was hypothesized that CTLA-4 blockade induced tumour specific activated memory T cells, which are capable of destroying tumour cells. It was also hypothesised that CTLA-4 blockade and the expression of STGFβRII governs a more activated T cells response, which although was not detected in the IFNγ production by Elispot may be detected in the ability of T cells to induce tumour regression *in vivo*. Finally, it was important to address the possibility that despite the existence of an activated T cells status, upon reaching the tumour microenvironment, the T cells response may face immunosuppressive factors that can induce T cells anergy or T cell suppression. In that case further expression of STGFβRII in the tumour environment with the adoptive transfer would give an additional advantage.

To test for this Balb/c mice were injected s.c. with $1x10^6$ CT26STGFβRII cells per mouse. When tumours were established, an average of ~ 18 mm^3, mice were assigned into groups of 5, and received splenocytes from section 6.3.2.1. Group 1 received splenotcytes from naïve control mice (non-tumour bearing) (Naïve spleens), group 2 received splenocytes from tumour bearing but untreated mice (-CTLA-4-Dox), group 3 received splenocytes from mice that received anti-CTLA-4 antibody only (CTLA-4-Dox), group 4 received splenocytes from mice that received both anti-CTLA-4 antibody and Dox (CTLA-4+Dox). To test whether activated T cells could have their activity suffer immunosuppression when reaching the tumour microenvironment, a fifth group was included which was treated as in group 4 (i.e. received splenocytes from mice that received both an anti-CTLA-4 antibody and Dox), but this group were also started on Dox to express STGFβRII from within the tumour (Figure 6.4). The expression of STGFβRII should therefore remove the immunosuppression effect of TGFβ faced by T cells, and thus should give this group an additional advantage in tumour regression when compared to group 4.

By day 12 post-splenocyte transfer and Dox administration, mice receiving splenocytes from naïve control mice showed an increase in tumour size when compared to all other groups

(Figure 6.4). This increase was significantly greater than that in mice receiving splenocytes from CTLA-4 and Dox treated (group 4 and group 5) ($p<0.05$) (Figure 6.4). This indicates T cells from CT26 tumour bearing mice can provide some tumour suppressive effect when compared to naïve T cells, however, a greater and significant ($p<0.05$) anti-tumour effect was seen with T cells post CTLA-4 blockade and STGFβRII expression.

By day 15 group 2, receiving splenocyte from tumour bearing but untreated mice, showed the greatest tumour volume with an average mean ~1.5 fold greater than group 3 (CTLA4-Dox), and ~ 2.2 greater than groups 4 and 5 (CTLA-4+Dox, CTLA-4+Dox (Dox)), however, this increase was not statistically significant ($P>0.05$) (Figure 6.4). Indicating that the significant activation of tumour specific T cells response as a result of CTLA-4 treatment that was observed by Elispot *in vitro* cannot be reflected *in vivo* with tumour regression.

Although groups 4 and 5 showed the lowest tumour volumes there was no significant difference between both groups and group 3. Similarly no statistical significance was seen between all 5 groups in terms of duration of survival.

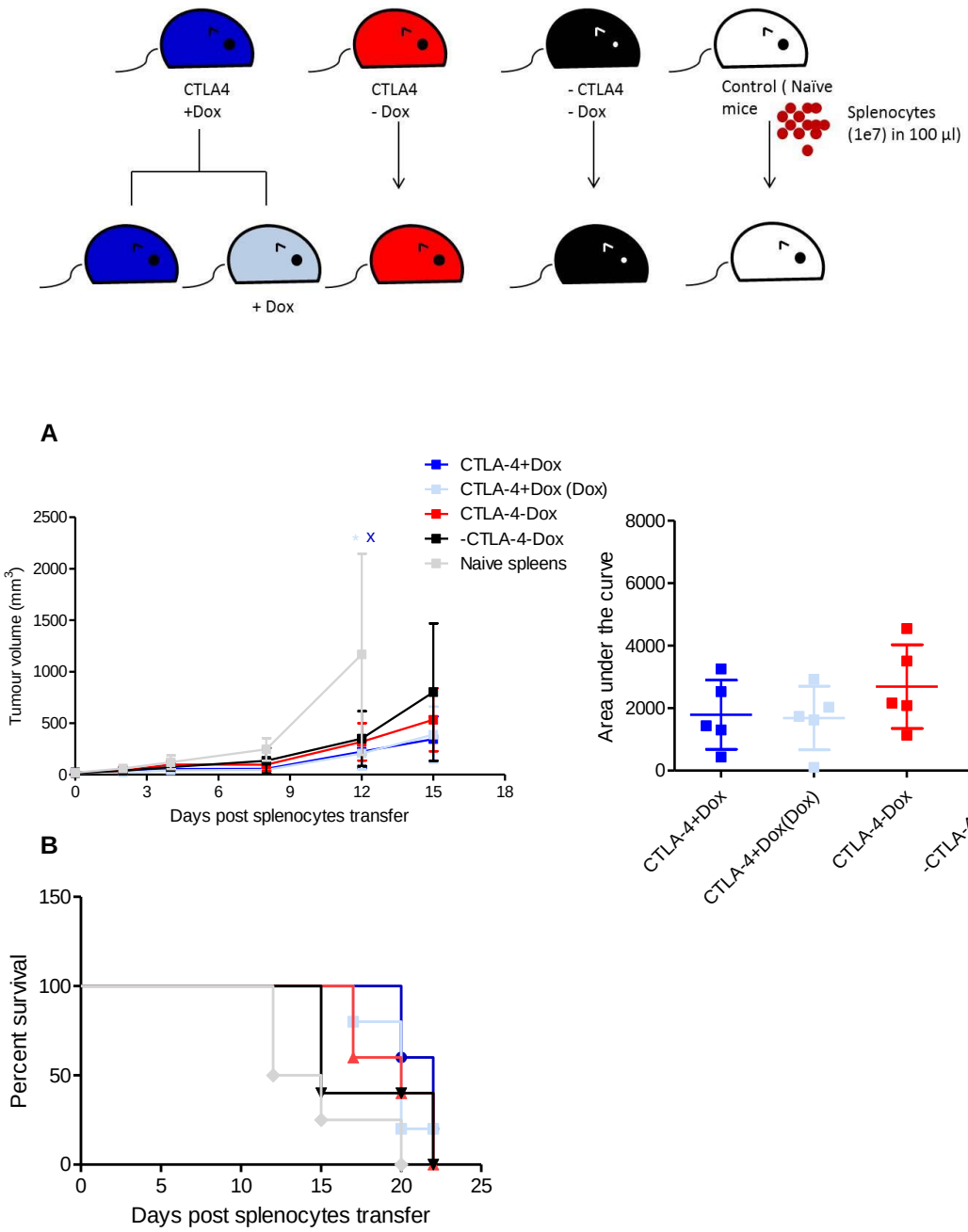


Figure 6.4 The effect of activated splenocyte adoptive transfer on tumour growth and animal survival

Balb/c mice with established CT26STGFβRII were injected i.v. with splenocytes (10^7 splenocytes per mouse) obtained from naïve non-tumour bearing mice (Naïve spleens), mice with CT26STGFβRII tumours but untreated (-CTLA-4-Dox), mice with CT26STGFβRII and treated with anti-CTLA-4 antibody (CTLA-4-Dox), mice with CT26STGFβRII and treated with anti-CTLA-4 antibody and Dox (CTLA-4+Dox). For one group Dox was administered to the recipient mice (CTLA-4+Dox (Dox)). N=.5, mean and SD shown, analysis performed using one way ANOVA for the area under the curve. *=p<0.05 relative to CTLA-4+Dox (Dox) group x=p<0.05 relative to CTLA-4+Dox group.

6.3.3 The effect of low dose CTLA-4 antibody treatment and STGFβRII expression on tumour growth and immune profile in higher tumour burden

6.3.3.1 The effect of low dose CTLA-4 antibody in combination with STGFβRII expression on tumour growth.

As seen in section 6.3.2 a full dose of CTLA-4 antibody treatment in mice with low tumour burden completely eradicated the tumour regardless of the expression of STGFβRII. Therefore the evaluation of any additive effect of CTLA-4 treatment and STGFβRII was not possible. A model in which a smaller dose of CTLA-4 antibody treatment is given to mice with higher tumour burden may allow evaluation of any additional effect of CTLA-4 treatment and STGFβRII.

Balb/c mice were injected s.c. with $1x10^6$ CT26STGFβRII cells per mouse. When tumours reached sizes of ~ 70 - 150 mm^3 (13 days post tumour implantation) they were equally divided into 4 groups receiving anti-CTLA-4 antibody and Dox (CTLA-4+Dox), anti-CTLA-4 antibody and no Dox (CTLA-4-Dox), only Dox (-CTLA-4+Dox), or left untreated (-CTLA-4 - Dox). Anti-CTLA-4 antibody was given i.p. as 1 dose of 100 µg per mouse. Tumour volumes were then monitored until a planned time (8 days post treatment).

As expected -CTLA-4 - Dox group showed the largest tumour volume, with mean of ~ 809 mm^3, in comparison to 527mm^3 for the CTLA4-Dox group, 469 mm^3 for the CTLA-4+Dox group, and 353 mm^3 for the –CTLA-4+Dox group (Figure 6.5). This however, was not statistically significant, indicating that lower doses of CTLA-4 antibody treatment in mice with a higher tumour burden is not sufficient to completely eradicate the tumours or significantly reduce tumour growth. No additive effect was detected with CTLA-4 blockade and the expression of STGFβRII. In fact the expression of STGFβRII alone displayed the lowest tumour volume mean and also demonstrated much less variation between mice (Figure 6.5). This is in accordance with results in chapter 5 in which expression of STGFβRII in established tumours displayed lower tumour volume mean than the non-Dox treated group, however, not statistically different.

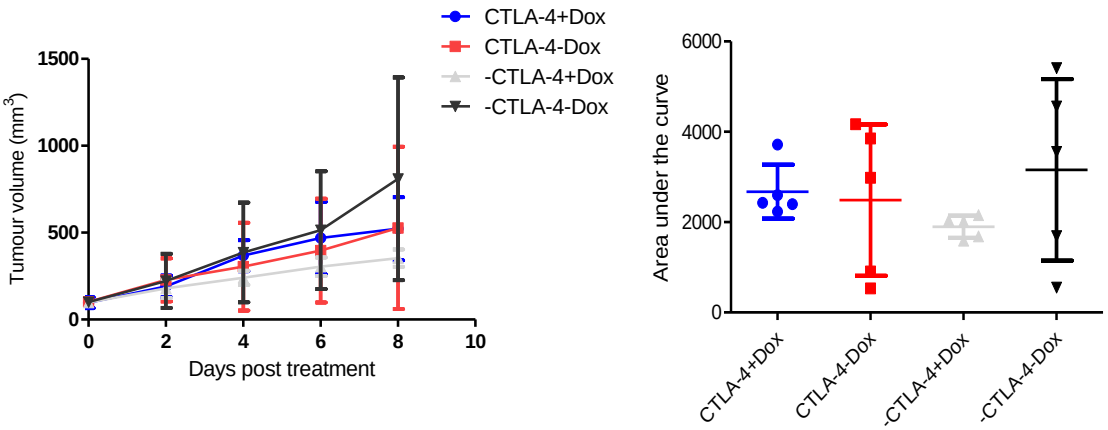


Figure 6.5 The effect of combining the expression of STGFβRII, and low dose of anti-CTLA-4 antibody on tumour growth

$1x10^6$ CT26STGFβRII cells per mouse were injected s.c. in Balb/c mice. When large tumours were established ~ 70-150 mm^3, mice were divided into 4 groups; and either received anti-CTLA-4 antibody and started on Dox (CTLA-4+Dox), anti-CTLA-4 antibody alone (CTLA-4-Dox), Dox alone (-CTLA-4+Dox) or left untreated (-CTLA-4-Dox). Tumour monitoring was performed every 2-3 days and was stopped 8 days post treatment). Graph represents n=5, mean and SD shown. Statistical analysis was performed using one way ANOVA of the area under the curve.

6.3.3.2 The effect of low dose CTLA-4 treatment and the expression of STGFβRII on the immune cytokine profile of established tumours

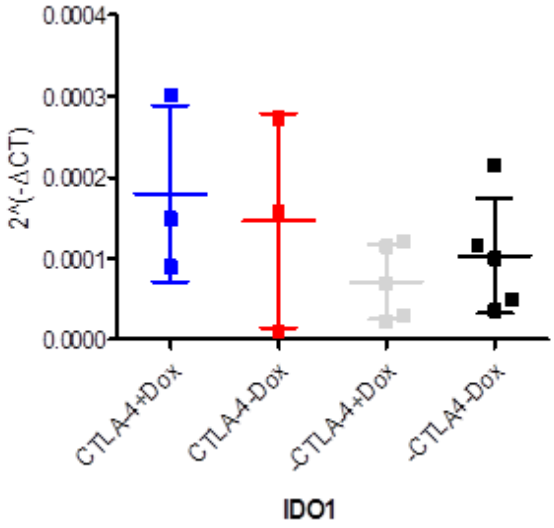
As tumours developed with the low dose of CTLA-4 treatment in section 6.3.3.1 it provided the opportunity to study the effect of CTLA-4 antibody treatment alone and in combination of STGFβRII expression on the immune cytokine profile in these tumours. Tumours from Balb/c mice in section 6.3.3.1 were extracted 8 days after CTLA-4 antibody treatment and Dox initiation to compare the RNA expression of these cytokines between all 4 groups. cDNA was obtained from total RNA and RT-QPCR was conducted using the RT2 profiler custom array panel.

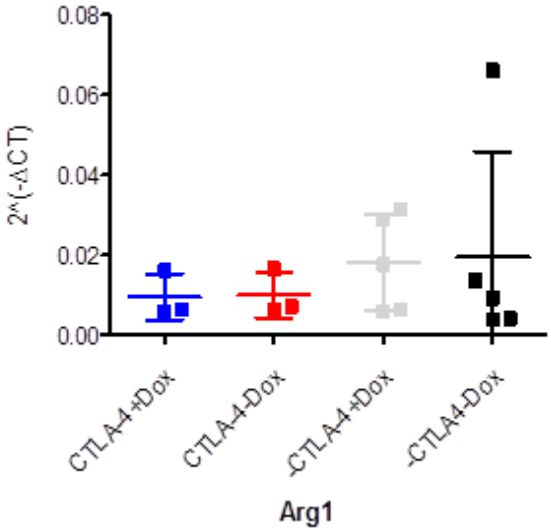
A significant decreased level of Tnsf9 (one way ANOVA) (p=0.02) in the CTLA-4+Dox when compared to –CTLA-4-Dox was detected, however, the expression of all other cytokines that were examined showed no significant differences between all 4 groups (Figure 6.6), however, this was not surprising as it reflects the non-significant differences in tumour growth between the treatment groups that was observed in Figure 6.5.

In concordance with section 5.3.2.1.2 when cytokines expression was compared between -CTLA-4+Dox and -CTLA-4-Dox groups only using a Student's T test, a significant decrease

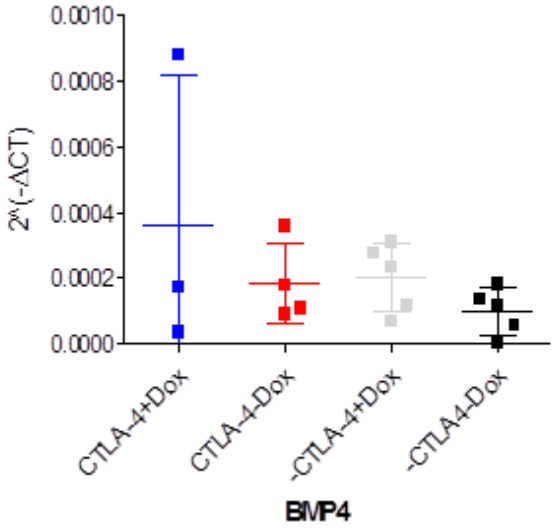
(p=0.009) in the levels of Tnsf9 and an increase in levels of Tnsf10 (P=0.047) could be detected.

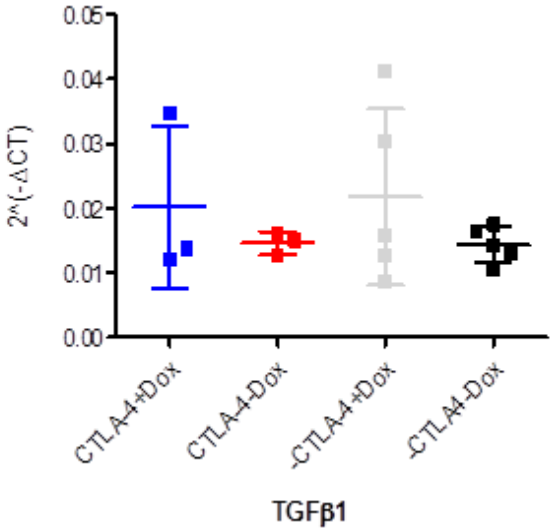
Metabolic regulators



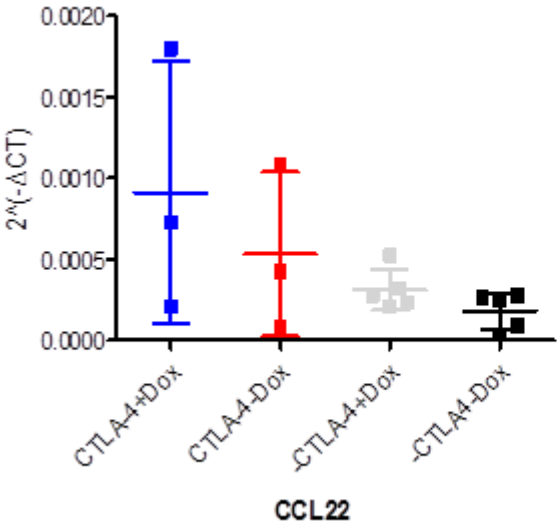


BMPs and TGFβ

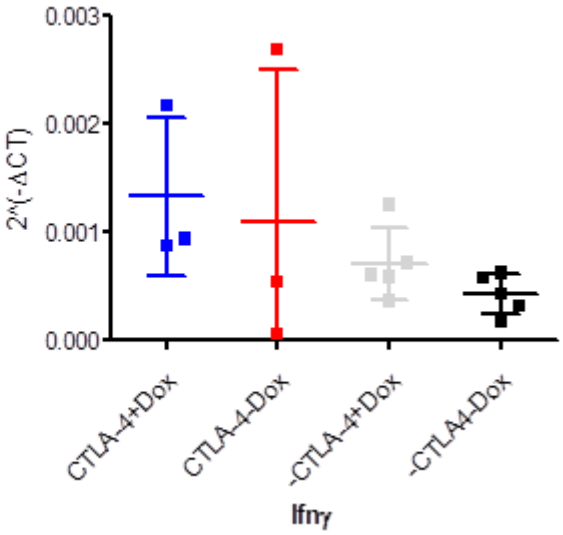




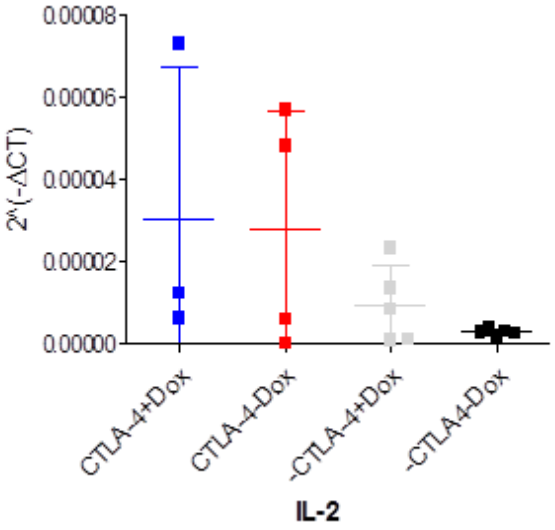
Chemokines



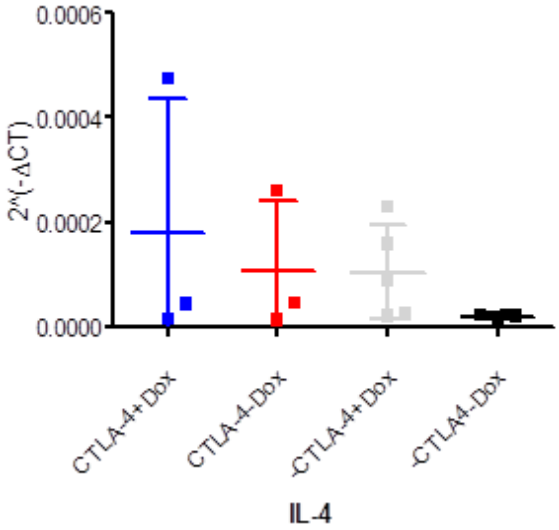
Interferons



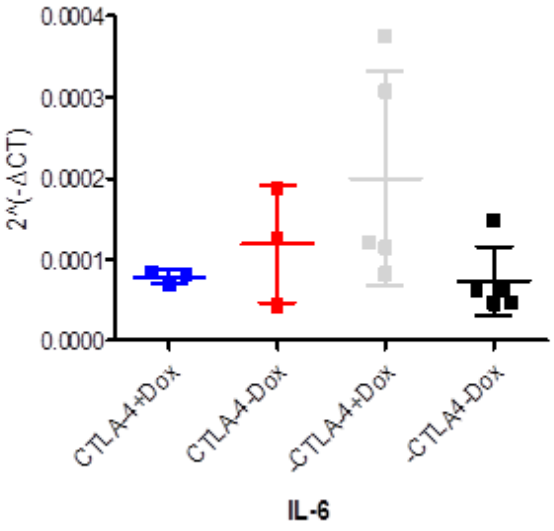
Interleukins



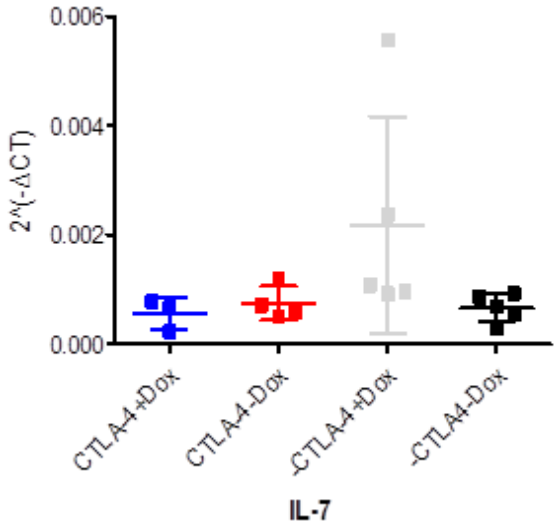
IL-2



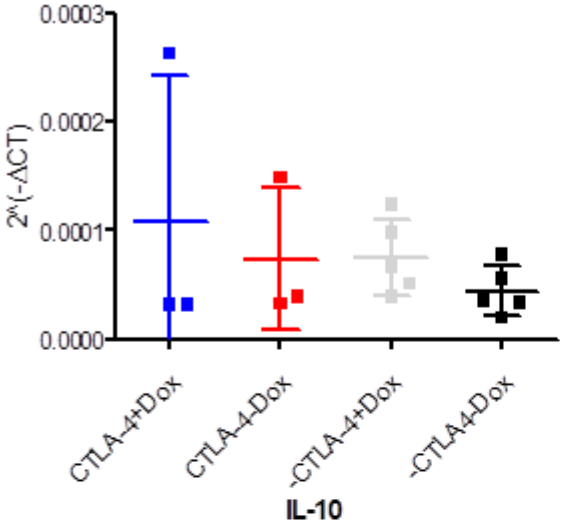
IL-4

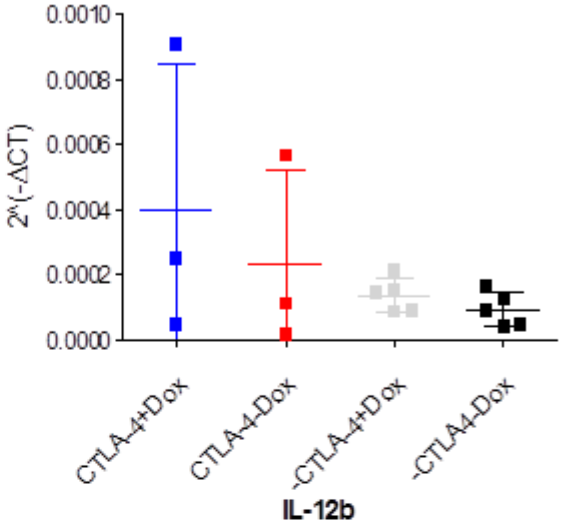


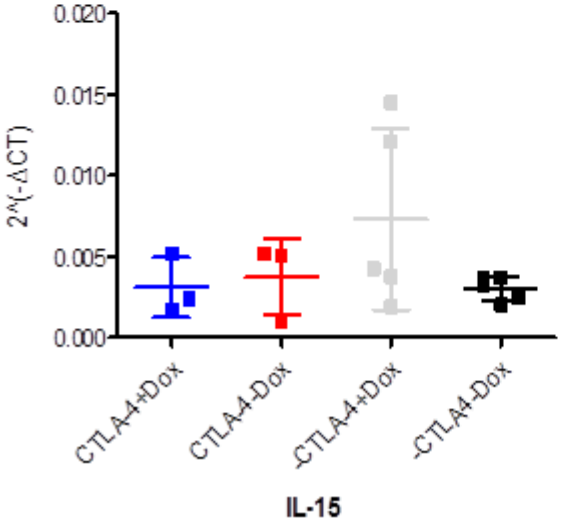
IL-6



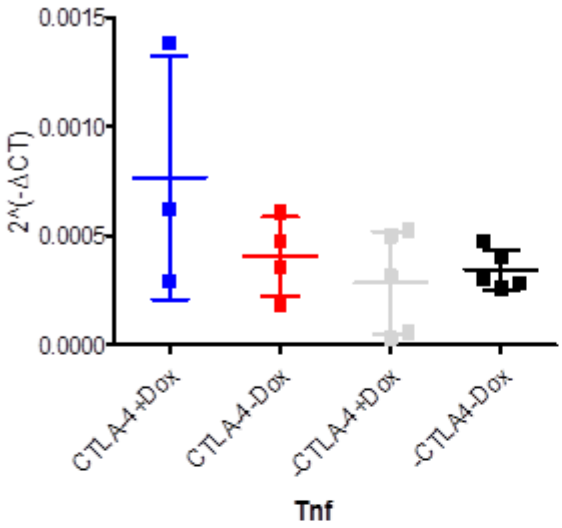
IL-7

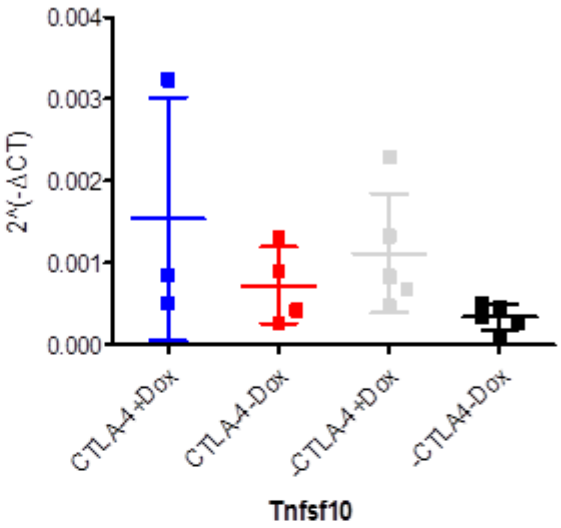

0.0003
0.0002
0.0001
0.0000
2^(-ΔCT)
CTLA-4+Dox
CTLA-4-Dox
-CTLA-4+Dox
-CTLA4-Dox
IL-10

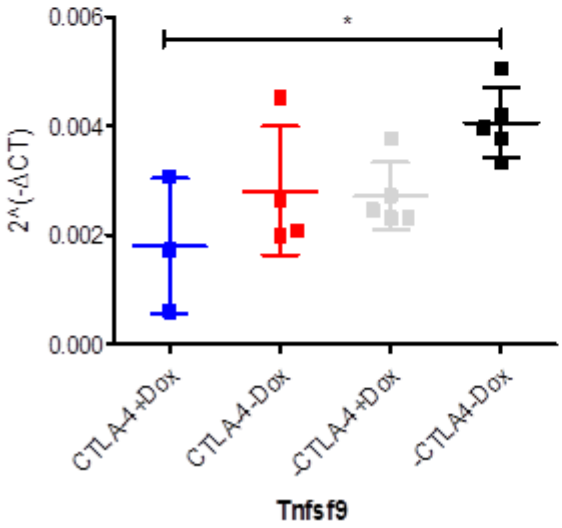

0.0010
0.0008
0.0006
0.0004
0.0002
0.0000
2^(-ΔCT)
CTLA-4+Dox
CTLA-4-Dox
-CTLA-4+Dox
-CTLA4-Dox
IL-12b

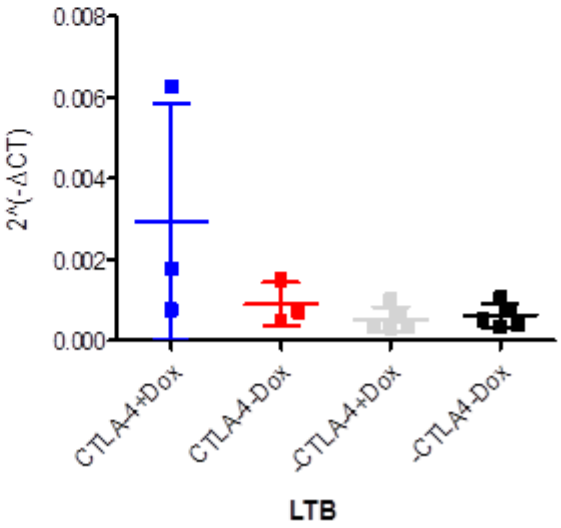

0.020
0.015
0.010
0.005
0.000
2^(-ΔCT)
CTLA-4+Dox
CTLA-4-Dox
-CTLA-4+Dox
-CTLA4-Dox
IL-15

Tnf superfamily









others

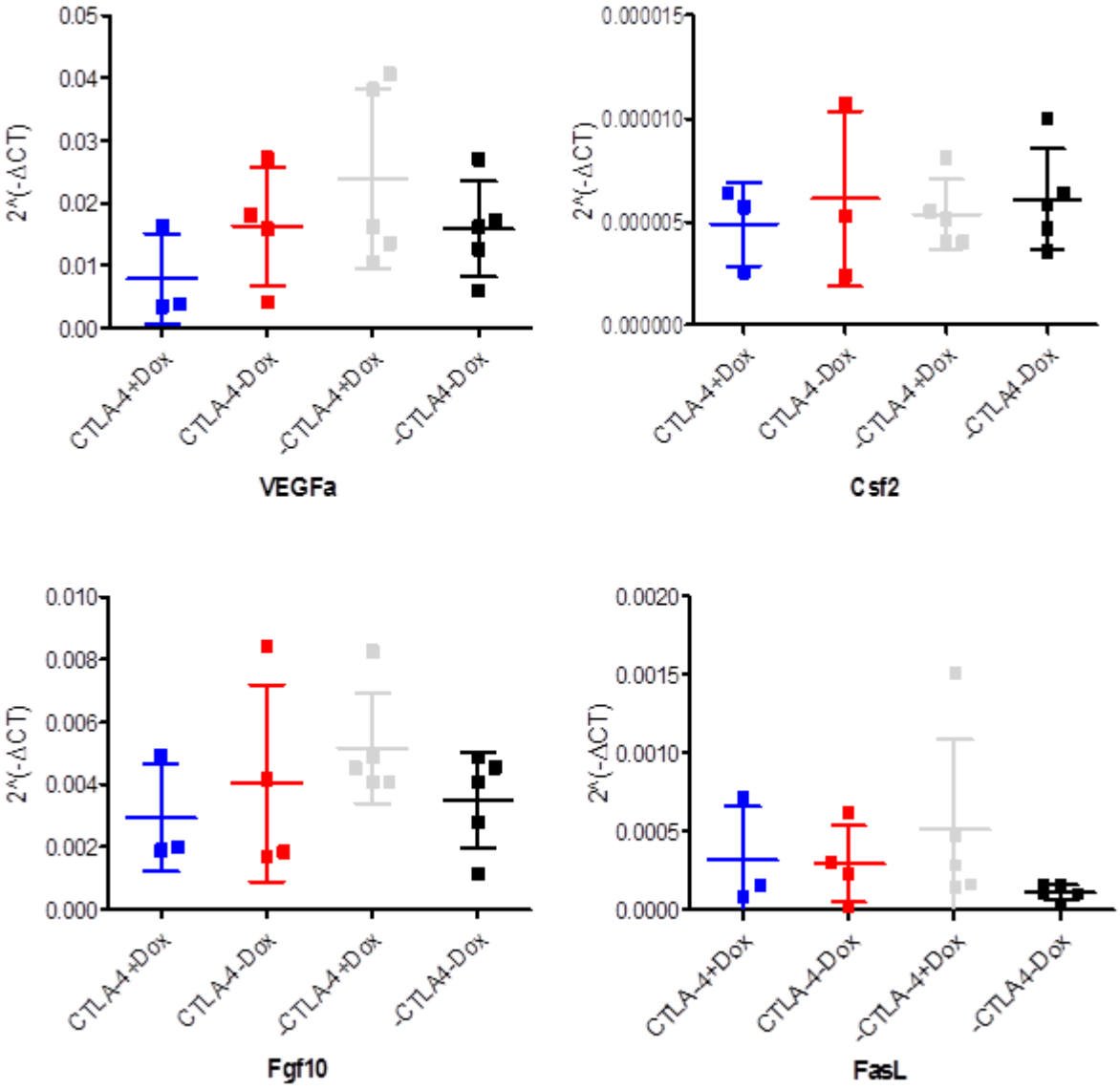


Figure 6.6 The effect of combining the expression of STGFβRII, and low dose of anti CTLA-4 antibody on tumour immune cytokines

Total RNA was extracted from CT26STGFβRII tumours from all 4 groups (CTLA-4+Dox), (CTLA-4-Dox), (-CTLA-4+Dox) (-CTLA-4-Dox). cDNA preparation and RT q-PCR was conducted as previously described. Data was plotted using the $2^{\wedge(-\Delta CT)}$).Mean and SD shown, n= (3-5), statistical analysis was performed using one way ANOVA.

6.4 Conclusion

Antibody blockade of CTLA-4 is one of the most promising immunotherapies in current clinical use. Due to its significant role in modulating T cells responses, the therapy has shown great efficacy in various tumour models as a monotherapy or in combination with other therapies such as chemotherapy, radiation, or immunotherapy (Grosso and Jure-Kunkel 2013).

Given that multiple mechanisms play a role in inducing tumour immunosuppression and immune tolerance, targeting more than one mechanism is a more promising approach to induce a greater anti-tumour effect. This has been the main reason behind combining CTLA-4 blockade with other immunotherapies that either express immune stimulatory cytokine e.g IL-2, or that aim to block other immune-checkpoints e.g. PD-1(Grosso and Jure-Kunkel 2013, Wolchok, Kluger et al. 2013).

Seeing the modest effect on tumour growth achieved with the expression STGFβRII on its own, the aim of this chapter was to study if any additional benefit could be achieved by CTLA-4 blockade and TGFβ antagonism.

To do this the effect of full dose of anti-CTLA-4 antibody in conjunction with STGFβRII expression on tumour growth was first studied in mice with small sized established tumours. By day 3 post initiation of CTLA-4 antibody treatment a clear reduction in tumour size could be seen in both groups that received the antibody treatment in comparison to control untreated mice (Figure 6.1). This was quickly followed by a complete eradication of tumours in both groups regardless of Dox administration. This showed that a full dose of anti-CTLA-4 antibody as monotherapy has a strong anti-tumour efficacy in established low tumour burden CT26 models. However, the strong anti-tumour effect observed made it hard to evaluate any additive effect that may be achieved with the combination of CTLA-4 blockade and STGFβRII expression.

Not many studies have evaluated the anti-tumour effect of CTLA-4 blockade on CT26 tumours. In one study, anti-CTLA-4 antibody therapy given simultaneously with CT26 tumours cells in Balb/c mice resulted in 90% survival in mice, however, this percentage dropped to 45% when the treatment was initiated in established tumours, indicating the importance of CT26 tumour burden in the determining the efficacy of CTLA-4 blockade (Pedersen, Buus et al. 2006)

When studying the lymphocyte population in the spleens of tumour bearing mice treated with anti-CTLA-4 antibody, it was clear that the treatment significantly ($p<0.001$) expanded both the CD8+, and the CD4+ population in comparison to control untreated mice (Figure 6.2). Previous studies have shown that CTLA-4 blockade can expand tumour specific CTL in the spleen and peripheral blood of tumour bearing mice (Fransen, van der Sluis et al. 2013). Furthermore, anti-CTLA-4 antibody treatment in glioma murine models was able to overcome the glioma induced reduction in CD4+ in spleens of mice, and restore it to levels found in naïve non-tumour bearing mice (Fecci, Ochiai et al. 2007).

To study the effect of CTLA-4 blockade and STGFβRII expression on tumour specific lymphocytes, spleens from mice treated with anti-CTLA-4 antibody alone or with Dox were stimulated with the CT26 antigen AH-1 and studied for IFNγ production by Elispot assay. Both groups treated with anti-CTLA-4 antibody showed a significant ($p<0.001$) increase in IFNy production, measured as SFC/10^6 splenocytes, when compared to untreated mice, or naïve non-tumour bearing mice (Figure 6.3). However, Dox administration did not provide any additional benefits.

To see if this strong tumour specific immune response could be translated *in vivo,* splenocytes from all groups were adoptively transferred into mice with established CT26 tumours and the ability of splenocytes to eliminate the tumour was measured. By day 12 post-splenocyte transfer, mice that received naïve splenocytes from non-tumour bearing mice had a larger tumour volume mean, and 50 % of the group had reached the humane sacrifice cut off point tumour volume (Figure 6.4). At this time point mice that received splenocytes from mice treated with CTLA-4 + Dox showed the lowest average mean tumour volume, which was significantly lower than naïve splenocytes receiving mice ($p<0.05$). By day 15, mice that received splenocytes from untreated tumour bearing mice showed the largest tumour volume, however, there were no significant differences between all groups.

It was hypothesised that despite CTLA-4 blockade generating an expansion of tumour specific lymphocytes, upon arriving within tumours these lymphocyte would be faced with immunosuppressive factors that might render them anergic. In this case the expression of STGFβRII might give an additional advantage to overcome any TGFβ induced anergy. To test

thisthe group of mice that received splenocytes from mice treated with anti-CTLA-4 antibody and Dox was further divided into two groups, a group that received Dox to trigger STGFβRII expression, and a group left untreated. However, Dox administration did not give any additive effects, and both groups showed similar tumour volumes.

There are 3 questions to discuss in this experiment. 1- Why was the significant difference between groups in the tumour specific IFNγ production (Elispot) *in vitro* not observed *in vivo,* this might be because although CTLA-4 blockade has the ability to induce some aspects of tumour specific T cell activation such as IFNγ production, it might still be limited in inducing other features of T cells activation e.g. the activation of cytotoxic gene expression such as perforim or granzyme B production. The overall effect would be a suboptimal tumour immune response *in vivo.* Another explanation could be that despite the generation and expansion of tumour specific T cells as a result of CTLA-4 blockade, these cells are faced with multiple immunosuppressive factors and mechanisms at the tumour site which renders them anergic or less functional. The expression of STGFβRII in the tumour site did not alter this situation, indicating that other immunosuppressive factors/ mechanisms might be involved in suppressing the generated tumour specific T cells.

The fact that the group that received splenocytes from mice treated with anti-CTLA-4 antibody and Dox showed the smallest tumour volumes might indicate that the expression of STGFβRII might have had an additive effect in inducing and expanding a tumour specific T cells population rather than reversing the activity of immunosuppressed T cells. This may be the result of the ability of STGFβRII to reverse the TGFβ induced immature, tolergenic, or immobolised DC. By reducing DC suppression a stronger initiation of tumour specific T cells response is expected.

The power of CTLA-4 blockade meant that it was not possible to evaluate if addition of STGFβRII expression to anti-CTLA-4 treatment could provide additional effect when a high dose of anti-CTLA-4 antibody was used. Therefore, a second experiment was designed in which a lower dose of anti-CTLA-4 antibody was used in larger tumours, in an attempt to minmise the strong anti-tumour response seen with the high dose treatment. Mice with established CT26STGFβRII tumours of ~ 70-150 mm^3 were therefore divided in 4 groups, and

treated with Dox only, one dose of anti-CTLA-4 antibody only, Dox and anti-CTLA-4 antibody, or left untreated.

As expected, tumours were not eliminated with the low dose of anti-CTLA-4 antibody. By day 8 post treatment the untreated group showed the largest mean of tumour volume, however there was no significant difference between all 4 groups and no additive effect with anti-CTLA-4 antibody and STGFβRII on tumour growth was detected.

The combination of STGFβRII and anti- CTLA-4 antibody therapy has been previously tested in one study using the AE17 murine mesothelioma in mice. In this study STGFβRII alone or anti-CTLA-4 antibody alone marginally improved survival. However, there was no difference with the combination therapy (Kissick, Ireland et al. 2012). The main breakthrough in this study came with the addition of a third therapy which was the anti-CD25 antibody for Treg elimination, the triple therapy showed a significant improvement in survival when compared to the untreated control ($p<0.0001$) or that of the double treatment ($p<0.0002$). This could support our conclusion that TGFβ antagonism is not sufficient to remove the effect of inhibitory factors on activated T cells.

Similar to the tumour growth studies, when studying the cytokine profile of these tumours, there was no significant difference detected between the 4 groups except for Tnsf9 which showed a significant decrease ($p=0.02$) in the CTLA4+Dox compared to -CTLA-4-Dox.(Figure 6.6). It is most likely that the decrease in Tnsf9 level is induced by the expression of STGFβRII since when comparing -CTLA-4+Dox and -CTLA-4-Dox groups (Student's T test) a significant decrease ($p=0.009$) was seen in the -CTLA-4+Dox groups, which is in concordance with results seen in section 5.3.2.1.2.

Overall this indicates that low doses of anti CTLA-4 antibody provide no substantial effect on tumour growth or cytokine profile of CT26 tumours, and similar to other reports, no additive benefit could be seen when a low dose of CTLA-4 antibody and the expression of STGFβRII.

Combinational immunotherapy is thought to be beneficial to broaden anti-tumour responses, and targeted immunotherapy can be combined in a rational way (Mellman, Coukos et al. 2011) that depends on mechanism of action and the sequential triggering of events to induce strong anti-tumour immune responses. One of the limitations in combinational therapy is the limited

understanding of the interactions between the combinations and the importance of timing. For example identifying the ideal timing for blocking TGFβ in combination with other therapeutic could give better insight (Flavell, Sanjabi et al. 2010). It might also be that long term expression of immunosuppressive inhibitors can induce selective pressure, ultimately selecting for immune tolerant tumour cells thus limiting the primary beneficial effect. By having an inducible model where TGFβ or IL-10 blockade tumour immunity can be regulated by various scheduling regimes and combinations can be tested which should provide better understanding. The testing platform developed here may provide more efficient means of identifying and optimizing combined immunotherapies.

7 Discussion

7.1 Preface

Cancer immunotherapy was identified as the 'Breakthrough of the Year' for 2013 by Science Journal. However, despite some promising results with current immunotherapies in several types of cancer, the number of patients that can benefit remains to quite limited (Lesterhuis, Haanen et al. 2011). Immunotherapy generally has shown the tendency to be less effective in patients with large tumour burdens (Lesterhuis, Haanen et al. 2011). This has been suggested to relate to a positive correlation between tumour burden and tumour induced immunosuppressive mechanisms (Lesterhuis, Haanen et al. 2011).

An important mechanism of tumour-induced immunosuppression is the secretion of soluble factors such as TGFβ and IL-10 into the tumour interstitium. Such agents have the ability to suppress a wide range of innate and adaptive immune cells (Mosser and Zhang 2008, Yang, Pang et al. 2010). While strategies for TGFβ blockade have been investigated previously, various questions remain to be answered. For example due to its pleiotropic effect, it is unclear which of the TGFβ activities is the most influential on tumour growth (Flavell, Sanjabi et al. 2010). In addition, little is known about the optimal timing of TGFβ blockade on its own or in combination with other targeted therapeutics (Flavell, Sanjabi et al. 2010). Such unanswered questions, and the current relatively poor understanding of the context dependence of TGFβ within the tumour microenvironment, could explain why clinical trials evaluating TGFβ blockade have failed to show clear benefits (Flavell, Sanjabi et al. 2010).

In order to address some of the current limitations with targeting immunosuppressive factors, this study aimed to design a new tumour model that would allow investigation of the effects of in situ antagonism of various soluble immunosuppressive factors ('immune-checkpoints'), both in early and in well-established tumours. My concept was to engineer murine CT26 colorectal carcinoma cells to express secreted cytokine-antagonists (specifically antagonists to TGFβ or IL-10) only when induced by the addition of Dox. This model should thus give precise control over when the antagonist would be expressed, and should be a useful tool in answering questions relating to tumour induced immunosuppression. In particular it might allow validatation of new immunosuppressive checkpoint targets within the tumour microenvironment. Crucially this approach could elucidate whether local antagonism of these immunosuppressive factors is sufficient for restoring tumour immunity, or whether a more systemic approach is superior. Thus,

the system should provide important insights into developing more effective and less toxic checkpoint inhibitors that can function locally rather that systemically.

7.2 Principle findings

Chapter 3:

- Lenti-X-Tet-On system shows strong reversible induction of transgene expression in CT26 cells *in vitro* and in CT26 tumours *in vivo*
- Transgene expression under the Lenti-X-Tet-On system is comparable to the CMV promoter *in vitro*

Chapter 4

- CT26 cells secret TGFβ and maintain an intact TGFβ downstream signaling
- CT26 cells are insensitive to the intrinsic anti-proliferative activity of TGFβ
- STGFβRII can be expressed and secreted from engineered CT26 cells expressing the protein under Dox control *in vitro*
- Secreted STGFβRII is biologically active and can efficiently inhibit the TGFβ induced downstream signaling *in vitro* (P-Smad2)
- STGFβRII is expressed in established CT26 tumours in Balb/c mice post Dox treatment
- The model can be utilised to express other immunosuppressive molecules antagonists such as SIL-10R

Chapter 5

- Early expression of STGFβRII from CT26 cells *in vivo* has a significant effect on tumour take rate and tumour growth
- Late expression of STGFβRII has no significant effect on tumour growth
- STGFβRII expression in established tumours can alter the cytokine profile increasing IL-2, IL-4, and TRAIL mRNA transcript levels, while significantly decreasing TGFβ, BMP4 and Tnsf9 (4-1BB)

- No difference in the percentage of CD8+ or CD11BGR1+ populations could be detected in tumours of mice receiving Dox (to express STGFβRII) and those which were not dosed with Dox

Chapter 6:

- In low tumour burden of CT26 tumours, anti-CTLA-4 antibody has a significant anti-tumour effect
- In high tumour burden and with low doses of anti-CTLA-4 antibody, CTLA-4 blockade has no significant effect on CT26 tumour growth
- In high tumour burden and with low doses of anti-CTLA-4 antibody, CTLA-4 blockade and STGFβRII expression resulted in a significant decrease in Tnfsf9 mRNA transcript levels in tumours.
- No additive effect was observed with STGFβRII expression and anti-CTLA-4 antibody on tumour growth
- CTLA-4 blockade can significantly increase tumour specific immune cells response (IFNγ production), however, the ability of these immune cells to eradicate established tumours following adoptive transfer is diminished.
- Adoptively transferred splenocytes from animals treated with anti-CTLA-4 antibody and Dox for STGFβRII expression can significantly reduce tumour growth when compared to adoptive transfer of naïve splenocytes

7.3 General discussion

Recent progress in our understanding of tumour immunity has led to the development of new immunotherapeutics that not only aim to stimulate an increase in tumour immunity but also aim to ‘take the brakes off’ the local tumour immune response, thought to relate to various tumour induce immunosuppressive mechanisms (Motz and Coukos 2013). Many molecules serve to down-regulate T cell function and thus act as immune-checkpoints within the tumour environment. However, recently unprecedented success has been seen with two T cell

checkpoint inhibitors (anti-CTLA-4 and anti-PD-1 antibodies) (Hodi, O'Day et al. 2010, Wolchok, Kluger et al. 2013).

As described in section 1.3.2, CTLA-4 acts as an inhibitory receptor on activated T cells which can induce inhibitory signals in these cells and compete with the CD28 co-stimulatory molecule (Grosso and Jure-Kunkel 2013). Thus the molecule ultimately decreases T cell activation and proliferation. The success with anti-CTLA-4 antibody was not only due to its clinical activity in a fraction of patients with advanced stage melanoma, but also by clear evidence of an infiltrating CD4+ and CD8+ T cell population in the tumour tissue studied (Hodi, O'Day et al. 2010, Perez-Gracia, Labiano et al. 2014). Thus linking the clinical activity with an evident T cell response (Perez-Gracia, Labiano et al. 2014).

Similarly, PD-1 receptor is an inhibitory surface receptor expressed on activated T cells, to maintain the dysfunction and chronic exhaustion of T cells that have been activated for prolonged periods (Perez-Gracia, Labiano et al. 2014). Tumour cells express PD-L1 and the expression has been linked with reduced immunogenicity of these cells. This has led to interest in anti-PD-L1 and PD-1 antibodies, which similar to ipilimumab (anti-CTLA-4 antibody) have shown clinical activity in various tumours including melanoma, renal cell carcinoma and colon cancer (Perez-Gracia, Labiano et al. 2014).

It is becoming clear that immune-checkpoints work in a non-redundant manner. They can inhibit T cell function, proliferation, and activation via various mechanisms and in different locations (Lymph node or tumour site). This has been the reason behind the concept of using combination immunotheraputics including checkpoint inhibitors, to generate stronger anti-tumour responses (Perez-Gracia, Labiano et al. 2014). The concept has led to investigation of the potential synergistic effect of anti-CTLA-4 and anti-PD-L1 antibody combination, which has shown promising results and distinct clinical responses from the monotherapies (Wolchok, Kluger et al. 2013), which has led to the combination being currently studied in a phase III clinical trial.

Overall, it is clear that immune-checkpoint inhibitors can empower tumour immune responses, and they represent an attractive approach for cancer therapy. One of the problems with the current inhibitors is the induction of widespread toxicities and side effects seen from systemic inhibition of such important immune regulators. Although manageable, a range of side effects

such as skin rashes, colitis, and other autoimmune complications are associated with these inhibitors (Wolchok, Kluger et al. 2013, Perez-Gracia, Labiano et al. 2014). This makes alternative therapies that target the protein locally a more attractive approach which can lead to faster development and better clinical efficacy of these inhibitors. Viruses represent the ideal agent to produce a therapeutic protein locally from infected cells, and oncolytic viruses may provide the ideal vector for amplified protein expression in a tumour-restricted fashion.

It is clear that a major part of success in the field of tumour immunotherapy has been through the development of various animal models (Budhu, Wolchok et al. 2014) . However there remain various limitations in these models which leave behind many unanswered questions. For example; what are the most important immune-checkpoint and immunosuppressive factors that will prove to be of clinical value when targeted, what is the best way to target these factors locally to avoid disruption of their systemic homeostatic functions (if any), when is the best time to target these factors, and finally what combinations can be used to enhance anti-tumour efficacy (Flavell, Sanjabi et al. 2010). The rapid improvement and progress in the field will therefore require appropriate pre-clinical models that can recapitulate the human clinical situation as precisely as possible (Budhu, Wolchok et al. 2014).

The new animal model presented in this thesis, allowing the regulated expression of immunosuppressive antagonists from CT26 cells only following establishment of tumours *in vivo*, provides an interesting method of achieving localised and temporally-regulated expression of proposed new checkpoint inhibitors. It should have various advantages compared to some of the other models. The model allowed us to evaluate inhibition of TGFβ signaling locally in immunocompetent mice, and in well-established tumours that resemble the clinical situation.

By having the ability to control the timing of TGFβ blockade, it was apparent that the clear clinical benefit came only from early blockade. the precise reason for this is not yet understood, although these data perhaps indicate that inhibiting TGFβ effects on early events during tumour growth and perhaps the effect on the innate immune system rather that the adaptive immune system. This was also supported by the fact that activated tumour specific immune cells generated by anti-CTLA-4 antibody could not eradicate established tumours following adoptive

transfer, even in the presence of Dox. Showing that TGFβ blocking on its own is not sufficient to reverse tumour induced T cell anergy.

7.4 Limitations

Despite the advantages of transplantable syngeneic models in immunocompetent mice they remain sub-optimal in recapitulating the multistep process of tumour immunity and tumour induced immunosuppression. Unlike spontaneous tumours, transplantable model do not undergo stages of immunoediting in a similar manner that is expected to occur in human tumours. Thus they might be limited in providing insight regarding the relative importance of immunosuppressive checkpoints (Budhu, Wolchok et al. 2014).

Although the Tet-On system has shown high levels of transgene expression, a potentially limiting factor in expressing a ligand trap checkpoint inhibitor such as STGFβRII is the requirement for sufficient amounts to compete with the wild type receptor. It is therefore plausible to believe that during low tumour burden with less recruitment of stromal and immune cells the expressed STGFβRII may be produced in sufficient amounts to compete with the wild type receptor, however as the tumour enlarges and more stromal cells are recruited which in turn can express TGFβ competitive inhibition of the wild type TGFβII receptor competitive might be harder to achieve.

Finally, as the levels of transgene expression are completely dependent on Dox, any factors that can limit Dox access into tumours would ultimately result in reduced transgene expression. This can be thought of in the case of larger tumours with poor vasculature and poor perfusion limiting access of the drug to the tumour core.

7.5 Future work

Designing strategies that can concurrently activate the immune system and reduce immune-suppression are thought to have potential in inducing effective anti-tumour immunity. Despite seeing limited effect with TGFβ blockade on its own, combination with immunostimulatory molecules and/or tumour vaccines might enhance anti-tumour effect. The model therefore can be used as well to express various immunostimulatory proteins or other immunosuppressive antagonist.

Since the model allows tight regulation of TGFβ blockade various timings of expression can be explored. For example comparing the tumour immune cytokine profile with early TGFβ blockade and late TGFβ blockade should give insight regarding which important factors are responsible for the TGFβ anti-tumour effect.

Alternative therapies that target the protein locally are an attractive approach. Viruses represent the ideal agent to produce a therapeutic protein locally from infected cells. Oncolytic viruses have developed to replicate and lyse cancer cells selectively (Cawood, Hills et al. 2012). Advances in genetic engineering have led to the exploitation of their intrinsic immunogenicity as pathogens and their utilisation for the delivery of various immunomodulating molecules (Cawood, Hills et al. 2012). Oncolytic viruses armed with immune-checkpoints inhibitors may therefore be an attractive approach to enhance anti-tumour immunity. An understanding of the best factors and combinations of factors and their levels and timings of expression will be crucial in engineering the most powerful and selective viruses possible.

The model introduced in this thesis should thus give precise answers to questions related to tumour induced immunosuppression, and may allow validation of immune-checkpoints inhibitors which can then be expressed in oncloytic viruses. This is a particularly important for species specific oncolytic viruses such adenoviruses which do not replicate in murine cells. Evidence of successful anti-tumour immune response from a surrogate preclinical model that can express immune-checkpoint inhibitors locally would thus validate the use of an armed oncolytic adenovirus for clinical trials.

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