Modulation of T regulatory activity for cancer therapy

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy (PhD) in the Faculty of Medical and Human Sciences.

2011

Christy Ralph

School of Medicine
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ABBREVIATIONS

\[^{1}H\]TdR tritiated thymidine
5T4 an oncofoetal antigen
APC allopohycyanin; a fluorochrome (FACS)
APC antigen presenting cells
B7 family of critical ligands which interact with receptors to regulate T lymphocyte activation and function, e.g. B7-1(CD80), B7-2(CD86)
BCG Bacille Calmette Guérin; vaccine against tuberculosis
CA19-9 tumour associated antigen; normal human serum range 0-31 U/mL
CCL22 chemokine ligand 22; a Th2 chemokine. Chemokines are organised into four families based on the arrangement of conserved cysteine residues in the N-terminal region: CC, CXC, C, CX3C.
CD152 see CTLA-4
CD25 the IL-2 receptor α-chain
CEA carcinoembryonic antigen, TAA (normal human serum range 0-3μg/L)
CI confidence intervals
CML chronic myeloid leukaemia
CP-675,206 tremelimumab, briefly ticilimumab (Pfizer), a fully humanised anti-CTLA4 monoclonal antibody
cpm counts per minute
CR complete response
CT computed tomography
CTLA4 cytotoxic T lymphocyte-associated antigen 4
DMSO dimethyl sulphoxide
DNA deoxyribonucleic acid
ECOG Eastern Cooperative Oncology Group
EU European Union
FACS fluorescence-activated cell sorting; flow cytometry
FasL Fas Ligand, an apoptotic agent and member of the TNF family which binds to cell surface receptor Fas (APO-1, CD95) leading to apoptosis.
FCS foetal calf serum
FITC fluorescein; a fluorochrome (FACS).
FM freezing mix
FoxP3 forkhead box protein 3; a marker of T regulatory lymphocytes
FSC forward scatter (in FACS analysis)
GCP Good Clinical Practice
GITR glucocorticoid-induced TNF receptor related gene
HER-2/neu human epidermal growth factor 2/neu; a transmembrane glycoprotein with intrinsic tyrosine kinase activity, a TAA and member of the epidermal growth factor receptor family
HHV-8 human herpes virus 8
ICH GCP International Conference on Harmonisation of Good Clinical Practice
IDO indoleamine 2,3-dioxygenase
IgG immunoglobulin G
IgV variable immunoglobulin-like (domain)
IL-2 interleukin-2
<table>
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<tr>
<td>IMDM</td>
<td>Iscove’s modified Dulbecco’s media; tissue culture media</td>
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<tr>
<td>INF-γ</td>
<td>Interferon-γ</td>
</tr>
<tr>
<td>IPEX</td>
<td>immune dysregulation, polyendocrinopathy, and X linked syndrome</td>
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<tr>
<td>IQ range</td>
<td>interquartile range</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide, a component of bacterial cell walls</td>
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<td>LSM</td>
<td>lymphocyte separation medium</td>
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<td>MAGE-1</td>
<td>melanoma antigen gene 1; a melanoma tumour associated antigen</td>
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<td>MHC</td>
<td>major histocompatibility complex molecules; part of the antigen processing machinery</td>
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<td>MHRA</td>
<td>Medicines and Healthcare Products Regulatory Authority</td>
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<tr>
<td>mIL2</td>
<td>murine interleukin-2</td>
</tr>
<tr>
<td>MVA</td>
<td>Modified vaccinia virus Ankara</td>
</tr>
<tr>
<td>NCI CTC</td>
<td>National Cancer Institute Common Toxicity Criteria</td>
</tr>
<tr>
<td>NHS</td>
<td>National Health Service</td>
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<tr>
<td>NK</td>
<td>Natural Killer cells; part of the innate immune response.</td>
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<tr>
<td>PB</td>
<td>permeabilisation buffer</td>
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<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PD</td>
<td>progressive disease</td>
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<td>PD1</td>
<td>programmed cell death 1; CD279; a broadly expressed regulatory cell surface receptor</td>
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<td>PE</td>
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<td>phytohaemagglutinin</td>
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<td>PR</td>
<td>partial response</td>
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<td>PS</td>
<td>Performance status; a measure of patient well being</td>
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<td>Regional Ethics Committee</td>
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<tr>
<td>RECIST</td>
<td>Response Evaluation Criteria in Solid Tumours</td>
</tr>
<tr>
<td>RPMI</td>
<td>tissue culture media utilising a bicarbonate buffering system; acronym derived from Roswell Park Memorial Institute where it was developed.</td>
</tr>
<tr>
<td>SD</td>
<td>stable disease</td>
</tr>
<tr>
<td>SEREX</td>
<td>serological analysis of recombinant cDNA expression libraries</td>
</tr>
<tr>
<td>SI</td>
<td>Stimulation index</td>
</tr>
<tr>
<td>SOP</td>
<td>Standard operating procedure</td>
</tr>
<tr>
<td>SSC</td>
<td>side scatter (in FACS analysis)</td>
</tr>
<tr>
<td>STAT-1</td>
<td>Signal transducer and activator of transcription (factor)-1: a transcription factor with a dedicated role in mediating INF-γ signalling.</td>
</tr>
<tr>
<td>SWOG</td>
<td>South Western Oncology Group</td>
</tr>
<tr>
<td>TAA</td>
<td>tumour associated antigen</td>
</tr>
<tr>
<td>TAP1</td>
<td>transporter associated with antigen presentation 1</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor; an inflammatory cytokine</td>
</tr>
<tr>
<td>Treg</td>
<td>natural regulatory T lymphocytes</td>
</tr>
<tr>
<td>TTP</td>
<td>time to (disease) progression (from screening to CT scan/ death)</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>Vs.</td>
<td>versus</td>
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ABSTRACT

The University of Manchester
Christy Ralph
Doctor of Philosophy (PhD)
Thesis Title: Modulation of T regulatory activity for cancer therapy.

Emerging evidence suggests the immune system has a role in preventing cancer, and in advanced cancer evidence of immune dysfunction is widespread. This project focused on cytotoxic T lymphocyte antigen 4 (CTLA4), a key negative regulator of T cell activation found on dedicated regulatory T cells (Treg) and activated T lymphocytes, and asked whether modulation of immune control with anti-CTLA4 blockade led to significant anti-tumour activity.

Clinical and laboratory investigation of anti-CTLA4 blockade using tremelimumab in a phase II trial of second-line therapy in advanced oesophageal and gastric adenocarcinomas was combined with an attempt to establish a suitable pre-clinical model based on therapeutic vaccination against the tumour associated antigen (TAA) 5T4.

Eighteen patients received tremelimumab. Most drug-related toxicity was mild but there was a single death due to bowel perforation. Four patients had stable disease with clinical benefit; one achieved a partial response after eight cycles (25.4 months) and remains well on study after four years.

Markers of regulatory phenotype, forkhead box protein 3 (FoxP3) and CTLA4, doubled transiently in CD4+CD25\textsuperscript{high} lymphocytes in the first month after tremelimumab before returning to baseline. In contrast, CTLA4 increased in CD4+CD25\textsuperscript{low/negative} lymphocytes throughout the cycle of treatment. Post-treatment expanded Treg expressed FoxP3 without interleukin-2 and their defining suppressive function was not abolished despite prolonged anti-CTLA4 blockade. De novo proliferative responses to TAA 5T4 (8 of 18 patients) and carcinoembryonic antigen (CEA; 5 of 15) were detected. Patients with a post-treatment CEA proliferative response had median survival of 17.1 months compared to 4.6 months for non-responders (p=0.002). Baseline interleukin-2 release after T lymphocyte activation was higher in patients with clinical benefit and toxicity.

Heterologous mouse 5T4 (m5T4) vaccination showed some evidence of weak therapeutic benefit, but all tumour models investigated had rapidly lethal kinetics. Specific m5T4 immune responses could be detected by serum antibody ELISA and IFN-\(\gamma\) ELISPOT assays in naive animals but were lower frequency than published responses to h5T4, and were further attenuated in tumour-bearing animals. The addition of anti-CTLA4 blockade did not result in significant augmentation of m5T4 specific immunity after vaccination in non tumour-bearing animals and combination treatment was ineffective as therapy in this autologous model.

Results are discussed in the context of emerging immunotherapeutics in melanoma and prostate cancer. In the absence of supportive data from the model system it would not be appropriate to pursue combination heterologous 5T4 vaccine with anti-CTLA4 blockade, but in view of the unusual durability of the best response to tremelimumab, and in vitro evidence of enhanced proliferative responses to relevant TAA, further investigation of drug activity may be warranted in metastatic gastric and oesophageal second-line treatment.
DECLARATION

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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Special mention and thanks to Dr Fiona Thistlethwaite and Dr Eyad Elkord for their support and practical guidance.
DEDICATION

... “and the great bay-window was
Spawning snow and pink roses against it
Soundlessly collateral and incompatible:
World is suddener than we fancy it.”

Louis MacNeice

Snow, Jan 1935 from Poems selected by Michael Longley, faber and faber 2001

For Ella, Adam, Eric and Neil Boyes who put up with a lot.

For SP, his anonymous five minutes.
CHAPTER 1: INTRODUCTION

1.1: Project outline

This project aimed to explore the modulation of T regulatory activity for cancer therapy. The primary research question was:

Does the modulation of immune control with anti-CTLA4 blockade lead to significant anti-tumour activity?

To address the question, this project combined the clinical and laboratory investigation of anti-CTLA4 blockade using the antibody tremelimumab in a phase II trial of second-line therapy in patients with advanced oesophageal and gastric adenocarcinomas, and attempted to establish and test a suitable pre-clinical model based on a therapeutic vaccination schedule against the oncofoetal tumour associated antigen (TAA) 5T4.

1.2: Background: oesophago-gastric cancer

1.2.1. Oesophago-gastric cancer: incidence and risk factors

Gastric and oesophageal adenocarcinomas are common tumours with poor prognoses. In 2007 age-standardised incidence in the United Kingdom (UK) was similar: 9.6 per 100,000 population for oesophageal carcinoma, and 8.9 per 100,000 population for gastric adenocarcinoma [1]. The UK age-standardised incidence of gastric cancer has fallen in the last 25 years, from 31.0 to 13.1 per 100,000 men; Helicobacter pylori infection which has a primary aetiological role in gastric cancer [2], and is strongly associated with social deprivation, has also been falling. Improved nutrition is also thought to have played a role in the falling incidence, although ironically obesity is emerging as a
significant risk factor. Gastric cancer incidence is twice as high in men and rises with age and cigarette smoking.

While the incidence of gastric cancer has been falling, that of oesophageal cancer has been rising: age-standardised UK incidence has risen from 8.8 to 14.4 per 100000 men between 1975 and 2007. Moreover, there has been a change in the pattern and histology of oesophageal cancer in white males from the predominance of proximal squamous carcinomas to that of distal adenocarcinomas. Oesophageal adenocarcinoma shares risk factors with gastric adenocarcinoma: incidence is more than double in males, and rises with age, obesity and cigarette smoking. *H.pylori* infection however has no aetiological role and may even be protective. Instead, chronic acid reflux leading to the development of Barrett’s metaplasia, a change in the lining of the lowest part of the oesophagus from a squamous to an intestinal endothelium, which has an important aetiological role in the development of adenocarcinomas of the distal oesophagus and gastro-oesophageal junction.

### 1.2.2. Oesophago-gastric cancer: treatment and survival

5 year survival of patients diagnosed with gastric cancer in England and Wales in 2000-2001 was 15% for men and 18% for women, and 5 year survival of patients diagnosed with oesophageal cancer in England and Wales in 2000-2001 was only 8% for both men and women.

In both gastric and oesophageal adenocarcinoma, only a minority of patients present with disease amenable to surgical cure, and even for these, 5 year survival with combination peri-operative chemotherapy and radical surgery is around 36% [3]. For the
majority, who present with advanced disease, median survival with combination cytotoxic chemotherapy is only 11 months [4]: a large randomized trial established the non-inferiority of substituting oxaliplatin (O) for cisplatin (C) and oral capecitabine (X) for infusional fluorouracil (F) in a three drug triplet also containing epirubicin (E), and demonstrated a reduced hazard ratio (HR) for death of the novel triplet EOX compared to established ECF of 0.80 (95% CI 0.66-0.97, p=0.02). There is no standard second line therapy. Although a number of active agents have been investigated in this setting there is no trial data confirming benefit over supportive care alone [5]. Response rates for active salvage therapies are around 20% but toxicity can be high [6]. Durable responses are very rare. There is a clear need for alternative therapeutic agents.

A recent meta-analysis of cancer risk in immune-compromised patients demonstrated a significant increase in incidence of gastric cancer in HIV/AIDS patients and transplant recipients, expressed as a standardized incidence ratios of 1.9 (95%CI 1.53-2.36) and 2.04 (1.49-2.79) [7]. There is also some evidence that oesophago-gastric cancer can respond to immunotherapy and non-specific immune activation is widely used in Japan and Korea as an adjuvant to surgery. Recent phase III trial data shows a 12% 15 year survival benefit of adjuvant chemo-immunotherapy over chemotherapy alone [8-10].

1.3: Cancer and the immune system

1.3.1. Cancer immunosurveillance

Over a century ago, Paul Ehrlich proposed that the immune system repressed the growth of cancer [11], and it was long considered that the immune system should be able to recognise and destroy cancer cells, but convincing evidence of this hypothesis was
hard to find. The theory fell out of favour during the 1960s and 1970s as a sequence of experiments in immunocompromised mice, such as the athymic nude mouse failed to demonstrate conclusive increased incidences in spontaneous or carcinogen-driven carcinogenesis [12]. Such immunocompromised mice do show a high susceptibility to virally-induced tumours, and increased rates of spontaneous lymphomas, but these were thought to be due to increased susceptibility to transforming viruses. In retrospect, the immune function of the experimental models was poorly characterised: crucially perhaps, athymic nude mice do have functional, detectable populations of αβ T cell-receptor bearing lymphocytes and NK cells, and are not therefore without a potential effector cell for immunosurveillance [13]. The strain of mouse may also alter sensitivity to carcinogens: repetition of methylcholanthrene tumour induction experiments with nude mice on a BALB/c [14] rather than CBA/H [12] background did show increased tumour formation compared to controls, indicating that the earlier results may have been compromised by the sensitivity of the control animals to the chosen experimental carcinogen.

There have always been rare instances of apparently immune-driven cancer regression, but there is a growing body of evidence that prolonged immunosuppression leads to increased risk of cancer in man. This is explained only in part by increased sensitivity to transforming viruses. For example, whilst transplant registries with long-term follow up of therapeutically immunosuppressed patients do show up to a 30 fold increase in standardised incidence ratios of tumours with a known viral aetiology, such as non-Hodgkin’s lymphoma (Epstein-Barr), Kaposi’s sarcoma (human herpes virus 8), and carcinomas of the genital tract (human papilloma viruses), there is also a 2 to 5 fold increase in cancers which do not have a viral aetiology, such as those of the lung, colon, larynx, bladder, and in men, of the prostate and testes[15].
Animal models provide more robust supportive data for immune control of cancer development, a theory termed cancer immunosurveillance [16]. First, endogenous interferon-γ (IFN-γ) protects the host from spontaneous, induced and transplanted tumour in experimental models [17]. This protection has three conditions: the host cells must produce IFN-γ, the tumour cells must be sensitive to IFN-γ, and the host animal must be able to mount a specific T cell immune response. Secondly, mice without perforin, a mediator of lymphocyte-dependent cell killing found in the cytolytic granules of cytotoxic T lymphocytes and NK cells, show an increased incidence of methylcholanthrine induced and spontaneous tumours [18].

Thirdly, the ability to define, and create specific immune dysfunction in animal models through genetic manipulation has allowed the investigation of potential specific mechanisms of cancer immunosurveillance. Schreiber's work with gene-targeted mice deficient in signal transducer and activator of transcription 1 (STAT1) or recombination activating gene 1 (RAG-1) or RAG-2, confirmed the presence of a cancer immunosurveillance mechanism dependent on IFN-γ and lymphocytes [19-20]. STAT1 is a transcription factor with an obligate and dedicated role in mediating signalling by IFN-γ and IFN-α/β receptors. STAT1 deficient mice are IFN-γ insensitive: outwardly developmentally normal, they succumb rapidly to exposure to microbial pathogens or viruses at doses which are sub-lethal in control animals, and they show increased rates of induced and spontaneous malignancy consistent with the earlier antibody-blocking experiments [20]. RAGs encode for enzymes involved in the repair of double-stranded DNA breaks, and are expressed exclusively in the lymphoid compartment. Mice without either enzyme are unable to rearrange lymphocyte antigen receptors, and are completely without T and B lymphocytes and NK cells. In methylcholanthrine tumour induction
experiments, RAG-2 deficient mice developed tumours earlier and with greater frequency (30/52) than control 129/SvEv mice (11/57). Spontaneous tumours were also more frequent in RAG-2 deficient mice: by 16 months all 12 RAG-2 deficient mice had occult evidence of neoplasia, including 6 adenocarcinomas of the lung or gastrointestinal tract compared to 2 of 12 control animals, both of which had benign tumours. Transplanted chemically induced tumours show similar growth kinetics in vivo in both types of animal for tumours originating in the immunocompetent controls. In contrast, tumours from the RAG-2 deficient mice, whilst growing with matching kinetics in other RAG-2 deficient mice, are frequently rejected by control animals (8/20) even at high levels of inocula (10^6 per mouse) [19].

This last result suggests that not just the rate but the nature of malignant neoplasms are altered by the immune environment in which they arise and develop. This process, the exertion of an immune evolutionary pressure on tumour development has been called “immunologic sculpting” [21], and has led to a revision of the original idea of immunosurveillance, to allow for the paradoxical emergence of less immunogenic tumours due to selection pressure. The theory of cancer immunoediting encompasses the processes of (1) recognition and potential elimination of tumour by the immune system, (2) equilibrium, during which the immune system exerts both control and selective pressure over the tumour, and (3) escape, during which the tumour grows inexorably despite an immunocompetent host [22].

1.3.2. The adaptive immune response

Any understanding of immune recognition and potential elimination of cancer is grounded in our understanding of the adaptive immune response. In health the immune
system recognises and eradicates pathogens and damaged cells without significant autoreactivity [23]. This requires a constant homeostatic balance between activation and regulation.

Classically, the immune system was conceived as divided into the innate and adaptive systems. Both systems involve humoral and cellular elements. Innate immunity forms a rapid, non-specific defence, involving, for example, mucosal barriers, complement activation, or phagocytosis of lipopolysaccharide (LPS) containing bacteria by macrophages or natural killer (NK) cells. In contrast, adaptive immunity requires priming, memory, and subsequent recognition of specific molecules or molecular fragments, termed antigens, in the individual organism. Adaptive immunity allows the development of a repertoire of tailor-made defences to potentially recurrent threats. These include antibody production and antigen-specific T cell activation. Although the mechanisms are shared, this adaptive immune repertoire will vary from organism to organism due to differences in history and context of antigen exposure.

The distinction between the innate and adaptive immune systems is useful but in vivo there is interaction between the two. Certain cytokines, such as IFN-γ, which is produced by both NK cells and by Th1 CD4⁺ and CD8⁺ lymphocytes play a key role in activation of both systems [24]. Recent evidence suggests that in some circumstances NK cells may share characteristics of T lymphocytes such as memory and longevity [25], whilst some cells of the adaptive system such as iNKT cells, γδ T cells and B1 cells show constitutive expression of activation markers and have a limited repertoire of antigen recognition in much the manner of innate immune cells [26].
The interaction with antigen is central to the adaptive immune response; antigen is in fact defined by its recognition by this adaptive immune response. The repertoire for such recognition is encoded in the receptors of T and B cells, and is generated by genetic recombination of genes\textsuperscript{a} [27]. Selection mechanisms reduce self-reactivity during ontogeny, but the potential for autoimmunity remains. Autoimmunity is held in check by homeostatic immune regulatory mechanisms, and by the importance of antigen context in the process of immune activation. Diversity of these naïve T cells maintains a capacity to respond to the multiplicity of potential microbial pathogens, but results in very low frequencies of cells which can respond to a given epitope [28]. Of the two major classes of lymphocyte, bone marrow derived B cells are responsible for specific antibody production which targets intact antigen on foreign cells or organisms, and thymic T cells interact with fragment antigen presented by specialised partner antigen presenting cells (APCs).

The presence of antigen is necessary but not sufficient for T cell activation. In acute infection, specialised APCs such as dendritic cells (DCs) act as a cornerstone of immune response. They can respond to “danger” signals, such as heat shock proteins, or materials released from damaged cells, and can recognise recurrent pathogenic motifs, e.g. bacterial cell wall LPS or double-stranded viral RNA through pattern recognition receptors, the Toll-like receptors (TLR 1-9). This recognition prompts co-ordinated antigen processing and antigen presentation. DCs mature and up-regulate antigen-presenting major histocompatibility (MHC) class I and class II molecules, a number of co-stimulatory molecules, and, depending on context, secrete different combinations of cytokines. Activated DCs migrate to secondary lymphoid organs and present antigen

\textsuperscript{a} The so-called generator of diversity (GOD).
bound to MHC to rare circulating antigen specific T cells. Antigen bound to MHC interacts with the T cell receptor (TCR), and in the presence of secondary co-stimulatory signalling, e.g. CD28 (T cell) with B7-1/B7-2 (CD80/86) on APCs, leads to a rapid cascade of effects including T cell activation and proliferation and cytokine release. During this primary immune response, the antigen-specific T cell population increases around 1000 fold [29]. T lymphocytes can be further distinguished by phenotype and function: CD8+ T cells recognise epitopes of 8-11 amino acids bound to ubiquitous MHC class I and are cytotoxic, whilst CD4+ T cells recognise epitopes of around 9-22 amino acids bound to MHC class II molecules after processing by specialist APCs, and can differentiate on activation into a range of cells with helper (e.g. Th1, Th2) and suppressor functions (e.g. Th17).

Activated T cells act as a second cornerstone of immune response, and can mediate direct cytotoxicity, or indirect cytotoxicity through cell recruitment and cytokines such as IFN-γ, tumour necrosis factor (TNF), and interleukin 2 (IL-2). As Fig 1.1 illustrates, the response of T cells to antigen is governed by the nature and presence of secondary signalling. In the absence of such secondary signalling, antigen presentation leads to T cell anergy and death. In the presence of regulatory secondary signalling, e.g. the high affinity interaction of cytotoxic T lymphocyte-associated antigen, CTLA4 (T cell) with B7-1/B7-2 (APC), antigen presentation leads to cell cycle arrest and the development of immune tolerance.
Naive CD4\(^+\) cells can differentiate on activation into a diverse range of cells, including the archetypal effector T helper (Th) cells, the fate and function of which is dependent on the local cytokine milieu and nature of presented antigen [31]. Classically, Type I effector Th cells (Th1) produce high levels of IL-2, TNF-\(\alpha\), and IFN-\(\gamma\) which supports the differentiation of further Th1 cells and suppresses Th2 differentiation. Th1 cells have a role in cell-mediated immunity to intracellular pathogens and tumour cells. Th2 cells in contrast are characterised by production of IL-5, IL-13, and IL-4, and coordinate humoral immunity, and eosinophilic response [32]. A classification of the key CD4\(^+\) lymphocyte subsets is shown in Fig 1.2; recent work suggests that some lineages may remain plastic, with the ability to change phenotype and cytokine secretion under specific circumstances [31].
**Fig 1.2: Diversity of CD4+ Lymphocytes**

Natural regulatory T cells (Treg) develop in an antigen-independent manner. Naive CD4+ T cells (labelled naive) differentiate on activation in the presence of antigen into a range of subtypes, the most relevant of which are shown with the key cytokines which they secrete. Blue outlines pro-activation phenotypes; red outlines regulatory phenotypes. Some natural killer T cells express CD4+ but are omitted for clarity. Key: Th, effector T helper lymphocyte; Tr1, T regulatory type 1 cells; iTreg, inducible regulatory T cells; TGF-β, transforming growth factor β; IFN-γ, interferon-γ; TNF-α, tumour necrosis factor α; IL, interleukin. Adapted from Kennedy et al [32].

Following acute infection, regulatory secondary signalling has a role in controlling the immune response once the pathogen is eliminated. Less than 10% of the generated antigen-specific T cells remain in a stable circulating memory pool after a contraction or cell death phase. These long-lived cells can expand rapidly in a secondary or recall response on re-presentation of the cognate antigen.
1.3.3. Tumour associated antigens (TAA)

Functional models of cancer immunoediting suggest that cancer can be recognised as sufficiently foreign to raise an immune response: van Pel et al discovered that specific immunity to spontaneous tumours could be induced by vaccinating mice with stably mutated tumour cells, and demonstrated tumour specific cytolytic T cell responses on in vitro re-stimulation of splenocytes [33]. The confirmed role of T lymphocytes has driven the search for potential target tumour associated antigens [34]. In man, the existence of such antigens was confirmed in 1991 when van der Bruggen identified MAGE-1 from malignant melanoma [35]. Tumour associated antigens (TAA) have been identified via the recognition of autologous tumour-specific T-cells from patients, and the characterisation of target antigens, and by in vitro techniques which identify potential antigens, either as over-expressed proteins [36] or MHC-bound peptides [37], and then attempt to generate T cell responses to these in culture using T cells from normal donors (‘reverse’ immunology). A further powerful technique which has identified over a hundred antigens uses sera containing patient antibodies to screen proteins encoded by cDNA libraries from tumours (SEREX: serological analysis of recombinant cDNA expression libraries) [38].

Tumour associated antigens can be classified in a number of different ways, based on distribution, origin, or function. Some are tumour specific, such as BCR/ABL in chronic myeloid leukaemia (CML), where the antigen, a fusion protein, is created by the specific reciprocal DNA translocation (9;22) which causes the malignancy [39]. Others show increased expression in malignancy but are present in normal mature tissues, for
example, the testis differentiation antibodies\textsuperscript{b} such as MAGE-1 [35, 40], the transmembrane glycoprotein HER-2/neu\textsuperscript{c} which has intrinsic tyrosine kinase activity and is found at low levels in normal epithelial cells but over expressed in up to 40\% of breast cancer [41-42], or tyrosinase, a lineage-specific antibody expressed in malignant melanoma and in normal melanocytes [43]. Oncofoetal antigens, for example, 5T4, are expressed in development and in malignancy; this link suggests a role in the shared features of these biological processes, such as tissue migration [44]. Viral tumour associated antigens, e.g. HHV-8 in Kaposi’s sarcoma, are derived from viruses, promote immortalisation of the infected cell line, and drive carcinogenesis [45-46]. TAA are recognised by the immune system: spontaneous TAA directed T cells can be demonstrated in the peripheral blood of cancer patients, but crucially they are often anergic [47]. With, for example, peptide vaccination, or peptide-pulsed dendritic cells, T cell responses against TAA can be stimulated both in vitro and in vivo, but in the human clinical setting, such responses have generally been too weak and transient to eradicate tumours.

1.3.3.1. Oncofoetal antigen 5T4

The oncofoetal antigen 5T4 was discovered by looking for shared properties of human trophoblast and cancer cells, and has been shown to be a tumour associated antigen [48]. 5T4 has limited distribution in normal adult tissue, but is found in both the tumour and

\textsuperscript{b} A large group of TAA\textsubscript{s} present in cancer and testis have been identified. Shared expression may be due to hypomethylation, constitutive in the testis, and acquired in cancer where it is thought to have a role in the development of genomic instability, and the switching on of genes which are normally silent.

\textsuperscript{c} HER2/neu is the target of the monoclonal antibody trastuzumab (Herceptin), which is licensed for use in the treatment of metastatic and adjuvant breast cancer.
surrounding stroma of a number of human cancers, including gastric, colon, and ovarian [49-50]. Expression has been shown to correlate with poor prognosis in these tumour types, but its precise role in malignancy remains to be determined. 5T4 is present in 41-52% primary gastric tumours [49, 51] where its presence correlates with lymph node metastases, intestinal morphology and poor prognosis [49]. It is a heavily glycosylated 72 kDa transmembrane glycoprotein with a large extracellular domain containing two leucine rich repeat areas (LRR) and associated cysteine-containing flanking regions separated by a hydrophilic sequence. There is a transmembrane region and small cytoplasmic tail. Sequencing shows 81% homology between the protein in mouse and man, and complete conservation of the transmembrane region [52-53].

5T4 has been investigated as a therapeutic target in cancer using both antibody targeted superantigen and vaccination. Established models using human antigen inserted into a mouse melanoma cell line B16.h5T4 have been translated into early clinical trials in renal and colorectal cancer [54-55].

1.3.3.2. Gastric and oesophageal tumour associated antigens

Circulating TAA shed into patient serum can be used in the clinic to monitor disease recurrence and response to treatment in a number of solid tumours, for example, carbohydrate antigen 125 (CA-125) in ovarian cancer [56]. Two routinely monitored TAA are commonly expressed in gastric and oesophageal adenocarcinomas, though neither have a role in diagnosis, and both can be raised in other malignancies and a number of non-malignant conditions. They remain research tools and are not recommended for routine clinical monitoring in these diseases [57]. Carcinoembryonic antigen (CEA), has established diagnostic and prognostic significance in colorectal
cancer [58], and is also widely expressed in oesophageal and gastric adenocarcinomas, being found in up to 86% of biopsies [59] and in the serum of nearly half of patients with advanced disease. CEA was first identified by Gold et al [60]; it is a 180kDa glycoprotein with a role in cell adhesion. It has been the target of both vaccination [61-62] and engineered T cell therapeutic approaches [63-64].

Carbohydrate antigen 19-9 (CA19-9) is raised in the serum of around 35% of patients with operable gastric cancer, where it has been shown to provide additional prognostic information [65]. In pancreatic cancer, changes in CA19-9 in the early weeks following chemotherapy appear predictive of response, and overall survival [66], although some studies suggest that transient surges in both serum CEA and CA19-9 can be seen in early assessment of around one in five patients who go on to have a formal CT response to chemotherapy [67].

1.3.4. Cancer and the immune system: cancer vaccines

The growing recognition of the multiple and complex ways in which tumours evade the immune system suggest the fundamental requirement of such evasion for tumour establishment, proliferation and metastasis. Vaccination is the most established tool by which we can prime the adaptive immune response, and has changed the relationship between man and infection over the last 50 years, leading to the eradication of smallpox in 1979 [68] and the widespread control within the developed world of many serious diseases such as measles, pertussis, and polio. Despite intermittent public health scares, vaccination, either with attenuated organisms, or engineered component parts, remains a remarkably safe procedure.
Tumour associated antigens (TAA) are attractive targets for vaccine based therapies. A number of vaccines against solid tumours – most commonly melanoma – have been developed as far as the clinic, but few are yet licensed for routine use. The choice of target, vector, and schedule are all important. Ideal targets would be highly expressed on the tumour (associated with intact antigen processing mechanisms) but have only negligible expression in normal tissues. Ideal vectors aim to provoke the recognition of antigen and promote a strong and lasting adaptive immune response. Many approaches have been tried [69-70].

1.3.4.1. Modified whole cell vaccines

Modified whole cell vaccines present multiple targets for immune activation, and have a breadth of approach which may increase response but risks normal tissue side-effects. Both personalised approaches which require removal and modification of the patients’ own tumour cells before re-inoculation, and those based on combinations of appropriate cell lines have been tried, but the technology is ponderous and has quality control issues. Both approaches have entered the clinic. For example, 214 patients with stage III melanoma have been treated with an autologous irradiated whole cell vaccine conjugated to the hapten dinitrophenyl (DNP) and given with the adjunct bacille Calmette-Guérin (BCG) in Phase II trials; 47% developed delayed-type hypersensitivity (DTH) to unmodified autologous melanoma cells, which was present 2-9 months after vaccination, and had a significant doubling in 5 year overall survival compared to those who did not, from 29.3 to 59.3% [71]. SWOG investigated the use of an allogeneic vaccine derived from two melanoma cell lines and given with the adjunct DETOX (Melacine) in the adjuvant setting [72]. Results failed to show benefit in disease free survival, but the trial was only powered to detect a 50% increase in median disease free survival, and sub-
group analysis showed that any benefit was restricted to patients with certain HLA class I haplotypes [73].

1.3.4.2. Peptide vaccines and viral vectors

Specific tumour associated antigens can be targeted alone, or in combination, by peptide vaccination, or by the presentation of the antigen within a viral vector. These techniques are acellular, and potentially more suitable for large scale use. Peptide vaccination is HLA class specific, and the vaccines have very short half-life. Many have been taken into the clinic, but clinically relevant responses are the exception. For example, a HER-2/neu E75 peptide vaccine stimulates specific immunity, defined by E75 CD8+ T cells, in clinically disease-free breast cancer patients, but immunity wanes with time, requiring booster inoculations, and the clinical benefit is as yet unreported [74].

Viral vectors include modified pox and adenoviruses. Modified vaccinia virus Ankara (MVA), is a recombinant attenuated pox virus which can break immune tolerance to TAAs and other targets, producing antibody and T cell responses, in mice [75] and man [55]. MVA was originally developed as a replication defective smallpox vaccine, and over a hundred thousand doses were given in West Germany in the 1970s without significant safety concerns [76]. Pre-exposure to the relevant virus can be a problem in the clinical setting, but there are some interesting results. For example, a phase I study of a recombinant vaccinia vaccine (rV-PSA) in prostate cancer patients with biochemical progression defined as rising prostate specific antigen (PSA) or metastatic disease showed that 13 of 33 patients had stable PSA and no evidence of progression at 6 months, 9 at 11 months, and 6 at 25 months[77].
1.3.4.3. Modified dendritic cell vaccines

Ultimately, dendritic cells are involved in the immune response to all vaccines. There has been considerable interest in pre-processing target antigens in modified dendritic cells, and using these as the vaccine vector. Sipuleucel-T is an active cellular immunotherapy consisting of autologous peripheral blood mononuclear cells (PBMC) including autologous antigen presenting cells activated ex-vivo with a recombinant fusion protein PA2024, which consists of prostatic acid phosphatase fused to granulocyte-macrophage colony-stimulating factor (GM-CSF). Two randomised, placebo controlled phase III trials in patients with hormone-refractory metastatic prostate cancer failed to show a significant improvement in time to progression but did show a significant improvement in overall survival, from 21.7 months in the control arm to 25.8 months in the vaccine arm, reflected by a 22% reduction in risk of death after active treatment (HR 0.78; P=0.03) [78-79]. The effect of sipuleucel-T on survival was observed consistently across sub-groups of patients, including those known to predict for poor outcomes, and persisted after adjustment for later treatment with docetaxel chemotherapy; these results have led to sipuleucel-T being licensed for use in the USA. Schlom et al [80] argue that vaccination is a dynamic process which alters response to subsequent treatment, and that patients can benefit even when vaccination alone is apparently unsuccessful by our current standard assessments.

1.3.4.4. Vaccination: practical considerations

Vaccination scheduling is complex, and alters with target and vector. Boosting with multiple vaccinations is usual, but can shift the dominant response from the target antigen towards the vector, or tip the response from augmentation towards tolerance. All the hindrances to immune recognition and activation in response to tumour, which will
be discussed in the next section, also apply in the context of vaccination. Heterologous prime boost schedules, in which different vectors are used to present the same antigen, can overcome some of these difficulties. For example, phase II randomised investigation of combinations of fowlpox (f) and vaccinia (v) vaccines targeting PSA in men with advanced prostate cancer showed a trend for improved progression-free survival in those treated with v-PSA prime followed by three f-PSA inoculations [81].

Several studies have reported correlations between antigen-specific immunity and clinical end-points, but many have not. A number of confounding factors must be considered in interpreting these results:

1. reported measures of antigen-specific immunity, both antibody levels and specific T lymphocyte are often investigated for convenience in the peripheral blood rather than at the tumour site;

2. few studies have investigated the presence and/or activity of regulatory T cells;

3. the subtype and avidity of antigen-specific T cells may be more important than their numbers;

4. the responses generated to the chosen specific antigen alone may not account for clinical benefit. The phenomenon of determinant spreading or “antigen cascade” has been observed with in patients with durable responses to single epitope vaccination approaches [82-83]. It is thought that it results from immune infiltration of the tumour by cytolytic vaccine-induced T cells, and subsequent cross-priming of higher avidity T cells to other TAA.

In the pre-clinical stage the choice of tumour model is also critical, and can affect the efficacy of vaccination. Some tumours are inherently more immunogenic than others,
and engineering to express xenogeneic antigens can greatly enhance potential response. Whilst allowing proof of principle experiments, such models fail to address the hindrances to immune recognition and activation which dominate the clinical picture in man.

1.3.5. Cancer and the immune system: hindrances to effective activation

Despite the growing evidence for immunoediting, and the identification of a multitude of tumour associated antigens which can be recognised by the patient’s immune system, once cancer is established, immune-driven cure is rare. Even with significant research investment into strategies such as vaccination or adoptive T cell transfer, effective tumour-directed immune activation remains the exception. A growing number of mechanisms are recognised which stand in the way of immune cure in established cancer.

Polly Matzinger proposed an alternative concept of the primary function of the immune system: not the classical separation of self and foreign, but rather the recognition of danger, i.e. actual or potential tissue damage, prompting the generation of an effective response [84-85]. This model is coherent with current understanding of tumour immunology: demonstrably, the immune system does recognise tumour, but fails to respond effectively. This failure to respond is complex and multi-factorial but can be considered in terms of (1) mechanisms of tumour evasion, (2) control of immune activation, and (3) acquired immune deficiencies, although these distinctions are artificial.
1.3.5.1. Mechanisms of immune evasion

The theory of immunoediting suggests that the immune system may exert selection pressure on a genetically unstable cancer; cancer cells thus selectively evade the immune system through the loss or reduction of necessary components of the antigen processing and presenting machinery which would allow immune activation. In practice, many tumour cells show down-regulation or loss of MHC molecules, without which antigen is not presented in a recognisable format to enable T cell activation [86-87]. Tumour cells also show loss of other components of the antigen processing equipment, e.g. antigen peptide transporter 1 (TAP1) in colorectal cancer [88], or reduced expression of co-stimulatory molecules, e.g. ligands of B7, so that even where antigen is presented it is unaccompanied by the necessary activation signals, and results in T cell anergy. Reduced MHC expression, a feature of both tumour and viral infection, should be detected by NK cells which can then act to lyse altered cells directly, but the mechanisms by which viruses and (potentially) tumour in turn evade NK cells are complex [89].

Tumour cells have also developed mechanisms by which they damage functional components of the immune system. For example, increased Fas ligand (FasL) expression on tumour cells results in T cell apoptosis on interaction [90].

1.3.5.2. Control of immune activation

Failure to respond to tumour as threat may be due in part to the nature and context of many TAA. Specificity remains an issue; the majority of TAA s are present in normal tissue at some stage of development, or in other disease states. Previous exposure to TAA in a benign context, for example, in foetal development, may result in T cell anergy and the development of tolerance. Unlike acute infection the majority of solid
malignancies in man are heterogeneous, and have probably existed for many years in a sub-clinical form. Chronic interaction of TAA and the immune system during tumour development, and the absence of danger signals due to tissue damage or foreign e.g. viral antigens, may tip the immune response towards tolerance rather than activation. Antigen tolerance is in part driven through the normal control mechanisms which exist to limit immune activation, and prevent unconstrained activation which would result in tissue damage and autoimmunity. Some of these mechanisms are developmental, e.g. thymic clonal deletion of self-reactive T and B cells [91], but most are triggered by immune activation. Tumour may interact with immune control mechanisms preventing effective activation. For example, tumour cells may secrete regulatory cytokines: the induction of transforming growth factor (TGF-β1) expression in an otherwise highly immunogenic murine tumour model suppresses immune response and leads to unopposed growth of the tumour [92]. In vivo, activated monocytes and macrophages are also a potent source of TGF-β1, which can in turn inhibit TNF-α, reduce dendritic cell migration and maturation, reduce class II MHC expression and result in tolerance to presented antigen [93].

Cells which control immune activation are also implicated in immune tolerance of cancer. For example, natural regulatory T cells (Treg) have been shown to be raised in both the tumour microenvironment and peripheral circulation in many human tumours, for example, breast, pancreatic [94], gastro-intestinal [95], and ovarian [96] carcinomas, and melanoma [97]. Curiel et al demonstrate that Treg inhibit specific TAA immunity in vitro and in vivo, allowing tumour growth. Consistent with these results, Treg levels are inversely prognostic in clinical practice. The demonstration of Treg migration towards chemokine CCL22, which is produced both by tumour cells and infiltrating macrophages, and found in ovarian tumour samples and malignant ascites, suggests that
tumour cells may have an active role in recruitment of Treg, which enable immune evasion [96].

Immature myeloid cells, the pre-‐cursors of granulocytes, monocyte/macrophages, and dendritic cells present self antigens to lymphocytes resulting in immune tolerance. This plastic group of cells, termed myeloid derived suppressor cells (MDSC), expand rapidly after tumour inoculation in animal models [98] where they have a role in suppressing antigen-‐specific T cell responses, potentially leading to vaccine failure [99]. In mice these cells are characterised as Gr-1⁺CD11b⁺ [100], but in man identification is hampered by the absence of a Gr-1 analogue, and these cells appear more heterogeneous. Distinct subtypes have been identified in cancer patients by a number of investigators using a combination of phenotypic and functional features. Patients with stage IV metastatic melanoma, for example, all showed significant expansion of a CD14⁺HLA-DR⁻/low monocyte subtype which were undetectable in healthy controls. In vitro work confirmed these cells suppress T cell responses via TGF-β secretion, an effect enhanced by subsequent vaccination with autologous tumour-derived heat shock protein peptide complex gp96 and low dose GM-CSF, which may have contributed to the lower frequency of immunogenicity observed compared to a previous study where vaccine was administered alone [101].

1.3.5.3. Acquired immune deficiencies

Advanced cancer leads to immune anergy and immunodeficiency through a number of systemic mechanisms, e.g. cancer cachexia and malnutrition, which in turn may be mediated by cytokine release [102], bone marrow infiltration, relative and absolute lymphopenia, functional deficiencies, and T cell apoptosis [103-104].
Two elements of the normal immune control mechanisms which prevent the activation of an effective immune response to tumour even in an apparently immuno-competent host are of particular interest in this work. Natural regulatory T cells (Treg) and the co-stimulatory molecule cytotoxic T lymphocyte-associated antigen 4 (CTLA4) which these cells constitutively express are both potential targets for future immunotherapy.

1.4: Natural Regulatory T cells (Treg)

Sakaguchi et al characterised a population of naturally occurring regulatory T cells (Treg), which are functionally distinct [105-106]. Depleting CD4+ splenic suspensions of CD5^{high} T lymphocytes, and transferring the CD5^{low} fraction to T cell deficient athymic Nude or severe combined immunodeficiency (SCID) mice resulted in the development of multiple organ autoimmune disease affecting the stomach, thyroid, ovaries, or testes. In contrast, co-transfer of undepleted CD4+ cells protected against subsequent autoimmune disease [105].

Refinements of these experiments identified CD25, the IL-2 receptor α-chain as a more useful defining marker for the relevant population of T cells: depletion of the CD4^{+}CD25^{+} fraction from splenic suspensions before transfer to athymic nude mice resulted in greater frequency, range, and severity of autoimmune disease [107]. The parallel recognition of an X-linked syndrome combining immunodeficiency and autoimmunity in mice (scurfy) and man (IPEX: immune dysregulation, polyendocrinopathy and X-linked syndrome) resulted in the identification of the ortholog defective genes, Foxp3 [108] and FOXP3 [109-110] respectively, which encode forkhead/winged-helix transcription factors. The transcription factor forkhead box P3 (Foxp3) is
the most specific marker of Treg identified to date and has a role in the development and function of these cells. Transduction of Foxp3 into CD25CD4+ cells using a retroviral vector converts them into Treg which express CD25, CTLA4, CD103, and GITR (glucocorticoid-induced TNF receptor related gene) and can suppress T cell proliferation in vitro and the development of autoimmune pathology in vivo [111].

That Treg suppress the activation and proliferation of other T cells is now accepted; precisely how they do this is less clear. There is some evidence for cytokine mediated activity in mouse models exploring the role of IL-10 [112] and TGF-β1 [113], but in vitro work suggests the dominant mode of action is through cell-to-cell T cell interactions [114]. Reductive transwell assays demonstrated that APC are not necessary for these direct cell interactions [115]. Treg also appear to be able to instigate “inside out” signalling to APC: they have been reported to down-regulate B7-1 and B7-2 expression on DC [116].

Alternative Treg, with differing phenotypes have been also been identified. Small populations of CD25 negative functional Treg occur naturally, and various “adaptive” inducible Treg can be created in experimental models in response to e.g. cytokines and TCR stimulation [117].

1.5:  CTLA4

1.5.1. CTLA4: expression and role

Cytotoxic T lymphocyte-associated antigen 4 (CTLA4) is a transmembrane protein which is constitutively expressed on the cell surface of CD4+CD25+ regulatory T cells,
and inducibly expressed by activated T cells and monocytes. CTLA4 is related genetically, structurally, and functionally to the co-stimulatory molecule CD28 which is also expressed by T cells. The relevant genes are closely related on chromosome 2q33 and the two proteins share a basic structure which includes a variable immunoglobulin-like (IgV) extracellular domain, a transmembrane component, and a short intracytoplasmic region with signalling motifs which allow multiple independent signals. Both proteins have an unpaired cysteine which allows dimerisation at the T cell surface, but immune-precipitation experiments suggest CTLA4 is mostly expressed in activated murine T cells as various monovalent forms with different levels of glycosylation [118]. Both CD28 and CTLA4 interact with the B7-1/B7-2 (CD80/86) receptors on antigen presenting cells (APC) as part of secondary signalling during antigen presentation, but to contrary, and balancing, effect [119].

CD28 has a number of apparently discrete T cell activating functions which are stimulated by interaction with B7-1/B7-2 on APC in the presence of T cell receptor (TCR) stimulation. CD28 has a role in upregulation of glucose metabolism, increased cytokine and chemokine release, T cell longevity, and sustained T cell expansion [120]. Unlike CTLA4, CD28 is constitutively expressed on most CD4$^{+}$ T cells, and around half of CD8$^{+}$ T cells.

Following T cell activation, CTLA4 is upregulated, moving rapidly from the cytoplasm to the site of APC and T cell interaction [121]. A higher affinity for their shared receptors puts a brake on CD28 mediated T cell stimulation, and instigates potent inhibition of T cells, leading to reduced TCR signalling, reduced IL-2 gene transcription and reduced T cell proliferation.
CTLA4 is a crucial inhibitor of T cell activation; knock-out mice develop fatal lymphoproliferation and autoimmunity including severe myocarditis and pancreatitis and die by 3-4 weeks of age [122]. Mice expressing only the extracellular domain of CTLA4 develop lymphadenopathy which does not progress to fatal lymphoproliferation, indicating that both ligand competition and intracellular signalling have a role in CTLA4 function [123].

The dominant inhibitory effect of CTLA4 may be via Treg, on which it is constitutively active, or via its direct cell-autonomous effects on activated T cells on which it is transiently upregulated. Like CD28 it may have discrete functions in these two populations of cells. CD28 ligation, for example, induces IL-2 secretion in conventional T cells, but not in Treg. There is evidence for inhibitory CTLA4 mechanisms by both routes. For example, in a SCID mouse model of autoimmune colitis, protective Treg function has been shown in vivo to be dependent on both signalling via CTLA4 and secretion of TGF-β [124]. In man, blockade of constitutive CTLA4 expression in CD4⁺CD25⁺ lymphocytes using a monoclonal antibody failed to abrogate suppressive activity of these cells [125].

Potential mechanisms of CTLA4 inhibitory effects include:

1) Via upregulation of TGF-β, and hence FoxP3 expression [126]. In murine models, TGF-β induced FoxP3 gene expression and transition of cells to a regulatory T cell phenotype with immunosuppressive potential to prevent asthma in a house-dust mite induced mouse model, and to inhibit antigen specific CD4⁺ T cell expansion in response to immunisation.
(2) Via B-7 dependent signalling altering tryptophan catabolism in dendritic cells (DC). In an allogeneic islet transplant model, binding of a soluble fusion protein of CTLA4 and immunoglobulin (CTLA4Ig) to B7 led to activation of the immunomodulatory enzyme indoleamine 2, 3-dioxygenase (IDO). This catalyses the initial and rate-limiting step in tryptophan catabolism; local deficiency in the cell microenvironment suppresses T cell activation [127]. Membrane-bound CTLA4 can also act via this mechanism, but indirect activation of IDO via IFN-γ secretion appeared more potent in Treg [128].

(3) Via regulation of Th1 versus Th2 response. CD4+ lymphocytes of CTLA4 knockout mice show a dominant Th2 response with high levels of IL-4 and IL-5 secretion [129].

1.5.2. CTLA4 as a viable target: pre-clinical

Pre-clinical studies demonstrated the ability of anti-CTLA4 monoclonal antibodies to unmask or promote endogenous immune responses to immunogenic tumour models. Alison’s group investigated the anti-CTLA4 antibody 9H10 in syngeneic modified murine colon carcinoma cell line 51BLim10 and demonstrated accelerated rejection of a B7 transfected cell line, rejection or reduced growth of a vector cell line, and regression of established tumours. Rejection of repeat tumour challenge suggests the establishment of immunologic memory [130]. Supporting results have been achieved in immunogenic model systems, such as syngeneic CSA1M fibrosarcoma, and OV-HM ovarian carcinoma using alternative antibody 4F10 [131]. Perhaps surprisingly, early treatment with anti-CTLA4 antibody 9H10 one week after tumour challenge results in 42% of animals rejecting syngeneic prostate cancer pTC1 which express little or no MHC I [132].
In many model systems, including B16, anti-CTLA4 blockade alone is of limited efficacy [133-136]. Hurwitz et al, for example, demonstrated that poorly immunogenic SM1 mammary carcinoma is resistant to anti-CTLA4 blockade even when combined with a B7 expressing SM1 vaccine. In contrast, anti-CTLA4 blockade combined with a vaccine of irradiated granulocyte-macrophage colony-stimulating factor (GM-CSF) expressing SM1 cells led to tumour regression in vivo via a mechanism which required both CD8⁺ and CD4⁺ T cells [137]. Similar results were achieved using the same approach in a B16 model, where tumour rejection was dependent on CD8⁺ and NK1.1⁺ but independent of CD4⁺ lymphocytes [138]. Despite limited single agent benefit, investigators have used anti-CTLA4 blockade to enhance the efficacy of a variety of immunotherapeutic approaches including GM-CSF-producing whole cell vaccines, a small molecule inhibitor (T22 peptide antagonist of CXCR4) [139], peptide vaccine [135], poxvirus vaccine [140] and xenogeneic DNA vaccine [134]. Immune-mediated efficacy has also been shown in combination with local radiotherapy in a model of metastatic breast cancer. Only animals treated with both local radiotherapy and anti-CTLA4 blockade showed improved survival and reduced number of lung metastases; the mechanism was again shown to be CD8⁺ dependent but CD4⁺ independent [141].

Two anti-CTLA4 antibodies have been used extensively in vitro and shown efficacy in vivo in murine models: 9H10 [142] and UC10-4F10 [118]. Both anti-CTLA4 antibodies used in murine models are hamster anti-mouse antibodies which may be immunogenic in mice. Toxicity in mouse models seems largely antigen or organ appropriate, e.g. vitiligo in combination with melanoma targeted vaccine [138], or prostatitis after combination therapy in the primary prostate tumours in transgenic (TRAMP) mice model [133]. These toxicities suggest that the anti-tumour response is, in part, directed to organ-specific normal tissue antigens. More detailed toxicity assessment of the parallel fully
human monoclonal antibodies in clinical development has been conducted in primates. Tremelimumab was administered to cynomolgus monkeys in escalating single dose and weekly schedules for one and six months; main toxicities seen with longer administration were skin rash, loose stool, reduced appetite, weight loss, and abnormalities of thyroid function. Dose-dependent lymphoid hyperplasia and mononuclear cell infiltration of organs were also found [143].

1.5.3. CTLA4 as a viable target: clinical experience

Two fully human anti-CTLA4 monoclonal antibodies have entered human trials:

(1) Ipilimumab (formerly MDX-010; developed by Medarex Inc, Bloomsberg, NJ and Bristol-Myers Squibb, Princeton, NJ).

(2) Tremelimumab (formerly CP-675,206, briefly ticilimumab; developed by Pfizer Pharmaceuticals Inc, New York).

1.5.3.1. Anti-CTLA4 antibodies: Isotype, dose, schedule

Ipilimumab is an IgG1κ antibody; the isotype mediates Fc domain functions such as antibody-dependent cellular cytotoxicity (ADCC) [144]. After initial phase I dose-defining studies, summarised in Table 1.1 the experimental dose was generally 3mg/kg by 90 min intravenous infusion every 3 weeks (Q3W) for four cycles (induction) with or without a maintenance dose, although one study investigated dose escalation from 3mg/kg or 5mg/kg up to maximum 9mg/kg in 46 patients with metastatic melanoma. Dose escalation resulted in increased toxicity, with 16 patients (35%) experiencing grade (G) 3/4 toxicity including diarrhoea (6) and pituitary hypophysitis (8), without any clear evidence of increase in objective response rates: 5 patients (11%) had partial responses
Initial studies of the drug included single fixed dose pilot protocols in hormone resistant prostate cancer [146] and previously vaccinated melanoma patients [147] rather than traditional dose-escalation studies; a later phase I/II study compared pharmacokinetics of ipilimumab derived from a more productive Chinese hamster ovary (CHO) cell line generated via plasmid transfection with ipilimumab derived from the original murine myeloma fusion hybridoma [148]. This study shows equivalent pharmacokinetics for 2.8mg/kg transfectoma and 3mg/kg hybridoma derived ipilimumab in a multiple dose cohort and then goes on to characterise pharmacokinetics, clinical responses and toxicity for various doses of the transfectoma derived drug in single and multiple dose schedules to support future clinical development of this version. The mean terminal phase half-life was 359 hours (15 days) after multiple doses of 10mg/kg ipilimumab.

Parallel investigation randomised pre-treated melanoma patients to 0.3, 3, or 10mg/kg ipilimumab Q3W followed by maintenance therapy every three months [149]. Pharmacokinetic modelling based on patient sample results showed that 0%, 30% and 95% of patients at escalating doses achieved target plasma trough antibody concentration $C_{\text{MIN}}$ of 20µg/mL which resulted in near complete receptor saturation in vitro. Unlike the earlier dose-escalation study this randomised double-blind study did demonstrate improved objective response rate (0%, 4.2%, and 11.1%) and a trend towards improved survival (median overall survival 8.6, 8.7 and 11.4 months) with increasing dose. Although higher rates of G3/4 drug-related toxicity were also seen with escalating dose (8%, 18%, and 27%) clinical experience has made management of toxicity safer; thus both 3mg/kg and 10mg/kg doses have been taken forward to phase III studies in melanoma.
Tremelimumab was developed as an IgG2 antibody to minimise complement activation, reduce the chance of antibody-dependent cell-mediated cytotoxicity directed against target lymphocytes, and limit the risk of cytokine release syndrome; the IgG2 isotype tends to act directly via antigen binding. Tremelimumab is highly selective for CTLA4, demonstrating over 500 fold greater selectivity than for CD28. In vitro studies in human blood confirmed the absence of non-specific cytokine release, and in competition binding assays tremelimumab blocked CTLA4 binding to B7-1 and B7-2 with average 50% inhibition concentrations of 0.65 and 0.50 nM respectively [143]. A plasma concentration of ≥ 30µg/mL was shown to be the minimum in vitro concentration which produced a significant increase in T cell activation.

Tremelimumab is thought to be cleared through the reticulo-endothelial system with kinetics similar to natural IgG2 antibodies; in a phase I single-dose escalation study in adults with solid malignancies (34/39 melanoma) terminal phase half-life was long at 22.1 days [150]. In this study, the majority of patients with clinical benefit (a composite of objective responses and lengthy disease stabilisation) achieved a plasma concentration greater than the target 30µg/mL four weeks after single treatment dose, and single doses up to 15mg/kg were administered safely resulting in breaking of immune tolerance to established melanomas and normal self-tissues. The protocol defined 10mg/kg as the maximum tolerated dose, but as most toxicity proved reversible, and 2 of the 5 patients treated with 15mg/kg with measurable disease had durable objective responses of greater than two years this dose was also investigated in subsequent studies. A combination phase I/II study showed similar pharmacokinetics after monthly dosing, with a mean terminal half life of 19.6 days, and a mean 26% increase in exposure by the fourth interval dosing [151]. In later sequential phase II cohorts, which do not allow formal comparison, objective responses occurred in equal numbers (8%) with 10mg/kg every
month (Q1M) and 15mg/kg every three months (Q3M), but the frequency of grade 3/4 adverse events appeared reduced from 27% to 13% in the less frequent schedule. Both dosing schedules have been investigated in further studies.

In addition to the isotype and pharmacokinetic differences between the two fully human anti-CTLA4 antibodies discussed, there will also be differences in affinity and epitope, which may result in variant downstream effects, and mean that laboratory data may differ between the two.

1.5.3.2. Anti-CTLA4 antibodies: Clinical responses.

The clinical experience with monoclonal anti-CTLA4 antibodies is summarised in Tables 1.1 and 1.2. The majority of trials have been in melanoma patients, but there has also been some investigation of efficacy in patients with advanced renal, ovarian, colorectal, and prostate carcinoma, and relapsed B cell lymphoma, and a recent study has investigated immunological end-points following neo-adjuvant administration of ipilimumab in patients subsequently treated with surgery for localised urothelial bladder cancer [152]. Durable objective response rates of between 5-20% have been achieved with anti-CTLA4 monotherapy in melanoma. A number of trials have explored the efficacy of anti-CTLA4 blockade in combination with vaccines (peptide or whole cell), established immunotherapeutics such as interleukin 2 (IL-2) [153], chemotherapy such as dacarbazine [154] or gemcitabine [155], and the aromatase inhibitor exemestane [156].

The data from animal models provide the rationale for anticipated synergy with these alternative therapeutic approaches, but whilst most have been tolerable, there are few indications in reported trials of dramatic benefit. Combination therapy with anti-CTLA4 blockade and IL-2 does not appear to increase expected objective response rates in
melanoma [153] whilst combination with exemestane in 26 patients with hormone-receptor positive advanced breast cancer did not result in a single objective response [156]; assessing any additive benefit in these small studies is complicated by the heterogeneity of approaches, and by the heavy pre-treatment of many of the patients.

A recently published multi-centre phase II study of ipilimumab (3mg/kg Q3W*4) ± dacarbazine (250mg/m²/day day1-5 Q3W*6) in 72 chemotherapy-naive patients with unresectable melanoma showed a trend towards increased objective response rate (14.3% vs. 5.4%), disease control rate (stable disease + PR +CR; 37% vs. 22%), and median overall survival (14.3 vs. 11.4 months) with combination therapy compared to anti-CTLA4 blockade alone without an unmanageable increase in toxicity [154]. Unfortunately the result is confounded as 13 patients cross over to the combination arm, and the study is underpowered to confirm the benefit in response and survival which is apparently seen.
Table 1.1: Clinical Experience of Anti-CTLA4 Antibodies: Ipilimumab

<table>
<thead>
<tr>
<th>Reference</th>
<th>Drug</th>
<th>Dose</th>
<th>Patients</th>
<th>Response</th>
<th>Duration (months)</th>
<th>Side effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Davis [157]</td>
<td>ipilimumb</td>
<td>Phase I 3mg/kg single dose</td>
<td>14 Hormone refractory prostate cancer (HRPC)</td>
<td>2/7 chemotherapy naïve had PSA response</td>
<td>3-5</td>
<td>4 rash (1 G3)</td>
</tr>
<tr>
<td>Tchekmedyian</td>
<td>ipilimumb</td>
<td>Phase I 3mg/kg single dose</td>
<td>17 Unresectable melanoma</td>
<td>2/17 PR</td>
<td></td>
<td>7/17 rash</td>
</tr>
<tr>
<td>Hodi [147]</td>
<td>ipilimumb</td>
<td>Phase I 3mg/kg single dose</td>
<td>9 Previous vaccine: 3 melanoma 2 ovarian (GMCSF secreting tumour) 4 melanoma (pep/DC)</td>
<td>3 necrosis 2 stable CA125 0</td>
<td></td>
<td>G3 transient ↑ LFT. G1 rash 3/3 melanoma. 1 tumour necrosis with haemorrhage</td>
</tr>
<tr>
<td>Weber [148]</td>
<td>ipilimumb</td>
<td>Phase I/II transfecotma derived A-S: 7.5, 10, 13, 20mg/kg A-MD: 2.8, 5mg/kg Q3W<em>4; vs. 3mg/kg hybridoma B:10mg/kg Q3W</em>4</td>
<td>88 Melanoma III/IV A-S: 30 A-MD: 34 B: 24</td>
<td>3PR, 1CR (4.6%) 14 SD</td>
<td>8, 21+, 23+, 24+ median duration 6.5M; range 3.3-31.5+</td>
<td>Common: Fatigue G1/2 41% Rash G1/2 40% Diarrhoea (all) 39% G3/4 19%: colitis (4), diarrhoea (6; inc. 1 perforation)</td>
</tr>
</tbody>
</table>

4 A-S: Group A, single dose protocol.
<table>
<thead>
<tr>
<th>Reference</th>
<th>Drug</th>
<th>Dose</th>
<th>Patients</th>
<th>Response</th>
<th>Duration (months)</th>
<th>Side effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maker [145]</td>
<td>ipilimumab</td>
<td>3mg/kg or 5mg/kg Q3W. Escalation to 9mg/kg did not increase ORR</td>
<td>46</td>
<td>5 PR</td>
<td>16+, 4, 13+, 9, 7.</td>
<td>16 G3/4: 6 diarrhoea, 8 hypophysitis, 1 nephritis, hepatitis, arthritis, dermatitis, anterior uveitis.</td>
</tr>
<tr>
<td>Wolchok [149]</td>
<td>ipilimumab</td>
<td>Phase II randomised: A: 0.3mg/kg vs. B: 3mg/kg vs. C: 10mg/kg Induction: Q3W *4 Maintenance: Q12W</td>
<td>217</td>
<td>5 PR</td>
<td>OS: med; 24M A: 8.6M; 18% B: 8.7M; 24% C: 11.4M; 30%</td>
<td>IRAE: all grades A: 19/72 (0% G3/4) B: 46/71 (7% G3/4) C: 50/71 (25% G3/4) Commonest G3/4 GI</td>
</tr>
<tr>
<td>Phan [159]</td>
<td>ipilimumab + HLA-A*0201 restricted peptide vaccine</td>
<td>3mg/kg Q3W 1mg gp100:210M sc 1mg gp100:288V sc</td>
<td>14</td>
<td>2 CR</td>
<td>11+ &amp; 12+ 15+ (update 29-32+)</td>
<td>6 G3/4: 3 dermatitis, 2 colitis, 1 hepatitis, 1 hypophysitis</td>
</tr>
<tr>
<td>Attia [160]</td>
<td>ipilimumab + HLA-A*0201 restricted peptide vaccine</td>
<td>3mg/kg Q3W or 3mg/kg loading dose then 1mg/kg Q3W 1mg gp100:210M sc 1mg gp100:288V sc 30+</td>
<td>56</td>
<td>2 CR</td>
<td>30+, 31+ 4, 6, 25+, 26+, 34+</td>
<td>14 G3/4: 8 colitis, 4 dermatitis, 1 each uveitis, hepatitis, hypophysitis. Less at 1mg/kg (19% vs. 31%, n.s.)</td>
</tr>
<tr>
<td>Reference</td>
<td>Drug</td>
<td>Dose</td>
<td>Patients</td>
<td>Response</td>
<td>Duration (months)</td>
<td>Side effects</td>
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<tr>
<td>Sanderson [161]</td>
<td>ipilimumab + multipeptide vaccine</td>
<td>Phase I I: 0.3, 1, 3mg/kg Q4W*6 then Q12W *2 + tyrosinase 370D sc; MART-1 27L sc; gp100 210M sc + Montanide ISA-51</td>
<td>19 HLA-A*0201 Melanoma resected III/IV</td>
<td>12/19 relapsed: 3/8 with IRAE 9/11 without</td>
<td>Median time to relapse 18.3 months</td>
<td>1 uveitis (G2; 1mg/kg) 5/5 diarrhoea at 3mg/kg (2 G2; 3 G3)</td>
</tr>
<tr>
<td>Maker [153]</td>
<td>ipilimumab + IL2</td>
<td>Phase I/II escalation, 0.1, 0.3, 1, 2, and 3mg/kg Q3W<em>3. II.2 720,000IU/kg Q8H</em>15 (cycle 2&amp;3)</td>
<td>36 Stage IV melanoma 24 given 3mg/kg</td>
<td>3CR 5PR (22%)</td>
<td>6 ongoing (11-19+)</td>
<td>5 G3/4: 4 enterocolitis 1 arthritis &amp; uveitis.</td>
</tr>
<tr>
<td>Hersh [154]  Fischkoff [162]</td>
<td>ipilimumab +/- dacarbazine (DTIC)</td>
<td>Phase II randomised I: 3mg/kg Q4W<em>4 DTIC:250mg/m² d1-5 Q3W</em>6</td>
<td>Vaccine/chemotherapy naïve unresectable metastatic melanoma: A: (alone) 37 B: (+DTIC) 35</td>
<td>A: 2PR (5.4%) B: 2CR, 3PR (14.3%)</td>
<td>A: 19+, 22+ B: (CR) 20+, 21+</td>
<td>A: G3 12.8% B: G3 22.9%</td>
</tr>
<tr>
<td>Weber [163]</td>
<td>ipilimumab +/- prophylactic budesonide</td>
<td>Randomised blind phase II I: 10mg/kg Q3W*4 +B: QD 9mg to week 12; 6mg to week 14; 3mg to week 16</td>
<td>115 III/IV melanoma (mix naïve/ previous chemotherapy)</td>
<td>Best ORR I : 15.8% B : 12.1%</td>
<td></td>
<td>IRAE: I: 84% all (39% G3/4) B: 81% all (41% G3/4) No benefit with budesonide</td>
</tr>
<tr>
<td>Reference</td>
<td>Drug</td>
<td>Dose</td>
<td>Patients</td>
<td>Response</td>
<td>Duration (months)</td>
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<tr>
<td>Yang [164-165]</td>
<td>ipilimumab</td>
<td>Phase II (Q3W) A: 1st 3mg/kg then 1mg/kg B: 3mg/kg repeated</td>
<td>Advanced renal: A: 21 (all failed IL2) B: 40 (broader entry)</td>
<td>PR</td>
<td>7, 8, 12, 17, 18, 21.</td>
<td>A: 3 G3 enteritis B: 14 ≥ G3 enteritis inc. 3 colonic perforation 2 hypophysitis 1 meningitis</td>
</tr>
<tr>
<td>Ansell [166]</td>
<td>ipilimumab</td>
<td>Phase I A: 3mg/kg then 1mg/kg Q4W<em>3 B:3mg/kg Q4w</em>4</td>
<td>Relapsed/refractory B cell lymphoma: A: 12 B: 6</td>
<td>1 CR 1 PR</td>
<td>31+ 19</td>
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</tbody>
</table>

**Table 1.1: Clinical Experience of Anti-CTLA4 Antibodies: Ipilimumab**

Table summarises reported phase I and II trials of ipilimumab as single agent or in combination therapy.

Abbreviations: MD multiple dose; M month; W week; D day; H hour; Q3W every 3 weeks; DC dendritic cell (vaccine); GMCSF granulocyte-monocyte colony stimulating factor; pep peptide; CA125 carbohydrate antigen 125; PSA prostate specific antigen; HRPC hormone refractory prostate cancer; ORR objective response rate; CR complete response; PR partial response; MR mixed response; SD stable disease; OS overall survival; G1-4 CTC toxicity grades; GI gastro-intestinal; inc. including; IRAE immune-related adverse events; LFT liver function tests; n.s. not significant.
Table 1.2: Clinical Experience of Anti-CTLA4 Antibodies: Tremelimumab

<table>
<thead>
<tr>
<th>Reference</th>
<th>Drug</th>
<th>Dose</th>
<th>Patients</th>
<th>Response</th>
<th>Duration (months)</th>
<th>Adverse events</th>
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<tbody>
<tr>
<td>Ribas [150]</td>
<td>tremelimumab</td>
<td>Phase I single dose: 0.01-15mg/kg</td>
<td>39 (29 measurable) Melanoma (34), renal cell (4), colon (1); metastatic or high risk of recurrence.</td>
<td>2 CR</td>
<td>25+ &amp; 35+</td>
<td>DLT (G3): 1 rash, 3 diarrhoea Also 1 each thyroiditis, panhypopituitarism, late hypothyroidism.</td>
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<tr>
<td></td>
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<td>MTD 10mg/kg</td>
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<td>2 PR</td>
<td>26+ &amp; 25+</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>4 SD</td>
<td>4-16+</td>
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<tr>
<td>Gomez-Navarro</td>
<td>tremelimumab</td>
<td>Phase I Q1M dose: 3-10mg/kg</td>
<td>Metastatic melanoma 28 89 44 45</td>
<td>26 SD</td>
<td>CR+PR: 4.7-28+</td>
<td>4 late DLT: hepatitis (2), rash, oedema. Common: fatigue, rash, itch, diarrhoea, nausea</td>
</tr>
<tr>
<td>[167] Camacho</td>
<td></td>
<td></td>
<td></td>
<td>1 CR</td>
<td>OS 9.97</td>
<td>G3/4 17% 13%</td>
</tr>
<tr>
<td>[151] Kirkwood</td>
<td></td>
<td>Phase II multiple dose: 1.10mg/kg</td>
<td></td>
<td>1 PR</td>
<td>OS 11.53</td>
<td></td>
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<tr>
<td>[168-169]</td>
<td></td>
<td>Q1M 2.15mg/kg Q3M*4</td>
<td></td>
<td>1 CR</td>
<td>8.9-29.8+</td>
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<td></td>
<td>3 PR</td>
<td>OS 10.0</td>
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<tr>
<td>Chung [170]</td>
<td>tremelimumab</td>
<td>Phase II 15mg/kg Q3M to PD</td>
<td>III/IV melanoma 251 (241 evaluable)</td>
<td>16 PR</td>
<td>G3/4: diarrhoea 28 (11%) fatigue 6 (2%)</td>
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<td></td>
<td></td>
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<td></td>
<td>(6.6%)</td>
<td>OS 10.0</td>
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<td>35 SD</td>
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<td>CBR 21%</td>
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<td>15 from enrolment. 21 (45%) survived 6M.</td>
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<td>Reference</td>
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<tr>
<td>Zatloukal [171]</td>
<td>tremelimumab maintenance after platinum chemotherapy (SD/ response)</td>
<td>Randomised phase II: 1.15mg/kg Q3M 2. Best supportive care</td>
<td>Advanced non-small cell lung cancer 87 1.44 2.43</td>
<td>PFS at 3 months 1. 2PR, 7SD 2. 0PR, 6SD</td>
<td>(months)</td>
<td>All G3/4 1. 9/44 (21%) 2. 0</td>
</tr>
<tr>
<td>Gordon [172]</td>
<td>tremelimumab (T) + sunitinib (SU)</td>
<td>Phase I dose escalation 6, 10, 15mg/kg Q3M + SU 50mg (4/2) or SU 37.5mg daily</td>
<td>Metastatic renal cell cancer 21</td>
<td>5/21 PR</td>
<td>6/21 on study ≥ 11M</td>
<td>DLT: 2/5 SU50+T6 1/6 SU37.5+T10 (sudden death) 3/6 SU 37.5 +T15: 2 acute renal failure, 1 dyspnoea</td>
</tr>
<tr>
<td>Ribas [173]</td>
<td>tremelimumab + DC vaccine (MART_{26,31} peptide) 1x10^7 i.d. Q3W*3</td>
<td>Phase I dose escalation 3,6,10mg/kg Q1M 10, 15mg/kg Q3M</td>
<td>16 Advanced melanoma IIIc/IV HLA-A*0201</td>
<td>2 PR 2 CR</td>
<td>9*-28+ 28+, 44+ *lung resolved; spinal met resected, tumour free 58M+</td>
<td>DLT (G3 diarrhoea, G2 hypophysitis) in 2/3 at 10mg/kg Q1M</td>
</tr>
<tr>
<td>Tarhini [174]</td>
<td>tremelimumab + IFN-α2B</td>
<td>Phase II T: 15mg/kg Q3M IFN: 20 MU/m² IV d1-5 Q1W*4 then 10 MU/m² SC TIW *8W</td>
<td>36 III/IV melanoma</td>
<td>3 CR 7 PR 12 SD</td>
<td>3, 9, 2-27+ 1.5-21 PFS 6.4M OS 15.9M</td>
<td>G3/4: neutropenia (6; 17%), hepatitis (4), anxiety (5), colitis (5), fatigue (12; 33%), rash (4).</td>
</tr>
<tr>
<td>Reference</td>
<td>Drug</td>
<td>Dose</td>
<td>Patients</td>
<td>Response</td>
<td>Duration (months)</td>
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<tr>
<td>Underhill [175]</td>
<td>Tremelimumab + PF-3512676 (TLR 9 agonist)</td>
<td>Phase I T 6,10,15mg/kg PF 0.05mg/kg</td>
<td>15 (ongoing) melanoma=12, mesothelioma =2, prostate =1</td>
<td>2 PR 5 SD</td>
<td></td>
<td>DLT: 2/6 T 15mg/kg (diarrhoea; vomiting)</td>
</tr>
<tr>
<td>Vonderheide [156]</td>
<td>Tremelimumab + exemestane 25mg a day</td>
<td>Phase I 3, 6mg/kg Q1M 6, 10mg/kg Q3M</td>
<td>26 Advanced hormone receptor positive breast cancer</td>
<td>11 SD 3-14</td>
<td></td>
<td>DLT: transaminitis (1); diarrhoea (4). MTD T 6mg/kg Q3M</td>
</tr>
<tr>
<td>Aglietta [155]</td>
<td>Tremelimumab + gemcitabine (G) after 4W G lead-in</td>
<td>Phase I T 6-15mg/kg Q3M G 1000mg/m² d1,8,15 Q1M</td>
<td>18 Untreated pancreatic cancer</td>
<td>2 PR</td>
<td></td>
<td>G3/4 AE: anaemia (4), ↑transaminases (3), ↑bilirubin (3). May be disease related</td>
</tr>
</tbody>
</table>

Table 1.2: Clinical Experience of Anti-CTLA4 Antibodies: Tremelimumab

Table summarises reported phase I and II trials of tremelimumab as single agent or in combination therapy. Abbreviations: T tremelimumab; SU sunitinib; DC dendritic cell (vaccine); MART melanoma-associated antigen recognised by T cells; TLR toll-like receptor; IFN interferon; MU mega-units; G gemcitabine; i.d. intra-dermal; IV intravenous; SC subcutaneous; M month; W week; Q3M every 3 months; TIW three times a week; CR complete response; PR partial response; SD stable disease; CBR clinical benefit rate (CR+PR+SD); PD disease progression; OS (median) overall survival; PFS (median) progression free survival; G1-4 CTC toxicity grades; DLT dose-limiting toxicity (usually ≥G3); MTD maximum tolerated dose.
Even in monotherapy trials, previous immunotherapy may play a role in response. Hodi et al [147] anticipated this when they conducted a phase I study of ipilimumab in previously immunised patients. Response was significantly altered by the nature of the previous vaccine. Melanoma patients previously treated with irradiated, autologous, GM-CSF producing tumour cells all developed extensive tumour necrosis with immune infiltrates containing CD4$^+$ and CD8$^+$ T cells and a brisk neutrophil response. In contrast, melanoma patients previously immunised with melanosomal antigens\textsuperscript{5}, delivered as modified peptide or via engineered autologous dendritic cells (DC), showed only lymphocytic tumour infiltrates with no CD4$^+$ cells and no necrosis. The prominent neutrophil response demonstrated in this study in both circulation and tumour was unexpected, and may be secondary to T cell activation. Crucially, in these patients the specific vaccine priming appears to have altered the balance of the effector immune response. A single patient, with subcutaneous and lung metastases from melanoma, initially treated with vaccinations of MART-1\textsubscript{27-35} peptide-pulsed immature DC, was subsequently enrolled in early phase studies of both ipilimumab and tremelimumab [176]. Although she had some initial, subjective increase in size of subcutaneous lesions, later immune analysis showed significant increase in MART-1 specific T cells by both tetramer and IFN-γ ELISPOT, with determinant spreading to additional melanoma antigens gp100 and tyrosinase in response to vaccine alone; subsequent CTLA4 blockade appears to have maintained frequency of generated specific T cells, but also led to the development of significant recall responses to previously exposed antigens (influenza, CMV, AFP). Clinically she developed slow resolution of all lesions, and had a complete response of over 2 years’ duration; the

\textsuperscript{5} Either autologous dendritic cells (DC) engineered to express gp100 and MART-1 (3), or modified gp100 peptide (1).
immunological results are used to suggest that vaccination primed her subsequent clinical response to anti-CTLA4 blockade, but until this hypothesis is addressed in a randomised clinical trial these results remain anecdotal. Disappointingly, the majority of the reported trials combining vaccination and anti-CTLA4 blockade have used concomitant administration rather than the post-vaccination schedule which appears to have benefitted this patient [160-161, 173].

Yang et al investigated toxicity and response to ipilimumab in small cohorts of melanoma patients who either had or had not received previous IL-2 therapy [165]. They found autoimmunity and objective responses in both cohorts, though responses seemed less durable in the patients who had received IL-2: 2 of 26 patients had partial responses (PR), both ≤ 8 months, compared to 3 of 14 patients with PR of 12-21 months duration. However, the study was not designed or powered to confirm differences between the cohorts, which showed a number of differences in patient characteristics: the cohort who had received IL-2 was younger (median 52 vs. 59 years), fitter (62% vs. 43% PS 0) and contained more women (38% vs. 14%). Further systematic investigation of the possible effect of pre-treatment on patient response to anti-CTLA4 blockade is still ongoing.

1.5.3.3. Anti-CTLA4 antibodies: Toxicity.

Toxicity has included a number of apparently auto-immune or inflammation mediated toxicities such as rash, colitis, and hypophysitis, which have been termed immune-related adverse events (IRAEs). Maculo-papular rash is commonly seen after anti-CTLA4 treatment. The rate of all skin reactions rises with clinically relevant three weekly doses of ipilimumab from 12.5% with 0.3mg/kg to around 45% with both 3
and 10mg/kg, whilst G3/4 skin reactions occur in 0%, 1.4%, and 4.2% with rising dose [149]. Rash is seen with similar frequency (34-36%) with tremelimumab 10mg/kg monthly and 15mg/kg three monthly [151]. Skin lesion biopsies from patients given either anti-CTLA4 antibody demonstrate dermal oedema with perivascular lymphocytic infiltrates [150, 160]. Most rash is mild and responds to topical therapy.

The commonest serious IRAEs are gastro-intestinal and include gastritis and a spectrum of bowel disturbance from diarrhoea to florid pan-colitis with the associated risk of perforation. Rates of gastro-intestinal adverse events are reported differently between trials, but appear similar: reported rate of all diarrhoea was 40% after 15mg/kg three monthly tremelimumab compared to 28% after 10mg/kg ipilimumab three weekly [151]. Both drugs demonstrate a dose dependent rise in serious (G3/4) gastrointestinal toxicity best illustrated by rates from the randomised double-blind study of ipilimumab: 0%, 2.8%, and 11% with 0.3, 3, and 10mg/kg ipilimumab three weekly respectively [149].

Beck et al [177] report a 21% incidence of enterocolitis (41 of 198 patients) in a pooled series of patients treated with ipilimumab in early clinical trials. All except one patient presented with diarrhoea; some had abdominal pain, fever, nausea or vomiting. Endoscopy showed gross abnormalities such as erythema or ulceration in just over half the patients. Thirty-nine patients had histological confirmation of colitis with three patterns of inflammatory infiltrates: neutrophils only (46%), lymphocytes only (15%), or mixed (38%). Neutrophils were associated with cryptitis and crypt abscesses; lymphocytes showed CD8+ infiltration of the crypts and CD4+ infiltration of the lamina propria. The majority of patients treated after establishment of a consistent toxicity treatment protocol responded to early treatment with supportive care and high
dose steroids (23 of 34), although a number relapsed (7) requiring further steroids or had refractory enterocolitis (7) which responded to a single dose of the anti-tumour necrosis factor-α (TNF-α) infliximab in all four treated cases. Nonetheless, 4 patients developed colonic perforation, two of whom died; in this review the mortality rate was 5% for patients who developed colitis, and 1% for all treated patients.

Autoimmune hypophysitis is a chronic inflammation of the pituitary gland which causes pituitary enlargement and hypopituitarism. It is a recognised but rare autoimmune condition, often associated with other autoimmune disease such as Hashimoto’s thyroiditis, and presents with headache, visual field defects and fatigue. Investigation may show pituitary enlargement by magnetic resonance imaging (MRI) and/or serum abnormalities of various pituitary hormones [178]. Hypophysitis is a recognised IRAE following anti-CTLA4 treatment; in a review Blansfield et al report a frequency of 5% in patients with metastatic melanoma (6 of 113) and 4% in patients with metastatic renal cancer (2 of 50) treated with ipilimumab [164]. This IRAE requires careful clinical review and can be hard to diagnose: patients developed a range of symptoms including fatigue, depression, insomnia, headache, arthralgia and impotence and had a range of endocrine abnormalities, although all had low thyroid hormone, low thyroid-stimulating hormone (TSH) and low cortisol. Presentation was after median of 12 weeks (range 9-24). All patients required withdrawal of anti-CTLA4, and all were treated successfully with appropriate hormone replacement with or without a short course of high dose steroids, but all required ongoing hormone replacement, including one patient with a complete response who was still under observation 26 months after treatment. Other endocrine abnormalities have been
reported following treatment with either anti-CTLA4 antibody, but all appear to occur with frequencies of less than 5%.

A randomised double-blind placebo-controlled phase II trial investigated the addition of a prophylactic non-absorbed oral steroid budesonide to 4 cycles of ipilimumab 10mg/kg every 3 weeks in patients with advanced melanoma but reported no improvement in the rate, severity, or pattern of IRAE: specifically the rate of grade ≥2 diarrhoea was no different, being 33% with budesonide and 35% with placebo [163].

It is unclear whether these IRAE are due to cross-reactivity with TAA or to a general enhancement of immunity resulting in a loss of control of autoimmunity. The gut and skin could be considered sites of maximal antigen exposure; the development of immune driven side-effects at these sites provides supportive evidence for the critical function of CTLA4 in immune control. Sanderson et al [161] looked at the expression in circulating PBMC of markers for T-cell homing receptors for skin (CLA and CCR4) and gastrointestinal mucosa (CCR9). Six months after anti-CTLA4 antibody and multiple melanoma peptide vaccination, CCR9 expression rose by 41% in CD4+ T cells, but individual response did not correlate with the development of autoimmunity in this small series of 13 patients.

There may be an association between IRAE and objective tumour responses after treatment with anti-CTLA4 antibody therapy in metastatic melanoma. A number of small studies have reported a relationship, but defined IRAE differently. Attia et al, investigating ipilimumab in combination with an HLA-A*0201 restricted peptide vaccine against gp100, report an objective tumour response rate of 36% (5 of 14) in
patients with grade III/IV toxicity, compared to 5% (2 of 42) in those without (P=0.008) [160]. A study of the immunobiologic effects of tremelimumab in patients with advanced melanoma treated with the drug in phase I and II studies at the M. D. Anderson Cancer Centre in Texas reported an objective tumour response rate of 33% (4 of 12) in those with grade II or higher dermatitis or diarrhoea, compared to 5.5% (1 of 18) in those without (P=0.0455) [179]. The correlation of IRAE and response seems somewhat undermined by a dose escalation study of ipilimumab which did not report an increase in objective response rate (11%) compared to published results, but did result in increased incidence of serious IRAE (35% G3/4). However, study design meant these results are not statistically significant, and the (albeit recent) historical controls are not ideal. Later randomised double-blind investigation of alternative doses of ipilimumab shows both IRAE and objective responses rise with increasing dose [149]. It is possible that the apparent relationship between IRAE and objective responses may be confounded by different dosing schedules; a recent abstract which summarised results for 571 patients treated with 15mg/kg tremelimumab suggests that most of the increase in IRAE seen in responders is due to their longer exposure to the drug [180].

1.5.3.4. Anti-CTLA4 antibodies: immunobiological effect.

T regulatory lymphocytes (Treg), which constitutively express CD4+CD25+ and CTLA4, and have a gatekeeper role in T cell inhibition and immune tolerance, are a possible target for anti-CTLA4 blockade. However, published results are equivocal. Maker et al demonstrate a rise not fall in Treg cell markers (defined by FoxP3 expression in CD4+CD25high lymphocytes) 3 weeks after ipilimumab. They conclude that CTLA4 blockade does not act through regulatory T lymphocytes, but has a direct
activating effect on all populations of lymphocytes, as demonstrated by increased HLA-DR expression in both CD4\(^+\) and CD8\(^+\) lymphocytes [145, 181]. In contrast, Reuben et al demonstrate a correlation between anti-tumour responses to tremelimumab and a number of immunomodulatory effects which do support a role for Treg: reduction in Treg at day 28 as defined by CD4\(^+\)CD25^{high} using flow cytometry, constitutive secretion of IL-10, raised IL-2 production, and a positive correlation between gene transcripts of FOXP3 and GITR [179]. These different results may be down to differences of technique or sample timing, or reflect genuine differences in the immunomodulatory effects of the two antibodies in clinical trials. Further work has subsequently been published, but will be discussed later in this project.

1.6: Summary of main research aims

1.6.1. Summary

In order to investigate modulation of T regulatory activity for cancer therapy this project explored whether the modulation of immune control with anti-CTLA4 blockade could lead to significant anti-tumour activity. Phase II clinical and laboratory investigation of anti-CTLA4 blockade using the antibody tremelimumab in patients with advanced gastric and oesophageal adenocarcinomas was combined with an attempt to establish and test a suitable pre-clinical model based on a therapeutic vaccination schedule against the tumour associated antigen 5T4.
1.6.2. A phase II trial of tremelimumab in advanced gastric and oesophageal adenocarcinoma

A phase II, single-centre, open-label, non-randomised study investigated the use of tremelimumab, a fully humanised anti-CTLA4 antibody, as a second-line therapy following platinum-based chemotherapy in patients with metastatic gastric and oesophageal adenocarcinomas. The primary end-point was clinical anti-tumour efficacy as described by the objective response rate. The secondary objectives were both clinical and laboratory.

The additional clinical objectives were:

1. To assess additional evidence of anti-tumour activity by:
   a. further characterisation of objective response (e.g. duration of response)
   b. progression-free survival
   c. overall survival
   d. and serum tumour antigen responses.

2. To characterise the safety profile and tolerability of tremelimumab in this setting.

The trial laboratory objectives were:

1. To assess changes to lymphocyte phenotype after CTLA4 blockade, and to identify potentially immunoregulatory phenotypes, including natural Treg.

2. To assess changes to lymphocyte function after CTLA4 blockade, looking particularly for any increase in specific anti-tumour activity, using responses to two relevant TAA: 5T4 and CEA.
3. To correlate any changes in lymphocyte phenotype and function with clinical outcome.

Clinical results are presented in Chapter 3 and laboratory results are presented in Chapter 4.

1.6.3. Murine model work

A murine model was established in order to investigate potential synergy between an autologous tumour therapeutic vaccination model targeted against murine 5T4 (m5T4) and modulation of immune regulation using anti-CTLA4 blockade.

The main aims of this work were:

1. To establish an active therapeutic vaccination model using the autologous murine oncofoetal antigen m5T4.
2. To develop an immunological measure of response to m5T4 vaccination.
3. To characterise response to vaccination against the antigen m5T4 in naive and tumour-bearing animals.
4. To attempt to modulate vaccination response using anti-CTLA4 blockade.
5. To test efficacy of combining anti-CTLA4 blockade with the therapeutic m5T4 vaccination model.

Murine model results are presented in Chapter 5.
2.1: A Phase II, open label, single arm study to evaluate anti-CTLA4 antibody tremelimumab in advanced gastric and oesophageal adenocarcinoma

2.1.1. Patients and Treatment

2.1.1.1. Regulatory and ethical considerations

The protocol [182] was developed in compliance with the European Union (EU) Clinical Trials Directive, the principle of the International Conference on Harmonisation of Good Clinical Practice (ICH GCP) guidelines and the Declaration of Helsinki. Approval was received from the Medicines and Healthcare Products Regulatory Authority (MHRA), The Christie NHS Foundation Trust Research and Development (R&D) department, and the North West Multicentre and South Manchester Research Ethics Committees (REC), prior to patient recruitment. All patients signed an informed consent before protocol enrolment and were treated in the Derek Crowther Unit, at The Christie (Manchester, UK).

A Data Safety Monitoring Board (DSMB) was convened on 24th April 2007 with two independent reviewers, Dr Paul Lorigan, Senior Lecturer in Medical Oncology, and Dr Juan W Valle, Senior Lecturer in Medical Oncology. An annual safety report was submitted to MHRA (July), and an annual summary to the main REC (April). An audit
of the consent process was undertaken by The Christie NHS Foundation Trust R&D department in May 2009.

2.1.1.2. Patient characteristics

Eligible patients were adults (≥18 years) with biopsy-proven locally advanced or metastatic gastric or oesophageal adenocarcinomas, who had previously received at least one cisplatin-based chemotherapy for advanced disease. They had an Eastern Cooperative Oncology Group (ECOG) performance status\(^6\) (PS) of 0 or 1, adequate bone marrow, hepatic and renal function (defined as neutrophil count ≥ 1.5 \(\times\) \(10^9\) cells/L, platelets ≥ 100 \(\times\) \(10^9\) cells/L, haemoglobin ≥ 9.0 g/dL, total bilirubin ≤ 2 \(\times\) upper limit of normal (ULN), and creatinine ≤ 170 µmol/L), and had recovered from all prior treatment-related toxicities. At least 4 weeks had elapsed since prior systemic therapy, and all patients had recovered from any treatment-related toxicity. The protocol allowed treatment 12 weeks after previous immunotherapy, but in fact none of the patients had received such treatment. Patients were excluded for any history of chronic inflammatory or autoimmune disease, colitis of any origin, uncontrolled cardiac or respiratory disease, and also for prior or concurrent malignancy within the last 5 years excepting basal cell carcinoma or \textit{in situ} carcinoma of the uterine cervix. Those who had required systemic steroids in the last four weeks, who were deemed likely to require them, or who had known brain metastases were also excluded.

Patients underwent pre-study computed tomography (CT) scan of the chest, abdomen, and pelvis within 28 days of enrolment. All patients had at least one measurable lesion

\(^6\) Appendix1
by Response Evaluation Criteria in Solid Tumours (RECIST) [183] and had persistent or progressive disease at screening.

2.1.1.3. Treatment

Tremelimumab (CP-675,206, previously ticilimumab, Pfizer, UK) was given by slow intravenous infusion (100 mL/hr) at a dose of 15 mg/kg every 90 days (a cycle). This dose was selected using Phase I and II toxicity and efficacy data from experience in patients with metastatic melanoma, renal cell carcinoma, and colorectal cancer [150]. In a Phase II trial of multiple dosing, the 15 mg/kg dose every 90 days showed equivalent efficacy but reduced toxicity compared with 10 mg/kg every 28 days [167]. Pharmacokinetic data shows that this dosing schedule results in serum anti-CTLA4 antibody levels above 30µg/mL throughout the 12 week cycle of treatment. Preclinical data had defined a minimum threshold for effective in vitro effects on T cell activation as 10-30 µg/mL [143].

2.1.1.4. Assessment

All patients had tumour assessment by CT scan of the chest, abdomen and pelvis at screening and every 90 days during therapy. The sum of the longest diameters of all marker lesions was calculated for each scan, and disease response was assessed according to RECIST as:

1. Complete response (CR) disappearance of all target lesions.
2. Partial response (PR) at least a 30% decrease in the sum of the longest diameter of all target lesions compared to the sum at baseline.
3. Disease progression (PD) (i.e. deterioration) at least 20% increase in the sum of the longest diameter of all target lesions recorded since treatment started, or the appearance of one or more new lesions, with reference to best previous CT scan.

4. Stable disease (SD) neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD.

All patients had clinical assessment of symptoms and toxicity prior to treatment, 15 days after treatment and then monthly after each cycle using the National Cancer Institute Common Toxicity Criteria (CTC), version 2 [184]. ECOG PS was recorded at all visits. Peripheral blood was collected on these visits for routine haematology and biochemistry, and for two serum tumour associated antigens (TAA):

- Carbohydrate antigen 19-9 (CA19-9): normal range <31 units/mL, and
- Carcinoembryonic antigen (CEA): normal range <3 µg/mL.

2.1.1.5. Retreatment

Patients with stable or responding disease on radiological assessment were retreated providing they had a PS 0 or 1, adequate bone marrow, hepatic and renal function, and had not developed any grade 4 drug-related adverse event, a grade 3 drug-related hypersensitivity or hepatitis, or a grade 2 uveitis. In the event of other drug-related toxicities patients could be retreated providing these had resolved to at least a grade 2 and/or were stable. The protocol anticipated a potentially slow mechanism of drug action, and allowed patients with asymptomatic disease progression after the first cycle of treatment to receive a single further dose.
2.1.2. Trial Objectives and Analysis

2.1.2.1. Clinical Objectives

The primary clinical objective was to assess the anti-tumour efficacy of intravenous tremelimumab administered at a dose of 15 mg/kg every 90 days to patients with advanced or metastatic gastric or oesophageal adenocarcinomas. The primary end-point was best objective response rate, defined as the percentage of patients with complete response or partial response. The clinical secondary end-points were:

1. To assess additional evidence of anti-tumour activity by:
   a. further characterisation of objective responses as measured by duration of tumour response
   b. progression-free survival
   c. overall survival
   d. serum tumour associated antigen (TAA) responses.

2. To characterise the safety profile and tolerability of tremelimumab as second line treatment of patients with advanced gastric and oesophageal adenocarcinoma.

Duration of objective response was measured from the date of enrolment to the date of disease progression or death. Progression-free survival (PFS) was defined as the time from the date of enrolment to the date of first documentation of disease progression, or to death from any cause. Overall survival (OS) was defined as the time from the date of enrolment to the date of death. In the absence of confirmation of death, survival time was to be censored at the last date the patient was known to be alive.
2.1.2.2. **Statistical analysis**

The null hypothesis was that the objective response rate in patients treated with tremelimumab does not exceed 20%. Sample size was calculated, using Gehan’s two stage design, to give an estimate of the response rate with 95% power and a standard deviation of 10%.

Survival analysis was performed using SPSS software (originally Software Package for the Social Sciences; now part of IBM). Progression free and overall survival were characterised both in terms of median survival and the probability of remaining progression free and/or alive at 3 months, 6 months and 12 months based on Kaplan-Meier estimates. Ranges and 95% confidence intervals (CI) were calculated using the Kalbfleisch and Prentice method [185]. Exploratory analyses of the effect of baseline characteristics and laboratory results were performed using Cox regression models.
2.1.3. Laboratory assays

2.1.3.1. Processing of Patient Samples

Collection: At each time point (pre-treatment, and 15, 30, 60, and 90 days after treatment) 45mL heparinised blood was collected by peripheral venesection, processed and stored for later assays. Additional written consent allowed the collection of screening blood for some patients (numbers 6-8 and 11-17). The samples were delivered at room temperature to the laboratory.

Reagents:

LSM  lymphocyte separation medium (Lymphoprep, Axis-Shield, Norway)
PBS  phosphate buffered saline
FM  freezing mix: 90% foetal calf serum (FCS; VWR, USA), and 10% dimethyl sulphoxide (DMSO; VWR)

Isolation of peripheral blood mononuclear cells (PBMC): The blood was layered in 10-12mL aliquots onto 10mL of LSM and centrifuged at 800g for 30 minutes at 20°C. The upper layers of plasma were removed using a wide-bore pastette, centrifuged to remove any debris, appropriately labelled and stored at -20°C. The interface layers of PBMC were removed using a fine-tipped pastette. Each aliquot was washed with 50mL PBS and centrifuged before pooling on resuspension in 10mL PBS. Viable cells were counted using trypan blue (0.4%, Sigma, Fife, UK) exclusion before a further wash with 40mL PBS.
**Storage:** PBMC were resuspended in FM at a concentration of $10^6$cells/mL, before immediate division into 1mL aliquots in pre-labelled cryovials (Nunc, Germany), and transfer to a freezing box at -80°C. After 24 hours, samples were transferred to liquid nitrogen (-180°C) for long term storage. All samples were labelled, recorded and tracked in line with the Human Tissue Act (2004) [178]. Labelling anonymised patient information, and used a code which identified the trial, patient number, cycle, days since treatment, and the type of sample, as shown in Fig 2.1.

**Thawing of PBMC:** Each cryovial of PBMC was quickly thawed in a water bath (37°C) and then immediately suspended dropwise in 10mL RPMI (Sigma-Aldrich, Ayrshire, UK) supplemented with 10% FCS, centrifuged for 5 min at 300g, and resuspended in an appropriate volume of media or PBS to determine concentration and viability using manual counting with a haemocytometer (C-Chip, Digital Bio, Seoul, Korea) and trypan blue exclusion. Viability and concentration were also determined using Guava Viacount™ reagent and counter (Guava Technologies, CA, USA). Briefly, for estimated cell concentrations between 1x$10^6$ and 1x$10^7$ cells/mL, 20μL cell suspension is mixed...
with 380μL Viacount Reagent in an eppendorf tube and rested for 5mins at room temperature before reading on the counter. Results are calculated automatically and data displayed in 2 dot plots: viability versus nucleated cells and forward scatter (FSC) versus viability.

2.1.3.2. Analysis of Lymphocyte phenotype (I)

Contemporaneous analysis of lymphocyte phenotype was performed within 24 hours at the National Blood Service Manchester Centre by Maria Hulston and Lesley MacDonald. Additional whole blood samples (3mL) were collected into BD vacutainers (BD Biosciences, CA, USA) containing 5.4mg Potassium EDTA. Absolute counts of T cell, B cell and natural killer cell populations were obtained contemporaneously by staining for the surface markers CD3 (anti-CD3 FITC; clone UCHT1), CD4 (anti-CD4 PE; clone 13B8.2), CD8 (anti-CD8 PE; clone B9.11), CD16 (anti-CD16 PE; clone 3G8), CD19 (anti-CD19 PE; clone J4.119), CD45 (anti-CD45 FITC; clone J33), CD56 (anti-CD56 PE; clone N901 (NKH-1); all Beckman Coulter, High Wycombe, UK), and CD25 (anti-CD25 PECy5; clone M-A251, BD Pharamingen, CA, USA). Briefly, 10μL of relevant antibodies were added to a BD Trucount™ tube (BD Biosciences) plus 50μL of whole blood. After 20 minute incubation the red blood cells were lysed by the addition of 1mL IOTest 3 Lysing solution (Beckman Coulter). Following a further 10 minute incubation cells were analysed on the flow cytometer (FC500; Beckman Coulter) without washing. The lymphocyte population was selected for analysis using either forward scatter (FSC) versus side scatter (SSC) or CD45 versus side scatter gating.
2.1.3.3. Analysis of Lymphocyte Regulatory Phenotype (II)

Cryopreserved PBMC were used to investigate changes in regulatory lymphocyte phenotype. Intracellular FoxP3 staining was done using a human regulatory T cell staining kit (eBioscience®, CA, USA). The technique was adapted to analyse intracellular CTLA4 and extracellular PD-1 staining which were performed in parallel.

For each time point, PBMC were thawed and counted as described then resuspended in PBS at 3x10⁶ viable cells/mL. Aliquots of 100µL (3x10⁵ PBMC) were stained with 4µL of the anti-human CD4 FITC (clone RPA-T4)/ anti-human CD25 APC (clone BC96) antibody cocktail for 20 minutes at 4°C in the dark. Cells were washed once with 3mL ice-cold PBS before fixing and permeabilising by 45 minute incubation in 1mL freshly prepared eBioscience® Fix/Perm buffer at 4°C in the dark. Cells were washed twice with 2mL ice-cold permeabilisation buffer (PB) before blocking with 1µL normal rat serum for 15 minutes at room temperature. Alternate tubes were stained, without washing, with 4µL rat IgG2a isotype control PE or 4µL anti-FoxP3 PE (clone PCH101) for 30 minutes at 4°C in the dark. Cells were again washed twice with 2mL ice-cold PB before suspension in 100-200µL of flow cytometry staining buffer.

This method was adapted for intra-cellular CTLA4 staining by substituting the following quantities and reagents for the second stain:

1. 2µL normal mouse serum (Dako, Glostrup, Denmark) for the blocking step
2. 2.5µL mouse IgG2a isotype control PE (Serotec, Oxford, UK)
3. 5µL anti-CTLA4 PE (clone BNI3, BD Biosciences).

For extracellular PD1 staining, the first labelling step of the method above was followed, but the cells did not require permeabilisation. After a second wash with 3mL
ice-cold PBS, alternate tubes were stained with 2.5μL mouse IgG1 isotype control RPE (Serotec) or 5μL anti-PD1 PE (anti-CD279, clone MIH4, BD Pharmingen) for 30 minutes at 4°C in the dark. Cells were washed twice with 2mL ice-cold PBS before suspension in 100-200μL of PBS for flow cytometry.

Cells were analysed using a FACSCalibur flow cytometer (Becton-Dickinson, Oxford, UK) and WinMDI 2.8 as previously described [186-187]. Lymphocytes were gated against forward and side scatter, and then against CD4 and CD25 to identify CD4⁺CD25<sup>high</sup> T lymphocytes shown in R3 gate (green arrow) in Fig 2.2 below. This gate was used to identify FoxP3⁺ (or CTLA4⁺) events against the isotype controls as shown by the blue arrow in Fig 2.2; removal of the CD4⁺CD25<sup>high</sup> gate also allowed assessment of CTLA4⁺ frequency in the CD25<sup>low/negative</sup> and CD4⁻ population.

A: Isotype control

B: FoxP3 labelled
Fig 2.2: Sample FACS analysis of FoxP3 phenotype: Patient 8, pre-treatment

Briefly, thawed PBMC were labelled with anti-CD4 FITC/anti-CD25 APC, permeabilised, and stained with anti-FoxP3 PE (B) or isotype anti-rat IgG2a PE (A) before analysis. Lymphocytes were selected using FSC and SSC (left hand plot, red arrow), CD4⁺CD25⁺ (middle plots, R3, green arrow) and CD4⁺CD25low/normal (R2) lymphocytes gated and FoxP3⁺ (R4, blue arrow) population identified within the R3 gate using the isotype control. In the example shown, absolute counts are: lymphocytes 35790, CD4⁺ 14365, FoxP3⁺ 811 (2.3% lymphocytes, 5.6% CD4⁺).

For extracellular PD1 analysis, histograms of CD4 against PD1 were plotted, and the isotype control of the latter was used to establish a 5% marker. This marker was used to assess the percentage of positive events at each time point, and the 5% limit was subtracted to give a final result.

2.1.3.4. Statistical analysis of Flow Cytometry

Absolute counts were converted into % of total lymphocytes and relevant lymphocyte compartment. Raw data was stored in Excel and analysed using StatsDirect. After assessment for parametric distribution (Shapiro Wilks), non-parametric pooled data was described by median and interquartile ranges, and presented as box and feather plots. Wilcoxon signed-rank (paired data) and Mann Whitney U tests (unpaired data) were used to test differences between lymphocyte phenotype before and after treatment, and a P value <0.05 was considered significant.

2.1.3.5. Lymphocyte Proliferation Assay to pooled peptides 5T4

Reagents:

IMDM Iscove’s media (Gibco®, Invitrogen, UK)

Human AB serum (Quest Biomedical, Norwich, NY)

DMSO dimethyl sulphoxide (VWR, Leicestershire, UK)
**Method:** To explore changes to in vitro responses to a relevant tumour associated antigen (TAA), overlapping 5T4 32-mer peptides (18 patients, 24 cycles) were used in a thymidine incorporation proliferation assay [188]. The oncofoetal antigen 5T4 is a 420 amino acid protein. Individual peptides of 32 amino acids, each overlapping by 12-15 amino acids, were designed to span the whole sequence of h5T4 and defined as A to X [188]. U, which spans the transmembrane region, is insoluble and was not used. 96 well round bottomed tissue culture plates (Falcon®, Becton Dickinson, NJ, USA) containing pooled 5T4 peptides were prepared in batches in advance and stored at -20°C. 6 pools of 4 peptides were prepared from stock to give a solution with 2µg of each of the four peptides in 40µL IMDM. 40µL of each pool (ABCD etc) were dispensed in an established pattern to give a final concentration of 10 µg/mL of each peptide in the established assay.

All time points for each patient were investigated in the same assay: cryopreserved PBMC were carefully thawed as described and 1.5 x 10^5 viable cells/well were cultured in IMDM supplemented with 10% human AB serum and 0.1 mg/mL penicillin and streptomycin (Invitrogen, Paisley, UK) with six pools of four overlapping 5T4 peptides spanning the whole sequence of human 5T4, and media controls with and without the appropriate concentration of DMSO which had been used to prepare peptide stocks [54, 189]. PHA-M at 2 and 5 µg/mL was used as a polyclonal stimulant. Each target was investigated using at least 3 replicates. Plates were incubated for 5 days at 37°C in 5% CO₂ before pulsing with 1µci/mL of tritiated thymidine [³H]TdR (Perkin Elmer, MA, USA) for an additional 18 hours prior to harvesting and counting with a Packard cell harvester and Topcount (Perkin Elmer). Cells were harvested on to
pre-wetted filter plates, dried and scintillated using 30µL/ well microscintillation fluid (Microscint™0, Perkin Elmer) before counting.

**Calculation:** Stimulation indices (SIs) were calculated by dividing the counts per minute of the test wells by the mean count per minute of the appropriate control wells; mean results are presented. A pre-treatment response was defined by a mean SI \( \geq 2 \). A post-treatment response was defined as a mean SI of \( \geq 2 \), with a doubling of pre-treatment response [54]. Patients with new post-treatment responses were considered responders.

2.1.3.6. **Lymphocyte Proliferation Assay to CEA protein**

In parallel thymidine incorporation proliferation assays, PBMC were cultured with purified CEA (Calbiochem, CA, USA). Preliminary investigation in cryopreserved PBMC from 8 patients with metastatic renal (n=2), colorectal (n=3) and pancreatic (n=3) cancer demonstrated no responses. 15 trial patients (17 cycles) were assayed. Preliminary assays used purified CEA at concentrations between 12.5ng/mL and 2µg/mL. In the optimised assay, PBMC were cultured with purified CEA at 100ng/mL, 200ng/mL, and, if sufficient cells, 300ng/mL. Each concentration was assayed in triplicate. Media was used as the negative control and PHA-M as the positive control.

2.1.3.7. **Variant Lymphocyte Proliferation assays**

Supplementary proliferation assays were conducted using the same method with both fresh unselected PBMC, and selected CD4^+^CD25^-^ (responders) with irradiated CD3
(accessory cells), in 3 patients with a diagnosis of advanced gastric or oesophageal cancer who had not yet received any treatment for their disease, and in patient 12 using pre-treatment blood from cycle 9, day 1 (24.2.09).

2.1.3.8. In vitro PBMC stimulation

To investigate changes in T lymphocyte responsiveness, cryopreserved PBMC were thawed and cultured at 1x10^6 per well for 48hrs in RPMI (Sigma-Aldrich) supplemented with 10% human AB serum in the presence of 1μg/mL anti-CD3 (OKT3, Janssen-Cilag, Buckinghamshire, UK) and 1μg/mL anti-CD28 (clone 37407, R&D Systems, MN, USA). Cells were pelleted by centrifugation at 300g for 5min and supernatant was collected and frozen at -20°C. At a later time point, total human IL-10 and IL-2 (n=15) were quantified using ELISA kits (Diaclone, Besançon, France), according to the manufacturers’ instructions.

2.1.3.9. Cytokine ELISA: IL-2

The human high sensitivity Interleukin-2 (IL-2) kit (Diaclone, Besançon, France) is a solid phase enzyme-linked immunosorbent assay (ELISA) with strips of wells precoated with monoclonal antibody specific for IL-2. All reagents were prepared with diluents extemporaneously in clean glassware according to the manufacturer’s instructions, including standard diluent buffer which was used to reconstitute standard IL-2 stock to give a solution of 60pg/mL. Serial 1:2 dilutions were prepared (60 to 1.87 pg/mL) for the standard curve. Two concentrations of the enzyme, Streptavidin-Horse radish peroxidise (HRP) were prepared from stock and washing buffer diluted 1:200 in distilled water.
Supernatant samples were thawed at room temperature, and diluted 1:25 or 1:50 with standard diluent buffer (or RPMI). 100μL of IL-2 standard dilutions, test samples, standard diluent control and media control were added to duplicate wells. Plates were incubated covered and with slow shaking at room temperature with the following sequence of reagents:

1. 50μL/well biotinylated anti-IL-2 for 1 hour.
2. 100μL/ well of the first streptavidin-HRP solution for 20 min.
3. 100μL/ well of amplifier solution for 15 min.
4. 100μL/ well of the second streptavidin-HRP solution for 20 min.

Plates were aspirated and washed three times after each step. Plates were developed by incubation in the dark with 100μL/ well ready-to-use tetramethylbenzidine (TMB). This reaction was stopped by rapid pipetting of 100μL/ well H₂SO₄. Plates were read within 30 minutes on an Emax precision microplate reader (Molecular Devices Corporation, Sunnyvale, CA) using 450nm as the primary wavelength and 650nm as the reference wavelength. Concentrations of test samples were calculated from the standard curve using Softmax Pro 4.0.

2.1.3.10. Cytokine ELISA: IL-10

The human IL-10 ELISA kit (Diaclone) was used according to the manufacturer’s instructions. Standard IL-10 stock was reconstituted with standard diluent buffer to give a solution of 400pg/mL IL-10. Serial 1:2 dilutions were prepared from 400 to 12.5pg/mL for the standard curve. Supernatant samples were defrosted at room temperature, and diluted 1:25 with RPMI. 100μL of IL-10 standard dilutions, test samples, standard diluent control and media control were added to duplicate wells.
Plates were incubated covered and with slow shaking at room temperature with the following sequence of reagents:

1. 50μL/well biotinylated anti-IL10 for 2 hours.
2. 100μL/well of streptavidin-HRP solution for 30 min.

Plates were aspirated and washed three times after each step. Plates were developed by incubation in the dark with 100μL/well ready-to-use TMB. The reaction was stopped by rapid pipetting of 100μL/well H₂SO₄, and plates were read as described above.

2.1.4. Additional in vitro human immunology methods

2.1.4.1. Intracellular FACS: IL-2 +/− FoxP3

PBMC were carefully thawed and plated at 2x10⁵ viable cells/well in 200μl complete medium in triplicate in a 96-well non-tissue culture plate pre-coated with 1μg/ml anti-CD3 (OKT3, Janssen-Cilag, Buckinghamshire, UK) and 1μg/ml anti-CD28 (clone 37407, R&D Systems, MN, USA) in the presence of 1μg/ml Golgi Plug (BD Biosciences) with or without 15μg/ml of tremelimumab. Polyclonal stimulation with PMA (50ng/mL) and ionomycin (500ng/mL; both Sigma) was used as an alternative to anti-CD3/anti-CD28. Plates were incubated for 6 hours in a humidified atmosphere of 5% CO₂ at 37˚C before staining for surface markers.

Triplicate wells were pooled to give est. 6x10⁵ cells/well. Cells were pelleted at each step by centrifuging at 300g for 5mins and decanting media/ buffer. Cells were washed with ample PBS then stained for 30 minutes at 4°C with a cocktail of 4μl CD3 PerCP (clone SK7), 4μl CD4 FITC (clone SK3) and 2.5μl CD25 APC-H7 (clone M-A251; all BD, NJ, USA). After washing with 200μl cold PBS cells were fixed and permeabilised by 45 minute incubation at 4°C with 100μl/well Fix/Perm buffer.
(eBioscience®). Cells were washed twice with permeabilisation buffer (PB). Non-specific binding was blocked by 15 minute incubation at room temperature with 1µl Rat serum (IL-2), before 30 minute incubation at 4°C without washing with 4µl FoxP3 Alexafluor 647 (clone 236A/E7; BD), and either 5µl IL-2 PE (clone MQ1-17H12; eBioscience®), or 1µl Rat IgG2a isotype control PE (eBioscience®).

Cells were washed twice with PB then resuspended in 200µl/well flow cytometry buffer. Samples were analysed using FACSCanto II (BD, NJ, USA) and FlowJo version 7.5.5 software (TreeStar, Ashland, OR, USA). Lymphocytes were selected using forward scatter versus side scatter followed by gating CD3+ T cells and applying this gate on a plot of CD4 against IL-2.

2.1.4.2. Functional Suppression assay

Cell fractions were isolated from PBMC using Dynabeads® Regulatory CD4+CD25+ T cell isolation kit (Invitrogen) according to the manufacturer’s instructions. In some experiments (as indicated) thawed PBMC were used to prepare accessory cells; all CD4+ subsets were generated from fresh cells within 24 hours of venesection.

Reagents:

i. Isolation buffer: PBS + 0.1% bovine serum albumin (BSA; Pierce, Thermo Scientific, IL, USA) + 2mM EDTA (Sigma-Aldrich, UK).

ii. Media: RPMI supplemented with 10% human AB serum (Quest Biomedical) and 0.1 mg/mL penicillin and streptomycin (Invitrogen).

All isolation steps were conducted in 15mL tubes. A Spiramix (Denley) was used to tilt and mix. A Dynal MPC-1 magnet (Invitrogen) was used for magnetic separation of
bead bound cells and supernatant by 1-3 minute incubation and careful pipetting of supernatant.

**Preparation of accessory cells (irradiated, CD3 depleted PBMC):**

5x10⁶ PBMC in 1mL isolation buffer were added to 25µl washed Dynabeads® CD3 and incubated with slow rotation at 2-8°C for 30 minutes. Supernatant containing CD3⁻ cells was collected by magnetic separation, washed with cold isolation buffer and cells resuspended in 5mL media. Resulting CD3⁻ cells were irradiated by X-ray source (3000rads= 30Gy), washed, counted, and resuspended in media at 5x10⁵/mL.

**Isolation of CD4⁺CD25⁻ Responder and CD4⁺CD25⁺ Regulatory T cells:**

All remaining PBMC were suspended in 500µl isolation buffer and incubated with 200µL FCS and 200µL antibody mix human CD4 at 2-8°C for 20 minutes. Cells were washed, resuspended in isolation buffer, and incubated with 1mL Depletion MyOne Dynabeads at room temperature with gentle tilting for 15 minutes. Bead-bound cells were discarded and supernatant containing bead-free CD4⁺ cells was collected and resuspended in isolation buffer at 1.5x10⁷ cells/mL. 200µL Dynabeads CD25 were added per 1.5x10⁷ cells, before incubation with tilting at 2-8°C for 25 minutes. The magnet was used to separate supernatant and bead bound cells. Supernatant contained CD4⁺CD25⁻ responder cells, which were washed, and resuspended at 5x10⁴/mL in 10% AB serum/RPMI, to be kept on ice until assay set up. Bead-bound CD4⁺CD25⁺ suppressor cells were gently washed then resuspended in 500µl RPMI/1% FCS and incubated with 80µl Detachabead at room temperature with tilting for 45 minutes. Supernatant containing CD4⁺CD25⁺ suppressor cells was collected by magnetic separation, washed twice in RPMI/1% FCS to remove any remaining beads, and resuspended at 5x10⁴/mL in RPMI + 10% AB serum.
Assay

The assay was established in a 96 well U bottomed tissue culture plate, using at least 3 replicates. All wells in a given assay contained equal numbers of accessory cells (50µl/well of 5x10^5/mL), and an equal concentration of anti-CD3 (OKT3, Janssen-Cilag; 50µl of 1µg/mL). Responders (CD4^+CD25^-) were added at fixed concentration (50µl/well of 5x10^4/mL) and suppressors (CD4^+CD25^+) were added with a serial 1:2 dilution (from 50µl/well of 5x10^4/mL) to give a range of responder: suppressor ratios. The volume of each well was made up to 200µl with media. Plates were incubated in 5% CO_2 at 37°C for 4-5 days, pulsed with 1µci/mL tritiated thymidine, and harvested 18 hours later on the Packard cell harvester. Harvested plates were scintillated and counted using Topcount as described in sections 2.1.3.5 & 6.

Results were expressed as mean counts per minute (cpm), and by percentage suppression where this was calculated for each responder to suppressor (R:S) ratio as:

\[1-(\text{cpm (each R:S ratio - S alone)/ cpm R alone})\times100,\]

where R= responder and S=suppressor.

2.1.4.3. Cytokine bead array

The assay was conducted using BD cytometric bead array (BD Biosciences, CA, USA) according to the manufacturer’s instructions. First, standards were prepared from supplied spheres and serially diluted 1:2. Next the Flex set capture beads were prepared. Prepared capture beads were washed before use then mixed and rested at room temperature for 15 minutes. Plasma samples were diluted 1:4 using kit diluents. The Flex set PE detection reagent was prepared and the assay was set up. 50µl of each dilution of standard and negative control (assay diluents alone) were transferred to the
top row of a 96 well U bottomed plate, and 25µl samples (serum/plasma) were added below. Prepared beads were vortexed, and 25µl added to each well. The plate was mixed at 500rpm on a digital shaker for 5 mins before 1 hour incubation at room temperature. Next, 25µl prepared PE detection reagent were added to each well, and the plate was again mixed at 500rpm for 5 mins before a 2 hour covered (dark) incubation at room temperature. 100µl wash buffer were added to each well, the plate was centrifuged at 200g for 5 minutes, and the supernatant discarded without disturbing the beads. The beads were washed again with 200µl wash buffer before final suspension in 75µl of wash buffer and analysis on a BD FacsArray™ bioanalyzer.

2.1.4.4. ELISA: h5T4-specific antibodies

Available plasma samples for the trial patients, who had signed the relevant addendum in the consent form, were sent to Oxford BioMedica (Oxford, UK) to measure 5T4 antibodies using their published method [190-191]. Briefly, 5T4-specific antibody responses were measured using a validated semi-quantitative enzyme-linked immunosorbant assay (ELISA). Polyclonal plasma, known to be positive for 5T4-specific antibodies, is used to establish a standard curve for each assay, and allows quantification of results using relative units (RU). The company have defined the criteria for a positive antigen-specific 5T4 antibody response by (1) defining background level of 5T4 antibody responses in plasma recovered from 50 nominally healthy volunteers such that less than 5% would be classified as positive, which is at a cut-off 12.77 relative units (RU) and (2) determining variation in 5T4 antibody responses in cancer patients not receiving 5T4 targeted therapy over time which allows determination of a relevant fold increase.
2.2: Modelling Immunotherapy

2.2.1. Cell culture

Reagents:

PBS  phosphate buffered saline
FCS  heat-inactivated foetal calf serum (FCS; VWR, USA)
FM  freezing mix: 90% FCS and 10% dimethyl sulphoxide (DMSO; VWR)

Unless otherwise stated cells were grown in either Corning® or Falcon tissue culture flasks (Corning Inc, USA and Becton Dickinson, USA) in a humidified atmosphere of 5% CO\textsubscript{2} at 37°C. Cell lines were tested for mycoplasma by PCR\textsuperscript{7} (Molecular Biology Core Facility, PICR) after being grown for at least 3 passages in antibiotic-free media. At all other times, media was supplemented with 0.1mg/mL penicillin and streptomycin (Invitrogen, Paisley, UK).

For long-term storage, samples were suspended in 1mL aliquots of FM in cryovials (Nunc, Denmark), and transferred to a freezing box at -80°C for at least 24 hours before long-term storage at -180°C in liquid nitrogen. Samples were thawed quickly by immersion in a 37°C water bath and immediately suspended drop-by-drop in 10mL of appropriate media supplemented with at least 10% FCS, before centrifuging at 300g for 5-10 minutes, and resuspension in appropriate media for culture.

2.2.1.1. Adherent Tumour cell lines

Adherent tumour cell lines were passaged when 80-90% confluent unless otherwise stated. Cells were washed with PBS, briefly incubated with trypsin EDTA

\textsuperscript{7} Polymerase chain reaction (PCR)
(0.5g/100mL, Sigma, Germany) until disaggregated, then washed in 10mL PBS or media, centrifuged at 300g for 5min, and suspended in an appropriate volume of media to allow re-seeding of a new flask.

The melanoma cell line B16.F10 [192] transfected with m5T4 cDNA in pCMVα (B16.m5T4) and control cells transfected with pCMVα alone (B16.neo; both gifts from K.Mulryian, PICR) [44] were grown in DMEM (Sigma) supplemented with 10% FCS and 2mM L-glutamine (Sigma). Cell lines were maintained under selection with 1mg/mL Geneticin® selective antibiotic (G-418, Invitrogen).

The glioma cell line GL261 [193] were a gift to the group from Dr Paul Walker, Switzerland. They were grown in RPMI (Sigma-Aldrich, Ayrshire, UK) supplemented with 10% FCS and 4mM L-glutamine. C127i (ATCC), a murine mammary tumour line derived from the RIII strain were grown in DMEM supplemented with 10%FCS and 4mM L-glutamine [194]. Cells were passaged when around 70% confluent.

CT26.wt (ATCC) is a lethal clone from an N-nitroso-N-methylurethane-induced BALB/c (H-2β) undifferentiated colon carcinoma cell line [195]. EMT6 (ATCC), is a clone of a transplantable murine mammary tumour EMT which arose after implantation of a hyperplastic mammary alveolar nodule [196]. Both were grown in DMEM supplemented with 10% FCS, and 2mM L-glutamine. TC-1 (ATCC), was derived from C57BL6 primary lung epithelial cells transformed to co-express human papilloma virus (HPV-16) antigens E6/E7, and the activated human c-Ha-ras oncogene [197-198], and was grown in RPMI supplemented with 10% FCS and 2mM L-glutamine.

2.2.1.2. Hybridomas

Hybridomas were cultured in suspension.
The hamster anti-mouse CTLA4 clone HB304, UC10-4F10-11 (4F10; Bluestone [118], ATCC) was initially cultured in DMEM supplemented with 20% FCS, 4mM \( L^- \) glutamine, 0.01mM MEM Nonessential amino acids (Gibco®, Invitrogen, Paisley, UK), and 0.05mM 2-mercaptoethanol (Gibco®). Once cells were established the FCS supplementation was cut serially to 10% then 5%. Cells were expanded and established in T175 flasks before sealing and leaving for 5-10 days. Supernatant was harvested, and viable cells re-established in fresh medium. Supernatant was stored at 4°C after the addition of 0.1% sodium azide.

2.2.2. Antibody purification and assessment

2.2.2.1. Antibody purification

All laboratory grade chemicals from BDH, VWR, UK unless otherwise stated.

Reagents:

i. Glycine 100mM: 0.75g glycine dissolved in 100mL distilled water. pH adjusted to 2.5 using NaOH. Sterile filtered and stored at 4°C.

ii. Tris 1M: 12.1g Tris (Sigma, Germany) dissolved in 100mL distilled water. pH adjusted to 9.1 with HCl. Sterile filtered and stored at 4°C.

After harvesting, hybridoma supernatant was centrifuged at 7148g for 20 minutes to remove cellular debris and pooled. Protein was precipitated by addition of a 45% solution of Ammonium sulphate (25.8g solid per 100mL), and pelleted by centrifugation (7148g for 20min), before resuspension in a minimal volume of PBS. The resulting solution was dialysed 4 times (minimum 2-3 hours each time) against a large volume PBS to remove the Ammonium sulphate before purification using a Sepharose protein G column (Hitrap Protein G HP 5mL, GE Healthcare, UK) and
peristaltic pump (model P-1, Pharmacia, Sweden). Antibody was eluted from the column using 100mM glycine (pH 2.5), and Tris buffer (pH 9.1) was immediately added sufficient to neutralise the pH. Protein concentration was assayed by spectrophotometry using a NanoDrop (ThermoScientific, DE, USA). Protein concentrations were calculated using the formula:

\[ \text{Protein concentration (mg/mL)} = 1.55A_{280} - 0.76A_{260} \]

where \( A_{280} \) and \( A_{260} \) are the absorbance at 280nm and 260nm respectively. Protein containing fractions were pooled. The column was washed with 10mL PBS and the collected unbound run-through reapplied to bind any remaining antibody. This procedure was repeated until the eluate no longer contained a significant amount of protein. The pooled product (4F10) was dialysed overnight against a large volume of PBS (i.e. 2-3 litres), and then sterile filtered.

**2.2.2.2. Endotoxin Testing: Limulus Amebocyte Lysate Test**

A Limulus Amebocyte Lysate (LAL) Test (Pyrogent® Plus; Cambrex, Lonza, Switzerland) was used to detect endotoxin within the processed antibody. In this test, which is used within the pharmaceutical industry, very small quantities of endotoxin result in coagulation of the Limulus lysate, derived from the blood of the horseshoe crab *Limulus polyphemus* [199]. The lysate sensitivity of the LAL test used throughout this work was 0.125 Endotoxin Units (EU)/mL.

All reagents (stored at 4°C) and test solutions were allowed to warm slowly to room temperature. All preparation was conducted within a class II tissue culture hood, and disposable sterile eppendorfs were used for dilutions. All solutions were prepared before all tests were started by adding 0.25mL of the appropriate test solution to a lysate vial with gentle mixing of the contents. Immediately after reconstitution, vials
were incubated for 60 minutes (±2 min) at 37°C without disturbance. After incubation, each vial was carefully removed and inverted 180°. A positive reaction was characterised by the formation of a firm gel which remains momentarily intact when the vial is inverted. A negative reaction was characterised by the absence of a solid clot even in the presence of increased viscosity.

**Positive controls:** All tests included a positive control prepared from lyophilised purified endotoxin from E. Coli strain 0111:B4 provided with the kit. This was reconstituted with clinical grade endotoxin-free water for injections (Baxter, UK) to provide stock solution of 1 EU/mL Control Standard Endotoxin (CSE) equivalent to FDA Reference Standard Endotoxin. Initial experiments confirmed lysate sensitivity of the test by serial dilution of CSE to give endotoxin concentrations of: 0.5, 0.25, 0.125, 0.06, and 0.03 EU/mL. Later experiments utilised two dilutions of positive control. These results were always read first.

**Negative control:** Clinical grade 0.9% sodium chloride (NaCl) solution (Baxter, UK) was used as a negative control in all experiments.

**Test solutions:** Test solutions were prepared by dilution with 0.9% NaCl solution used as negative control. As the test volume was fixed (0.25mL), aliquots of processed antibody were initially screened for endotoxin at 1:50 dilution (6µl in 294µl 0.9% NaCl) before further investigation at between 1:3 and 1:10 dilution.

### 2.2.2.3. Endotoxin Removal

Endotoxin was removed from contaminated samples using Detoxi-Gel™ Endotoxin Removing Columns (Pierce Biotechnology, Thermo Scientific, IL, USA) according to the manufacturer’s instructions [200]. Immobilized polymixin B is used to bind and remove pyrogens from solution. Briefly, the resin-filled column (1mL volume) was
regenerated before each use by washing with 5mL of 1% sodium deoxycholate (Thermo Scientific, ibid.) followed by 5mL clinical grade 0.9% NaCl (Baxter, UK) to remove the detergent. The resin-filled column was equilibrated by washing with a further 3-5mL clinical grade 0.9% NaCl, before application of the sample in 1mL aliquots. Each aliquot was incubated for at least 1 hour before collection by gravity into clean universal. All solutions were at physiological pH to reduce non-specific binding. Every third aliquot was screened for endotoxin at 1:50 dilution to determine when the column required regeneration. All aliquots used in vivo had protein concentration estimated by spectrophotometry and were tested independently for endotoxin as described.

2.2.2.4. Protein concentration

Formal protein concentration was also measured using the DC Protein Assay (Bio-Rad Laboratories, Hertfordshire, UK) according to the manufacturer’s instructions. This is a simple colorimetric assay based on colour change of Coomassie Brilliant Blue G-250 dye in response to different protein concentrations [201]. Protein concentration was assayed against a standard curve established with bovine standard albumin (BSA; Pierce, Thermo Scientific, IL, USA) from stock 2.0mg/mL in 0.9% NaCl solution containing sodium azide. A multi-well microtiter plate was marked and loaded with:

i. BSA 2.0mg/mL. Concentration estimate from spectrophotometry used to plan standard curve.

ii. PBS and dialysate control.

iii. Antibody purification product at different dilutions.

Serial dilutions of the BSA were performed to establish a standard curve, and 5µl from each well were transferred into a fresh well for the addition of 25µl Reagent A and
200µl Reagent B. The plate was agitated for 15 minutes before reading on a multi-well microtiter plate reader (Molecular Devices) at λ=650 nm. The protein concentration of the processed antibody was read against the standard curve.

2.2.2.5. Characterising Antibody: Silver stain gel

Polyacrylamide gel electrophoresis (SDS-PAGE) was used to investigate the purity of the processed antibody. Samples were prepared in non-reducing PAGE loading buffer (Thermo Fisher), heated to 100ºC for 3 minutes and loaded on to a non-denaturing pre-cast 10% polyacrylamide gel (BioRad) and run in Lamelli buffer (25mM Tris-base, 192 mM glycine, 0.1% SDS) at 120V for 1.5 hrs at 200mA. Molecular weight markers (Amersham International, UK) were run adjacent to the samples.

Following electrophoresis, the gels were silver stained using the ProteoSilver™ Silver Stain Kit (Sigma, Missouri, USA) according to the manufacturer’s instructions. The kit utilises silver nitrate which binds selective amino acids under weakly acidic or neutral pH conditions. Protein bound silver ions are reduced by formaldehyde at alkaline pH to form metallic silver. The method is highly sensitive, and can detect 0.1ng of BSA/mm². Briefly, gels were fixed for 20 minutes (Fixing solution: 50mL ethanol, 10mL acetic acid, 40mL ultrapure water) before washing (10 min) with 30% ethanol then ultrapure water, and treatment (each 10 min) with sensitizer then silver solutions. Finally gels were briefly washed with ultrapure water (1-1.5 min) and developed using extemporaneously prepared developer solution. Stop solution was added after 3-7 minutes. Gels were washed for 15 minutes with ultrapure water and could be stored wet. All steps were carried out at room temperature on an orbital shaker at 60-70rpm.
2.2.2.6. Characterising Antibody: Blocking Flow cytometry assay.

Murine splenocytes (C57BL/6, H-2b) were used to characterise processed 4F10 product by demonstrating CTLA-4 specific binding in a blocking flow cytometry experiment. Splenocytes from 6-8 week old untreated animals were processed as described in section 2.2.6.2, then suspended in PBS at 5x10^6 cells/mL. Cells were washed twice with ice-cold PBS between steps.

Aliquots of 5x10^5 splenocytes were first incubated for 30 minutes at 4ºC with:

- **positive control**: 1.5µl of 100µg/ml directly conjugated Armenian hamster monoclonal anti-CTLA4 (1B8) PE (abcam, Cambridge, UK),
- **negative control**: left unlabelled (no anti-hamster isotype available),
- **test samples**: processed anti-CTLA4 (4F10) antibody at 1:50 (2µl, 5.2µg), 1:100 (1µl, 2.6µg), and 1:200 (0.5µl, 1.3µg).

After washing, the controls were fixed in 100µl 0.1% formaldehyde in PBS, and the test samples incubated at 4ºC for 30 minutes with the same concentration of 1B8 PE, before further washing and fixation. Samples were analysed with a FACSCalibur flow cytometer and WinMDI 2.8. Cells were gated using forward scatter versus side scatter. Histograms of FL2 were plotted, and a 1% marker set on the PBS control was used to assess the percentage of positive events with each sample.

2.2.3. Flow cytometry: m5T4 expression analysis

Adherent cells were trypsinised and washed twice in PBS. Cell concentration was adjusted to 2x10^6/mL. Cells were suspended and washed in cold PBS and all steps were performed on ice. 100µL of suspended cells (2x10^5) were labelled with antibodies for 30 minutes at the following concentrations:

i. 9A7 (G Smethurst) 1:100; isotype rat IgG1 (Dako, Glostrup, Denmark) 1:100;
ii. B5C9 (D Shaw; DS) 1:100 and 1:400; isotype mouse IgG1 (Dako) 1:100;
iii. B3F1 (2.3mg/mL, 1.8mg/mL 13.12.06, DS) 1:500; isotype mouse IgG2α (Dako) 1:100;
iv. P1H10 (DS) 1:250 (no mouse IgG2β isotype available so PBS used).

Cells were washed twice before secondary labelling with RbαRat FITC (Dako) 1:40 or polyclonal rabbit anti-mouse immunoglobulins FITC (Dako) 1:40 for 30 minutes. Cells were washed twice and fixed in 100µL 0.1% formaldehyde in PBS. Cells were analysed on a FACSCalibur flow cytometer. Using WinMDI 2.8, cells were gated using forward scatter versus side scatter, and histograms of FL1 plotted. A 5% marker set on the isotype control was used to assess the percentage of positive events with each antibody, and subtracted from the percentage of positive events to give a final result.

2.2.4. Vaccine used

2.2.4.1. MVA.m5T4

The modified vaccinia virus Ankara (MVA) strain MVA.m5T4 was constructed, processed and titred within the Immunology group as described by Mulryan et al [202] using the MVA transfer vector plasmid plW-22 [203-204]. Additional supplies of vaccine were received from Oxford Biomedica (Oxford, UK). Control strain expressing Escherichia coli LacZ alone was constructed and processed using the same methodology [202].

2.2.4.2. Titration of MVA virus

Viral diluent: 100mL PBS with 1% FCS and 5mL 20% BSA.
BHK-21 cells were plated at 1x10⁵/well in a six well tissue culture plate and cultured for 24 hours, until monolayers were 80% confluent. Cells were incubated with serial dilutions of MVA (e.g. 10⁻¹ to 10⁻⁸) in 1mL viral diluent at 37°C for 45 minutes. The monolayer was washed 3 times with complete media to remove excess virus. Media was refreshed and cells incubated for a further 24 hours before titre was determined by:

i. Beta-galactosidase (β-gal) expression using X-Gal staining kit (Imgenex, CA, USA) according to the manufacturer’s instructions. *LacZ* gene codes for the hydrolase enzyme β-gal which acts on the colorimetric substrate X-Gal to create a blue die [205].

ii. m5T4 expression using B3F1 antibody. Briefly, monolayer cells were fixed by 2 minute incubation with 1mL 1:1 acetone methanol mix, then labelled sequentially with:

   a. 1:400 B3F1 in 1mL PBS for 45 min,

   b. 1:400 rabbit anti-mouse immunoglobulin HRP (Dako) in 1mL PBS/3% FCS for 1 hour.

All incubations were at room temperature, and the monolayer was washed twice with PBS between each step. Stain was developed using Vector Nova Red Substrate Kit (Vector Labs, CA, USA).

Equivalent titres were obtained using both methods, as illustrated in Fig 2.3 below.
Fig 2.3: Titration of MVA.m5T4 (Oxford Biomedica)

Figure shows light microscopy of BHK-21 cells cultured for 24 hours after incubation with MVA.m5T4 before titre determination by β-gal expression (blue) and m5T4 expression using B3F1 and rabbit anti-mouse immunoglobulin HRP (brown). Two concentrations are illustrated: 10⁴ above and 10³ below. Titre of this batch of MVA.m5T4 was 2.5 x 10⁸/mL by both methods. Illustration thanks to D.J.Burt.

2.2.4.3. Recombinant adenovirus expressing m5T4

A recombinant replication defective adenovirus expressing m5T4 (pAd.m5T4) was constructed, and processed within the Immunology group [206-207] using the Cre-lox recombination methodology first described by Hardy et al [208]. Viral titre was determined by BioRad DC Protein assay described in section 2.2.2.4. Absorbances were read at 650nm and titre calculated as:

1mg/mL = 3.4x10¹² particles = 3.4x10¹⁰ pfu/mL

As described by Ali et al [207]. pAd.GFP, used as a control vaccine, was grown in 293 cells and processed using the same methods.
2.2.5. In vivo experiments

2.2.5.1. Animals

BALB/c and C57BL/6 (B6) mice (female, 6-8 weeks old unless otherwise stated) were purchased from Harlan and housed under specific pathogen free conditions. All experimental procedures were conducted in accordance with the British Home Office guidelines. Experiments were conducted under British Home Office Project licences 40/2666 (expired Oct 08) and 40/3200 (expires Sept 13) by personal licensees Christy Ralph (40/8692) with support and supervision from Kate Mulryan (40/5695).

2.2.5.2. Kinetics of tumour models

Two groups of seven C57BL/6 mice were challenged with $5 \times 10^5$ tumour cells/200µl subcutaneously in the right flank, using either B16.m5T4 or TC-1. Simultaneously two groups of seven BALB/c mice were challenged with $5 \times 10^5$ tumour cells/200µl subcutaneously in the right flank, using either CT26.wt or EMT6. Tumours were measured in two dimensions three times a week from day 5, and animals were sacrificed when tumour reached an area of 1.24cm$^2$ or if they showed signs of suffering.

2.2.5.3. Active prime-boost vaccination schedule

Two groups of 10 C57BL/6 mice were challenged with $5 \times 10^4$ B16.m5T4 cells /200µl subcutaneously in the right flank. A specified number of days later (first experiment day 5; later experiments day 1), animals were vaccinated with either MVA.m5T4 (active vaccine) or MVA.LacZ (control vaccine) at a dose of $1 \times 10^7$pfu/100µl. Seven days later they received boost vaccination with either pAd.m5T4 (active) or pAd.GFP.
(control) at a dose of 1x10^9 pfu/100µl. All vaccines were given 50:50 by subcutaneous (sc) and intramuscular (im) routes.

In the initial experiment a further control group of 5 C57BL/6 mice were challenged with 5x10^4 B16m5T4 cells/ 200µl sc in the right flank, but did not get either active or control vaccines (tumour control).

In the later experiment two groups of 10 BALB/C mice were also challenged with 5x10^4 CT26.wt cells /200µl subcutaneously in the right flank and then treated with active or control vaccination using the doses and schedule above.

Tumours were measured in two dimensions three times a week from day 3, and animals sacrificed when tumour reached an area of 1.24cm^2 or if they showed signs of suffering.

Serum was harvested on days 4, 12 and 19 by pooling blood from tail bleeds for 5 animals in each group, and by terminal cardiac puncture following halothane anaesthesia in a number of animals at the time of sacrifice. B16.m5T4 and CT26.wt used for the in vivo tumour challenge were assessed contemporaneously for m5T4 expression using flow cytometry as described in section 2.2.3. In addition, tumour m5T4 expression was reassessed ex vivo after the second experiment. Animals from each experimental group were culled on day 18. Tumours were dissected post-mortem from the subcutaneous site in the test animals, harvested into PBS and weighed. Cells from each tumour were plated in 3mls DMEM supplemented with 10% FCS, 2mM L-glutamine, and 0.1 mg/ml penicillin and streptomycin in a six well plate, as both small tumour lumps and disaggregated suspension. They were cultured for 9 days until established in T75 flasks. They were trypsinised, and m5T4 expression was assessed by flow cytometry with the anti-m5T4 antibody B5C9.
2.2.5.4. Therapeutic combination immunotherapy model

Groups of 8 BALB/c animals were challenged with $5 \times 10^4$ cells CT26.wt sc in the right flank on day 0 and treated with:

i. nothing: tumour control.

ii. m5T4 heterologous prime boost vaccination alone: day 2 MVA.m5T4 (1$\times$10$^7$pfu/100µL sc & im) followed by day 9 pAd.m5T4 (1$\times$10$^9$pfu/100µL sc & im).

iii. anti-CTLA4 antibody alone: two doses of clone 4F10 (100µg in 100µL 0.9% NaCl intra-peritoneally, ip) on days 1 and 4.

iv. combination immunotherapy: MVA.m5T4/ pAd.m5T4 and peri-prime anti-CTLA4 antibody using the same agents, doses and schedules described above.

All animals were assessed throughout the experiment and tumours measured bi-dimensionally three times a week. Animals were sacrificed when surface area exceeded 1.24cm$^2$, or if they showed signs of suffering. Single animals in each group were sacrificed on day 16 for planned analysis of m5T4 specific immune responses.

2.2.5.5. Statistical analysis of in vivo experiments

Pseudo-survival was analysed in SPSS using standard Kaplan-Meier plots and the log rank test to test statistical differences between active therapy and control groups. In all experiments p<0.05 was considered significant.

Tumour growth was calculated by mean tumour surface area for each group. In some analyses, where indicated, sacrificed animals are censored at maximum tumour surface area. Graphs were plotted against time with error bars showing standard deviation (SD). Differences between groups were assessed using unpaired T test; p<0.05 was considered significant.
2.2.6. Assessment of murine immune responses

Immune responses were investigated in detail in non-tumour bearing animals. Animals were treated with different schedules of

i. Vaccines (all 50:50 sc & im):
   a. single pAd.m5T4 at 1x10⁹ pfu/100µl
   b. single and homologous boost MVA.m5T4 at 1x10⁷ pfu/100µl
   c. heterologous boost MVA.m5T4 (1x10⁷ pfu)/pAd.m5T4 (1x10⁹ pfu)
   d. heterologous boost pAd.m5T4 (1x10⁹ pfu)/ MVA.m5T4 (1x10⁷ pfu).

ii. anti-CTLA4 antibody (clone UC10-4F10-11) 100µg ip alone or in combination with vaccination. Anti-CTLA4 antibody was processed as described in section 2.2.2; additional commercially processed purified NA/LE anti-CTLA4 antibody (clone UC10-4F10-11; BD Pharmingen™) was also used where indicated at same dose and schedule. Purified NA/LE hamster IgG1, K isotype control (BD Pharmingen™) was used at dose of 100µg ip.

Animals were monitored for toxicity and sacrificed at between 1 and 6 weeks after treatment. Spleens and serum were processed and used to investigate immune responses as described below.

2.2.6.1. Preparation of serum samples

Serum was processed from whole blood. Samples were collected either by pooling venous tail bleeds for a group (3-5 animals) or by terminal cardiac puncture following halothane anaesthesia. Whole blood samples were allowed to settle at 37°C for 1 hour and then centrifuged at 300g for 5 minutes. Serum was separated from the red cell pellet, labelled and stored at -20°C for later assay.
2.2.6.2. Preparation of splenocytes

Murine splenocytes were prepared for all flow cytometry and in vitro experiments using the same protocol and reagents. Spleens were harvested immediately after death using an aseptic technique, and processed within a few hours to generate a single cell suspension of splenocytes. After physical disruption of the spleen by mashing through a 100µm nylon cell strainer (BD Falcon) in a small volume PBS and red cell lysis by 5 minute incubation at room temperature using buffered 0.1M ammonium chloride (BD Pharm Lyse™), cells were washed and resuspended in 20mL RPMI with 10% FCS before manual counting of viable cells using trypan blue exclusion (0.4%, Sigma) and a haemocytometer [202]. Cells were washed with ice-cold PBS between each step.

2.2.6.3. ELISA: m5T4 Antibodies

A mouse 5T4-IgG Fc fusion protein (Angela Eastham), consisting of the extracellular domain of m5T4 and the Fc of human IgG1 was used in an ELISA to detect anti-5T4 serological responses using the published method [202]. The mouse 5T4-IgG Fc fusion protein, consisting of the extracellular domain of m5T4 and the Fc of human IgG1, had been made using the eukaryotic expression vector sig-pIg [202]; it was used to coat ELISA plates at 1.0 µg/ml in 0.1M (pH9.5) carbonate buffer overnight. Plates were washed three times with PBS containing 0.05% (v/v) Tween 20 (PBS-T) between steps. Non-specific binding was blocked by incubation with 2% dried non-fat milk powder (Marvel, Premier Brands, UK) for 2 hours at 37°C. Triplicate doubling dilutions of sera (initially 1:250 to 1:32000, subsequently 1:10 to 1:1280) in PBS-T were incubated on the plates for 1 hour at 37°C before detection using HRP-labelled rabbit-antimouse immunoglobulin (1:1500; Dako) and acidified 3,3’5,5’-tetramethylbenzidine (TMB; Sigma). Absorbance was measured at \( \lambda_1 = 450 \text{ nm} \) and \( \lambda_2 = 650 \text{ nm} \) using a
precision multi-well plate reader (Molecular Devices), and the reading is given by $\lambda_1 - \lambda_2$.

**Controls:** All experiments included serum from untreated animals (negative control) and from known matched historical serum (BALB/c or C57BL/6 and C57BL/6^{m5T4-/-}; positive controls).

### 2.2.6.4. ELISPOT: murine Interferon-γ (IFN-γ)

A solid-phase enzyme linked immunospot assay (ELISPOT) allows sensitive enumeration of cytokine-producing cells in a single cell suspension [209]. During culture secreted cytokines are captured by coated antibody, avoiding diffusion or protease degradation in supernatant, or binding to soluble membrane receptors. After cell removal, the captured cytokines are revealed by tracer antibodies and appropriate conjugates [210].

Murine 5T4 (m5T4) shares an 81% amino acid sequence with the human homologue [211]. Individual peptides of 30 amino acids, overlapping by 15-22 amino acids, were designed to span the whole sequence of m5T4 and defined as 12 to 37, 23 and 34, the latter of which spans the transmembrane region, are insoluble and were not used. Sequences are shown in **Fig 2.4** below.

Binding buffer was made by mixing the following solutions 1:2, bicarbonate buffered to adjust pH to 9.6, filter sterilised and stored at 4°C:

- Solution 1: 0.1M Na$_2$CO$_3$IOH$_2$O
- Solution 2: NaHCO$_3$
Fig 2.4: Peptides of m5T4.

Amino acid sequences using standard nomenclature.

Prepared splenocytes were assayed in 96 well tissue culture plates (Immubilon P; Millipore, MA, USA) using the Murine IFN-γ ELISPOT kit (Diaclone, Besançon, France).
France) according to the manufacturer’s instructions. Plates were pre-coated with capture antibody for incubation overnight at 4°C. Non-specific binding was blocked by 3 washes with 200µl/ well PBS and incubation with 200µl RPMI + 10% FCS/ well at room temperature for at least 1 hour. Peptides of m5T4 were diluted in media to an appropriate concentration, and dispensed in triplicate in 50 µl/ well: stock solutions [0.2mg/mL], stored at -80°C, were thawed and diluted in RPMI to 90µg/mL to give a final concentration of 30µg/mL in the standard assay. Serial 1:2 dilutions allowed titration of response to between 30 and 1.87µg/mL peptide.

Splenocytes were prepared as described, and a single cell suspension in RPMI supplemented with 10% FCS, 2mM L-glutamine, and 0.1 mg/mL penicillin and streptomycin was made at an appropriate concentration (5x10⁶/mL unless otherwise stated), and 100µl/ well was dispensed. Controls were included on each plate:

**Negative:** Media alone

**Positive:** Concanavalin A (Con A; Sigma) is a lectin lymphocyte mitogen derived from Jack bean *Canavalia ensiformis*. Stock of 0.01mg/mL used at two doses of 2µl and 5µl per well.

**Additional negative** (where space allowed): non-reactive peptide (e.g. 12, 14)

Plates were incubated without moving in a humidified atmosphere of 5% CO₂ at 37°C for 24 – 48 hours before development.

When supernatant was saved for future ELISA, 50 µl/ well was removed by careful pipetting; otherwise, plates were emptied by flicking over a sink and careful tapping on absorbent paper. Plates were fixed by 10 minute incubation at 4°C with 100µl/ well 0.1% Tween (Sigma) in PBS, also used for all subsequent washes. After washing, reconstituted detection antibody was dispensed, and plates sealed and incubated for 90 minutes at 37°C. Plates were washed three times before 100µl/ well streptavidin-
alkaline phosphatase (diluted 1/5000 in 1% BSA in PBS) was dispensed and plates sealed and incubated for 60 minutes at 37°C. Plates were again washed three times before the development step which used ready-to-use BCIP/NBT and was stopped when spots became apparent after 2 – 10 minutes by multiple washes with distilled water. Plates were dried overnight before automated reading using a computer-assisted video-imaging analysis ELISPOT Plate Reader (AID Diagnostika GmbH, Strassberg, Germany). Triplicate wells were used to generate mean, standard deviation and standard error of mean results for each condition.

2.2.6.5. ELISA: Murine IL-2 (mIL-2)

A solid-phase sandwich ELISA was performed using the Murine IL-2 ELISA Kit (Diaclone) according to the manufacturer’s instructions. A monoclonal antibody specific for mIL-2 is pre-coated onto the wells provided. Briefly, the antigen and a biotinylated monoclonal antibody specific for mIL-2 are simultaneously incubated. The revelation steps include Streptavidin- horse radish peroxidise (HRP) and TMB as chromogen.

All reagents were prepared extemporaneously in clean pyrogen free glassware by combining with the appropriate diluents. Standards were prepared for each assay by serial 1:2 dilution of 500 pg/mL mIL-2 stock. Standard diluent buffer was assayed in blank wells. Media (RPMI + 10% FCS) was spiked with known concentration of mIL-2 standard stock to assess recovery. All incubations were carried out with the plate covered at room temperature (18-25°C). All washes used 3 times 300 µl/well washing buffer. 100µl of standards, blanks, controls and samples were added in duplicate to an appropriate number of wells. 50µl biotinylated anti-mIL2 was added to all wells and plates were incubated for 3 hours. Plates were washed before incubation with
streptavidin-HRP solution for 30 minutes. Plates were washed before 15-20 minute development in the dark by ready to use TMB; the colour development reaction was stopped by rapid pipetting of H$_2$SO$_4$ solution.

The absorbance of each well was read using a precision multi-well plate reader (Molecular Devices) with 450 nm as the primary wavelength and 620 nm (610-650) as the reference wavelength. A linear standard curve was plotted using the standard dilutions of mIL-2, and used to quantify mIL-2 in each sample. Mean values and standard deviations were calculated from duplicates.
CHAPTER 3: CLINICAL TRIAL RESULTS

3.1: Aims and objectives

Gastric and oesophageal adenocarcinomas are common tumours with poor prognoses. The majority of patients in the UK present with advanced disease, and have a median survival with combination cytotoxic chemotherapy of only 10 months [4]. There is no standard second-line therapy. Although a number of active agents have been investigated in this setting, there is no trial evidence confirming survival benefit over supportive care alone, and durable responses are rare [5]. A phase II, single-centre, open-label, non-randomised study was set up to investigate the use of tremelimumab, a fully humanised anti-CTLA4 antibody, as a second-line therapy for patients with metastatic gastric and oesophageal adenocarcinomas. The primary end-point was clinical anti-tumour efficacy as described by the objective response rate. The secondary objectives were both clinical and laboratory. The laboratory secondary objectives will be addressed in the next chapter. The clinical secondary objectives which will be addressed in this chapter were:

1. To assess additional evidence of anti-tumour activity by:
   a. further characterisation of objective responses (e.g. duration of response)
   b. progression-free survival
   c. overall survival
   d. serum tumour antigen responses.

2. To characterise the safety profile and tolerability of tremelimumab in this setting.
### 3.2: Patients and Treatment

#### 3.2.1. Patient characteristics

<table>
<thead>
<tr>
<th>Patient #</th>
<th>Age</th>
<th>Sex</th>
<th>Primary</th>
<th>Survival from diagnosis (m)</th>
<th>Sites of disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>41</td>
<td>M</td>
<td>O</td>
<td>10.27</td>
<td>L, N, liver, lung</td>
</tr>
<tr>
<td>2</td>
<td>49</td>
<td>M</td>
<td>O</td>
<td>24.60</td>
<td>L</td>
</tr>
<tr>
<td>3</td>
<td>63</td>
<td>M</td>
<td>GOJ</td>
<td>11.30</td>
<td>L, N</td>
</tr>
<tr>
<td>4</td>
<td>37</td>
<td>F</td>
<td>G</td>
<td>12.37</td>
<td>Duodenal mass, ascites, peritoneal, pelvic</td>
</tr>
<tr>
<td>5</td>
<td>55</td>
<td>M</td>
<td>O</td>
<td>7.33</td>
<td>L, N, liver, lung, bone</td>
</tr>
<tr>
<td>6</td>
<td>39</td>
<td>M</td>
<td>G</td>
<td>12.53</td>
<td>L, N</td>
</tr>
<tr>
<td>7</td>
<td>63</td>
<td>M</td>
<td>GOJ</td>
<td>11.93</td>
<td>L, N, lung</td>
</tr>
<tr>
<td>8</td>
<td>43</td>
<td>F</td>
<td>G</td>
<td>21.17</td>
<td>L, omental, serosal, ascites</td>
</tr>
<tr>
<td>9</td>
<td>57</td>
<td>M</td>
<td>G</td>
<td>10.53</td>
<td>L, N, pleural effusion, ascites</td>
</tr>
<tr>
<td>10</td>
<td>75</td>
<td>F</td>
<td>G</td>
<td>5.23</td>
<td>L, N, liver</td>
</tr>
<tr>
<td>11</td>
<td>49</td>
<td>M</td>
<td>O</td>
<td>7.67</td>
<td>L, adrenal, chest wall, muscle</td>
</tr>
<tr>
<td>12</td>
<td>67</td>
<td>M</td>
<td>O</td>
<td>12.83</td>
<td>L, N, liver, lung, adrenal, pleural, bone</td>
</tr>
<tr>
<td>13</td>
<td>75</td>
<td>M</td>
<td>GOJ</td>
<td>7.73</td>
<td>L, N, lung, pleural effusion</td>
</tr>
<tr>
<td>14</td>
<td>61</td>
<td>F</td>
<td>G</td>
<td>37.37</td>
<td>N, splenic</td>
</tr>
<tr>
<td>15</td>
<td>68</td>
<td>M</td>
<td>GOJ</td>
<td>6.27</td>
<td>L, N, liver, lung</td>
</tr>
<tr>
<td>16</td>
<td>41</td>
<td>M</td>
<td>GOJ</td>
<td>8.77</td>
<td>L, N</td>
</tr>
<tr>
<td>17</td>
<td>62</td>
<td>M</td>
<td>O</td>
<td>20.70</td>
<td>L (pericardium and diaphragm)</td>
</tr>
<tr>
<td>18</td>
<td>53</td>
<td>M</td>
<td>GOJ</td>
<td>7.50</td>
<td>L, liver, chest wall</td>
</tr>
</tbody>
</table>

**Table 3.1: Patient Demographics and Disease Burden at Screening.**

*Detail derived from screening data and review of clinical notes. “Survival is calculated from the diagnosis of advanced disease by biopsy or imaging to date of trial screening in months (m). Other*
Abbreviations: Gender M, male, F, female; Primary site O, oesophageal, G, gastric, GOJ, gastro-oesophageal junction; Sites of disease L, local; N, lymph node. Blue shading indicates patients with stable disease at first scan; red shading indicates the patient who developed fatal toxicity.

Between September 2006 and April 2007 18 patients were recruited and treated at the Christie Hospital, Manchester, UK. Individual characteristics are shown in Table 3.1.

In summary, the majority of the patients were male (n=14). The median age was 56 years, with a range from 37 to 75. All patients had metastatic oesophageal (n=6), gastric (n=6) or gastro-oesophageal junctional (n=6) adenocarcinomas. Median time from the diagnosis of advanced disease to screening was 10.5 months (95% CI 8.4 - 12.7 months).

At screening most patients had an ECOG performance status (PS) of 1 (n=14) and had disease-related symptoms. Only four patients had a PS of 0, of whom two remained in full-time employment throughout the trial (patients 4 & 16). Pre-treatment CT scans showed a significant burden of disease. A majority of the patients had local involvement visible on CT scan (n=16), measurable lymph node metastases (n=12), and/or visceral metastases (n=11) at screening. Three patients, all with gastric primaries (patients 4, 8, & 9), had infiltrative involvement of the bowel and evidence of ascites at screening.

As shown in Table 3.2 all patients had previously received treatment with appropriate cisplatin-based chemotherapy in a metastatic disease setting, and three had also received second-line chemotherapy. All patients had also received a fluoropyrimidine, either infusional 5-fluorouracil (10 patients) or oral agents capecitabine (7 patients) or S-1 (TS-1®, Taiho Pharmaceutical Co. Ltd., Tokyo, Japan), an unlicensed agent administered as part of a clinical trial. 5 patients had prior surgery with curative intent;
patients 12 and 18 also received neo-adjuvant chemotherapy, and patient 4 was treated with adjuvant chemo-radiation after gastrectomy. Patient 11 received 3 cycles of cisplatin and 5-fluorouracil with the intention of proceeding to potentially curative surgery but his tumour was deemed inoperable due to infiltration of the left main bronchus. The objective response rate to first line chemotherapy in the metastatic setting for these patients was only 28% (2 complete responses, 3 partial responses), with a 72% disease control rate (responses and stable disease).

<table>
<thead>
<tr>
<th>Previous treatment</th>
<th>No. of patients (n=18)</th>
<th>Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surgery</td>
<td>5</td>
<td>4,10,12,14,18</td>
</tr>
<tr>
<td>Chemoradiotherapy</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Adjuvant/ Neoadjuvant chemotherapy</td>
<td>4</td>
<td>11,12,18</td>
</tr>
<tr>
<td>Palliative 1st line chemotherapy</td>
<td>18</td>
<td>ECF (6) CF (4) MCX (4) ECX (3) CS-1 (1)</td>
</tr>
<tr>
<td>Palliative 2nd line chemotherapy</td>
<td>3</td>
<td>6,8,14</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>therapy</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Epirubicin; C, Cisplatin; F, 5Fluorouracil; M, Mitomycin; X, Capecitabine; S-1, an oral fluoropyrimidine; D, Docetaxel ; Ir, Irinotecan.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2: Previous treatment.

3.2.2. Patients declined trial entry

Simple demographics of the further 30 screened patients who did not enter the trial were similar to those who did; 70% were male, and median age was 61 years (range 29-76). Table 3.3 summarises the reasons patients were declined entry into the trial.
Entry was most commonly declined on the grounds of poor performance status (n=13), or patient preference (n=4). Two patients had a histological diagnosis of squamous cell carcinoma of the oesophagus, and one had a concomitant malignancy chronic lymphocytic leukaemia (CLL) which affects B lymphocytes.

<table>
<thead>
<tr>
<th>Reason for trial exclusion</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECOG PS&gt;2</td>
<td>13</td>
</tr>
<tr>
<td>Abnormal biochemistry</td>
<td>1</td>
</tr>
<tr>
<td>Patient declined</td>
<td>4</td>
</tr>
<tr>
<td>Squamous histology</td>
<td>2</td>
</tr>
<tr>
<td>Steroids</td>
<td>2</td>
</tr>
<tr>
<td>No RECIST lesion</td>
<td>2</td>
</tr>
<tr>
<td>Alternative treatment</td>
<td>2</td>
</tr>
<tr>
<td>History of colitis</td>
<td>1</td>
</tr>
<tr>
<td>Concomitant malignancy</td>
<td>1</td>
</tr>
<tr>
<td>Death during screening</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>30</td>
</tr>
</tbody>
</table>

Table 3.3: Summary of reasons screened patients were declined entry into the phase II trial of tremelimumab.

3.2.3. Treatment and follow up

Twelve patients received only a single cycle of tremelimumab. Five patients received two cycles of tremelimumab, and a single patient remains on treatment after eleven cycles (36.9 months). There were no dose reductions. No patients were lost to follow up and all were assessed for toxicity. A single patient was not objectively assessed for response, but had symptomatic progression by 3 months.
3.3: Toxicity

3.3.1. Toxicity: summary

Adverse events were recorded at baseline and at regular clinical review at least 2, 4, 8, and 12 weeks after each cycle of treatment. Grade was determined by NCI Common Toxicity Criteria (CTC) version 2. All adverse events without another sufficient explanation were deemed to be probably or possibly drug-related.

<table>
<thead>
<tr>
<th>CTC Toxicity</th>
<th>grade</th>
<th>cycle 1 (n=18)</th>
<th>cycle 2 (n=6)</th>
<th>cycle 3+ (n=1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Itch</td>
<td>1</td>
<td>8</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Rash</td>
<td>1</td>
<td>4</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Fatigue</td>
<td>1</td>
<td>5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Arthralgia</td>
<td>1</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nausea</td>
<td>1</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dizziness</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>Hypertension</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Atrial fibrillation</td>
<td>2</td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>AST increase</td>
<td>3</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Eosinophilia</td>
<td>1</td>
<td>6</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.4: Common and serious treatment-related toxicities for patients with metastatic gastric and oesophageal cancer receiving tremelimumab

*Toxicity data was analysed on 8.12.09 and table shows data for all 33 completed cycles of tremelimumab. N indicates number of patients. Only one patient received more than 2 cycles of tremelimumab. Abbreviation: AST, aspartate aminotransferase.*

All serious or common tremelimumab-related toxicity is summarised by cycle in Table 3.4 and shown graphically by patient in Fig. 3.1. Most toxicities were grade 1. There was one toxic death from bowel perforation complicating diarrhoea (grade 5). There
was no drug-related bone marrow toxicity. Anaemia was common, but apparently disease related, increasing with disease progression: 44% patients had mild (G1/2) anaemia at baseline, 77% by the end of treatment, and there were 3 episodes of gastrointestinal haemorrhage, one of which proved fatal. Post mortem examination in this patient, who died unexpectedly at home, showed an ulcerative gastric tumour with clotted blood in the gut. Three patients experienced no drug-related toxicity. The commonest toxicities were immune mediated but mild [151, 160]: itch (50% of patients); rash and eosinophilia (both 33%); and diarrhoea and fatigue (both 28%). Despite the single fatality, tremelimumab was well tolerated by most patients.

Fig 3.1: Common and serious treatment-related toxicities after tremelimumab by patient number affected.

Patients with metastatic oesophageal and gastric adenocarcinomas received tremelimumab until symptomatic disease progression. Adverse event data was collected and assessed as described in the methods. Toxicity (worst grade) is presented by the number of patients affected. AST: aspartate aminotransferase. Updated 8.12.09
3.3.2. Toxicity: Rash

The rash was an itchy maculopapular eruption, generally distributed on the torso and upper arms, and is illustrated in Fig. 3.2 which shows symmetrical distribution across the back of patient 15. Photographs show recurrence of rash during second cycle of treatment, and were taken 56 days after second cycle of tremelimumab.

Fig 3.2: Tremelimumab-related rash.
The rash usually appeared within two to four weeks after infusion of tremelimumab, and was managed when necessary with simple emollients and mild steroid cream. It resolved in all patients but recurred in subsequent cycles of treatment. Patient 12, for example, had documented grade 1 itch in 6/9 cycles, associated with visible rash in 3/9 cycles, despite prophylactic use of emollients.

### 3.3.3. Toxicity: Diarrhoea

Most diarrhoea was mild and self-limiting but a single patient (number 11) developed colitis which initially responded to systemic steroids, but relapsed and was complicated by fatal bowel perforation, which has been described as a rare class toxicity with anti-CTLA4 blockade [177].

![Leucocyte count changes with toxicity (patient 11)](image)

**Fig 3.3: Leucocyte count changes with toxicity (patient 11)**

*Leucocyte counts are derived from clinical blood results undertaken at the Christie hospital laboratory. Normal ranges are: neutrophils 2.0-7.5x10⁹/L, lymphocytes 1.5-4.0x10⁹/L, monocytes 0.2-0.8 x10⁹/L. Time line shows days from tremelimumab treatment. Graph also shows duration of diarrhoea symptoms (all grades) and arrows indicate initiation of systemic corticosteroids.*
Fig 3.3 shows the initial neutrophilia, and later lymphophilia associated with diarrhoea symptoms in patient 11. Diarrhoea began around 22 days after tremelimumab but was initially mild and managed as an out-patient with loperamide and oral rehydration. On deterioration, the patient was admitted on day 35, and had an immediate response to systemic corticosteroids with cessation of all symptoms. Diarrhoea recurred almost immediately on stopping steroids, and worsened rapidly, occurring 7-10 times a day. Unfortunately the patient failed to contact the clinical team as instructed. At emergency admission four days later, he was dehydrated and shocked with a blood pressure of 89/60 and tachycardia of 130. He was treated with intravenous fluids, broad spectrum antibiotics and re-introduction of steroids. His clinical condition initially stabilised, then deteriorated 36 hours later on day 53 with the onset of new abdominal pain. CT scan confirmed clinical suspicion of bowel perforation, showing marked dilatation of the colon, intra-mural gas in the right hemi-colon and free air in the peritoneal cavity. The patient had a surgical review and was transferred to the High Dependency Unit, but after extensive discussion with him and his family, was managed conservatively with fluids, antibiotics and symptomatic measures. He died two days later (day 55).

Repeated microscopy and culture of both stool and blood did not identify an infectious organism, and testing for Clostridium difficile toxin was negative. Post mortem examination showed multiple areas of haemorrhage and mucosal ulceration throughout the large bowel from caecum to rectum, with friable bowel wall and at least 3 small perforations with copious blood and faecal stained fluid in the peritoneal cavity. Tumour was confined to the known sites of disease: locally in the oesophagus infiltrating into the bronchus, adjacent lymph nodes, and in metastatic subcutaneous
and adrenal deposits. There was no evidence of tumour in the large bowel, and histology of the bowel showed features consistent with mucosal ulceration and active chronic inflammation.

3.3.4. Toxicity: Transaminitis

The other two tremelimumab toxicities greater than grade 3 were transient. Patient 4 had asymptomatic transaminitis (G3) as illustrated in Fig. 3.4; serum bilirubin peaked on day 56 of the first cycle of tremelimumab at 42 umol/L, alkaline phosphatase (Alk.Phos.) at 792 IU/L, and aspartate aminotransferase (AST, also called aspartate transaminase) at 499 IU/L, before resolving spontaneously to baseline within 10 days.

Fig 3.4: Transient transaminitis after tremelimumab (patient 4).

*Biochemistry results are derived from clinical blood tests undertaken at the Christie hospital laboratory. Graph plots serum Alkaline phosphatase (AlkPhos; normal range 25-110 IU/L) and aspartate aminotransferase (AST; normal range 5-45 IU/L) on the left axis and serum bilirubin (Bili; normal range 1-20 umol/L) on the right axis against time after first tremelimumab treatment. Patient was re-treated on day 71.*
Patient 4 had no evidence of viral hepatitis or liver metastases, and signs of liver inflammation did not recur on re-treatment (day 71). Imaging (both ultrasound and CT scan) showed mild dilatation of the common bile duct and some mucosal thickening at the duodenum which probably represented recurrent disease, but the latter was also present on baseline investigations. As Fig 3.4 shows, both aspartate aminotransferase and bilirubin levels were only elevated during this brief episode. In contrast, alkaline phosphatase and gamma glutamyl transpeptidase (GGT; normal range 0-65 IU/L) were elevated prior to treatment in this patient, probably reflecting disease involvement. Patient 4 had evidence of disease progression on CT scan at day 159 after a second cycle of treatment, and developed rapidly progressive disease after withdrawal from the study, which is reflected by rapidly rising alkaline phosphatase after day 190.

3.3.5. Toxicity: Cardiovascular effects

Tremelimumab caused infusion-related hypertension in three patients. Patient 12 required anti-hypertensive medication after cycle 4 when his blood pressure was sustained at 193/102; the other episodes resolved spontaneously within a few hours. Two patients had new onset of atrial fibrillation with no clear precipitant, though as both episodes occurred towards the end of the treatment cycle, causality remains unclear.
3.4: Response

3.4.1. CT Responses

In order to address the primary objective of the trial, which was to assess anti-tumour efficacy of tremelimumab in second-line treatment of metastatic gastric and oesophageal cancer, objective responses were assessed by Response Evaluation Criteria in Solid Tumours (RECIST) [183] using repeated CT scans after each cycle of tremelimumab.

**Fig 3.5A** shows axial CT scan of the pelvis at two levels pre-treatment (left: i, ii) and after one cycle of tremelimumab (right: iii, iv). The patient is supine, and the bony pelvis can be used for orientation. A large adnexal metastasis can be clearly seen in the right hand side of the pelvis on all images, and is indicated by a red arrow in the upper two images. Oral contrast (white) can be seen within loops of bowel, and is more prominent after reduction in the size of the metastasis.
There were no objective responses by RECIST at the end of the first cycle of tremelimumab. However, 4 patients had stable disease with clinical benefit, including weight stabilisation, pain resolution, cessation of vaginal bleeding from a pelvic metastasis, and return of libido. One patient (12) went on to achieve durable benefit with incremental reduction in tumour burden with each cycle of treatment and a RECIST partial response after 25.4 months (8 cycles) of treatment. **Fig 3.5.A** illustrates the reduction in size of a large pelvic metastasis (13.1 x 8.8 cm) after a single cycle of tremelimumab for patient 4. **Fig 3.5.B** shows maximum dimension of this RECIST marker lesion on serial CT scanning before, during and after the trial. Tremelimumab was given on days 5 and 76 after screening. Following symptomatic disease progression at the end of the second cycle of treatment, the patient was taken off study on day 167 and referred for pelvic surgery. Measurements from the pre-
operative scan suggest there was accelerated tumour growth after withdrawal of tremelimumab.

3.4.2. Survival

Median time to progression was 2.83 months (95% CI 2.65-3.02), and median overall survival was 4.83 months (95% CI 4.06-5.59) reflecting the rapid course of progressive disease in the majority of patients. A minority of patients survived longer than expected: 12 month survival was 33% (95% CI 14-55 by Kalbfleisch and Prentice method), and 15 month survival was 17% (95% CI 4-37%). Median overall survival for those patients with stable disease at first scan was 12.2 months (95% CI 4.6-19.8; p=0.085). The one patient with a durable response remains alive, well, and on treatment, after 36.9 months (31/12/09); he remains well more than four years after screening (Jan 2011).

Two exploratory survival comparisons were performed to provide a context for the trial overall survival results in this poor prognosis setting. For initial comparison Kaplan Meier survival is plotted in Fig 3.6 for those patients who entered the trial (solid line) and those declined entry at screening (dotted line). Median overall survival for the latter group of patients was reduced, at 3.3 months (95% CI 2.4-4.1, log rank p=0.028). Poorer survival of these patients probably reflects the fact that many were appropriately excluded due to poor fitness (i.e. PS≥2). Of the six patients in this group who survived for more than six months, two had declined trial entry, two had no measurable disease due to complete response to palliative chemotherapy, one was on long-term steroids for respiratory disease and only one was excluded due to poor PS.
Fig 3.6: Overall survival (1)

*Shows Kaplan Meier survival plot for the patients who entered the trial (solid line) and those declined entry (dotted line). One patient with apparently synchronous CLL and metastatic gastric cancer was excluded from the analysis. Survival was censored on 8.12.09 for the one survivor. Median overall survival was 4.8 months (95% CI 4.1-5.6) for the treated patients and 3.3 months (2.4-4.1) for the untreated patients (log rank p=0.028).*

Information derived from a contemporary audit of second-line chemotherapy for advanced gastric and oesophageal cancer prescribed between June 2001 and May 2007 at the Christie hospital was also used for comparison. Patients with advanced gastric and oesophageal cancer treated with second line chemotherapy within the Christie were initially identified through pharmacy records before careful scrutiny of the notes. 20 patients were identified by the audit: one was excluded from further analysis as first line therapy was high risk adjuvant consolidation after radical gastrectomy and omentectomy, and one because he was treated within the tremelimumab trial.

Audit patients treated with second line chemotherapy had comparable demographics to the trial patients: median age was 65 years (range 38-80), 89% were male, and
primary sites were gastric (n=8), junctional (n=3) and oesophageal (n=7). Performance status using the Karnofsky scale (KP) [212] was recorded for 16 of the patients as: 90 (n=3), 80 (n=10), 70 (n=1), 50 (n=1) and 30 (n=1). Karnofsky performance status 70-90 corresponds to ECOG PS 0-1 [213], and so with two exceptions the patients were judged to have comparable fitness to those who entered the trial of tremelimumab. Audit patients had received first line treatment with ECF (n=10), MCF (n=5), or MX, MCX, Cis5FU (1 each). Objective response rate was 33% (6 PR) and disease control rate 50% (9 of 18).

![Fig 3.7: Overall survival (2)](image)

**Fig 3.7: Overall survival (2)**

Kaplan Meier survival plot for the patients treated with tremelimumab in the trial (solid line) and those treated with 2nd line cytotoxic chemotherapy identified in a contemporary audit (dotted line). Survival was censored on 8.12.09 for the one survivor. Median overall survival was 4.8 months (95% CI 4.1-5.6) for the patients treated with tremelimumab and 5.0 months (3.1-6.9) for the patients treated with chemotherapy (log rank p=0.44).

Most patients received a combination of irinotecan and infusional fluorouracil given by the modified de Gramont regime (15), whilst single individuals received MCX,
MCF, and irinotecan combined with capecitabine. Objective response rate to second line chemotherapy was only 6%: 1 PR for 12 patients with documented CT results and 4 with clinical deterioration which made scanning inappropriate. One patient had chemotherapy stopped after two cycles following a myocardial infarction, but survived 5.8 months. The final patient survived 7.8 months. Three trial patients and two identified in the audit went on to have further third-line systemic therapy.

Comparison of the survival curve for tremelimumab trial patients with that for patients treated with second line chemotherapy (Fig 3.7) shows no significant difference in median survival between the two groups (log rank p=0.44). At 6 months, survival was the same for both groups (44%; 95% CI 22-65), but at 12 month survival was lower for the 2nd line chemotherapy patients at 11% (95%CI 2-30) compared to those treated with tremelimumab (33%; 95% CI 14-55). Of interest, only 3 of 10 audit patients who survived beyond six months had a primary diagnosis of oesophageal adenocarcinoma, and none of these survived beyond 9 months. These exploratory survival analyses must be interpreted with caution given the small number of patients in all groups and the absence of randomisation, and mindful of the risks of selection bias and interpretation of retrospective case review data, but do provide a near contemporaneous context for the interpretation of patient survival within the trial. Importantly, on the basis of the available evidence, patients do not appear to have been disadvantaged by trial enrolment. Only a randomised trail design could be used to prove this.

3.4.3. Serum Tumour antigen responses

Serum tumour associated antigen (TAA) levels were used as a supplementary method for assessing response. In a number of solid tumours circulating TAA levels are
routinely used to monitor disease recurrence and treatment response in, e.g. CA-125 for ovarian cancer [65]. Although still an exploratory tool for assessing disease burden in advanced oesophageal and gastric cancer, quantification of serum TAA circulating in the peripheral blood is simple and reproducible, and seemed a pertinent measure in an immunotherapy trial. Two serum TAA levels were measured monthly throughout the trial:

Carbohydrate antigen 19-9 (CA19-9) and

Carcinoembryonic antigen (CEA).

Questions remain over the sensitivity and specificity for both markers, and they are not diagnostic as both can be raised in other malignancies and a number of non-malignant conditions, such as autoimmune pancreatitis, cholestasis and interstitial lung disease in the case of CA19-9. However, in pancreatic cancer, changes in CA19-9 in the early weeks following chemotherapy appear predictive of response, and overall survival [66]. CEA has established diagnostic and prognostic significance in colorectal cancer [58] where measurement has become part of the routine assessment of advanced disease during treatment and remission [214-215]. CEA is also widely expressed in oesophageal and gastric adenocarcinomas, being found in up to 86% of biopsies [59] and in the serum of nearly half of patients with advanced disease.

Elevated serum tumour associated antigen levels were found in 13 patients pre-treatment: 8 CA19-9 in 11 patients (median 131 units/mL, range 35-15091 units/mL), and CEA in 6 patients (median 86 µg/L, range 12-2537 µg/L). Marginal elevation of

8 Only two patients never showed raised serum levels of either tumour antigen throughout the trial; both had gastric cancer with peritoneal distribution of disease and malignant ascites.
CEA is seen in some smokers. Patient 2, a heavy smoker, had CEA levels of 4-5 µg/L in all blood samples which were not judged to be clinically significant.

Fig 3.8: Serum CA19-9 after tremelimumab

Serum TAA CA19-9 was measured monthly from peripheral venous blood samples of patients with advanced metastatic gastric and oesophageal cancer treated in the phase II trial of tremelimumab. Normal range at the Christie hospital biochemistry laboratory was 0-31 units/mL. Graphs show results for all patients with elevated CA19-9 except 12: top graph (A) shows levels above 5000U/mL, bottom (B) those below 400U/mL.

Fig 3.8 and Fig 3.9 show changes in serum CA19-9 and CEA respectively with tremelimumab for all patients except 12 which are presented in section 3.4.4.2.
the first cycle of tremelimumab serum tumour antigen levels increased in 10 of these
patients and decreased in 3. For the minority of patients with increased serum levels of
both TAs, changes occurred in parallel. One patient (number 18) developed
increased serum CA19-9 and CEA de novo by day 60 of the first cycle. Two patients
(2 & 4) had mildly increased levels of CA19-9 alone at day 60 but later samples were
within the normal range. Median overall survival for patients with stable or falling
serum tumour antigens was 12.20 months (95% CI, 7.84-16.59) compared to 4.6
months (95% CI, 3.45-5.75) for those whose levels rose (log rank p=0.03).

Fig 3.9: Serum CEA after tremelimumab

Serum TAA CEA was measured monthly in peripheral venous blood samples of patients with
advanced metastatic gastric and oesophageal cancer treated in the phase II trial of tremelimumab.
Normal range at the Christie hospital biochemistry laboratory was 0-3µg/L. Graph show results for
all other patients with elevated CEA except 12.

3.4.4. Patient 12

In this study one patient (12) has experienced a remarkably durable objective response.
He remains alive, well, and on treatment, after 36.9 months (31/12/09). His clinical
results are discussed below. Such long-lived benefit is remarkable in this poor
prognosis group. Literature review did not identify any reports of spontaneous remission in metastatic oesophageal adenocarcinomas. No clinical features at screening identify this patient as unusual, and some, such as the presence of multiple visceral metastases, have been reported to predict poor prognosis [216]. Review of pathology from his primary surgery (Ivor Lewis oesophagectomy) shown in Fig 3.10 did not identify any unusual features: resected tumour was a moderately to poorly differentiated mucinous adenocarcinoma, stage pT2 N1. His best response to first-line chemotherapy was only stable disease, and at screening he was underweight with a body mass index of 16.2, and had symptomatic disease progression with recurrence at his surgical anastamosis, nodal, liver, lung, adrenal, and bone metastases, and increasing serum CEA (2537 µg/L) and CA 19-9 (2333 units/mL).

![Fig 3.10: Histology of primary oesophageal adenocarcinoma: patient 12](image)

**Fig 3.10: Histology of primary oesophageal adenocarcinoma: patient 12**

*Pictures show haematoxylin and eosin stained sections of the oesophagectomy specimen for patient 12. Left hand picture low magnification view of the tumour shows glandular architecture and mucin secretion. Right hand picture shows higher magnification view of tumour cells and reactive lymphocytes. Pathology review and photographs thanks to Dr Bipasha Chakrabarty, Consultant Histopathologist, at the Department of Histopathology, the Christie NHS Trust.*

3.4.4.1. **Patient 12: CT scan response**

Patient 12 was one of four individuals who had stable disease by RECIST on CT scan after the first cycle of tremelimumab. He continued to have repeat imaging after each
cycle of treatment. He achieved an objective partial response (i.e. at least a 30% reduction in the sum of the longest diameter of target lesions with reference to baseline pre-treatment scan) incrementally over 24.8 months, as shown in Fig 3.11 B. This response has proven durable (>12 months) and is still ongoing.

![CT scan response after tremelimumab (patient 12)](image)

**Fig 3.11: CT scan response after tremelimumab (patient 12)**

*A* shows pre-treatment (left) and recent (right; post cycle 10, 32.4 months) axial CT scans of the abdomen for patient 12 illustrating the reduction in bulky intra-abdominal disease (red arrow). *B* shows cumulative reduction in measurable disease with 12 cycles of tremelimumab.
3.4.4.2. Patient 12: Serum tumour marker response

As part of the histology review for patient 12, new immunohistochemistry of the primary oesophageal tumour removed during surgery with curative intent prior to trial involvement showed strong staining for CEA as shown below. At the time of screening, patient 12 showed high serum levels of both CEA and CA19-9.

![Image of Immunohistochemistry of primary oesophageal adenocarcinoma.](image)

**Fig 3.12: Immunohistochemistry of primary oesophageal adenocarcinoma.**

Photograph show immunostained section of the oesophagectomy specimen for patient 12. Tumour (left; red arrow) stains strongly for CEA using M7072 antibody (Dako) and there is some positive staining of the bowel lumen (right) which acts as an internal control. Pathology review and photographs thanks to Dr Bipasha Chakrabarty, Consultant Histopathologist, at the Department of Histopathology, the Christie NHS Trust.

Patient 12, who went on to achieve durable objective benefit after tremelimumab, had the most marked fall in serum tumour antigen levels, as shown in **Fig 3.13.** CEA and CA 19-9 both peaked at day 30 of the first cycle of tremelimumab, at 3071μg/L and 2785 units/mL respectively before falling to 56μg/L and 34 units/mL respectively by the end of the first cycle. By the end of the second cycle serum levels of both markers were just above the normal threshold, and this effect has been sustained out to beyond 36 months.
Fig 3.13: Patient 12: serum tumour antigen response

Serum TAA CA 19-9 and CEA were measured monthly in peripheral venous blood after treatment with tremelimumab. Normal ranges: CA19-9 0-31 units/mL, CEA 0-3 µg/L.

3.5: Summary

This phase II study of anti-CTLA4 antibody tremelimumab as a second line therapy in patients with metastatic oesophageal and gastric adenocarcinomas had a negative primary end point: the objective response rate in this cohort of patients was only 5%. However, there was clinical benefit with evidence of disease control in a small cohort of patients, as assessed by stable disease at 3 months on CT scan (4 of 18) and/or decline or stabilization of serum TAA (5 of 18). In this study, the one partial response was achieved incrementally over 24.8 months, with very different kinetics from cytotoxic chemotherapy responses, and has proven remarkably durable.

Median overall survival was 4.83 months for all patients, and 12.2 months for those who had stable disease following tremelimumab. The one patient with a durable
response was alive, well, and on treatment, after 36.9 months, when the data was analysed (31/12/09), and remains so now nearly 4 years after he entered the trial: this response is so unusual in this poor prognosis disease that despite its isolation it does provide anecdotal evidence of anti-tumour activity with tremelimumab.

Tremelimumab was well-tolerated in the majority of these patients, despite increasingly symptomatic disease. The single treatment-related death was due to bowel perforation complicating auto immune colitis, a recognized complication of CTLA-4 blockade with a frequency of 4 of 700 patients (0.6%) at doses of ipilimumab of 3 mg/kg or more [217]. This study is clearly too small to characterize whether the frequency of severe colitis and perforation varies with tremelimumab and gastro-esophageal primary disease but there was no increased frequency of diarrhea (all grades) or evidence of upper gastro-intestinal inflammation at gastroscopy, which several patients required for management of dysphagia.

Despite the attractions of randomized control trials, an open-label, non-randomised phase II design was appropriate, achievable, and ethical as a means of investigating a novel immunotherapy approach in this poor prognostic group of patients. With the low but remarkably durable response to tremelimumab observed, practical clinical use of this agent in patients with metastatic and oesophageal adenocarcinomas would need to be targeted to potential responders. Such targeting of tremelimumab to potential immune responders requires a better understanding of the effects and kinetics of CTLA4 blockade in treated patients, an area explored in the next chapter.
CHAPTER 4: TRIAL LABORATORY RESULTS

4.1: Brief introduction

The phase II trial of anti-CTLA4 antibody tremelimumab as second-line therapy in patients with metastatic gastric and oesophageal cancer included planned laboratory investigation of changes in lymphocyte phenotype and function after anti-CTLA4 blockade. The main aims were:

1. To assess changes to lymphocyte phenotype after anti-CTLA4 blockade, and to identify potentially immunoregulatory phenotypes, including natural Treg.
2. To assess changes to lymphocyte function after CTLA4 blockade, looking particularly for any increase in specific anti-tumour activity, using responses to two relevant tumour associated antigens (TAA): 5T4 and CEA.
3. To correlate any changes in lymphocyte phenotype and function with clinical outcome.

4.2: National Blood Service (NBS) Results

4.2.1. Pre-treatment phenotype

4.2.1.1. Pre-treatment absolute counts

Absolute granulocyte, monocyte and lymphocyte phenotype counts were investigated contemporaneously. Paired fresh samples, i.e. pre- and post-treatment, were available for the last 12 patients (nos.7-18). Pre-treatment absolute counts were available for a further three patients (4-6) but are excluded from later pooled analyses as discussed in
section 4.2.2. Median absolute counts at baseline for all available samples (n=15) are shown in Table 4.1 below by phenotype: median absolute lymphocyte count was 1.02 x10^9/L (IQ range 0.90-1.28), compared to 1.10 x10^9/L (IQ range 0.92-1.40) for those patients (n=13) with paired samples. Range (0.66-3.00 x10^9/L) was unchanged.

| Cell type                  | Absolute count *10^9/L | Baseline |  |  |
|----------------------------|------------------------|----------|  |  |
|                            | median                 | IQ range | patient 11 | patient 12 |
| Granulocytes               | 4.42                   | 2.90 – 6.08 | 7.113 | 4.76 |
| Monocytes                  | 0.64                   | 0.33 – 0.73 | 0.649 | 0.729 |
| Lymphocytes                | 1.02                   | 0.90 – 1.28 | 3.003* | 1.0185 |
| CD19⁺                      | 0.07                   | 0.04 – 0.12 | 0.24 | 0.014** |
| CD16⁺CD56⁺                 | 0.19                   | 0.16 – 0.26 | 0.225 | 0.136 |
| CD8⁺                       | 0.31                   | 0.19 – 0.46 | 0.848* | 0.487 |
| CD4⁺                       | 0.41                   | 0.35 – 0.65 | 1.809* | 0.406 |
| CD4⁺CD25<sub>high</sub>    | 0.020                  | 0.016 – 0.034 | 0.097* | 0.025 |

Table 4.1: Baseline leucocyte count

Results are shown for contemporaneous analysis of leucocyte phenotype by flow cytometry. Median and inter-quartile (IQ) range are shown for patients 4-18; individual values are shown for patients 11 and 12. Key: * highest count; ** lowest count.

4.2.1.2. Variation in pre-treatment absolute counts

Seven patients, who had all signed additional consent, had two pre-treatment samples analysed. Fig 4.1 shows screening and pre-treatment results for the patients for whom two baseline samples were available. These were taken a median of 7 days apart (range 2-14 days), and show a small amount of variation but no significant differences between the earlier and later pre-treatment samples. For example, mean change in absolute lymphocyte count was +0.01x10^9/L (range -0.21 to +0.12 x10^9/L) or 0.9%.
Fig 4.1: Variation in baseline lymphocyte phenotype

Absolute cell counts for paired screening (S) and pre-treatment (T) samples for seven trial patients (numbers 11-17) with two baseline samples available are shown. NBS data is used; lymphocyte phenotype markers shown in the key.

4.2.1.3. Pre-treatment phenotype: patients 11 and 12

Baseline counts for two patients are shown in both figures. Patient 11, who went on to develop fatal toxicity, had the highest pre-treatment absolute lymphocyte, CD3⁺CD8⁺, CD3⁺CD4⁺ and CD4⁺CD25^{high} counts of all patients investigated, although two patients had higher pre-treatment relative CD4⁺CD25^{high} expression (5.2% and 3.3% of total lymphocytes for patients 4 and 6 compared to 3.2% for patient 11). In contrast, patient 12, who went on to have durable benefit from treatment, had average absolute counts of all these lymphocyte markers prior to treatment, but the lowest count of CD19⁺ lymphocytes (i.e. B lymphocytes; 0.014 x10⁹/L).
4.2.1.4. Pre-treatment phenotype: variation with age

The four patients with the lowest pre-treatment CD19$^+$ counts were aged 62-75 years; in contrast the three patients with the highest CD19$^+$ counts were all under 50 years of age (patients 4, 11, and 16; 0.25, 0.24 and 0.28 $10^9$/L respectively). As shown in Fig 4.2 below, splitting the group by median age (56 years), demonstrates a significant difference in median baseline CD19$^+$ count: 0.12 $10^9$/L for younger patients versus 0.04 $10^9$/L for older patients (p=0.0037 by Mann Whitney U).

![Chart showing baseline variation CD19$^+$ between patients by age.

Fig 4.2: Baseline variation CD19$^+$ between patients by age.

Absolute CD19$^+$ count for 15 trial patients with metastatic gastric and oesophageal adenocarcinoma prior to trial treatment derived from contemporaneous flow cytometry data split by median age (56 years); individual results (♦) and medians (−) are shown. Lowest result is for patient 12.

There were no significant differences in absolute granulocyte, monocyte, total lymphocyte, or CD4$^+$, CD8$^+$, and CD16$^+$CD56$^+$ lymphocyte subsets by age. However, the subset of CD4$^+$CD25$^{high}$ lymphocytes was significantly different with age, with median 0.034 $10^9$/L for younger patients compared to 0.016 $10^9$/L for older patients (p=0.009).
4.2.2. Changes in phenotype with tremelimumab

Variation in absolute granulocyte, monocyte, and lymphocyte counts with anti-CTLA4 blockade was investigated for the last 12 patients (7-18) where paired pre- and post-treatment samples were available: data were pooled for day 0 (n=12), day 15 (n=10), day 30 (n=12), day 60 (n=9), and day 90 (n=8). Pre-treatment absolute counts were available for a further three patients (4-6) but are excluded from pooled analyses due to missing post-treatment samples (5) or outlying results due to proven bacterial sepsis (6) or toxicity (4).

There was no significant change in median granulocyte or monocyte count with tremelimumab. Total lymphocyte count, and CD8⁺ lymphocyte count (median 0.35 x10⁹/L) did not vary with tremelimumab, but median CD4⁺ lymphocyte count rose transiently from 0.39 x10⁹/L to 0.72 x10⁹/L at day 15 (p=0.009). Within this population, there was an increase in CD4⁺CD25⁺ cells from 0.02 x10⁹/L to 0.035 x10⁹/L at day 15 (p=0.003) which was maintained until day 30 before falling. Results are shown in detail in Table 4.2 below.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Median absolute count *10⁹/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>Day 15</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>4.36</td>
</tr>
<tr>
<td>Monocytes</td>
<td>0.54</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>1.10</td>
</tr>
<tr>
<td>CD19⁺</td>
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</tr>
<tr>
<td>CD16⁺CD56⁺</td>
<td>0.19</td>
</tr>
<tr>
<td>CD8⁺</td>
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</tr>
<tr>
<td>CD4⁺</td>
<td>0.39</td>
</tr>
<tr>
<td>CD4⁺CD25⁺</td>
<td>0.020</td>
</tr>
<tr>
<td>CD4⁺CD25⁺</td>
<td>0.107</td>
</tr>
</tbody>
</table>

Table 4.2: Summary changes in leucocyte phenotype (NBS data)

Median absolute cell counts for baseline pre-treatment, and post-treatment (day 15 and 30) samples are shown for twelve trial patients (numbers 7-18) with paired samples available. NBS data obtained from contemporaneous flow cytometry is used. Significance was tested by Wilcoxon signed-rank, and is shown where p≤0.05.
Absolute CD4⁺CD25⁺ and CD4⁺CD25^{high} counts rose with tremelimumab in all patients except patient 11, as is illustrated for CD4⁺CD25^{high} counts in Fig 4.3 below. Patient 12, who went on to have substantial clinical benefit from treatment, appears to show a sustained rise in CD4⁺CD25^{high} lymphocytes for 60 days after first infusion, in contrast to most of the other patients for whom samples are available.

**Fig 4.3: Absolute CD4⁺CD25^{high} count with tremelimumab (7-18)**

*Individual and median absolute CD4⁺CD25^{high} cell counts for the first cycle of tremelimumab are shown for twelve trial patients (numbers 7-18) with paired samples available. NBS data obtained from contemporaneous flow cytometry was used, but re-analysed to gate CD4⁺CD25^{high} lymphocytes. Counts for patient 11 (◊) and 12 (♦) are shown.*

### 4.2.3. Changes in phenotype with tremelimumab toxicity: patient 4

As discussed in the clinical results, patient 4 developed transient, asymptomatic elevation of bilirubin and a number of liver enzymes in keeping with an immune-mediated transaminitis which has been described after CTLA-4 blockade [147]. Serum bilirubin peaked at 42umol/L, alkaline phosphatase at 792 IU/L, and aspartate aminotransferase (AST) at 499 IU/L on day 56 of the first cycle of tremelimumab, before resolving spontaneously to baseline within 10 days. **Fig 4.4** illustrates that
transaminitis was accompanied by significant rises in absolute counts of CD3⁺CD8⁺, CD3⁺CD4⁺, CD4⁺CD25<sup>high</sup>, and CD16⁺CD56⁺ lymphocytes. Absolute lymphocyte count rose from 0.96 to 2.07 x10⁹/L and the percentage of CD4⁺CD25<sup>high</sup> lymphocytes rose from 5.2 to 42.9%. It is likely in this context that CD4⁺CD25<sup>high</sup> lymphocytes represent recently activated rather than regulatory cells. Small rises in absolute counts of monocytes and CD19⁺ lymphocytes were also seen at the same time, and are omitted for clarity. A smaller rise in granulocytes (from 3.08 to 3.99 x10⁹/L) did occur, but as numbers remained within the normal range, and subsequently rose to similar levels later in the trial, the relationship with transaminitis is less convincing. These results support an immune-mediated or inflammatory aetiology for this tremelimumab toxicity.

**Fig 4.4: Lymphocyte phenotype changed with toxicity in patient 4**

NBS data obtained from contemporaneous flow cytometry is plotted against days after first tremelimumab infusion for patient 4. Duration of CTC grade 3 liver toxicity is shown by bold line; the patient was re-treated on day 71 with no recurrence. Samples from days 15 & 30 were missed.
4.2.4. Changes in phenotype with multiple cycles of tremelimumab: patient 12

As discussed in the clinical results, only a minority of patients (6) received more than a single cycle of tremelimumab, and only one (patient 12) received more than two. He remained on tremelimumab after eleven cycles (32.7 months), which allowed more detailed analysis of changes in leucocyte phenotype with the 90 day cycle of treatment. 39 samples were available in total: for day 0 (n=8), day 15 (all day 14, n=5), day 30 (days 28-30, n=9), day 60 (days 51-63, n=10) and day 90 (days 78-91, n=7).

![Fig 4.5: CD3⁺CD4⁺ lymphocyte count with tremelimumab: patient 12](image)

*Absolute counts (NBS data) obtained from contemporaneous flow cytometry are plotted against days after tremelimumab infusion (treatment cycle) for patient 12. First cycle (□) and subsequent cycles (▲) are shown. All 39 available samples are illustrated.*

For patient 12, absolute lymphocyte count, CD3⁺ lymphocyte count, and CD3⁺CD4⁺ lymphocyte count all follow the same pattern of change with the cycle of tremelimumab treatment, but this pattern is most marked in CD3⁺CD4⁺ lymphocyte counts illustrated above in Fig 4.5. Absolute lymphocyte counts were normally distributed for this patient, allowing illustration of mean results. CD3⁺CD4⁺ counts...
rose significantly from a mean 0.47 x10^9/L on the day of treatment to 0.63, 0.65, and 0.60 x10^9/L on days 15, 30 and 60 (p=0.012, p=0.002, and p=0.007 respectively by unpaired T test) before falling slightly at the end of treatment. In contrast, CD3\(^+\)CD8\(^+\) lymphocytes do not rise significantly, being mean 0.47 x10^9/L on the day of treatment compared to 0.55 x10^9/L thirty days later (p=0.08). **Fig 4.6** below shows the pattern of change in CD4\(^+\)CD25\(^{high}\) lymphocytes, which rise on day 15 of the first cycle but do not show a significant rise in mean count across all cycles of treatment (0.023 to 0.030 x10^9/L at day 15, p=0.058); results for day 15 have to be interpreted cautiously as there are fewest samples for this time-point.

**Fig 4.6: CD4\(^+\)CD25\(^{high}\) lymphocyte count with tremelimumab: patient 12**

*Absolute counts (NBS data) obtained from contemporaneous flow cytometry are plotted against days after tremelimumab infusion (treatment cycle) for patient 12. First cycle (□) and subsequent cycles (▲) are shown. All 39 available samples are illustrated.*
4.3: Regulatory lymphocyte phenotype

4.3.1. Pre-treatment regulatory lymphocyte phenotype

Baseline samples were obtained for later flow cytometry analysis of regulatory lymphocyte phenotype for all patients. FoxP3$^+$ in CD4$^+$CD25$^{\text{high}}$ subset was initially used to define regulatory T lymphocytes (Treg). Increased frequency of Treg have been documented in the peripheral blood of cancer patients [186]. Treg frequency, defined by CD4$^+$CD25$^{\text{high}}$FoxP3$^+$ in baseline samples for trial patients with gastric and oesophageal adenocarcinomas were compared with normal volunteers (n=9) and patients with renal cell carcinoma (n=6). Results are shown below in Fig 4.7.

![Figure 4.7: Treg in peripheral blood: cancer patients and healthy volunteers](image)

Flow cytometry analysis of thawed, cryopreserved PBMC, defined Treg by CD4$^+$CD25$^{\text{high}}$FoxP3$^+$. Figure shows results expressed as a percentage of total lymphocytes for trial patients with gastric and oesophageal adenocarcinoma (n=18) compared to normal volunteers (n=9) and patients with renal cell cancer (n=6). Individuals (♦), including patient 12 (♦), and medians (▬) are shown for each group.

Median Treg (FoxP3$^+$) was significantly higher for the trial patients than normal volunteers both as a percentage of total lymphocytes (1.43 vs. 0.27; p=0.0006 by Mann Whitney U test, median difference 1.05 (95% CI 0.33-2.12)) and as a percentage of
CD4⁺ lymphocytes (3.42 vs. 0.91; p=0.0015). The small group of renal cancer patients also had higher median Treg than the normal volunteers (0.73 vs. 0.27 % total lymphocytes; p=0.025), but there was no significant difference between the two groups of cancer patients. Duplicate baseline results from screening and pre-treatment bloods were only available for four patients (11, 13, 14 & 16) but showed no significant change in Treg between the two time-points (median 0.75 v. 0.70 % total lymphocytes by FoxP3).

An exploratory analysis of the small group of trial patients detected no differences in Treg numbers by primary site (gastric/ oesophageal/ junctional), disease burden (presence/ absence of visceral metastases), or survival from diagnosis of metastatic disease. As Fig 5.8 shows, a trend towards difference was noted when the group was split by median age, but unexpectedly, the younger patients had higher Treg (median 2.24 vs. 0.55 % lymphocytes; p=0.053).

Fig 4.8: Treg in peripheral blood: baseline analysis of trial patients by age.

Flow cytometry analysis of thawed, cryopreserved PBMC, defined Treg by CD4⁺CD25⁺FoxP3⁺. Figure shows results expressed as a percentage of total lymphocytes for trial patients with gastric and oesophageal adenocarcinoma (n=18) split by median age (56 years). Individuals (♦) and medians (▬) are shown for both groups.
4.3.2. Changes in regulatory lymphocyte phenotype with tremelimumab

For comparative analysis of regulatory phenotype results from the first cycle, data were pooled for day 0 (n=18), day 15 (n=17), day 30 (n=17), day 60 (n=13), and day 90 (n=9). For each patient, cryopreserved PBMC were thawed and analysed by flow cytometry on the same day. The gating used to determine CD4⁺CD25high and CD4⁺CD25low/negative and determine CTLA4⁺ lymphocytes in both subsets is shown in Fig 4.9 using patient 12 as an example.

Fig 4.9: Flow cytometry analysis of peripheral blood mononuclear cells.

Patient 12 is shown as an example: lymphocytes are gated against forward and side scatter, then against CD4⁺CD25high (R3) and CD4⁺CD25low/negative (R2). The upper right quadrant shows CD4⁺CTLA4⁺ lymphocytes in the gated population as a percentage of total lymphocytes. Pre-treatment levels of CTLA4⁺ lymphocytes (i) rise in both populations at day 15 (ii).

As Fig 4.10 shows, there was a strong correlation between the proportion of CD4⁺CD25high lymphocytes expressing FoxP3 or CTLA4 (tau=0.45, p<0.0001).
Fig 4.10: Treg: CD4⁺CD25^{high} lymphocytes expressing FoxP3 or CTLA4.

Flow cytometry analysis of cryopreserved PBMC, defined Treg in CD4⁺CD25^{high} by FoxP3 (y axis) and CTLA4 (x axis) for first cycle (n=76). Graph shows correlation between the two markers.

The changes in patient lymphocyte phenotype with tremelimumab treatment are summarized in Fig 4.11-4.12. 1.4% lymphocytes were Treg pre-treatment, defined by both FoxP3 and CTLA4 markers in CD4⁺CD25^{high} subset, and rose transiently to 3% and 3.2% respectively on day 15 (p<0.005) before returning to baseline by day 60.

CTLA4 is also found on activated T cells. A more sustained rise with tremelimumab was seen in these CD4⁺CD25^{low/negative}CTLA4⁺ lymphocytes, from 5.0% lymphocytes pre-treatment to 8.9–10.4% from day 15 to 90 (p<0.005; Fig 4.11 Bii). This population may represent potential effector lymphocytes.
**A** FoxP3 expression

![Box plot showing FoxP3 expression over treatment cycle (days)](image)

* *p<0.05

**Bi** CTLA4 expression in CD4+CD25^{high}

![Box plot showing CTLA4 expression in CD4+CD25^{high}](image)

**Bii** CTLA4 expression in CD4+CD25^{low/negative}

![Box plot showing CTLA4 expression in CD4+CD25^{low/negative}](image)

**Fig 4.11: Flow cytometry analysis of PBMC with tremelimumab: FoxP3 and CTLA4.**

Flow cytometry analysis of cryopreserved PBMC defined Treg in CD4^{+}CD25^{high} by FoxP3 (A) and CTLA4 (Bi), and assessed CTLA4 in CD4^{+}CD25^{low/negative} subset (Bii). Graphs show changes in lymphocyte phenotype after treatment with tremelimumab for all patients. Pooled data are expressed as box and whisker plots of median expression with quartile range and minimum and maximum results marked. Statistical significance is by Wilcoxon signed-rank test.

**4.3.3. Changes in regulatory lymphocyte phenotype: patient 12**

For patient **12**, who had the best response to tremelimumab, and was the only patient to receive more than two cycles of treatment, regulatory phenotype was analysed in
thawed PBMC for the first three cycles of treatment over two consecutive days: A (n=5), B (n=4) and C (n=3). The results for CTLA4 are shown below in Fig 4.12. The pattern of change is variable for CD4^+CD25^{high}CTLA4^+ lymphocytes, but the percentage of these cells does rise transiently above baseline at early time points (day 15 or 30) in both cycles for which data is available. A clear pattern is seen in CD4^+CD25^{low/negative}CTLA4^+ lymphocytes with the cycle of tremelimumab: CD4^+ CD25^{low/negative}CTLA4^+ lymphocytes rise from baseline to day 30 and then decline, but only to near baseline after the first cycle. Mean results are 3.96% of lymphocytes at baseline, rising to 5.94%, 8.69%, 7.47% and 6.12% at days 15, 30, 60 and 90 respectively. This result is consistent with the pattern of change seen across the first cycle of treatment in all patients. It is also consistent with the pattern of change seen in absolute CD3^+CD4^+ lymphocyte counts across 11 cycles of tremelimumab for patient 12 by contemporaneous flow cytometry as discussed in section 4.2.4.

(1) CD4^+CD25^{high}
Fig 4.12: Flow cytometry analysis of patient 12 PBMC with tremelimumab: CTLA4.

Flow cytometry of cryopreserved PBMC analysed CTLA4+ after cell permeabilisation in (1) CD4+CD25^{high} and (2) CD4+CD25^{low/negative} lymphocytes. Results are shown for available samples from patient 12 for first three cycles of tremelimumab treatment labelled A (black marker), B (grey marker), and C (white marker).

4.3.4. Further characterization of FoxP3+ T regulatory lymphocytes

Analysis of regulatory markers FoxP3 and CTLA4 in CD4+CD25^{high} lymphocytes in this study of patients with metastatic oesophageal and gastric adenocarcinomas show transient but significant rises in the population of these putative Treg with anti-CTLA4 blockade using tremelimumab. However, in man, neither marker is restricted to Treg lymphocytes: FoxP3 can be transiently induced in effector CD4+ lymphocytes after activation, with peak expression at 4-6 days after in vitro activation [218]. In contrast to activated effectors, Treg do not produce effector cytokines such as IL-2 and IFN-γ [219]. It has recently been shown that induced FoxP3+ lymphocytes which expressed high levels of effector cytokines were not functionally suppressive, and that
the combination of FoxP3 and cytokine staining is therefore useful for further distinguishing primary Treg from activated effector lymphocytes [220]. Therefore, the ability of in vivo expanded FoxP3⁺ lymphocytes to produce effector cytokine IL-2 was assessed to further characterise these cells. Matched frozen PBMC from four patients (nos. 11, 12, 15 & 16) pre-treatment and 15 days post-treatment were thawed and activated with either plate bound anti-CD3/CD28 or PMA and ionomycin in the presence of Golgi Plug. After 6 hours, cells were stained for surface markers and FoxP3 and IL-2 as described in section 2.1.4.1.

Below, Fig 4.13 shows representative flow cytometry plots for two patients: patient 12, who had durable clinical benefit from treatment, and patient 15, who experienced asymptomatic disease progression by the end of the first cycle of tremelimumab, and went on to receive a further cycle of treatment, surviving 17.1 months from trial screening. As expected, prior to treatment, IL-2 secreting lymphocytes did not express FoxP3, and FoxP3 positive cells did not secrete any IL-2, confirming they were bona fide primary Treg.

Samples taken 15 days after tremelimumab showed stable or increased FoxP3⁺IL2⁻ Treg with no detectable levels of FoxP3⁺IL2⁻ lymphocytes in any samples. Pooled results for all four patients showed a trend towards increased FoxP3⁺IL2⁻ Treg with tremelimumab treatment from mean 6.0% lymphocytes pre-treatment to 7.2% 15 days after first infusion (paired t test p=0.010).
Fig 4.13: Analysis of FoxP3 expression and IL-2 release in PBMC.
Flow cytometry analysis was performed in cryopreserved PBMC stimulated with plate bound anti-CD3/28 antibodies for 6 hours before surface and intracellular markers were determined. Plots show representative results for patient 12 (above) and 15 (below). A(12) and A(15) show gating strategy and percentage CD3+CD4+ and CD3+CD4- (i.e. CD8+) in pre-treatment (i) and day 15 (ii) samples for patients 12 and 15 respectively. B(12) and B(15) show percentages of FoxP3+IL2 Treg, FoxP3IL2-Teff and FoxP3+IL2+ lymphocytes in CD3+CD4+ (1st and 3rd columns) and CD3+CD4- (2nd and 4th columns) for pre-treatment (i) and day 15 post-treatment (ii) samples. B(12) and B(15) show results for isotype stain (first row), and FoxP3 and IL2 staining following in vitro stimulation in the absence (second row) and presence (third row) of 15µg/mL tremelimumab.

Furthermore, addition of 15µg/mL tremelimumab in vitro did not reduce the proportion of FoxP3+IL2 Treg in either pre- or post-treatment samples, which were mean 6.0% vs. 6.3% at day 0 and 7.2% vs. 7.5% at day 15 in the absence or presence of in vitro drug. There was a trend towards increased IL2 secretion with the addition of 15µg/mL tremelimumab in vitro in both CD3+CD4+ and CD3+CD4- (i.e. CD8+) lymphocytes in the baseline samples: from 2.0% to 2.9% and 2.6% to 3.3% respectively (paired t tests: p=0.055 and p=0.070), which would be in keeping with published results in normal donors which show a more robust effect at 24 hours [221].

4.3.5. Changes in regulatory lymphocyte phenotype with tremelimumab: PD1

Programmed cell death 1 (PD1) is a cell surface protein which shares structural and functional features with CTLA4 and CD28 [119, 222]. It has a role in the regulation of lymphocyte activation: expression is induced by T cell receptor (TCR) and B cell receptor signalling, and enhanced by tumour necrosis factor. PD1 is inducibly expressed on a wide range of haematopoietic cells including CD4+, CD8+, and NK T cells, B cells, and monocytes. PD1 interacts with two ligands:

(1) PD-L1, which is ubiquitously expressed on T cells, B cells, mast cells, macrophages and non-haematopoietic cells such as those of the epithelium;
(2) PD-L2, which has more restricted expression on DC and macrophages. The interaction of PD1 and its ligands has a role in the induction and maintenance of peripheral T cell tolerance and may protect peripheral tissues from autoimmune attack. One group reported increased expression after CTLA4 blockade in those who experienced autoimmunity without clinical benefit compared to other groups [179].

Additional flow cytometry analysis using samples from the first eight trial patients investigated the frequency of cell surface PD1 with tremelimumab in thawed cryopreserved PBMC; samples for patient five were not viable after analysis. For comparative analysis, data were pooled for day 0 (n=7), day 15 (n=5), day 30 (n=7), and day 60 (n=7). Results are shown in Fig 4.14 below.

**Fig 4.14: Flow cytometry analysis of PBMC with tremelimumab: PD1.**

*Flow cytometry analysis of cryopreserved PBMC assessed PD1 in total lymphocytes without permeabilisation. Graph shows change in lymphocyte phenotype after treatment with tremelimumab for patients 1-8. Pooled data are expressed as box and whisker plots of median expression with quartile range and minimum and maximum results marked. No significant differences.*
There was wide variation in baseline PD1: median 5.8% lymphocytes, range 2.2-18.6%. Although differences were not statistically significant, the same pattern of change described for the other regulatory markers was again seen, with a rise in PD1 following tremelimumab treatment, from median 5.8% lymphocytes to 16.8% and 13.0% on 15 and 30 days after tremelimumab respectively.

4.4: Lymphocyte function: proliferative response to TAA

4.4.1. Lymphocyte proliferative responses to 5T4

Lymphocyte proliferative responses to 5T4 were investigated in all study patients using pooled peptides. From the first cycle of treatment, at least 3 time points were available for all patients, and a complete data set (5 time-points) was available for 10. From the second cycle of treatment, 1 (n=2), 3 (n=1) or 4 (n=2) time-points were available.

Fig 4.15: Lymphocyte proliferative responses to 5T4.
Graph shows example proliferative response to 5T4 peptides for patient 12, with de novo responses to QRST at days 120 (SI 4.82) and 150 (SI 2.82). Days after second cycle of tremelimumab (B) are shown in brackets. Error bars show SEM. SI= stimulation index.
6 of 18 patients had pre-existing baseline responses, and 8 of 18 had at least one de novo post-treatment response. Clinical characteristics were similar for those with and without baseline responses, except that more were female: 3 of 6 compared to 1 of 12. There were small but not significant increases in baseline median absolute counts of lymphocytes (1.24 compared to 0.99 x10^9/L), monocytes (0.64 vs. 0.44 x10^9/L), and granulocytes (5.0 vs. 3.5 x10^9/L) in those with pre-existing responses, but no difference in absolute counts of CD4^+CD25^{high} lymphocytes, nor of FoxP3 or CTLA4 within this subset. Those with pre-existing responses to peptides of 5T4 appeared to have higher baseline CD19 counts: 0.175 vs. 0.043 x10^9/L. Pre-treatment responses occurred with equal frequency against all the peptide pools except IJKL and VWX. Post-treatment responses occurred against all peptide pools, but were most frequent against QRST (5/18), IJKL and VWX (3/18 each). The timing of post-treatment responses was complex: median time to first post-treatment response was 35 days but the range was large (19–120 days), and three patients did not develop a de novo post-treatment response until after day 90, including patient 12 whose results are shown in Fig 4.15 above. Responses were most frequent around 30 days after tremelimumab, with 3 responses at this time after the first cycle and 2 responses after the second cycle.

4.4.2. Lymphocyte proliferative responses to CEA

Lymphocyte proliferative responses to CEA were investigated in 15 patients (all except 3, 5 & 6) where material was available using the whole protein. From the first cycle of tremelimumab, at least 3 time points were available for 14 patients, and a complete data set (5 time-points) was available for 7 patients. The second cycle of
tremelimumab was also investigated in three patients, using 2 (n=1), or 4 (n=2) time-points.

Fig 4.16: Lymphocyte proliferative responses to CEA: patient 17

_Graph shows example proliferative response to CEA protein for patient 17. Error bars show SEM. Insufficient cells from day 28 for CEA 300ng assessment. Dotted line at stimulation index 2._

2 of 15 patients had pre-treatment responses, and 5 of 15 had post treatment responses to CEA. Only one patient showed both pre- and post-treatment responses. Patient 17 had strong pre-treatment responses to 200ng and 300ng CEA with stimulation indices (SI) of 2.55 and 2.48 respectively; as Fig 4.16 shows, these responses were augmented at day 28 (100ng CEA, SI 3.91) and 56 (100ng CEA, SI 3.38; 200ng, SI 3.75⁹; 300ng, SI 5.31). For all patients, post-treatment responses were most frequent mid-cycle: 3 responses occurred 30 days after first cycle, 2 at 60 days after the first cycle, and 1 at 30 days after the second cycle of tremelimumab. In the

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⁹ This result fails to meet pre-determined criteria for response: SI ≥ 2 and > 2 x baseline, but is included as a clear dose response can be seen.
subset of patients with raised serum CEA at baseline (n=5), none had a pre-treatment proliferative response to CEA, but two developed a proliferative response post-treatment at day 30 (nos. 7 & 12). In both cases this apparently precedes a fall in serum CEA, albeit of very different magnitudes: 6% for patient 7 and 85% for patient 12. **Fig 4.15** shows responses to 5T4 and **Fig 4.17** shows responses to 200ng and 300ng CEA during the first two cycles of treatment for patient 12 who had durable benefit from treatment: significant responses to CEA occur at day 30 of both cycles of tremelimumab, and to 5T4 at day 30 and 60 of the second cycle. Only three patients had post-treatment responses to both TAAs, and whilst only one of these experienced durable benefit from treatment, the other two (nos. 7 & 15) did have stable visceral disease on CT scan and survived for 7.27 and 17.20 months respectively.

![Graph showing lymphocyte proliferative responses to CEA: patient 12.](image)

**Fig 4.17: Lymphocyte proliferative responses to CEA: patient 12.**

*Graph shows example proliferative response to CEA protein for patient 12, with responses to 200ng at day 30 of the first (SI 3.24) and second cycles (SI 2.91). At the later time point a strong response was also seen to 300ng (SI 4.53). Days after second cycle of tremelimumab (B) are shown in brackets. Error bars show SEM.*
4.4.3. Proliferative responses to TAA and survival

The patients were stratified post-hoc by first cycle post-treatment proliferative responses to CEA in order to explore any relationship between the in vitro data and outcome. Median survival for the responders was 17.1 months (95% CI 7.9-26.2 months) compared to 4.6 months (95% CI 3.9-5.3 months) for the non-responders (p=0.002) as shown in Fig 5.18 below. Parallel stratification by first cycle post-treatment proliferative responses to any pool of 5T4 showed no relationship with overall survival.

![Graph](image)

Fig 4.18: Overall survival by lymphocyte proliferative response to CEA

*Graph shows Kaplan-Meier overall survival (in months) by de novo post-treatment lymphocyte proliferative response to CEA (n=15). Responders (n=5) are shown by bold line; non-responders (n=10) by dotted line. Single surviving patient (12) censored in Nov 2009.*

4.5: Lymphocyte function: antibody response to TAA

Translational work within the group had previously explored the development of 5T4 specific antibodies in cancer patients treated with a 5T4-targeted vaccine [54]. Early work in prostate cancer patients has suggested the development of anti-NY-ESO
antibodies may be clinically relevant after treatment with ipilimumab [223]. As the development of such anti-tumour antibodies targeting a relevant TAA is a potential mechanism or marker of immune efficacy of anti-CTLA4 blockade, available plasma samples for the patients who had signed the relevant addendum in the consent form, were sent to Oxford BioMedica to measure 5T4 antibodies using their published method [190-191]. They have defined the criteria for a positive antigen-specific 5T4 antibody response by (1) defining background level of 5T4 antibody responses in plasma recovered from 50 nominally healthy volunteers such that less than 5% would be classified as positive, which is at a cut-off 12.77 relative units (RU) and (2) determining variation in 5T4 antibody responses in cancer patients not receiving 5T4 targeted therapy over time which allows determination of a relevant fold increase. Samples were available for 16 patients for day 0 (n=16), day 15 (n=15), day 30 (n=16), day 60 (n=12) and day 90 (n=11). Results are shown in Table 4.3 below.

<table>
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Table 4.3: Antigen-specific 5T4 antibody responses after tremelimumab

Table shows antigen-specific 5T4 antibody responses before (day 0) and after tremelimumab treatment for 16 patients with metastatic gastric and oesophageal adenocarcinomas. Individual and mean values
for each time point are included; relative units (RU) are used. \( D \) = day (after treatment), \( R \) = responder phenotype defined by result >12.77 RU and fold change from baseline.

Eight of the sixteen patients had baseline antigen-specific 5T4 antibody responses above the normal serum threshold of 12.77 RU, but there was no apparent relationship between baseline responses and any clinical features such as age, survival from diagnosis to trial entry, or subsequent progression free or overall survival. All but three patients had a rise in antigen-specific 5T4 antibody response at day 30, albeit of different strengths; mean response rose slightly from 29.69 RU to 34.48 RU (t test \( p=0.027 \)). Five of the patients are identified by established criteria as having a responder phenotype: nos. 6, 10, 13, 14 and 18. All of these individuals had progressive disease at first CT scan, and none received a second cycle of tremelimumab; only one (patient 14) survived beyond 4.83 months, which was the median overall survival for the trial. Thus it does not appear that the development of antigen-specific 5T4 antibody responses reflects treatment response either in terms of objective measurement or overall survival in these patients with gastric and oesophageal adenocarcinomas treated with anti-CTLA4 blockade.

4.6: Lymphocyte function: cytokines

4.6.1. Effect of tremelimumab on cytokine release after in vitro stimulation

To explore the effect of tremelimumab on T lymphocyte responsiveness, PBMC were stimulated with anti-CD28 and anti-CD3 before harvesting supernatant for cytokine ELISA (n=15). Median baseline IL-10 release was 1254 pg/mL (IQ range, 581–2479) and, as Fig 4.19 shows, did not change with treatment.
Fig 4.19: Post-stimulation IL-10 with tremelimumab

Graph shows post-stimulation IL-10 measured by ELISA (pg/mL) with tremelimumab treatment for available patients (n=15). Pooled data are expressed as box and whisker plots of median expression with inter-quartile range (box) and minimum and maximum results (lines) marked.

Analysis of the results shows no significant pattern of change in either cytokine with treatment, although median pre-treatment IL-2 increased from 153 pg/mL (IQ range, 64 – 419) to 328 pg/mL (IQ range, 143-646) at day 30 (p=0.15) as shown below in Fig 4.20.

Fig 4.20: Post-stimulation IL-2 with tremelimumab

Graph shows post-stimulation IL-2 measured by ELISA (pg/mL) with tremelimumab treatment for available patients (n=15). Pooled data are expressed as box and whisker plots of median expression with quartile range (box) and minimum and maximum results (lines) marked.
Fig 4.21 shows changes in post-stimulation IL-2 and IL-10 across two cycles of tremelimumab for patient 12 who achieved durable benefit from treatment: there is no clear pattern of change in IL-2 release, although all post-treatment results are lower than baseline, but IL-10 release does appear to rise with both cycles, from baseline 1826pg/mL to 2832 and 2397pg/mL on day 60 first cycle and day 30 second cycle respectively. In the absence of a clear pattern for the group, these changes may just reflect the influence of inter-current events, such as presence of side effects, or recent immune exposure of infectious agents, rather than any direct effect of the drug.

![Graph showing cytokine levels](image)

**Fig 4.21: Post-stimulation cytokines with tremelimumab: patient 12**

Post-stimulation IL-2 and IL-10 measured by ELISA (pg/mL) with tremelimumab treatment are shown for patient 12 across two cycles of treatment. Timing of re-treatment is indicated by the arrow.

### 4.6.2. Further analysis of baseline cytokine release after in vitro stimulation

Median pre-treatment IL-2 release was higher for those who later had stable disease at first CT scan (399 pg/mL; IQ range 304–611) compared to those with disease progression and no significant toxicity (73 pg/mL; IQ range 17–136, p=0.054 by Mann Whitney U test). Median baseline IL-10 release was also marginally higher for
those who later had stable disease at first CT scan (med 1599 pg/mL; IQ range 1348-2152) compared to those with disease progression (925 pg/mL; IQ range 392-3062) but the difference does not approach significance due to the wide spread of results for those with subsequent disease progression. Baseline cytokine release is shown for all tested patients in **Fig 4.22** (IL-2) and **Fig 4.23** (IL-10) below.

**Fig 4.22: Baseline post-stimulation IL-2**

Baseline post-stimulation IL-2 measured by ELISA (pg/mL) by objective clinical response to tremelimumab treatment (n=15). CT PD = disease progression and CT SD = stable disease on first post-treatment CT scan. Results for patient 11 (red) and 12 (white) are highlighted. Median result for all patients with disease progression was 77 pg/mL; IQ range 31-268.

**Fig 4.23: Baseline post-stimulation IL-10**

Baseline post-stimulation IL-10 measured by ELISA (pg/mL) by objective clinical response to tremelimumab treatment (n=15). CT PD = disease progression and CT SD = stable disease on
first post-treatment CT scan. Results for patient 11 (red) and 12 (white) are highlighted. Three patients released >4000 pg/mL IL-10: 1, 3, and 11.

As illustrated in Fig. 4.24 below, the four patients with the highest IL-2 levels included two with stable disease at first scan (nos. 2 & 12) and the patient who developed fatal toxicity (no. 11). Moreover, when median pre-treatment IL-2 release after T cell activation was used to divide patients with high and low IL-2 responses, there was an increased time to progression (5.1 months (95% CI 1.1-9.0) vs. 2.8 (95% CI 2.6-2.9), Mantel Cox p=0.014) and a trend towards increased overall survival (median 12.2 months vs. 4.7, p=0.27) with high baseline IL-2.

Fig 4.24: Baseline post-stimulation IL-2 release against overall survival

4.6.3. Cytokine release ex vivo

To explore the effect of tremelimumab on T lymphocyte responsiveness ex vivo, cytokine concentrations in thawed plasma samples were assessed using a sensitive flow cytometric cytokine bead array. Results are available for 10 patients at baseline (n=8),
and 15 (n=8), 30 (n=9), 60 (n=4) and 90 (n=5) days after first infusion of tremelimumab. Cytokines investigated included Th1 cytokine IFN-γ, Th2 cytokines IL-4, IL-5 and IL-10, and IL-2 which has a broader role in lymphocyte activation. Only low levels of cytokines were detected from plasma separated from un-stimulated PBMC. At baseline, IL-5 (3/8), IL-4 (4/8), IL-10 and IL-2 (both 5/8) and IFN-γ (6/8) were detected with increasing frequency; two patients (4 and 14) did not have detectable baseline levels of any of these cytokines. Mean concentrations for those with detectable levels were 5.3 (IL-5), 5.6 (IL-4), 5.9 (IL-10), 19.2 (IL-2), and 23.2 pg/mL (IFN-γ). Patient 12, who went on to have durable benefit from tremelimumab, had the highest baseline levels of IL-10, IL-2, and IFN-γ at 8.0, 24.4 and 56.6 pg/mL respectively. There were no patterns detected in IL-5, IL-4 or IL-2 levels with tremelimumab. Changes in IFN-γ and IL-10 are shown in Fig 4.25 below.
Fig 4.25: Plasma cytokine levels with tremelimumab

Ex-vivo cytokines were measured in stored plasma using cytokine bead array (n=10). A shows IFN-γ and B shows IL-10 for all available samples and group mean (line). Patient 12 results are highlighted.

The number of patients with detectable IFN-γ appeared to fall with the treatment cycle: from 6/8 at baseline to 3/8 by day 15 and 0/5 by day 90. In contrast, the number of patients with detectable IL-10 rose initially from 5/8 at baseline to 8/8 at day 15 before falling again to 5/9 at day 30; mean IL-10 (all tested) rose from 3.7 to 6.3pg/mL (unpaired t test p=0.067). These results are low compared to mean serum IL-10 of 20.1pg/mL (SD ± 6.9) documented in patients with gastric cancer [224]. Changes seen may be due to concurrent events, or to disease progression, and detection of cytokines in the plasma may be influenced by the presence and levels of...
other proteins such as fibrinogen, but the apparent rise in IL-10 would be in keeping with the demonstrated rise in regulatory lymphocyte phenotype including Treg by CD4+CD25\textsuperscript{high}FoxP3\textsuperscript{+} and CD4+CD25\textsuperscript{high}CTLA4\textsuperscript{+} 15 days after tremelimumab, as this cytokine has a role in control of immune activation and may be secreted by monocytes, DC, B lymphocytes, and T lymphocyte sub-populations including Treg [225]. Further investigation of cytokine release ex vivo would require access to serum samples.

4.7: Lymphocyte function: suppression assay

The preceding work has demonstrated a rise in Treg phenotype, defined both by CD4+CD25\textsuperscript{high}FoxP3\textsuperscript{+} and CD4+CD25\textsuperscript{high}CTLA4\textsuperscript{+}, in patients with gastric and oesophageal adenocarcinomas treated with tremelimumab. The demonstrated failure of these lymphocytes to secrete IL-2 suggests that they are bona fide natural Treg, but ideally confirmation requires the demonstration of functional suppressive capacity [220]. In vitro suppression assays are well established but technically demanding and require large numbers of viable lymphocytes. Effector (Teff), regulatory (Treg) and accessory cell populations were identified and divided using magnetic bead cell sorting before recombination in culture at known proportions, and proliferation was assessed by \textsuperscript{3}H-thymidine incorporation.

Fresh PBMC were obtained with formal consent from three patients with recent diagnoses of advanced gastric (1) or oesophageal (2) adenocarcinoma who had not yet received treatment due to concerns about the effect of platinum based chemotherapy on lymphocyte count. All the patients were male, and of similar ages to those recruited into the trial (50, 55, and 75 years). Absolute lymphocyte counts obtained from the
Christie haematology laboratory showed significant variation: 0.9, 1.3 and 4.6 x10⁹/L, although the highest count occurred in a patient with a significant history of psoriatic arthropathy, and other evidence of inflammation such as a raised platelet count (1088 x10⁹/L).

<table>
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Table 4.4: Yields from magnetic cell sorting

Table shows number of viable cells for three patients at each stage of magnetic cell sorting prior to establishing in vitro suppression assay. Scientific notation is used: 4.00E+06 = 4.00 x10⁶. Teff, effector T cells; Treg, regulatory T cells; Y %, percentage yield.

Table 4.4 above records the numbers of viable cells at each stage of magnetic cell sorting. CD3 depletion generated accessory cells which were irradiated prior to culture; mean yield was 29% unsorted PBMC (range 13-53%). CD4⁺ cells were generated by negative selection; mean yield was 38% unsorted PBMC (range 17-71%). The high yields for both negative selections for the second patient may reflect either technical problems with the assay or the presence of unusually high numbers of CD3-CD4⁺ cells such as monocytes. CD4⁺ cells were then used to generate CD4⁺CD25⁻ lymphocytes by negative selection, and CD4⁺CD25⁺ lymphocytes by positive selection: mean yields were 80% (range 65-95%) and 0.6% (range 0-0.9%) of CD4⁺ cells respectively. Due to the low yields of accessory cells, normal donor accessory cells were generated from frozen PBMC and buffy coats, and suppression assays were set up using these allogeneic accessory cells which generate a mixed lymphocyte response. The best of three experiments is shown in Fig 4.26 below.
Fig 4.26: Suppressive activity of CD4⁺CD25⁺ Treg in vitro: untreated patient

In vitro suppression assay was established using allogeneic accessory cells (CD3⁺ irradiated) after magnetic bead sorting of Teff (CD4⁺CD25⁻) and Treg (CD4⁺CD25⁺) from fresh PBMC. Cells were cultured for 4 days and pulsed with ³H-thymidine 18 hours before harvesting. Best of three results shown; the patient was a 75 year old gentleman with recurrent oesophageal adenocarcinoma with coeliac nodal metastases. Results are presented as (A) counts per minute (CPM) and (B) % suppression. Error bars show SEM.
As Fig 4.26 shows, bead-sorted CD4⁺CD25⁺ displayed a significantly reduced proliferative response compared to CD4⁺CD25⁻ Teff as would be expected for an anergic Treg population. These Treg were able to significantly suppress Teff proliferation at different Teff: Treg ratios: by 39% at 1:0.125 to 55% at 1:1, with mean counts per minute (CPM) falling from 9169 (1:0) to 5556 (1:0.125; t test p=0.12) to 4142 (1:1; t test p=0.037).

The same assay was established for patient 12 using fresh PBMC obtained just prior to his 9th cycle of tremelimumab, to investigate whether his prolonged treatment with an anti-CTLA4 antibody had abrogated the suppressive function of his Treg lymphocytes. Results are presented below in Fig 4.27, which shows that the bead-sorted CD4⁺CD25⁺ lymphocytes have maintained functional characteristics of Treg even after prolonged treatment with anti-CTLA4 antibody as (1) they are anergic, failing to proliferate despite the stimulation of allogeneic accessory cells and (2) they were able to significantly suppress Teff at different Teff: Treg ratios: by 17% at 1:0.5 to 76% at 1:1, with mean counts per minute (CPM) falling from 4620 (1:0) to 3829 (1:0.5; t test n.s.) to 1098 (1:1; t test p=0.016). Cautious comparison with the results for the untreated patient show less evidence of effective suppression with Teff: Treg ratios between 1:0.125 and 1:0.5, which may just reflect the high level of well to well variation in the results. However, effective suppression by Treg is clearly not abolished after prolonged exposure to tremelimumab as measured ex vivo by suppression of Teff with equal ratios of Treg (76% vs. 55% for untreated patient). Direct comparison of these results is difficult because of intra- and inter-assay variation.
Fig 4.27: Suppressive activity of CD4⁺CD25⁺ Treg in vitro: patient 12

In vitro suppression assay was established using allogeneic accessory cells (CD3⁻; irradiated) after magnetic bead sorting of Teff (CD4⁺CD25⁻) and Treg (CD4⁺CD25⁺) from fresh PBMC. Cells were cultured for 4 days and pulsed with [³H-thymidine 18 hours before harvesting. Best of two results shown; the patient was a 70 year old gentleman with metastatic oesophageal adenocarcinoma who had received 8 cycles of treatment with tremelimumab. Results are presented as (A) counts per minute (CPM) and (B) % suppression. Error bars show SEM.
4.8: Summary results

To recap, the main aims of this work were:

1. To assess changes to lymphocyte phenotype after anti-CTLA4 blockade, and to identify potentially immunoregulatory phenotypes, including natural Treg.
2. To assess changes to lymphocyte function after CTLA4 blockade, looking particularly for any increase in specific anti-tumour activity, using responses to two relevant tumour associated antigens (TAA): 5T4 and CEA.
3. To correlate any changes in lymphocyte phenotype and function with clinical outcome.

This work has demonstrated changes in lymphocyte phenotype with anti-CTLA4 blockade using tremelimumab in patients with advanced gastric and oesophageal adenocarcinomas. Absolute CD4$^+$ and CD4$^+$/CD25$^{high}$ lymphocyte subsets increased transiently with treatment, and parallel changes were seen in relative Treg further defined using FoxP3 and intracellular CTLA4, which they constitutively express [124]. Both markers suggest the proportion of Treg rises in a reproducible temporal pattern after tremelimumab. Additional flow cytometry demonstrates these cells do not secrete IL-2, supporting their definition as natural Treg. Suppression assay data confirms the presence of functional suppressor CD4$^+$CD25$^{high}$ Treg even after prolonged treatment with anti-CTLA4 blockade. These results support the idea that CTLA4 blockade is not acting primarily through Treg.

In contrast, CD4$^+$/CD25$^{low/negative}$/CTLA4$^+$ lymphocytes show a sustained relative rise after tremelimumab. As CTLA4 expression is a late event in lymphocyte activation, these cells may represent a population with potentially sustained activation. This
inference is supported by development of de novo in vitro lymphocyte proliferative responses to two tumor associated antigens, 5T4 and CEA, after tremelimumab. Within the cohort investigated, the development of a response to CEA after tremelimumab correlated with survival. Interestingly, the patient with a durable clinical response developed recurring post-treatment lymphocyte proliferative responses to both TAA after tremelimumab. These results need to be interpreted with caution, and would require confirmation in a further cohort of patients.

Analysis of prognostic factors shows a relationship between immune-related adverse events and clinical responses to CTLA4 blockade [226]. Both toxicity and responses seem to occur in a minority of potential immune responders. In this small study, pre-treatment IL-2 release after T cell activation seems to identify both those patients with potentially beneficial and serious toxic responses to tremelimumab, but would require prospective investigation for confirmation.
CHAPTER 5: IMMUNOTHERAPY MODEL RESULTS

5.1:  Brief Introduction

5.1.1. Use of murine models

To complement the clinical and laboratory investigation of immunomodulation by anti-CTLA4 blockade in patients with advanced cancer, this project aimed to establish and test a suitable pre-clinical model. Safety and efficacy data from animal models still underpins early phase clinical trials of novel agents in man. Murine models have obvious advantages both financial and practical. Mice have been research tools in both oncology and immunology for many decades. Arguably more is understood about the mouse immune system than the human [227].

5.1.2. 5T4 as an immunotherapeutic target in murine models

The oncofoetal antigen 5T4 was discovered by looking for shared properties of human trophoblast and cancer cells, and has been shown to be a tumour associated antigen [48]. Sequencing shows 81% homology between the protein in mouse and man [52-53]. Established models using human antigen inserted into a mouse melanoma cell line B16.h5T4 were initially used to investigate a 5T4 targeted vaccine strategy. Recombinant viruses based on the attenuated modified vaccinia virus Ankara (MVA) strain were constructed to express human 5T4 (MVA.h5T4), murine 5T4 (MVA.m5T4) and control Escherichia coli LacZ [44]. Triple vaccination with MVA.h5T4
showed significant benefit in both protection and therapy models using two syngeneic
tumour cell lines transfected with h5T4 cDNA.

Parallel work using MVA.m5T4 in a subcutaneous B16 melanoma model transfected
to express m5T4 cDNA showed significant benefit in protection experiments, with
median survival of 43 days for double MVA.m5T4 (iv) versus 25 days for MVA.LacZ
(p=0.00024). This regime did not however demonstrate significant benefit in a therapy
model [202]. This is in keeping with the greater difficulty in breaking tolerance to a self
antigen in an autologous model system. Vaccination with two to three doses of
MVA.m5T4 (either iv or im) in naive animals can be shown to break tolerance, with
the induction of specific m5T4 antibodies, but at much lower titres (≤ 20 000) than
parallel h5T4 antibodies (>128 000) following MVA.h5T4.

Repeat vaccination, required to overcome pre-existing tolerance in a self antigen
model can result in anti-vector immunity which interferes with the desired TAA
specific response, or prompt tolerance rather than enhancing activation. This has led
to the development of diversified prime boost schedules using alternative vectors,
which demonstrate enhanced T lymphocyte immunity and anti-tumour responses
[228-229].

Using this approach, Ali et al [207] demonstrated benefit in both protection and
therapy experiments in the B16 human 5T4 model by combining replication defective
adenovirus (Ad.h5T4) and a retrovirally transduced immortal dendritic cell line
(DC.h5T4). However, the sequence which showed greatest protective benefit
(DC.h5T4 then Ad.h5T4; 5/7 animals tumour free, p=0.0001) had no efficacy as
therapy. In contrast, greatest therapeutic benefit was seen with the sequence Ad.h5T4
then DC.h5T4 (median survival 26 vs. 19 days, p=0.0049) which had delayed but not prevented tumour growth in the protection experiment. Within this model, B16.h5T4 tumour growth is not ignored, and appears to drive a Th2 response to h5T4 which may lead to the induction of regulatory lymphocytes, altering the immune milieu, and therefore the response to the vaccine components. These results from the human 5T4 expressing mouse tumour model system suggest that active tumour therapy models may be more relevant for the evaluation of cancer vaccines than prophylactic regimes, and that moreover benefit in one type of experimental vaccination model does not necessarily predict for benefit in the other.

5.1.3. Rationale for use of an autologous murine model

Heterologous antigens such as OVA or h5T4 have established proof of principle for many immunotherapeutic approaches and vaccine schedules in cancer models. They can stimulate robust immune responses and allow measurement of specific responses, such as antibody generation, CD8+ T cell cytotoxicity, or cytokine secretion, with relative ease. However, such foreign antigens are by their nature poorer models of the chronic antigen exposure seen in the clinic in advanced malignancy. In man many target tumour associated antigens are also found at low levels in normal or developing tissues; such exposure may prime regulatory mechanisms leading to tolerance rather than immune activation. These regulatory mechanisms may be targeted using anti-CTLA4 blockade. This project aimed to explore the potential of anti-CTLA4 blockade in an autologous murine m5T4 system where these regulatory mechanisms may already be functioning.
5.1.4. Aims of murine model

The main aims of this work were:

1. To establish an active therapeutic vaccination model using the autologous murine oncofoetal antigen m5T4.
2. To develop an immunological measure of response to m5T4 vaccination.
3. To characterise response to vaccination against the antigen m5T4 in naive and tumour-bearing animals.
4. To attempt to modulate vaccination response using anti-CTLA4 blockade.
5. To test efficacy of combining anti-CTLA4 blockade with the therapeutic m5T4 vaccination model.

5.2: Characterising components of an immunotherapy model

5.2.1. Tumour cell lines

The first step in establishing a therapeutic vaccination model was to choose an appropriate tumour model. This needed to be available, syngeneic, and show stable and high levels of m5T4 antigen expression. Ideally, the tumour would grow slowly but reliably in vivo, allowing sequencing of therapy. Previous work within the group had concentrated on the B16 melanoma model [202, 207], but B16 melanoma is poorly immunogenic and rapidly lethal, allowing only a brief window for efficacy of therapy. A panel of available alternative cell lines was therefore assessed for suitability in a therapeutic autologous model.
5.2.1.1. m5T4 expression

First, expression of the autologous antigen m5T4 was assessed by flow cytometry using a panel of antibodies raised against m5T4 in the 5T4KO mice [53]. The putative binding sites for the antibodies raised against m5T4 in 5T4KO mice occur within the distal leucine rich repeat, LRR (B3F1) and proximal LRR (B5C9 & P1H10) areas. Flow cytometry enabled detection of the antigen at the cell membrane where it may be recognised by a primed immune system. Cell lines exhibit some heterogeneity, and expression of m5T4 can vary with cell development and behaviour. Repeated flow cytometry looked for reproducible and high levels of m5T4 in cell lines grown in vitro.

Fig 5.1: Specificity of Anti-m5T4 antibodies

Cells were labelled with anti-m5T4 antibody (white) or isotype control (red), and counterstained with polyclonal rabbit anti-mouse immunoglobulins FITC. Histograms show mean fluorescence against
events. The 5% marker established using the isotype control is shown (M1), and cell type and anti-m5T4 antibody used label each histogram.

Specificity of the antibodies B5C9 and B3F1 is shown in Fig 5.1. The top two plots (A&B) show B5C9 labelling in the transfected cell lines B16.m5T4 (86%) and negative control B16.neo which contains an empty pCMVα vector (0%). The lower two plots (C&D) show B3F1 labelling B16.m5T4 (90%) and B16.neo (0%). This result confirms that the antibodies made in the 5T4KO mice recognise B16.m5T4 cells but not B16.neo cells. Having established the specificity of these antibodies, B5C9 and B3F1 were then used to look for m5T4 by flow cytometry in a panel of cell lines. B16.m5T4 derived from the melanoma cell line B16-F10, and A9.m5T4, derived from the immortalised lung fibroblast cell line A9 [44] were used as positive controls, and B16.neo were used as a negative control. Illustrative results are shown in Fig 5.2 and summarised in Table 5.1 below.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Strain</th>
<th>Origin</th>
<th>% cells m5T4+ by antibody:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>B3F1</td>
</tr>
<tr>
<td>A9.m5T4</td>
<td>C3H/An</td>
<td>lung fibroblast</td>
<td>86</td>
</tr>
<tr>
<td>B16.m5T4</td>
<td>C57BL/6</td>
<td>melanoma</td>
<td>90 ±3</td>
</tr>
<tr>
<td>B16.neo</td>
<td>C57BL/6</td>
<td>melanoma</td>
<td>0</td>
</tr>
<tr>
<td>C127i</td>
<td>RIII</td>
<td>mammary ca</td>
<td>88±8</td>
</tr>
<tr>
<td>CT26.wt</td>
<td>BALB/c</td>
<td>colon ca</td>
<td>88±3</td>
</tr>
<tr>
<td>EMT6</td>
<td>BALB/c</td>
<td>mammary ca</td>
<td>93</td>
</tr>
<tr>
<td>GL261</td>
<td>C57BL/6</td>
<td>glioma</td>
<td>38±23</td>
</tr>
<tr>
<td>TC1</td>
<td>C57BL/6</td>
<td>lung epithelial</td>
<td>45±11</td>
</tr>
</tbody>
</table>

Table 5.1: m5T4 in murine cell lines
Flow cytometry assessment of % trypsinised adherent cells m5T4+ using antibodies raised in the m5T4KO mouse. Cumulative results from six experiments summarised by mean ± standard deviation where available.
As Table 5.1 shows, similar results were obtained with all three anti-m5T4 antibodies. A high percentage of two stably transfected cell lines which served as positive controls were consistently m5T4⁺. As Fig 5.2 shows, a low and variable percentage of the glioma GL261 cells were m5T4⁺ (11-54%) and this was therefore excluded as a candidate cell line for the vaccination model.

GL261

Fig 5.2: m5T4 in murine tumour cell lines by flow cytometry.

Cells were labelled with anti-m5T4 antibody (white) or isotype control (red), and counterstained with polyclonal rabbit anti-mouse immunoglobulins FITC. Histograms show mean fluorescence against events. The 5% marker established using the isotype control is shown (M1), and cell type and anti-m5T4 antibody used label each histogram.

Four tumour cell lines showed a high percentage m5T4⁺ cells, comparable to the levels found in the two m5T4 transfected cell lines. These were:

GL261

CT26.wt
(1) C127i, a murine mammary carcinoma cell line derived from the inbred RIII strain, which frequently develop spontaneous hormone-dependent mammary tumours. Unfortunately, it did not prove possible to acquire the RIII strain, and so this cell line was not investigated in vivo.

(2) EMT6 a clonal isolate of cell line EMT, a murine mammary tumour line cloned from a hyperplastic mammary alveolar nodule implanted in a BALB/c animal [196].

(3) CT26.wt is a lethal clone from an N-nitroso-N-methylurethane-induced BALB/c (H-2^d) undifferentiated colon carcinoma cell line [195].

(4) TC-1, a tumourigenic immortalised cell line, was derived from C57BL6 primary lung epithelial cells transformed to co-express human papilloma virus (HPV-16) antigens E6/E7, and the activated human c-Ha-ras oncogene [197-198].

The two mammary cancer cell lines were known to have high percentage of m5T4^+ cells by flow cytometry using the established 9A7 antibody [44].

In summary, a high proportion of cells from four alternative cell lines grown in vitro (C127i, EMT6, CT26.wt, and TC1) were labelled for m5T4 on more than two occasions using antibodies raised in the m5T4KO mouse. The presence of the antigen at the cell surface of the majority of these cells suggested they may be suitable candidates for the autologous vaccination model, and they were next investigated for tumour kinetics in vivo. It is of course possible that m5T4 expression alters when cell lines grow in vivo, an issue which is addressed in later in vivo work.
5.2.1.2. Tumour growth kinetics in vivo

The rapid growth of the B16.m5T4 tumour model in vivo limits the potential efficacy of therapeutic immunotherapy, and makes scheduling of combination therapies extremely difficult. The growth kinetics of available alternative cell lines which had been identified as showing high and consistent proportion of m5T4⁺ cells were investigated in vivo. **Fig 5.3** shows results by survival rate.

All animals in the experiment developed measurable subcutaneous tumours by day 7. Animals were sacrificed at a pre-defined endpoint when visible tumours reached a surface area of greater than 1.24cm². As the figure shows, all animals were sacrificed between 9 and 18 days after tumour inoculation. Tumour growth kinetics and median survival were the same for all cell lines investigated.

**Fig 5.3:** In vivo growth kinetics of alternative cell lines.

*Groups of 7 mice were challenged with 10⁵ tumour cells on day 0 to assess subcutaneous in vivo growth kinetics of alternative m5T4⁺ cell lines against B16.m5T4. Tumour growth was measured in two dimensions and mice were sacrificed when surface area exceeded 1.24cm².*

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In conclusion, none of the alternative m5T4+ tumour cell lines showed a slower growth rate than B16.m5T4 in vivo. It is possible that m5T4+ is largely found in cell lines with rapid proliferation, although this inference is contradicted by in vitro work in A9 fibroblasts which showed increased doubling times (most marked in sub-optimal conditions) after transfection with m5T4 [44]. The established B16.m5T4 and a candidate alternative cell line (CT26.wt) were therefore chosen to investigate an autologous m5T4 vaccination model on pragmatic grounds, and in an attempt to minimise the number of animals required. The cell line TC1 was excluded due to recurrent problems with yeast infestation, and (perhaps unnecessarily) because it has been engineered to co-express foreign antigens from human papilloma virus (HPV-16; antigens E6/E7), and the activated human c-Ha-ras oncogene. CT26.wt was chosen largely because, being a gastro-intestinal adenocarcinoma, it presented the best parallel with the focus of the clinical trial in advanced gastric and oesophageal adenocarcinomas.

5.2.1.3. CTLA4 expression

CTLA4 expression has been described in a number of human tumour cell lines by flow cytometry and RT-PCR, including the melanoma line MEL-1 and the colorectal lines HCT-8 and CACO-2 [230]. As this would clearly have a bearing on efficacy of anti-CTLA4 blockade in a therapeutic tumour model, cell membrane CTLA4 was assessed by flow cytometry in B16.m5T4 and CT26.wt as shown in Fig 5.4. Neither cell line showed any cell membrane CTLA4 by this method. Positive labelling, although with different gating, was seen in mouse lymphocytes as shown in Fig 5.7.
Fig 5.4: Analysis of cell surface CTLA4 in tumour cell lines by flow cytometry.

*Cells grown in vitro were labelled with CTLA4 PE. Histograms plot mean fluorescence (FL2) against events and show stained cells (black) overlying isotype control (red).*

Although no surface labelling of CTLA4 was seen by flow cytometry, it is possible that intracellular CTLA4 was present. With hindsight this could have been assessed by antibody labelling after fixation and permeabilisation. Despite some evidence that cells can take up sufficient antibody to neutralise intracellular targets such as the phosphatases of regenerating liver (PRL), oncogenic protein tyrosine kinases [231], most antigen targets of therapeutic monoclonal antibodies are cell surface molecules, and most therapeutic interaction occurs at the cell membrane. The absence of cell surface CTLA4 in these tumour lines suggests that, even if the antigen is found intracellularly, direct effects of anti-CTLA4 blockade on the tumour cells are likely to have only a minor role in any therapeutic response.

5.2.2. Anti-CTLA4 antibody

5.2.2.1. Antibody processing

In order to have sufficient anti-CTLA4 antibody for in vivo experiments to look for modulation of immune responses to m5T4 vaccination in mice with and without tumours, and to explore therapeutic efficacy of combined therapy, a commercially
available hybridoma was purchased. This clone UC10-4F10-11 (HB-304\textsuperscript{TM} ATCC [232-233]) has shown efficacy as a single agent in restricted tumour-bearing stages in CSA1M fibrosarcoma and OV-HM ovarian cancer [131] and in combination with subtherapeutic melphalan [136], peptide vaccination [135] or Treg depletion by low-dose cyclophosphamide and/or anti-CD25 antibody depletion [234].

Culture of UC10-4F10-11 yielded 1.77 litres of supernatant which was stored at 4\degree C after the addition of 0.1% sodium azide. Processing of ~400mL UC10-4F10-11 supernatant produced two pools: around 10mL of solution (A) with a protein concentration of 2.62mg/mL and around 15mL (B) with a protein concentration of 0.84mg/mL. Total protein yield was therefore 38.8mg/400mL, or 0.097mg/mL supernatant. Purity was assessed using silver staining of gel electrophoresis which is a very sensitive method of protein detection. Result is shown in Fig 5.5 which shows a single band at 150 kDa corresponding to the molecular weight of unreduced hamster IgG.

![Fig 5.5: Silver stain gel of putative antibody](image)

\textit{Fig 5.5: Silver stain gel of putative antibody}

\textit{Following SDS-PAGE gel electrophoresis of putative antibody, gel was stained using ProteoSilver\textsuperscript{TM} Silver Stain Kit according to the manufacturer's instructions. Ladder shows marker bands (kDa). Aliquots A1.1 (1.19mg/mL) and A1.3 (0.6mg/mL) were run at 1:20 and 1:10 dilutions respectively.}
5.2.2.2. Endotoxin levels

Processing of antibody from a hybridoma is vulnerable to contamination with endotoxin at multiple stages, from plasmid DNA, during culturing, and (despite precautions) from equipment such as tubing used during purification. Endotoxins – classically structural components of live bacteria released on lysis, e.g. lipopolysaccharides (LPS) -are ubiquitous, making production of pharmaceutical grade antibody within an open laboratory a considerable challenge. Nonetheless, within the proposed in vivo autologous model, endotoxin contamination would pose both a theoretical safety risk to the animals, and significantly confound any evidence of immunomodulation.

A sensitive Limulus Amebocyte Lysate (LAL) test (Cambrex, Lonza, Switzerland) was used to detect endotoxin within the processed antibody. In this test, which is used within the pharmaceutical industry, very small quantities of endotoxin result in coagulation of the Limulus lysate, derived from the blood of the horseshoe crab *Limulus polyphemus*. The lysate sensitivity of the LAL test used throughout this work was 0.125 Endotoxin Units (EU)/mL. Initial testing of processed antibody showed significant endotoxin contamination: 16 EU/mL, or 6.1 EU/mg antibody.

Endotoxin was removed using a Detoxi-Gel™ Endotoxin Removing Column (Pierce, Thermo Scientific, MA, USA). Immobilised polymixin B removes pyrogens by binding lipid A domains, and can be regenerated with 1% deoxycholate solution. Best results were achieved by incubating sample within the column for at least one hour, and collecting run off by gravity; as a result, aliquots were less than 1mL in volume.
Endotoxin levels were screened in every third aliquot, and detailed estimation undertaken before in vivo use. Example results are shown in Table 5.2 below.

<table>
<thead>
<tr>
<th>aliquot</th>
<th>Conc (mg/mL)</th>
<th>Vol (mL)</th>
<th>Total (mg)</th>
<th>Endotoxin (EU/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1.1</td>
<td>1.19</td>
<td>0.4</td>
<td>0.476</td>
<td>1:3 &lt;0.32</td>
</tr>
<tr>
<td>A1.2</td>
<td>1.69</td>
<td>0.6</td>
<td>1.014</td>
<td>1:3 &lt;0.22</td>
</tr>
<tr>
<td>A1.3</td>
<td>0.6</td>
<td>0.7</td>
<td>0.42</td>
<td>1:10 &lt;2.08</td>
</tr>
<tr>
<td>A1.4</td>
<td>1.3</td>
<td>0.9</td>
<td>1.17</td>
<td>1:10 &lt;0.96</td>
</tr>
<tr>
<td>A1.5</td>
<td>1.15</td>
<td>1.0</td>
<td>1.15</td>
<td>1:10 &lt;1.09</td>
</tr>
<tr>
<td>A1.6</td>
<td>1.21</td>
<td>0.9</td>
<td>1.089</td>
<td>1:10 &lt;1.03</td>
</tr>
</tbody>
</table>

Table 5.2: Aliquots of anti-CTLA4 antibody used in vivo

Endotoxin was removed from aliquots using Detoxi-Gel™ Endotoxin Removing Columns (Pierce, Thermo Scientific) according to the manufacturer’s instructions. Protein concentration was estimated by spectrophotometry and endotoxin levels were tested using Limulus LAL technique (Cambrex).

There is no definite consensus on acceptable endotoxin limits within pre-clinical animal models, and in fact there is no standardisation of the units used for endotoxin testing in this setting even within the pharmaceutical industry. Endotoxin limits change with dose volume, formulation, and dose timing, and with animal species and size. Using the FDA threshold pyrogenic human dose (5 EU/kg), a safety threshold of 6 EU/mg drug has been derived [235]. In practice, levels of <1 EU/mg drug are considered insignificant. However, testing to this threshold can absorb a significant volume of antibody. All anti-CTLA4 antibody aliquots used in vivo were negative when tested at 1:10 (<1.25 EU/mL), and both selected aliquots were also negative when tested at 1:3 (<0.375 EU/mL). Known endotoxin levels were therefore well within the safety limit, and (in all but one small aliquot) proven to be around or below the lower 1 EU/mg threshold.
5.2.2.3. Characterising Antibody: Blocking Flow cytometry assay.

It is usual to confirm the presence of antibody which can interact with known antigen using a direct method such as sandwich ELISA or flow cytometry with a second anti-immunoglobulin labelling step. With titration, both methods allow quantification of antibody and more accurate estimation not just of protein yield but of potentially active antibody. Restricted access to purified antigen and difficulty defining a consistent CTLA4+ cell population prevented the use of these techniques.

Instead, an alternative directly conjugated anti-CTLA4 antibody (1B8 PE, abcam) was used in a blocking flow cytometry assay to assess the specific binding of the UC10-4F10-11 anti-CTLA4 antibody processed from the hybridoma.

**Fig 5.6: CTLA4 Flow cytometry assay**

Plots show mean fluorescence (CTLA4 PE) against events when mouse splenocytes were labelled with **A**: negative control (black; PBS) and commercial anti-CTLA4 PE (navy; clone IB8, abcam). **B** shows that staining is blocked by first incubating with 4F10 hybridoma product at concentrations of 1:100 (pale grey) and 1:200 (dark grey).

As shown in **Fig 5.6.A** the directly conjugated anti-CTLA4 stained 42% of gated splenocytes but blocking with the hybridoma product at dilutions of 1:50 (0.53µg/mL), 1:100 (0.26µg/mL) and 1:200 (0.13µg/mL) reduced staining to near
background levels as shown in B, demonstrating that the antibody product is specific for the target CTLA4.

The methodology used shows specificity but does not allow a useful estimate of the concentration of active antibody. For pragmatic reasons, later in vivo work equates the protein concentration with activity of the processed anti-CTLA4 antibody. As this assumption remains unproven, a commercially processed anti-CTLA4 antibody was also utilised in vivo to allow comparison of effect.

5.3: Assessment of immune responses

5.3.1. Aims

In parallel with establishing a therapeutic vaccination model using the autologous murine oncofoetal target antigen m5T4, this project aimed to develop an immunological measure of response to m5T4 vaccination. A robust immunological measure of response could potentially serve as a surrogate end-point for further assessment of immunomodulation of vaccine response, and allow characterisation of response to m5T4 vaccination in naive and tumour-bearing animals. Two main techniques were used to assess immune response:

1. serum ELISA for m5T4 specific antibody, and
2. IFN-γ ELISPOT for m5T4 peptide responses.
5.3.2. Antibody responses

5.3.2.1 Antibody responses to heterologous boost vaccination in tumour-bearing mice

The ability of an autologous heterologous boost pAd.m5T4/ MVA.m5T4 vaccination schedule to stimulate immunity in vivo was tested in two strains of m5T4+ tumour-bearing mice. Immunity was assessed by ELISA measurement of anti-m5T4 serological responses against an m5T4-IgG Fc fusion protein [202]. Initial results are shown in Fig 5.7 below.

![Graph showing antibody responses](image)

**Fig 5.7: m5T4 serological responses in tumour bearing C57BL6 mice (I)**

*Mice were challenged with sc tumour on day 0, and treated with active vaccine (black; MVA.m5T4; pAd.m5T4) or control vaccine (brown; MVA.LacZ; pAd.GFP) sc&im on days 5 and 12, or nothing (day 4 blue; day 18 aqua). Day 4 serum was processed from pooled tail bleeds for 3 animals; all day 18 serum was processed after terminal cardiac puncture of single animals. All sera assayed in triplicate at each dilution. Figure shows mean and SD. Representative one of two assays.*

As Fig 5.7 shows, all animals appeared to show a higher m5T4 antibody response 18 days after m5T4+ tumour challenge compared to 4 days. The animals which received active vaccination do show the highest responses, but the result is only marginally in
favour of the active vaccine schedule, and the levels are lower than expected from the published work in non tumour-bearing animals.

There are recognised differences in antibody responses in different strains of mice, so the experiment was repeated using alternative BALB/C strain. Additional control sera from untreated C57BL6 and known positive historical sera from non tumour bearing animals treated with MVA.m5T4 *2 were included. For completeness, naive BALB/C sera should also have been included, and historical MVA.m5T4/pAd.m5T4 but these were not available at the time. All sera were derived from terminal cardiac puncture at least three weeks after vaccination schedule commenced.

Fig 5.8: m5T4 serological responses in tumour bearing mice (II)
Mice were challenged with sc tumour on day 0, and treated with active vaccine (black, bold line; MVA.m5T4; pAd.m5T4) or control vaccine (grey, dotted line; MVA.LacZ; pAd.GFP) sc&im on days 1 and 8. C57BL6 results shown as triangles; BALB/C results shown as squares. Control untreated C57BL6 sera shown (unfilled, bold) and historical sera from non tumour-bearing animals treated with MVA.m5T4*2 im (red). All sera assayed in triplicate; mean and SD shown. A titre line was calculated at twice the level of naive response. Representative one of two assays.
As Fig 5.8 shows there was no difference between anti-m5T4 antibody titre following active compared to control vaccination in tumour-bearing animals in either C57BL6 melanoma or BALB/C colon tumour models. The antibody titre is low in vaccinated tumour-bearing animals (>1:600). Similar titres in control vaccinated animals (>1:300) suggest this weak antibody response may be stimulated by the presence of subcutaneous m5T4+ tumour rather than vaccination. With or without active vaccine, tumour-bearing BALB/C animals do demonstrate marginally higher antibody levels than C57BL6 strain, which may be due to inherent differences between their immune systems, or to differences in timing of sera collection: days 25-28 compared to day 38 respectively.

Substantially higher anti-m5T4 antibody titres are seen in historical control non tumour-bearing C57BL6 and BALB/C animals (>1:4000), with the highest titres seen following homologous prime boost vaccination with MVA.m5T4 in C57BL6^5T4-/- knock out animals (>1:10000). C57BL6^5T4-/- animals show no embryonic or adult expression of m5T4 and show a strong antibody response to m5T4, which is effectively a foreign rather than self antigen for this strain. Serum was used in these experiments as a strong positive control. The other historical sera also showed higher antibody titres but the protocol of vaccination was different: non tumour-bearing animals had received two vaccinations with MVA.m5T4 on day 0 and 21, which was demonstrated to be sufficient to elicit maximum antibody titres on day 28 irrespective of route (im or iv) [202].

The repeated failure of the active vaccine protocol to elicit higher serological responses than the control vaccine protocol could be due to failure of the vaccines, to inferiority of the heterologous prime boost compared to the homologous prime boost, or to altered immune response in tumour-bearing animals due to prior and/or ongoing
antigen exposure, or to the possibility that any antibody produced is absorbed by m5T4 \(^+\) tumour cells.

Different batches of both vaccines had been used in repeat experiments, making failure of vaccines less likely. Titres of available batches of MVA.m5T4 were reassessed by \(\beta\)-galactosidase and m5T4 expression using B3F1 antibody as described in the methods. Vaccine used in the published work was acquired from Oxford Biomedica and titre by these methods confirmed stated titre from the company. Viral titres of pAd.m5T4 were assessed by BioRad DC Protein assay, and were confirmed within the group by infection of m5T4 knockout (KO) embryonic stem cells and embryonic fibroblasts during experimental work on CXCL12 chemotaxis [53].

5.3.2.2. Antibody responses to vaccination in non-tumour-bearing mice

The potential difference between heterologous (MVA.m5T4/pAd.m5T4) and homologous (MVA.m5T4*2) prime boost protocols was next explored in non-tumour-bearing animals. BALB/C strain were used as these had previously shown marginally higher antibody levels.

As Fig 5.9 shows, specific anti-m5T4 antibodies are detectable after both homologous (MVA.m5T4*2) and heterologous (MVA.m5T4/ pAd.m5T4) prime boost vaccination in non-tumour-bearing BALB/C animals. Antibody titre is actually slightly higher in the heterologous than the homologous prime boost (detectable at dilutions of >1:4200 versus >1:3200 where titre is expressed as twice the naive serum result). Despite some increased background response in animals which received control vaccine (detectable at dilutions of <1:500) over naive animals, there was clear antibody response to both vaccine schedules.
Fig 5.9: m5T4 serological responses in non tumour-bearing BALB/C mice (I). Mice were vaccinated with prime boost vaccination schedule (day 1, day 8, sc&im route): A (♦) MVA.m5T4*2; B (■) MVA.m5T4/pAd.m5T4; or C (■) control MVA.LacZ/pAd.GFP. Control naive and historical MVA.m5T4 homologous boost were included. Day 28 responses are shown. All assays were assessed in triplicate. A titre line was calculated at twice the level of naive response. Error bars show standard deviation.

There was no difference in m5T4 antibody titre between days 14 and 28 following control or homologous MVA.m5T4 vaccination, but as Fig 5.10.A shows, there was augmentation of antibody response to heterologous MVA.m5T4/pAd.m5T4 prime boost between days 14 and 28. Given the rapid growth in vivo of all m5T4+ tumours investigated, these kinetics may impair the potential therapeutic efficacy of the vaccine protocol.

Although less marked, there is still a difference between current and historical vaccination response (Fig 5.9). Two variations in protocol may account for this: route, and boost vaccination timing. In the past all vaccine was given by intramuscular (im) route; in the current protocol dose was split 50:50 between intramuscular and subcutaneous routes to limit the risk of haematoma formation. In the past, boost was delayed until three weeks after prime vaccination; given the rapid in vivo growth
kinetics of m5T4⁺ tumours, such a schedule would be impractical for a therapeutic model as the majority of animals would not survive until boost vaccination.

A: MVA.m5T4/ pAd.m5T4

B: MVA.m5T4 x2; MVA.LacZ/ pAd.GFP

Fig 5.10: m5T4 serological responses in non tumour-bearing BALB/C mice (II).

Mice were vaccinated with prime boost vaccination schedule (day 1, day 8, sc & im route): A (♦) MVA.m5T4*2; B (■) MVA.m5T4/ pAd.m5T4; or C (■) control MVA.LacZ/ pAd.GFP.
Control naive and historical MVA.m5T4 homologous boost were included. Graphs show day 14 and 28 responses. All assays performed in triplicate. Error bars show standard deviation.

In summary, both homologous MVA.m5T4*2 and heterologous MVA.m5T4/pAd.m5T4 vaccination can stimulate immunity to m5T4 in wild type mice, as demonstrated by moderate serological responses in non-tumour-bearing animals. However serological responses appear attenuated in tumour-bearing animals, but ELISA detection of circulating serum antibody cannot distinguish between antibody detection and response. It remains possible that tumour-bearing animals have impaired response to vaccine, or that alternatively, antibody is produced but cannot be detected in the serum as it is absorbed by the m5T4+ tumour. An alternative, and potentially more sensitive, assay of immunity which could be used to detect responses in tumour-bearing animals, was explored next.

5.3.3. Cytokine ELISPOT responses

Cytokine ELISPOT allows characterisation of the immune response at single cell level, and is considered one of the most sensitive methods for demonstrating antigen specific immunity [209]. Interferon gamma (IFN-γ) is a soluble cytokine secreted by cells of the innate and adaptive immune systems as part of the immune response; downstream effects include increased activation of macrophages, NK cells, and antigen presenting cells (APCs), increased MHC class I expression, and suppression of Th2 immune response. NK and natural killer T cells both secrete IFN-γ as part of the innate immune response; both CD4+ and CD8+ T lymphocytes secrete IFN-γ once antigen-specific immunity develops [24].

The amino acid sequence of m5T4 is shown in Fig 5.11. Potential cytotoxic T lymphocyte (CTL) epitopes can be identified by computer-based prediction and
supported by testing of the recognition pattern of vaccine generated T cells [188]. The repertoire of m5T4 specific immune responses was investigated by in vitro re-stimulation using overlapping 30 mer peptides spanning the whole sequence of m5T4.

MPGAGSRGSPAGDGRLLRLARLALVLGLGWVSASAPSSSVPSS
TSPADFLASGSAQMPAERCAPACECSEAARTVKCVNRNLLE
VPADLPPYVRNLFLTNQMTVPAGAFARQPLADLEALNLS
GNHLKEVCAGAFEHLPLRRLDHNLPSLNLSASAFVGSNAS
VSAPSPLEELILNHIVPPEDQRQNGSFEGMVAEGMVAAALAR
SGLALRGLTRLELASNHHLFLPLRDLLAQLPSLRYLDLRNNSL
VSLTYASFRNLTHLESLLHLEDNALKVLHNSTLAEWQGLAHVK
VFLDNNPWVCYMADMVWLVKETEVPDKARLTCAFPEKMR
NRGLLDLNSSDLDCDAVLPQSLQTSYVFLGIVLALIGAIFLL
VLYLNRSKGIKKWMHNIRDACRDHMGEHYHYEINA DPRTLNL
SSNSDV

Fig 5.11: Mouse 5T4 Amino Acid Sequence

5.3.3.1 IFN-γ responses to pAd.m5T4

Initial experiments explored the m5T4 specific immunity of single pAd.m5T4 in non-tumour-bearing animals. All assays contained media alone as a negative control and the polyclonal stimulant Concanavalin A as a positive control; where the latter is not
illustrated, results were too strongly positive to quantify. As Fig. 5.12 shows IFN-γ responses were seen within 7 days of single pAd.m5T4 vaccination. Responses were seen to a limited repertoire of m5T4 peptides: 17, 19, 21, 33, 35, 36, and 37. Strongest responses were seen to peptides 21 and 33: mean 179 and 126 spots/well compared to 26 spots/well for media. As shown, the majority of peptides (12-16, 18, 20, 22-32) do not generate an IFN-γ response after single pAd.m5T4 vaccination.

![Graph](image)

**Fig 5.12:** IFN-γ response to m5T4 peptides after single pAd.m5T4.

Elispot response to 30mer peptides (labelled 12-37) spanning sequence of m5T4 after single pAd.m5T4 (sc & im). Figure shows typical result 7 days after immunisation (one of three). 5x10⁵ splenocytes plated per well. M= media control. All wells assayed in triplicate; error bars show standard deviation.

ELISPOT is able to detect low frequency events: if, as is intended, each spot represents a single activated cell, only around 0.03% splenocytes have responded to peptide 21 in the assay illustrated above.

---

10 Defined by mean spots per well peptide ≥ 2 x mean spots per well media control
The timing of IFN-γ ELISPOT responses after single pAd.m5T4 vaccine was investigated using a single batch of vaccine. For clarity, Fig 5.13 summarises responses to positive peptides, media control, and two illustrative negative peptides (12, 18) with time. ELISPOT responses developed within 7 days of pAd.m5T4 vaccination and were still detectable at least two weeks later. Fairly consistent responses were seen to peptides 17, 19, 33, and 35, but the magnitude of the responses is not high, generally within the range of 2-5 x media control. In contrast, positive control wells cultured with concanavalin A were between 40-80 x media control. By day 19, some responses appear attenuated, such as those to peptides 17, 33 and 35. A clear response (nearly 3 times background) is still seen to peptide 19. These different results may also reflect animal to animal variation, or assay to assay variation.

![Fig 5.13: IFN-γ response to m5T4 peptides with time after single pAd.m5T4.](image)

ELISPOT response to peptides of m5T4 after single pAd.m5T4 (sc & im). For clarity the figure shows results for positive peptides (17, 19, 21, 33, 35, 36) and two negative peptides (12&18). Mean spots per well test results were divided by mean spots per well for media controls and expressed as standard units. MED, media control. All wells assayed in triplicate; error bars show SD. Positive control using concanavalin A (not shown) were between 40-80 standard units.
Titration of positive IFN-γ response by serial dilution of the relevant peptide allows assessment of frequency and affinity; a high affinity response would still be seen with lower concentration of relevant peptide, and may therefore be more relevant in vivo. Titration of demonstrated IFN-γ responses was difficult, but is illustrated in Fig. 5.14. As shown, responses to peptide 19 were the most robust, having the highest frequency (21-34 spots/ 5 x 10^5 splenocytes) and little titration with serial dilution of peptide suggesting a higher affinity response.

**Fig 5.14: IFN-γ dose response to m5T4 peptides after single pAd.m5T4**

IFN-γ ELISPOT responses to selected 30mer peptides of m5T4 after single pAd.m5T4 (sc & im) are shown. 5x10^5 splenocytes plated per well. All wells assayed in triplicate; error bars show standard deviation. Key shows peptide concentration. Solid line indicates background response of cells incubated in media (3±0.58 spots/well). Assay illustrated was one of two conducted 14 days after vaccination.

In contrast, the other peptide responses detected, to peptides 17, 33 and 35, were of very low frequency (9-17 spots/ 5 x10^5 splenocytes), and those to peptides 17 and 35 were lost with titration. The low frequency of detectable responses is not unexpected with an autologous antigen, but affects reliability of the assay. In this experiment no convincing responses were seen to peptides 21 or 37. A low frequency response is
seen to lower concentrations of peptide 36. This result was confirmed on repetition and may reflect some inhibitory or toxic effect of the higher concentration of peptide 36 which was not seen with the other peptides used.

These results show that m5T4 specific immune responses can be detected at low frequency in non tumour-bearing animals by IFN-γ ELISPOT after single pAd.m5T4 vaccination, and identify a number of specific peptides which can be used to detect m5T4 specific immune responses. These peptides are illustrated in Fig 5.15. Several contained sequences predicted to be MHC class I or II peptide binders from analysis of the m5T4 protein sequence using the validated algorithm RANKPEP [236], although this analysis must be interpreted cautiously as a number of predicted sequences also showed no evidence of responses in these assays.

Fig 5.15: m5T4 peptides which define IFN-γ response in BALB/C splenocytes.

Standard nomenclature is used, to describe amino acid sequence. Peptide 17 is part of the extracellular distal leucine rich repeat domain. Underlined amino acids form part of the transmembrane region. Highlighted sequences are predicted to be MHC.
Despite use of a standard method and careful assessment of cell viability and concentration, there is some assay to assay variation in the frequency and pattern of peptide responses and low frequency results need to be interpreted cautiously even after repetition. Assay to assay variation reflects both variation between individual mice, and inevitable variation in complex multi-step assay particularly in the development stage.

### 5.3.3.2 IFN-γ responses to MVA.m5T4

Having characterised the peptides which identify m5T4 specific response after pAd.m5T4 vaccination, IFN-γ responses to MVA.m5T4 were investigated in non tumour-bearing mice. Results of the first experiment are shown in **Fig 5.16** below.

The control vaccine schedule did not result in any specific peptide responses, but no peptide-specific responses were identified after active homologous MVA.m5T4*2 either despite strong positive control which demonstrated in vitro polyclonal activation with concanavalin A. The sera of these animals had shown evidence of m5T4 antibodies after vaccination (**Fig 5.9**).
A: MVA.m5T4 x 2 d33

B: MVA.LacZ + pAd.GFP d33

**Fig 5.16**: IFN-γ response to selected m5T4 peptides after MVA.m5T4

ELISPOT response to selected 30mer m5T4 peptides (14,17,19,21,29,30,33,35-37) one month after: **A** homologous MVA.m5T4*2 and **B** heterologous control MVA.LacZ/ pAd.GFP vaccination. One of two assays illustrated.

Initial concern about vaccine batch led to repeat experiment using MVA.m5T4 from an alternative source (Oxford Biomedica) and the full range of m5T4 peptides. Responses were investigated at an earlier time point when maximal results had been seen after pAd.m5T4 vaccine. As **Fig 5.17** shows, this experiment supported the
earlier result. No responses were seen to any of the m5T4 peptides after single MVA.m5T4 vaccine.

**Fig 5.17: IFN-γ response to all m5T4 peptides after MVA.m5T4**

ELISPOT response to all 30mer m5T4 peptides 7-15 days after single MVA.m5T4 (Oxford Biomedica). One of two assays illustrated. MED, media control; Con A, concanavalin A control.

The absence of IFN-γ responses to specific m5T4 peptides was unexpected. After repetition with MVA.m5T4 vaccine from an alternative source, and detection of specific antibody responses, primary vaccine failure seems unlikely. There is some evidence that pAd.h5T4 induces Th1 polarising immune responses which can overcome the Th2 polarised response to growing B16.h5T4 [207]. It may be that pAd.m5T4 also induces a Th1 polarised response, which is dominated by IFN-γ, whilst MVA.m5T4 may in contrast induce a Th2 polarised response which could be dominated by alternative cytokines IL-5 and IL-10. Ali et al used two methods to investigate polarisation of immune response to human antigen h5T4: elisa for h5T4 specific antibodies using HRP labelled Goat anti-mouse IgG1 and IgG2a polyclonal antibodies, and cytokine elisa following in vitro re-stimulation with dendritic cells.
transfected with h5T4 [207]. Had time allowed, Th1/Th2 polarisation of immune responses to the autologous antigen m5T4 following pAd.m5T4 and MVA.m5T4 could have been compared in non tumour-bearing animals using these methods, although determining response may have been more difficult given the lower antibody titres and reduced frequency of IFN-γ responses demonstrated in the autologous model. Control vaccines with pAd.GFP and MVA.LacZ and naive animals would be necessary controls.

5.3.3.3 IFN-γ responses to heterologous prime boost m5T4 vaccination.

The IFN-γ ELISPOT m5T4 peptide assay was next used to investigate responses in non tumour-bearing animals following heterologous prime boost vaccination with MVA.m5T4 and pAd.m5T4. Vaccines were given on day 0 and day 8 using the same routes and doses as previous experiments, to investigate whether the order of the component vaccines affected the frequency or pattern of m5T4 specific responses. The result is shown in Fig 5.18 below. All peptides were tested. To compare the assays, mean peptide spots/well was divided by mean media spots/well and a response defined as ≥ 2. For clarity, the graph shows media controls, all peptides with two or more responses, a negative peptide (12), and peptide 33 which has been illustrated in the assays discussed above. All concanavalin A responses were too dark to quantify.
Fig 5.18: IFN-γ response to m5T4 peptides after prime boost vaccination.

ELISPOT to m5T4 peptides (labelled 12-37) after heterologous prime boost vaccination (sc&im) on days 0 and 7 with either pAd.m5T4/MV/A.m5T4 (AM) or MV/A.m5T4/pAd.m5T4 (MA) using 5x10⁵ viable splenocytes/ well. Figure shows results for two individual animals for each schedule 2 weeks after first immunisation. MED, media control. All wells assayed in triplicate; error bars show standard deviation. Selected peptides are shown for clarity. One of two assays illustrated.

As Fig 5.18 shows all animals had similar background response to media alone (median 14, range 11-22 spots/ well) but there was considerable variation between the frequency and pattern of responses seen in individual animals. By pre-defined response criteria, two animals, one treated according to each schedule, showed some response to many of the peptides (between 10 and 22), and two showed only infrequent responses (to 1 or 2 peptides only) but no clear difference in the frequency or pattern of responses by heterologous boost schedule emerged. Highest responses are seen to peptides 35 (median 53 range 21-70 spots/well), 18 (median 36.5 range 23-55 spots/well), 17 and 36 (both median 35.5 spots/well). However, as illustrated, responses are seen to a range of other peptides, suggesting that animals AM1 and MA1 may be showing some non-specific immune response within the assay. Immune response prompted by the combination of the vaccine vectors is possible but was not
shown during previous experiments with MVA.LacZ/pAd.GFP where repeated assays showed no response above baseline for a range of m5T4 peptides (14, 17, 19, 21, 29, 30, 33, 35 and 36). The absence of responses to media alone, or to negative peptides (12, 15) refutes the possibility of contaminant in the assay, and makes non-specific response, e.g. to FCS supplementing media, unlikely. The results probably reflect the difficulty of using even this sensitive assay to assess responses to autologous antigen.

5.3.4. Conclusion

Two methods were used to demonstrate immunity after m5T4 vaccination:

(1) serum ELISA for m5T4 specific antibody, and

(2) IFN-γ ELISPOT for m5T4 peptide responses.

Both methods could be used to demonstrate responses in non tumour-bearing animals, albeit with low titre or frequency in comparison to parallel work within the group using the foreign antigen h5T4. Serum antibody responses were attenuated in tumour-bearing animals and unlikely to be useful in the planned therapeutic model. Specific IFN-γ m5T4 peptide responses can be identified after single pAd.m5T4 or heterologous prime boost vaccination which incorporates this vaccine, but frequency and pattern of responses shows considerable individual and assay variation, and required further testing in tumour-bearing animals.
5.4: Combining anti-CTLA4 blockade with m5T4 vaccine schedule

5.4.1. Aims

Anti-CTLA4 antibody was combined with m5T4 vaccine in non tumour-bearing animals, and IFN-γ m5T4 peptide responses were used to look for modulation of the immune response.

5.4.2. IFN-γ responses after anti-CTLA4 antibody alone

Anti-CTLA4 blockade may allow the development of immune response to autologous antibodies; in early phase clinical trials in man, most dose-limiting toxicities had an auto-immune aetiology [150, 161]. Specific m5T4 peptide responses in non tumour-bearing animals were therefore first investigated after treatment with anti-CTLA4 antibody alone by screening IFN-γ responses to peptides 15 (consistently negative) and 35 (positive). Results are shown in Fig 5.19 below. Naive and anti-CTLA4 antibody treated animals had similar number of total splenocytes (0.918 vs 0.752 x10⁸ respectively).

<table>
<thead>
<tr>
<th>A</th>
<th>Naïve</th>
<th>anti-CTLA4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide 35</td>
<td><img src="image-url" alt="Image" /></td>
<td><img src="image-url" alt="Image" /></td>
</tr>
<tr>
<td>Con A</td>
<td><img src="image-url" alt="Image" /></td>
<td><img src="image-url" alt="Image" /></td>
</tr>
</tbody>
</table>
Fig 5.19: IFN-γ response to m5T4 peptide 35 after anti-CTLA4 antibody.

Animals were treated with single dose 100μg anti-CTLA4 antibody ip or nothing (naïve) and responses tested 1 week later. A shows representative responses to peptide 35(above) and the positive control concanavalin A (below) for naïve (left) and anti-CTLA4 antibody treated (right) animals. B shows mean response (spots/well) and standard deviation on a logarithmic scale.

The logarithmic scale allows comparison of media control (1 vs. 5 spots/well for naïve and anti-CTLA4 treated animals respectively) and positive control concanavalin A (404 vs. 314 spots/well respectively). Control responses are of similar magnitude and as expected, there is no specific response to peptide 15. However there is a weak response to peptide 35 in the animal treated with anti-CTLA4 antibody (shown in A upper right, mean 15 spots/well). This response has to be interpreted cautiously due to its low level, but seemed to support investigation of combination of anti-m5T4 vaccination with anti-CTLA4 antibody.
5.4.3. Combining m5T4 vaccine and anti-CTLA4 antibody

Anti-CTLA4 antibody was combined with single pAd.m5T4 vaccine in non tumour-bearing animals, and IFN-γ m5T4 peptide responses were used to look for modulation of the immune response. Published work supports the use of 3 doses 50-100 µg of intraperitoneal (ip) anti-CTLA4 antibody (both 4F10 and 9H10 clones) for proven anti-tumour efficacy [130, 135], whilst single dose 100 µg ip 4F10 is sufficient to antagonise CTLA4 function in an vivo model of allergic encephalitis [237] and enhance host response to Leishmania donovani [238]. As a compromise, two doses of 100µg anti-CTLA4 antibody given 3 days apart were compared using the schedules illustrated in Fig 5.20 below.

![Fig 5.20: Schedule for combining pAd.m5T4 and anti-CTLA4 antibody.](image)

Pairs of animals were treated with single dose pAd.m5T4 sc & im alone or in combination with two doses of 100mcg anti-CTLA4 antibody ip either pre-vaccination (A), peri-vaccination (B) or post-vaccination (C). Control animals received pAd.m5T4 combined with control hamster IgG using the peri-vaccination schedule, or were left completely untreated.
5.4.3.1. IFN-γ responses after combination therapy

The established IFN-γ ELISPOT protocol was used to investigate whether there was enhancement of specific m5T4 peptide responses to pAd.m5T4 vaccination with the addition of anti-CTLA4 antibody in non-tumour bearing BALB/c animals. To allow single plate investigation of responses in all animals in order to minimise variation within the assay, a limited range of peptides which had shown consistent responses after pAd.m5T4 were chosen:

Plate A: media/ 19/ 33/ 35;

Plate B: media/ 17/ 36/ concanavalin A.

To look for early responses which might be relevant in the therapeutic model, initial investigation was made 8 days after pAd.m5T4 vaccination. Results which could be counted are summarised below.

![Graph showing IFN-γ response](image)
Fig 5.21: IFN-γ responses after pAd.m5T4 and anti-CTLA4 antibody: day 8.

Animals were treated with single pAd.m5T4 sc & im alone (■) or in combination with two doses of 100mcg anti-CTLA4 antibody ip either pre-vaccination (■), peri-vaccination (■) or post-vaccination. Control animals received pAd.m5T4 and isotype control hamster IgG using the peri-vaccination schedule (hatched), or were left untreated (discussed below). All tests conducted in triplicate. Error bars show SEM. Best of three results shown: A shows Plate A, B shows Plate B.

Fig 5.21.A shows strong responses to peptides 19 (mean 50 spots/well), 33 (42 spots/well), and 35 (52 spots/well) after single pAd.m5T4 alone in contrast to a minimal response to media alone (3.7 spots/well). All results were significant by paired Ttest (p<0.05) not adjusted for multiple analyses. In B this animal shows a minor response to peptide 17 (11.7 compared to 6.3 spots/well), but not to peptide 36.

After pAd.m5T4 and peri-vaccination control hamster IgG antibody splenocytes show a high background IFN-γ response when plated with media alone (107 spots/well in A, 75 spots/well in B) but still demonstrate a rise with peptide 33 (165.3 spots/well, T test p=0.009). In contrast, all the animals treated with combination of pAd.m5T4 and anti-CTLA4 antibody show high background IFN-γ response (>100 spots/well) when
plated with media alone, but show no enhancement when re-stimulated with any of the chosen m5T4 peptides. Results for both animals treated with anti-CTLA4 antibody post vaccination were so strong in this assay as to be unquantifiable.

Contamination of the assay media or peptides may have explained this result, but control naive splenocytes (not shown) in the same two plates show <1 spot/test well in media and against all selected m5T4 peptides but do show a robust response to concanavalin A (120 spots/well, ± SEM 18.4). It is possible that assay results are due to contamination of the anti-CTLA4 antibody used in vivo, but this seems unlikely as the aliquot used was tested for endotoxin before use (<0.32 EU/mg), and the same result was seen after treatment with locally and commercially processed anti-CTLA4 antibody; all wells unquantifiably dark.

In an attempt to reveal any peptide specific effect the assay was repeated using 1x10^5 splenocytes/well to titrate demonstrated responses. Background IFN-γ responses of splenocytes in media alone are summarised in Table 5.3 along with responses to representative m5T4 peptides 19 and 35.

### Table 5.3: IFN-γ responses after pAd.m5T4 and anti-CTLA4 antibody: titration

Surplus splenocytes from experiment in Fig 5.19 were suspended in FM and stored in liquid nitrogen. They were carefully thawed and viable cells were resuspended for plating at 1x10^5/well in an IFN-γ ELISPOT against selected peptides m5T4. All tests were carried out in triplicate and results above combine two plates. Results for additional peptides 17, 33 and 36 were of the same magnitude.
Briefly, despite titration, none of the splenocytes from animals treated with combination therapy demonstrate specific responses to selected peptides of m5T4. The m5T4 peptide specific response after single pAd.m5T4 is also lost after titration. Given the low frequencies of responses seen in previous assays this result is not unexpected. Despite freeze/thaw, all splenocytes except those of the naive animal show a response to concanavalin A (range 32-133 mean spots/well) indicating that they remain viable in the assay. Splenocytes of the animal treated with commercial anti-CTLA4 antibody after pAd.m5T4 vaccination continue to show a higher background IFN-γ response against media alone (mean 57.2 spots/well), but do not demonstrate any enhanced specific responses against the selected m5T4 peptides.

The non-specific response may be due to enhancement of an immune response to the adenoviral vaccine vector following anti-CTLA4 blockade, or to re-exposure to a hamster immunoglobulin. In retrospect, additional controls of single and repeated ip dosing of anti-CTLA4 alone and in combination with control vaccine pAd.GFP could have been included to explore these possibilities.

In order to investigate the time course of this non-specific IFN-γ response and ascertain whether m5T4 specific responses could be revealed at a later time point, the experiment was repeated 29 days after pAd.m5T4. To titrate any responses splenocytes were plated at two concentrations: 5x10⁵/well and 2.5x10⁵/well. There are no specific peptide responses in the former experiment: background response to media alone remains high (>65 spots/well) in all animals given anti-CTLA4 antibody, and none of control animals (naive, vaccine only, and vaccine combined with control
hamster antibody) show any significant specific peptide responses although all respond to concanavalin A. The naive animal, for example, shows media response of 3.4 spots/well and concanavalin A response 86 spots/well. Results from the latter experiment are shown in Fig 5.2.

![Fig 5.2: IFN-γ responses after pAd.m5T4 and anti-CTLA4 antibody: day 29](image)

Animals were treated with single pAd.m5T4 sc & im alone (4) or in combination with two doses of anti-CTLA4 antibody ip either peri-vaccination (1) or post-vaccination (3.1, local or 3.2 commercial anti-CTLA4). Control animals received pAd.m5T4 and isotype control hamster IgG using the peri-vaccination schedule (2), or were left untreated (discussed above). 2.5x10^4 splenocytes plated per well. All tests conducted in triplicate. Error bars show SEM. One of two results shown. There was only room on the plate for investigation of responses to peptide 36 in the two illustrated animals.

No peptide specific responses are seen after pAd.m5T4 alone at this later time point. Titration of the assay allows demonstration of specific m5T4 peptide responses but only in animals given combination pAd.m5T4 and anti-CTLA4 antibody treatment. A similar pattern is seen after both peri-vaccination (1) and post-vaccination (3.1 & 3.2) anti-CTLA4 antibody with two animals (both treated with commercially processed anti-CTLA4) showing modest increases in IFN-γ spots with all three peptides tested,
with a doubling of background response to peptide 35. The animal treated with anti-CTLA4 processed locally (3.1) shows stronger responses to peptides 19, 35, and 36, all of which are more than double background response to media. All animals showed similar responses to concanavalin A (range 47-92 spots/well).

In conclusion, IFN-γ ELISPOT does not demonstrate of enhanced m5T4 peptide responses after combination of pAd.m5T4 vaccine with anti-CTLA4 antibody. On the contrary, all specific responses were lost on investigation one week after vaccination, and low frequency responses could be demonstrated one month after vaccination, but only on titration of the established assay. It is possible that weak vaccine responses may be more durable following combination therapy, but this would have required confirmation with a repeat assay, and it seems questionable whether these weak responses are relevant in vivo. This work does show the difficulty of demonstrating robust reproducible in vitro responses against an autologous target.

5.4.3.2. Immune effect of combination therapy: total splenocytes

ELISPOT shows consistent non-specific IFN-γ responses after combination pAd.m5T4 and anti-CTLA4 antibody, and some effect after vaccine and hamster immunoglobulin. In order to characterise this effect further, total splenocytes harvested were compared for all animals included in the experiment described in Fig 5.20. Below, Fig 5.23 shows total splenocytes both 8 and 29 days after treatment. BALB/c animals have increased total splenocytes after pAd.m5T4 vaccination compared to naive controls in keeping with earlier results. This result is sustained four weeks after vaccination. No significant difference is seen at either time point with the addition of anti-CTLA4 antibody.
Fig 5.23: Total viable splenocytes after pAd.m5T4 and anti-CTLA4 antibody.

Pairs of mice were treated with single pAd.m5T4 sc & im alone (■) or in combination with two doses of anti-CTLA4 antibody either pre-vaccination, peri-vaccination or post-vaccination (data pooled; wide hatch). Control animals received pAd.m5T4 and control hamster IgG peri-vaccination (aham IgG; narrow hatch), or were left untreated (□). Spleens were harvested 8 and 29 days after vaccination and processed and counted using established protocol. Summary results are presented above.

Animals treated with pAd.m5T4 and control anti-hamster IgG antibody only show increased number of splenocytes at the earlier time point. The addition of anti-CTLA4 antibody does not appear to increase the total number of splenocytes above the response seen to pAd.m5T4 alone, but it may have altered the phenotype of these cells. Had time allowed, changes in cell phenotype could have been investigated using flow cytometry; again animals treated with anti-CTLA4 alone would have been important controls.

5.4.3.3. Cytokine ELISA after combination therapy

Because of the difficulties titrating the established IFN-γ ELISPOT after combination immunotherapy, an alternative cytokine ELISA was used. Supernatant harvested after
in-vitro re-stimulation with m5T4 peptide 19 (a necessary step in the established assay) was used to look for changes in murine IL-2 release after pAd.m5T4 ± anti-CTLA4 antibody combination therapy.

![Graph](image)

**Fig 5.24: mIL-2 Elisa after pAd.m5T4 and anti CTLA4 blockade: day 29**

*Animals were treated with pAd.m5T4 sc & im in combination with two doses of anti-CTLA4 antibody either peri-vaccination (1) or post-vaccination (3). Anti-CTLA4 was processed locally (3.1) or commercially (1 and 3.2). Control animals received pAd.m5T4 alone (4) or in combination with control hamster IgG (2), or were left untreated (5). mIL-2 release after in vitro restimulation with m5T4 peptide 19 was assessed by Elisa. All tests conducted in duplicate; error bars show standard deviation.*

As **Fig 5.24** shows, naive splenocytes (5) show low mIL-2 release in media (31 pg/mL) and no increase in the presence of m5T4 peptide 19. The same result is seen 29 days after single pAd.m5T4 alone (4) or in combination with hamster IgG (2). In contrast, all animals treated with a combination of pAd.m5T4 and two doses of anti-CTLA4 show very high mIL-2 release in media in vitro (range 1990-2808 pg/mL). Two show no increase in the presence of m5T4 peptide 19, but one animal treated
with peri-vaccination anti-CTLA4 antibody (I), shows slightly enhanced mIL2 release after re-stimulation with peptide 19 from 2134 to 2988 pg/mL, which is probably not significant. This assay suggests the non-specific activation after combination therapy seen in repeated IFN-γ ELISPOTs is not the result of some technical problem with that assay, but a real ex-vivo effect in this experiment.

5.5: In vivo tumour experiments (I)

Two autologous m5T4 tumour models were investigated using active heterologous prime boost vaccination in the therapeutic setting. The autologous target was chosen to allow investigation of the role of regulatory immune features such as CTLA4, and to create a more relevant model to parallel the clinical reality of patients with chronic exposure to autologous tumours, many of which express oncofoetal antigens such as 5T4. The use of heterologous prime boost vaccination has the proven advantage in many settings of boosting vaccine response without creating tolerance or a dominant anti-vector response. The choice of vaccine schedule and doses in this work is based on investigation of anti-m5T4 immunity [202] and exploratory work in protection and therapy [239]. Investigation of antibody responses showed evidence of m5T4 immunity in non tumour-bearing animals, but detection was attenuated in tumour-bearing animals. IFN-γ responses to specific m5T4 peptides were detected after pAd.m5T4 and heterologous prime boost, but not after MVA.m5T4. With hindsight, further investigation of the vaccination route and schedule in tumour-bearing animals would have been appropriate. Two alternative vaccine routes have shown benefit in alternative tumour models: intravenous and intra-tumoural [240] but both require high
vaccine titres for small volume inoculation which would have presented practical difficulties with the proposed reagents.

With these caveats, the two autologous m5T4 tumour models investigated were transfected B16.m5T4 and spontaneously m5T4 expressing CT26.wt.

5.5.1. B16 melanoma therapeutic vaccination model.

Initial investigation in the existing B16.m5T4 model compared active heterologous prime boost vaccination (MVA.m5T4/ pAd.m5T4) with control vaccination (MVA.LacZ/ pAd.GFP) after subcutaneously tumour challenge. A further control group received no therapy. Therapy commenced on day 5 by which time 17/25 animals had visible tumour. There was a brief delay in tumour growth in vaccinated animals, only 6/10 having measurable tumours by day 15 compared to 14/15 control animals, but this did not result in a significant difference in mean tumour area (Fig 5.25) or in median survival as shown in Fig 5.26 below.

![Graph showing tumour growth](Image)

**Fig 5.25:** Tumour growth after therapeutic MVA.m5T4/pAd.m5T4 vaccination: B16 model

*Graph shows mean tumour area following subcutaneous challenge (day 0) after active (♦) or control (♦) prime boost vaccine schedule, or no treatment (◊). Tumours were measured in two dimensions and*
animals sacrificed when area exceeded 1.24cm². Mean tumour area was calculated for each group; after day 18 culled animals were censored at last measurement. Overlapping error bars are omitted for clarity.

![Graph showing survival probabilities](image)

**Fig 5.26: Survival after therapeutic prime boost vaccination: B16 model (I)**

Graph shows Kaplan Meier survival for active vaccine (bold) against control vaccine (dotted); the smaller group of animals left without treatment showed same survival as after control vaccination.

Median survival was 22 days (95% CI 18.9-25.0) for active vaccination to 20 days (95% CI 17.5-22.5) for control vaccination (log rank, Mantel Cox, p=0.244). Median survival of control tumour group was also 20 days (95% CI 17.9-22.1). This experiment showed no benefit for MVA.m5T4/pAd.m5T4 in this therapeutic tumour model.

### 5.5.2. Comparison of B16 and CT26 therapeutic vaccination models.

Next, investigation compared active heterologous prime boost vaccination (MVA.m5T4/ pAd.m5T4) with control vaccination (MVA.LacZ/ pAd.GFP) in both B16 melanoma and CT26.wt colon cancer tumour models. Therapy commenced 24
hours after tumour inoculation before visible tumour growth to see if this would benefit vaccinated animals.

5.5.2.1. B16 melanoma model (II)

This experiment showed a small early delay in B16.m5T4 tumour growth with active vaccination (mean tumour area day 17: 0.09 vs. 0.29 cm$^2$) but no survival advantage: median survival 33 days (95% CI 24-42) to 32 days (95% CI 30-34). Control survival was longer than expected from the first experiment, where median survival had been 20 days for both control vaccination and untreated animals. **Fig 5.27** shows uncensored Kaplan Meier survival data.

![Graph showing survival data](image)

**Fig 5.27: Survival after therapeutic prime boost vaccination: B16 model (II)**

*Graph shows no difference in Kaplan Meier survival for active vaccination (bold) compared to control vaccination (dotted); log rank (Mantel cox) p=0.788.*

Two animals (both housed in the second cage) did not develop subcutaneous tumours which may reflect under dosing at inoculation step; in contrast, median survival of the
first cage of control treated animals was 21 days (95% CI 17-25), which is more in keeping with earlier data. Although censoring the second cage of control animals suggests a small benefit to active vaccination given shortly after tumour inoculation (Mantel Cox p=0.035), this is not confirmed by rates of tumour growth: as Fig 5.28 shows there is no convincing difference between the patterns of tumour growth seen after active and control vaccination.

In summary, there is no convincing evidence of therapeutic benefit of heterologous prime boost vaccination with MVA.m5T4/ pAd.m5T4 in the B16.m5T4 model.

5.5.2.2. CT26 adenocarcinoma model

In contrast the same heterologous prime boost schedule showed a small but significant benefit over control vaccination (MVA.LacZ/ pAd.GFP) in the CT26.wt model. This was shown by a transient delay in tumour growth with active vaccination, with significant differences in mean tumour area between active and control vaccine treated animals seen at days 14 (0.20 vs. 0.43cm²; T test p=0.034) and 17 (0.37 vs. 0.96 cm²; p=0.003) as shown in Fig 5.29.
**Fig 5.29: Mean tumour growth in therapeutic vaccination models**

Graph shows mean tumour area following sc challenge (day 0) after active (■) or control (■) vaccine schedule as detailed in the methods. Tumours were measured in two dimensions every 2 days; mean tumour area was calculated for each group until the first animal was culled. Error bars show SD.

This transient delay in tumour growth translates into a small improvement in median survival as shown in **Fig 5.30**. Median survival was 21 days (95% CI 19.8-22.2) for active vaccination compared to 19 days (95%CI 18.0-20.0) for control vaccination (log rank Mantel Cox p=0.042).

**Fig 5.30: Survival after therapeutic prime boost vaccination: CT26.wt model**

Graph shows Kaplan Meier survival for active vaccination (bold) against control vaccination (dotted).
In summary, there is evidence of effect for heterologous prime boost MVA.m5T4/pAd.m5T4 in the CT26.wt model but therapeutic efficacy is slight even when vaccination commences shortly after tumour challenge. This model with demonstrable benefit was chosen to explore the combination of vaccination and anti-CTLA4 blockade in an attempt to improve poor therapeutic efficacy. Results could have been confirmed by repetition, but this was balanced against the resource requirements and the attempt to minimise the number of animals used in this work. Use of this model was also preferred as expression of m5T4 in this model is inherent rather than an engineered over-expression, and a gastro-intestinal adenocarcinoma provided a parallel model for the phase II clinical trial in gastric and oesophageal adenocarcinomas. The main challenge was to demonstrate evidence of m5T4 immunity using the established techniques, without the benefit of m5T4 knockout animals on the BALB/C background.

5.5.3. Antigen expression ex vivo

To explore a possible mechanism for poor efficacy of therapy, m5T4 antigen expression was assessed in ex-vivo tumours. As Fig 5.31 shows, there was no loss of antigen expression to account for therapeutic failure of active vaccination schedule. Ex vivo tumour cells showed similar expression of m5T4 by flow cytometry after active and control vaccination: 89% and 92% respectively. Both show a similar frequency of m5T4+ cells to control B16.m5T4 maintained in tissue culture throughout the experiment (82%). Similar result was obtained for CT26.wt which was 90% m5T4+ in tissue culture and 80% m5T4+ ex vivo after control vaccine schedule; tumour obtained after active vaccine became contaminated and could not be assessed.
Fig 5.31: m5T4 expression of ex-vivo B16.m5T4.

Subcutaneous tumours were dissected post-mortem after MVA.m5T4/ pAd.m5T4 (active vaccine; below) or MVA.LacZ/ pAd.GFP (control vaccine; above), expanded for a week in tissue culture, and m5T4 expression assessed by flow cytometry using antibody B3F1 and isotype control mouse IgG2a. Left plot shows B16.m5T4 maintained in tissue culture (TC) throughout the experiment.

5.6: In vivo combination immunotherapy (II)

5.6.1. Design

Despite the difficulty demonstrating modulation of m5T4 vaccination response using anti-CTLA4 blockade in non tumour-bearing animals, the efficacy of combining anti-CTLA4 blockade with m5T4 vaccination was tested in a therapeutic model. Extrapolating from these immune assay results suggests any benefit was likely to be marginal, so a simple design was used to test whether combining anti-CTLA4 antibody with m5T4 vaccination enhanced the previously observed minor therapeutic benefit, whilst minimising the number of animals used in the experiment. Following subcutaneous CT26.wt tumour challenge, animals were treated with MVA.m5T4/ pAd.m5T4 combined with peri-prime anti-CTLA4 blockade. Control animals received
no treatment, and alternative treatment groups received vaccination alone or anti-CTLA4 antibody alone. Efficacy was assessed by analysis of tumour growth and survival. Immunity was assessed by IFN-γ ELISPOT and by IL-2 cytokine ELISA response to m5T4 peptides.

5.6.2. Results: Tumour growth

![Graph showing tumour growth](image)

**Fig 5.32: Tumour growth after m5T4 vaccination and anti-CTLA4 blockade**

Graph shows group mean subcutaneous tumour surface area calculated from bi-dimensional measurements against days after tumour challenge for animals left untreated (□) or treated with MVA.m5T4/ pAd.m5T4 alone (■), anti-CTLA4 antibody alone (■), or a combination of the two (■). After day 16, measurements are censored for culled animals. Standard deviation error bars are shown for two groups only for clarity.

There was no significant difference in mean tumour area with prime boost vaccination or combination therapy compared to untreated animals, as shown in Fig 5.32 above. However slightly slower mean tumour growth was seen in animals treated with CTLA4 blockade alone; the individual tumour growth curves for this group, which show some slowing of growth between days 14 and 18, and tumour regression in 1/8 animals are illustrated against those for untreated animals in Fig 5.33 below.
**Fig 5.33: Individual tumour growth after anti-CTLA4 blockade**

Graphs show individual tumour surface area calculated from bi-dimensional measurements against days after tumour challenge for animals left untreated (left; □) or treated with anti-CTLA4 antibody alone (right; ■).

### 5.6.3. Results: Survival

There was no survival benefit after either prime boost vaccination or combination therapy compared to untreated animals. Median survival was 18 days in all three groups with 95% confidence intervals of 15.2 - 20.8, 16.6 - 19.4 and 16.8 - 19.2 days respectively. Kaplan Meier survival curves for all groups are shown in **Fig 5.34.A**.

![Survival curves for different treatment groups](image-url)
**Fig 5.34:** Survival after therapeutic anti-CTLA4 antibody and m5T4 vaccination.

*Kaplan Meier survival after tumour challenge for animals left untreated (...) or treated with MVA.m5T4/ pAd.m5T4 alone (-----), anti-CTLA4 antibody alone (---------), or a combination of the two (-----). A illustrates all groups; B shows tumour control vs. anti-CTLA4 treatment alone for clarity.*

However, the delay in tumour growth seen in animals which received anti-CTLA4 antibody alone does translate into a small improvement in survival, with median overall survival increased by four days to 22 days (95% CI 21.2-22.8; log rank p=0.012 compared to untreated animals). Kaplan Meier survival curve for anti-CTLA4 antibody treated animals is shown against untreated animals only in **Fig 5.34.B** for clarity. Using an alternative method confirms this survival benefit from anti-CTLA4 antibody alone: 20 day survival, calculated by Kalbfleish and Prentice method, is 75% (95% CI 32-93) after treatment with anti-CTLA4 antibody alone compared to 14% (95% CI 1-46) after no treatment, 25% (95% CI 4-56) after vaccine alone, and 13% (95% CI 1-42) after the combination.
5.6.4. Results: IFN-γ ELISPOT

A planned analysis of immune responses was undertaken 16 days after tumour challenge, which corresponds to 14 days after MVA.m5T4 prime and 7 days after pAd.m5T4 boost vaccination when m5T4 peptide specific responses could be detected in non tumour-bearing animals. Non-specific responses to negative control media alone and positive control concanavalin A are shown in Fig 5.35. There was a significant increase in IFN-γ response in control media after any immunotherapy compared to untreated tumour-bearing animals: mean 60 spots per well after m5T4 vaccination alone (p=0.005 by unpaired T test, not adjusted for multiple analyses), 70 after anti-CTLA4 antibody alone (p=0.001), and 127 after combination therapy (p=0.005) compared to 33 after no immunotherapy. There was also a significant increase in detected IFN-γ response to polyclonal stimulation with concanavalin A after all tested immunotherapies compared to untreated tumour-bearing animals: mean 1116 spots per well after m5T4 vaccination alone (p=0.001), 1295 after anti-CTLA4 antibody alone (p=0.004), and 1009 after combination therapy (p=0.005) compared to 322 for untreated animals challenged with CT26.wt.

Naive splenocytes included as extra control on second plate had very low mean responses to media and all m5T4 peptides of ≤ 13 spots per well, with a relatively high standard deviation (±9.3 spots/ well) but did respond to concanavalin A (163±24.7).
**Fig 5.35: IFN-γ response after combination m5T4 vaccination and anti-CTLA4 antibody 16 days after tumour challenge: controls**

Graph shows mean spots per well from IFN-γ ELISPOT established 16 days after tumor challenge in untreated (red ■) animals, and after MVA.m5T4/ pAd.m5T4 alone (blue ■), anti-CTLA4 antibody alone (purple ■) or both combined (blue ■). A control non-tumour bearing animal was included (□). Cells were plated at 1x10⁵/well. Controls are shown. All tests were carried out in triplicate. Error bars show SD.

However, as **Fig 5.36** shows, there is no evidence of specific responses in any of the tumour-bearing animals on re-stimulation with selected m5T4 chosen for consistent negative (peptide 15) and positive (peptides 17, 19, 33, 35 and 36) responses following pAd.m5T4 vaccination in non-tumour-bearing animals.
Fig 5.36: IFN-γ responses to specific m5T4 peptides after combination m5T4 vaccination and anti-CTLA4 antibody 16 days after tumour challenge.

All tests were carried out in triplicate. Graph shows mean results for media control and selected m5T4 peptide wells. X axis shows treatment group (from left to right): tumour alone, MVA.m5T4/pAd.m5T4 vaccine alone, anti-CTLA4 antibody alone, and combination treatment. Error bars show SD.

In an attempt to detect specific responses despite high background IFN-γ, vaccine and anti-CTLA4 combination treated splenocytes were titrated on a second plate to $4 \times 10^5$/well with same result. In summary, m5T4 specific responses could not be detected in tumour-bearing animals treated with MVA.m5T4/pAd.m5T4 vaccine, anti-CTLA4 antibody, or combination therapy by this method.

5.6.5. Results: Cytokine ELISA

A complementary cytokine ELISA (IL-2) was also used to assess potential m5T4 specific responses. ELISA has the advantage of quantification using a standard curve, and allows easier titration by dilution than the ELISPOT which is titrated by splenocyte concentration on the day of cell harvest. The main disadvantage is the loss of specific cell responses. Supernatant was collected after in vitro re-stimulation of
splenocytes of animals from the in vivo tumour therapy experiment with selected peptides of m5T4, stored, and used for later mIL-2 cytokine ELISA.

Fig 5.37: Combination immunotherapy tumour model: day 16 murine IL2 Elisa

Supernatant harvested after 48 hours in vitro re-stimulation with selected m5T4 peptides was used in a mouse IL-2 ELISA. X axis shows treatment group (left to right): tumour alone, MVA.m5T4/pAd.m5T4 prime boost vaccination, anti-CTLA4 antibody alone, and combination immunotherapy. Control naive splenocytes were also included. All tests except naive control were carried out in triplicate and graph shows mean results; error bars show SD.

Higher IL-2 was detected after incubation of splenocytes in media ± selected m5T4 peptides in animals treated with any immunotherapy than in naive animals or those only challenged with tumour. For example, Fig 5.37 shows that for media alone, mean IL-2 was 37 pg/mL (i.e. per 5x10^5 splenocytes) after vaccine alone, 111 pg/mL after anti-CTLA4 antibody alone, and 115 pg/mL after the combination compared to 16 pg/mL in naive animals and 6 pg/mL in tumour-bearing animals.

There are no responses to peptides 17 and 19, but all animals show a small increase in mean IL-2 detected after incubation with peptide 35: 55 pg/mL after vaccine alone, 148 pg/mL after anti-CTLA4 antibody alone, 180 pg/mL after the combination, 25 pg/mL in naive animals and 20 pg/mL in tumour-bearing animals. The magnitude of
increase is highest for the untreated tumour-bearing animal (3.3 x media result), and is the same for all other animals, including the naive one (1.3-1.6 x media result). Whilst a response to peptide 35 in the untreated tumour-bearing animal may reflect exposure to m5T4 expressed on the CT26.wt tumour, the presence of the same relative result (albeit at lower frequency) in the naive animal casts doubt on the specificity of responses generated to this peptide.

In conclusion, IL-2 ELISA supports the results of the IFN-γ ELISPOT, with increased background responses in all animals treated with immunotherapy. There is no convincing evidence of specific m5T4 immunity after vaccination with or without anti-CTLA4 blockade in tumour-bearing animals.

In retrospect, including vaccine treated (pAd.m5T4 and combination MVA.m5T4/ pAd.m5T4) non tumour-bearing animals in the experiment would have been of interest. With additional time and resources, this method could have been extended to additional m5T4 peptides (e.g. 15, 33, 36).

5.6.6. Conclusion

This final set of experiments demonstrated no efficacy from combination immunotherapy with heterologous MVA.m5T4 prime/ pAd.m5T4 boost vaccination and anti-CTLA4 blockade in a therapeutic model. There was however a small therapeutic benefit seen in animals treated with anti-CTLA4 blockade alone, both in terms of slower mean tumour growth, and a small survival benefit. No m5T4 specific responses were seen by IFN-γ ELISPOT, but there was increased ex vivo IFN-γ detected in all animals treated with immunotherapy. Alternative IL-2 ELISA again demonstrated increased ex vivo cytokine responses in animals treated with
immunotherapy, and modest responses to peptide 35 alone which were also seen in naive and tumour-bearing untreated animals. The negative results from this investigation of combining anti-CTLA4 blockade with a m5T4 prime/boost vaccination schedule will be considered in detail in the discussion chapter which follows.

5.7: Brief discussion

To recapitulate, the main aims of this work were:

1. To establish an active therapeutic vaccination model using the autologous murine oncofoetal antigen m5T4.
2. To develop an immunological measure of response to m5T4 vaccination.
3. To characterise response to vaccination against the antigen m5T4 in naive and tumour-bearing animals.
4. To attempt to modulate vaccination response using anti-CTLA4 blockade.
5. To test efficacy of combining anti-CTLA4 blockade with the therapeutic m5T4 vaccination model.

In summary these aims were met in part. Heterologous MVA.m5T4/ pAd.m5T4 vaccination showed some evidence of weak therapeutic benefit, but the search for an alternative tumour model with more favourable growth kinetics was unsuccessful. Specific m5T4 immune responses could be detected by serum antibody ELISA and IFN-γ ELISPOT assays in naive animals but were much lower frequency than published responses to h5T4, or to known responses in m5T4 knock out animals, and were further attenuated in tumour-bearing animals. The addition of anti-CTLA4
blockade did not result in any demonstrable augmentation of m5T4 specific immunity shortly after vaccination in non tumour-bearing animals; however, m5T4 responses were detected one month after combination therapy by assay titration. Given the timing of these responses and the rapidly lethal tumour model available, the therapeutic failure of combination m5T4 vaccination and anti-CTLA4 blockade was unsurprising, and has been discussed in detail above.

The attempt to establish a useful autologous model is creditable but difficult, and with hindsight it may have been wise to utilise a parallel heterologous model to further validate some of the techniques, and to elucidate the differences between immune responses to self and foreign antigen in the context of anti-CTLA4 blockade.

Confirming that, even with a heterologous prime boost schedule, m5T4 vaccination alone or in combination with anti-CTLA4 blockade, shows no significant therapeutic benefit in the autologous model, in contrast to benefit seen using similar approaches in the heterologous h5T4 model, is believable, and problematic if we believe this is a truer parallel of the clinical situation.
CHAPTER 6: DISCUSSION

6.1: Brief introduction

To briefly recap, this project aimed to explore the modulation of T regulatory activity for cancer therapy, and asked whether the modulation of immune control with anti-CTLA4 blockade led to significant anti-tumour activity. Clinical and laboratory investigation of anti-CTLA4 blockade using the antibody tremelimumab in a phase II trial of second-line therapy in patients with advanced oesophageal and gastric adenocarcinomas was combined with an attempt to establish and test a suitable pre-clinical model based on a therapeutic vaccination schedule against the oncofoetal tumour associated antigen (TAA) 5T4.

6.2: Clinical trial: clinical results

6.2.1. Clinical aims

As part of this project, a phase II, single-centre, open-label, non-randomised study investigated the use of tremelimumab, a fully humanised anti-CTLA4 antibody, as a second-line therapy for patients with metastatic gastric and oesophageal adenocarcinomas [241]. The primary end-point was clinical anti-tumour efficacy as described by the objective response rate; clinical secondary objectives aimed to assess additional evidence of anti-tumour activity and characterise the safety profile and tolerability of the drug in this setting.
6.2.2. Primary end-point: objective response rate

The primary end-point of this phase II trial was negative: the objective response rate for tremelimumab as a second line treatment for metastatic esophageal and gastric adenocarcinomas was only 5% (1 of 18), but there was clinical benefit with evidence of disease control in a small cohort of patients, as assessed by stable CT scan (4 of 18) and/or decline or stabilization of serum TAA (5 of 18). The one partial response was achieved incrementally over 24.8 months, with very different kinetics from cytotoxic chemotherapy responses, and has proven remarkably durable, with the patient remaining alive, well, and on treatment over 4 years after treatment began. No demographic or clinical features identified this patient as unusual at screening, and some such as the presence of multiple sites of visceral metastases, including in the liver, have been reported to predict poor prognosis [216, 242].

6.2.3. Secondary end-point: survival

Despite the low objective response rate in this trial, median overall survival at 4.8 months (95% CI 4.1-5.6) was comparable to near contemporaneous local audit data of second line chemotherapy where median overall survival was 5.0 months (3.1-6.9), and similar to that reported in a 2005 review of 12 phase II trials of second line chemotherapy for gastric cancer, where average median survival was 5.6 months (range 2.5-11) [5]. At 12.2 months, survival for patients who had stable disease at 3 months on CT scan following tremelimumab compares favorably with that of chemotherapy responders whose mean survival was 9.1 months (range 5.5-12 months). Despite a proliferation of phase II studies of potentially active
chemotherapeutics such as oxaliplatin, docetaxel, irinotecan, mitomycin and fluorouracil-based drugs including S1, and some preliminary evidence of disease stabilization with targeted agents such as cetuximab and sunitinib, recent review of second line therapy in advanced gastric cancer reported similar results [243].

6.2.4. Responses after CTLA4 blockade: assessment, kinetics and durability

Both of the anti-CTLA4 antibodies in clinical development, tremelimumab (previously CP-675,206 and briefly ticilimumab) and ipilimumab (MDX010) have shown interesting anti-tumor activity in melanoma. Both antibodies are fully humanized but they have different isotypes: tremelimumab is IgG2, ipilimumab IgG1κ. This results in different pharmacokinetic properties, and along with preliminary clinical data has led to different dosing schedules: ipilimumab is administered every 3 weeks [217], tremelimumab using either monthly or 3 monthly dosing schedules [151].

Objective response rates for both drugs have been modest at around 10-15% when measured using classical CT response criteria. However, some responses have been reported either late, or after apparent disease progression: an analysis of kinetics of objective responses to ipilimumab in 41 of 269 patients with stage III or IV melanoma in five trials, reported late onset complete response (CR) and partial response (PR) occurring after 10-106 weeks and 5-62 weeks respectively [244], and published case reports demonstrate CR after apparent clinical, serological and radiological disease progression [245]. This unusual time course of responses has led to concern that reliance on established CT response guidelines such as RECIST or WHO [246], developed to validate rapid responses to traditional cytotoxic chemotherapy, will underestimate the activity of such agents. Increasing experience with targeted therapies

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suggests that modest responses or durable stable disease are valid surrogate end-points of meaningful clinical outcomes such as progression free survival [247]. Durable disease stabilization in a phase III study of the mTor\(^{11}\) inhibitor temsirolimus in patients with poor prognosis renal cancer translated into a significant 3.6 month survival advantage over control interferon α (hazard ratio for death 0.73; 95% CI 0.58-0.92; p=0.008) [248]. In contrast, for example, classic RECIST responses have been shown to correlate poorly with survival of gastrointestinal stromal tumour (GIST) patients treated with the tyrosine kinase inhibitor imatinib [249].

Hodi’s group characterised responses in a large cohort of melanoma patients treated in three randomized phase II trials of ipilimumab as occurring in four patterns: (a) shrinkage of baseline lesions (b) durable stable disease (c) late response after apparent disease progression and (d) reduction in total disease burden despite appearance of new (often nodal) lesions all of which were associated with favourable survival. In the prospective cohort of 227 patients 22 of 123 patients with progressive disease by WHO criteria at 12 weeks went on to have later evidence of partial responses (5) or durable stable disease (17). These findings have led Hodi to propose an alternative “immune related response criteria” derived from modification of WHO criteria to allow incorporation of the measurements of new lesions into the sum of tumour burden, such that they do not necessarily define disease progression [250-251]. Where responses have occurred in melanoma with anti-CTLA4 blockade, many are durable. For example, in an early NCI trial of ipilimumab +/- peptide vaccine with some intrapatient dose escalation, 9 of 23 CT responses were sustained beyond 2 years [226], and

\(^{11}\) Mammalian target of rapamycin
4 of 8 responders in the original tremelimumab phase I/II studies have achieved durable benefit for more than 2 years [151].

6.2.5. Toxicity

Toxicity seen in patients with metastatic gastric and oesophageal cancer treated with tremelimumab in this small phase II study was similar to that described in the available literature. Immune related adverse events (IRAE) were common but generally mild, with the exception of one grade 3 transaminitis which resolved spontaneously and one fatality due to fulminant colitis and bowel perforation. Evidence of immune activation, as documented by increased T cell activation markers (CD4⁺CD25⁺) and lymphocytosis respectively, support an immune mediation in these events. There was no evidence of unexpected or increased frequency of observed toxicity and treatment was generally well tolerated despite evidence of rapid disease progression in the majority of patients. The unusual toxicity profile of novel immunotherapeutics presents a clinical challenge in both diagnosis and management; one patient with an appropriate clinical presentation was screened for hypophysitis by serum hormone profile and MRI imaging but had developed a solitary metastasis in the cerebellum.

6.2.6. Anti-CTLA4 antibody therapy in gastrointestinal adenocarcinoma

The other published trial of anti-CTLA4 antibody treatment in gastrointestinal adenocarcinoma using tremelimumab in patients with metastatic colorectal cancer who had exhausted all standard chemotherapies also reported a solitary objective response (1 of 43 patients) which occurred 9 months into treatment and lasted for a further six months [170]. Despite the poor response rate, survival at six months (45%) was higher
than expected for this heavily pre-treated group, but it is not possible to draw any conclusions from this result as the study shared some of the design limitations of that discussed in this project.

6.2.7. Trial design considerations

This single-arm phase II study of tremelimumab as second line treatment for patients with advanced gastric and oesophageal adenocarcinomas has limitations due to the small number of patients, the non-randomised design, and the chosen efficacy end-point of objective RECIST response. Sample size was calculated to detect a response rate of approximately 20% with 95% power using Gehan’s two-stage design, and was powered to give an estimate of response rate with a standard deviation of 10%. Disappointingly, the objective response rate for tremelimumab in this study is only 5%, and in fact the only objective response occurs outside the predicted 12 month time-line for completion of the study. Supportive evidence for clinical effect was seen in clinical responses with disease stabilization, in serum TAA responses, and in survival data which compares favourably with published and contemporaneous audit data. With hindsight, a survival end-point may have been more illuminating, but much of the data which brings into question the use of established response criteria in immunotherapy has emerged since the trial was designed. A survival end-point would probably require a randomized control arm which raises ethical and recruitment issues in a situation with no proven standard of care. Appropriate control arms would be either best supportive care or a cytotoxic chemotherapy regime with evidence of disease activity, but recruitment is difficult as patients and doctors tend to have established preferences. Few of the patients who entered the trial, many of whom had already survived beyond median predictions, would have accepted randomization to
no treatment. Several of those screened for (8 of 48) or entering (2 of 18) the trial had prompted their own referrals when details became available through CRUK website.

Despite the attractions of randomized control trials, an open-label, non-randomised phase II design was appropriate, achievable, and ethical as a means of investigating a novel immunotherapy approach in this poor prognosis group of patients. With the low but remarkably durable response to tremelimumab observed, practical clinical use of this agent in patients with metastatic and oesophageal adenocarcinomas would need to be targeted to potential responders. Such targeting of tremelimumab to potential immune responders requires a better understanding of the effects and kinetics of CTLA4 blockade in treated patients.

6.3: Clinical trial: laboratory results

6.3.1. Laboratory aims

The phase II trial of tremelimumab as second-line therapy in patients with metastatic gastric and oesophageal cancer included planned laboratory investigation of lymphocyte phenotype and function after anti-CTLA4 blockade and aimed to identify and assess potentially immunoregulatory phenotypes, including natural Treg, and to look for evidence of specific anti-tumour activity, using responses to two relevant tumour associated antigens (TAA): 5T4 and CEA. This work also aimed to investigate correlation between laboratory and clinical outcomes.
6.3.2. Immune phenotype of trial patients

The immune system changes in individuals and populations with age and disease; a first step in any investigation of changes with a specific treatment with time is the need to characterise consistent pre-treatment results. Lymphocyte phenotype was interrogated using both contemporaneous flow cytometry which allowed validated quantification of well defined lymphocyte sub-groups, and later intracellular staining for two markers of regulatory T cell phenotype, FoxP3 and CTLA4, which investigated changes in relative proportions of these cells with treatment. In the absence of a control group in the trial, two methods were used to further describe baseline phenotype. Comparison, where available, showed no significant variation in absolute phenotype (n=7) nor relative Treg (n=4) between screening and pre-treatment samples taken a median of 7 days apart. However, trial patients did have significantly higher Treg defined by FoxP3\(^+\) than normal healthy volunteers (1.43 vs. 0.27 \% total lymphocytes; \(p=0.0006\)), whilst there was no significant difference between results for trial patients and a small cohort of patients with metastatic renal cancer. These findings are in keeping with published results in many tumour types, including gastro-intestinal [95], and renal cancer [186], where higher Treg are found in cancer patients and appear to be associated with poor prognosis.

Age-related changes in the immune system have been investigated as a possible cause of age-related disease; a large study of the well elderly (65+) in Cambridgeshire demonstrated a significant effect of ageing on total lymphocytes and CD4\(^+\), CD3\(^+\) and CD19\(^+\) subsets [252] which has been confirmed in nonagenarians [253] and is supported by work in animals [254]. Huppert et al establish normal ranges for CD19\(^+\) lymphocyte counts in the well elderly which are significantly higher than median
counts seen in the patients with advanced gastric and oesophageal adenocarcinomas who entered this trial: for men (65+) median $0.19\times10^9$/L (IQ range 0.12-0.28) compared to $0.07\times10^9$/L for trial patients [252]. Compared to the normal ranges established in the study of the well elderly, the patients in the trial also have low median absolute lymphocyte, CD8$^+$ and CD4$^+$ counts of 1.02, 0.31, and 0.41 $\times10^9$/L compared to 2.13 (IQ range 1.72-2.72), 0.54 (0.38-0.76) and 0.95 $\times10^9$/L (0.70-1.11).

Whilst these differences could be due to technique, the methods used in both studies were very similar. If real, the differences could reflect relative impairment of the adaptive immune system due to advanced cancer or previous cytotoxic treatment. Confirmation would require an additional cohort of patients, and was outside the scope of this study.

6.3.3. Changes in immune phenotype with tremelimumab

Targeting of tremelimumab to potential immune responders requires a better understanding of the effects and kinetics of CTLA4 blockade in treated patients. It is clear that CTLA4 blockade can release useful anti-tumour immunity in a minority, but the mechanisms involved are complex. CTLA4 blockade may act by suppressing Treg control of lymphocyte activation, or more directly on activated lymphocytes which transiently express the target. Maker et al showed an increase in relative FoxP3 gene expression by PCR after ipilimumab treatment in a small group of patients with metastatic melanoma (n=10), and no loss of the suppressive activity of CD4$^+$CD25$^+$ lymphocytes in vitro in a mixed cohort of patients with metastatic melanoma and renal cancer (n=5) [181]. In contrast, Reuben et al documented significant reductions in Treg and constitutive IL-10 production accompanied by a significant increase in activated IL-2 in patients with metastatic melanoma who developed objective
responses after treatment with tremelimumab [179]. They defined Treg by flow cytometry as CD3⁺CD4⁺CD25^high. Natural Treg remain hard to characterize; even FoxP3 is not limited to these cells alone, and therefore in this study Treg were initially assessed by both FoxP3 and intracellular CTLA4. Both methods suggest that Treg numbers rise rather than falling, and show a clear temporal pattern in relation to tremelimumab treatment. These results support the idea that CTLA4 blockade is not acting primarily through Treg. Whilst these results appear to contradict those of Reuben, the use of additional, arguably more specific markers of Treg phenotype, make direct comparison difficult. Additionally, timing of investigation in relation to drug dosing appears important: the decrease in CD3⁺CD4⁺CD25^high lymphocytes described by Reuben is only seen transiently 28 days after starting therapy; in contrast, the rise in Treg by CD4⁺CD25^highFoxp3⁺ and CD4⁺CD25^highCTLA4⁺ described in this trial was found 15 and 30 days after anti-CTLA4 blockade, but also returns to baseline by day 60. Finally, Reuben is describing a significant change in lymphocyte phenotype for a small group of melanoma patients with objective evidence of disease response. The solitary objective response in this trial makes similar investigation impossible, but the same pattern of a transient rise in Treg by FoxP3⁺ was seen for patients both with and without disease progression at first CT scan: from 1.4 to 2.6 % total lymphocytes (PD) and from 2.3% to 4.7% (SD).

The difficulty of defining Treg by phenotype alone, now there is evidence that even FoxP3 can be transiently induced in effector CD4⁺ lymphocytes after activation, with peak expression at 4-6 days after in vitro activation [218], and that FoxP3 expression can be induced without functional T cell regulatory differentiation [255] was addressed by intracellular staining for the effector cytokine IL-2 which, in contrast to activated
effectors, functional Treg do not produce [219-220]. Both before and after tremelimumab, IL-2 secreting lymphocytes did not express FoxP3, and FoxP3+ lymphocytes did not secrete any IL-2, suggesting they were bona fide primary Treg. In vitro suppression assay established after magnetic sorting of fresh PBMC in the patient with durable benefit from treatment clearly demonstrates that functional suppression of Teff by Treg is not abolished despite prolonged treatment with tremelimumab, a result which is supported by the work of Maker et al [181].

In the absence of a control group, it is possible that the changes in phenotype described in this study are merely defining change with disease progression. However, the patterns of transient increase in Treg, and where more sustained evidence of increase in CD4+CD25low/negativeCTLA4+ are seen, the replication of results for three cycles of treatment for patient 12, make this less likely. The rise in Treg in this study is also supported by later work which describes a transient fall in Treg by CD4+CD25+CD62L+ 72 hours after ipilimumab followed by a rebound rise above baseline at 28 days, a bimodal pattern of response which was also observed in CD4+ and CD4+CD25+ cells [256]. The early transient fall in Treg does not seem to have been replicated by other groups [257]. It was not possible to investigate early changes in lymphocytes in response to CTLA4 blockade in this study due to the timing of blood sampling, which was a compromise between research data and the practical and ethical implications for the patients and clinical team.

Finally, a study of prostate cancer patients treated with escalating doses of ipilimumab with a fixed dose of GM-CSF, which defined Treg by CD4+CD25+FoxP3+, and assessed CTLA4+, supports the work presented here, by demonstrating a dose-
dependent rise in Treg 4 weeks after treatment, and sustained levels of both cell surface and intracellular CTLA4 in these cells [219]. Using a surface capture antibody labelling technique, levels of CTLA4 detected in FoxP3+ lymphocytes after 6 hour incubation were very similar to those obtained by permeabilisation and intracellular labelling, which suggests that the majority of intracellular CTLA4 can translocate to the cell surface over this time. This result supports the relevance of assessing intracellular CTLA4; had time allowed, a surface capture antibody labelling technique could have been employed to confirm the relationship between intracellular and cell surface CTLA4 in PBMC from the gastric and oesophageal trial patients.

6.3.4. Evidence of immune activation

6.3.4.1. Phenotypic evidence of activation

Whilst transient early phenotypic changes may reveal (or even drive) evidence of effect of anti-CTLA4 blockade, it seems likely that more sustained changes in lymphocyte phenotype and function would be required for durable anti-cancer effects. In contrast to Treg, CD4+CD25low/negative lymphocytes show a sustained rise in CTLA4, a pattern which is seen in pooled first cycle results for all patients, and during repeated cycles of treatment for patient 12. As CTLA4 expression is a late and usually terminating- event in lymphocyte activation, such cells may represent a population with potentially sustained activation. This inference is supported by published evidence of expansion of CD4+CD25+CD69+ [219] and HLA-DR expression in CD4+ and possibly CD8+ lymphocytes [181] after anti-CTLA4 blockade, along with restoration of ex-vivo TCR-driven T cell proliferation in lymphopaenic patients with advanced melanoma [258]. Comin-Anduix et al used cluster analysis of changes in T cell activation (HLA-DR)
and memory markers (CD45RO) after tremelimumab to identify a common group of melanoma patients with evidence of clinical effect, i.e. response and/or inflammatory toxicity [259].

6.3.4.2. Functional evidence of activation

The inference that expanded CD4+CD25<low/negative>CTLA4+ lymphocytes may represent activated Teff is supported within this work by the development of de novo in vitro lymphocyte proliferative responses to two relevant tumour associated antigens, 5T4 and CEA, after tremelimumab treatment. Within the cohort investigated (n=15), the development of a response to CEA after the first cycle of tremelimumab correlated with survival: median overall survival was 17.1 vs. 4.6 months (p=0.002). Inevitably, the surviving patients had more PBMC samples to investigate, but the majority of new responses to CEA occurred by day 30 when samples were available for the entire cohort. Interestingly, the patient with a durable clinical response developed recurring post-treatment lymphocyte proliferative responses to both tumour associated antigens after tremelimumab. These results need to be interpreted with caution, and would require confirmation in a further cohort of patients.

In pre-clinical models, CTLA4 blockade has been shown to enhance detectable T cell responses to tumour associated antigens in combination with xenogeneic vaccination schedules [134]. Identifying parallel responses against relevant tumour associated antigens in human subjects is more problematic. Despite recurring evidence of intra-tumoural lymphocytic infiltrates in anti-CTLA4 responders [147, 260], previous investigators did not detect any trend in post-treatment levels of circulating antigen-specific CD8+ T cells against melanoma specific antigens (MART1, gp100 and
tyrosinase) after monthly single agent tremelimumab [259], or enhancement of response over vaccination alone when ipilimumab was combined with gp100 peptide vaccination [159-160]. There was no increase in circulating vaccine specific CD8+ T cells in a mixed cohort of cancer patients given ipilimumab after experimental vaccine failure despite evidence of tumour regression in 2 of 4 patients with non-Hodgkin’s lymphoma, one of whom developed significant lymphocytic infiltration of CD8+ and predominantly CD4+ lymphocytes on post-treatment tumour biopsy [256]. In contrast, biopsy from a patient who went on to develop a complete response showed enrichment of MART1 specific CD8+ T cells in the tumour micro-environment compared to the peripheral blood. High throughput screening has also identified de novo induction of NY-ESO-1 antibodies in a small number of patients with prostate cancer treated with ipilimumab, which, like the in vitro proliferative data from this study, imply the induction of anti-tumour specific lymphocytes by CTLA4 blockade [223].

The use of the long-established radioactive thymidine incorporation assay allows detection and relative quantification of overall lymphocyte response to specific stimuli, such as TAA, as measured by proliferation, but does not allow easy characterisation of that proliferative response by lymphocyte sub-type or effector cytokines. Enhanced proliferative responses to relevant TAA after anti-CTLA4 blockade, measured using pooled synthetic peptides or purified antigen, imply the presence not just of responsive lymphocytes in the peripheral circulation, but of intact antigen-processing machinery. Although this may not be strictly necessary with peptides it is still likely there is some antigen processing occurring for maximal responses as CD8+ T cells recognise short epitopes of 8-11 amino acids presented by MHC class I whilst CD4+
lymphocytes do recognise longer epitopes of 9-22 amino acids presented by MHC class II. The assay provides a global measure of anti-TAA immune response, but does not provide insight into the mechanism of potential in vivo anti-tumour effect. The use of pooled peptides or whole protein and the inclusion of monocytes in the unselected PBMC avoided the need to define specific epitopes which will vary with HLA. Although the majority of trial patients were Caucasian, they showed a wide range of HLA subtypes; for example, only 7/12 expressing the common HLA-A*0201 subtype. The failure of published ELISPOT and tetramer assays to identify tumour specific CD8+ lymphocytes after anti-CTLA4 blockade, even in the context of known relevant epitopes such as after vaccination, highlights the difficulty of measuring immunological responses where the mechanism of action of immunotherapy is poorly understood.

6.3.4.3. Limitations of PBMC

The ability to detect anti-tumour specific lymphocytes by CTLA4 blockade requires further investigation in man, but is hampered by ethical and practical considerations. The widespread investigation of changes in PBMC is a pragmatic response to the difficulty of sampling lymphocyte populations in serial tumour biopsies where the cells of most interest are likely to be found, but presumes that changes in the peripheral circulation will be revealing with regard to mechanism of action and anti-tumour effect.
6.3.4.4. Changes in tumour-infiltrating lymphocytes with anti-CTLA4 blockade

Recent work by Sharma’s group has begun to address these issues. They have treated two cohorts of 6 patients with high grade pT1-T2 operable bladder cancer with two cycles of ipilimumab (either 3mg/kg or 10mg/kg) prior to radical cystectomy [152]. Pre-operative anti-CTLA4 blockade proved tolerable, although 3 of 6 patients treated with the higher dose had surgery delayed due to the development of grade 2-3 diarrhoea. Serial sampling of peripheral blood allowed investigation of lymphocyte phenotype with treatment, which could be correlated with investigation of lymphocyte subsets in post-treatment pathological specimens. Control tissue was obtained from bladder tumours of 10 untreated patients, and 4 normal ureters from untreated patients. No consistent change in FoxP3 expression in peripheral blood lymphocytes was found with ipilimumab, but treated bladder cancer specimens showed a greatly reduced proportion of FoxP3+CD4+ lymphocytes compared to untreated cancers (mean 7 ± 4% vs. 67 ± 25%, p<0.05) comparable with levels seen in non-malignant tissues (mean 8 ± 5%). The group did find increased expression of inducible co-stimulator (ICOS) in both peripheral and tumour CD4+ lymphocytes, cells which could produce IFN-γ and recognise the NY-ESO-1 tumour antigen, suggesting this marker may identify potential anti-tumour effector cells [261]. Subsequent work has shown increased CD4+ICOS+ lymphocytes in both incidental adenocarcinomas and non-malignant prostate tissues samples from patients treated with ipilimumab compared to untreated controls, suggesting similar immunologic effects of anti-CTLA4 blockade can be seen in both normal and malignant tissues, which may make serial assessment more practical, but perhaps undermining the potential specific anti-tumour role for these cells [262].
The key limitation of this approach is the absence of any correlation with clinical response. Study design makes response assessment problematic as patients are only fully staged post-operatively, and may be down-staged due to initial diagnostic transurethral resections. Although 4/12 patients demonstrated a change from positive to negative urine cytology ± fluorescent in situ hybridisation (FISH) analyses of characteristic chromosomal abnormalities found in invasive bladder cancer after anti-CTLA4 blockade, the significance of these changes is not yet clear [152].

6.3.4.5. ICOS

ICOS is a T lymphocyte cell surface marker which shares structural features with CD28 and CTLA4 and is expressed on both effector and regulatory T cells; it has been implicated in the interactions of T and B cells, in germinal centre formation, in the regulation of balance between Th1 and Th2 cytokines, in the response to self antigen, and in the fate and function of effector and regulatory CD4+ lymphocytes [263-264]. ICOS seems necessary for T cell activation and function, and may signal through the phosphoinositide 3-kinase (PI3K) pathway to enhance T cell survival [265]. Recent work suggests that primary mutations of the ICOS gene may account for the full spectrum of autoimmune, inflammatory and malignant complications which can occur in human common variable immunodeficiency (CVID), which is characterised by hypogammaglobulinaemia and was previously thought to be a B cell disease [266]. The relevance of ICOS in malignancy remains to be determined, but had time allowed, it would have been interesting to determine whether CD4+ICOS+ lymphocytes also rose in the peripheral blood of the trial patients with advanced gastric and oesophageal adenocarcinomas after tremelimumab, and to link this analysis with more conventional
markers of both T and B cell activation such as CD44, CD69, HLA-DR, CD62L and CD23.

6.3.4.6. Clinical evidence of activation

Immune-related adverse events (IRAE) such as rash, colitis, hypophysitis, transaminitis and uveitis, seen as commonly recurrent toxicities after anti-CTLA4 blockade with both ipilimumab and tremelimumab provide clinical evidence of immune activation. Several small early studies suggested a relationship between such IRAE and anti-cancer response in metastatic melanoma [160, 179], and although the correlation was somewhat undermined by a dose escalation study of ipilimumab which reported increased IRAE (35% G3/4) without an improved objective response rate (11%) compared to published results, later combined analysis of prognostic factors in patients treated at the National Cancer Institute (Bethesda, MD) showed a clear relationship between IRAE and clinical responses to ipilimumab [226]. This effect may still be confounded by different dosing schedules and prolonged treatment in responders; a recent abstract which summarised results for 571 patients treated with 15mg/kg tremelimumab suggests that most of the increase in IRAE seen in responders is due to their longer exposure to the drug [180].

In this study of patients with gastric and oesophageal adenocarcinomas there was no clear relationship between serious IRAE and clinical benefit; in fact, the most severe IRAE was seen in a patient with no evidence of clinical benefit, whilst the patient with a durable objective response has avoided all but mild IRAE (grade 1 rash). There was however, clinical evidence of immune activation with toxicity, as demonstrated by the development of lymphocytosis with both grade 3 transaminitis (patient 4) and fatal
colitis (patient 11), the latter of which showed initial response to immunosuppression with high dose systemic corticosteroid.

Both toxicity and responses seem to occur in a minority of potential immune responders. In this small study, pre-treatment IL-2 release after T cell activation appears to identify both those patients with potentially beneficial and serious toxic responses to tremelimumab, but would require prospective investigation for confirmation.

6.4: Model work

6.4.1. Vaccine strategy

Early clinical trials of ipilimumab and tremelimumab in metastatic melanoma and other adult solid tumours suggest the objective response rate of anti-CTLA4 monotherapy is around 5-10%. Within pre-clinical animal models, therapeutic efficacy has been enhanced by combination with a variety of vaccine approaches. This project therefore aimed to (1) establish an active therapeutic vaccination model using the autologous murine oncofoetal antigen m5T4, and (2) develop an immunological measure of response to m5T4 vaccination in order to (3) characterise responses to m5T4 vaccination in naive and tumour- bearing animals before (4) attempting to modulate vaccination responses and efficacy using anti-CTLA4 blockade.

These aims were met in part. Heterologous MVA.m5T4/ pAd.m5T4 vaccination showed some evidence of weak therapeutic benefit, but the search for an alternative
tumour model with more favourable growth kinetics was unsuccessful as all the m5T4 expressing tumours investigated grew in vivo with similar rapidly lethal kinetics. This is perhaps consistent with evidence from histological studies in human tumours, where h5T4 expression appears to correlate with poor prognosis [49].

The choice of vaccine schedule and doses in this work was based on investigation of anti-m5T4 immunity which showed that vaccination with two or more MVA.m5T4 gave equivalent antibody titres by intra-muscular and intravenous routes, and resulted in significant protective immunity to B16.m5T4 tumour challenge, but was not therapeutic compared to control vaccine when given after tumour inoculation [202]. Later exploratory work in protection and therapy using combinations of MVA.m5T4 and pAd.m5T4 suggested heterologous boost combinations gave improved protection to tumour challenge over homologous boost, and that MVA.m5T4/pAd.m5T4 had weak efficacy as therapy [239]. With hindsight, further investigation of the vaccination route and schedule in tumour-bearing animals would have been appropriate. For example, comparison of heterologous MVA.m5T4/pAd.m5T4 with single and homologous boost pAd.m5T4 in the therapeutic setting would have been logical after the results of ELISPOT investigation of m5T4 specific responses, and establishing an effective protection model, whilst a further step removed from an advanced cancer setting, would have allowed the exploration of additive effect of anti-CTLA4 blockade in a model with convincing efficacy. Two alternative vaccine routes have shown benefit in alternative tumour models: intravenous and intra-tumoural [240] but both require high vaccine titres for small volume inoculation which would have presented practical difficulties with the proposed reagents. Recent work has also focused on improving immunogenicity by novel vaccine delivery e.g. transient disruption of cell
membranes by brief electroporation at vaccine delivery site enhanced the uptake of DNA vaccines by 10-100 fold [267].

6.4.2. Assessment of immune response to autologous antigen

Investigation of antibody responses showed evidence of m5T4 immunity in non tumour-bearing animals by anti-m5T4 antibody ELISA, but detection was attenuated in tumour-bearing animals. IFN-γ responses to specific m5T4 peptides were detected after pAd.m5T4 and heterologous prime boost, but not after MVA.m5T4. These responses were not detected in tumour-bearing animals during the final combination therapy experiment, but the conclusion that they were absent is somewhat weakened by the omission of vaccinated non tumour-bearing control animals.

In fact, the demonstration of convincing vaccine-induced m5T4 specific responses in wild type animals, though possible, is problematic throughout this work, which is in keeping with failure to demonstrate m5T4 specific cytotoxicity by chromium release assay after pAd.m5T4 in wild type compared to m5T4 knock out animals, failure of prophylactic efficacy of pAd.m5T4 in wild type compared to m5T4 knock out animals [268], and the lower frequency and reduced repertoire of IFN-γ m5T4 peptide ELISPOT responses in wild type compared to m5T4 knock out C57BL6 animals, and the failure to generate significant responses using a thymidine incorporation proliferation assay [269].
Fig 6.1: IFN-γ splenocyte response to m5T4 peptides in pAd.m5T4 immunised C57BL6 mice.

Graph shows mean result for 3 wild type (WT) animals and a single m5T4 knock out (KO) animal after immunisation with single pAd.m5T4. Thanks to H. Jiang and F. Castro.

Fig 6.1 shows the lower frequency and reduced repertoire of IFN-γ m5T4 peptide responses in wild type compared to m5T4 knock out C57BL6 animals: only the peptide 26 response, later shown to be CD8⁺ specific, is still seen, and this is 37 vs. 208 spots per well. Given the difficulty of demonstrating convincing, reproducible m5T4 specific responses in non tumour-bearing animals due to the low frequency of m5T4 specific splenocytes (generally 35-100 per 5x10⁵ splenocytes), the loss of these responses in tumour-bearing animals may be due to loss of these cells, to increased regulation of responses, or to re-distribution of cells away from the spleen, perhaps towards the tumour itself or to draining lymph nodes or the peripheral circulation. Where a high affinity epitope can be demonstrated, labelling cells with an epitope-specific tetramer can allow demonstration of such antigen-specific cells using immunohistochemistry and/or flow cytometry, but in the absence of such an epitope, investigating these possibilities by the established ELISPOT would have required large numbers of animals. It did not seem appropriate to pursue this in the absence of significant therapeutic benefit.
Arguably, poor immunogenicity, reflected by such low frequency or low avidity responses to vaccination and which may be the consequence of natural exposure of the immune system to many TAAs such as 5T4, underlies the recurrent failure of the vaccination approach in cancer therapy. Despite the demonstration that even late stage patients can respond to vaccine and generate long-lived T cell memory [270], objective benefit is rare. Avidity is influenced by MHC-peptide affinity and modulated by multiple factors such as co-stimulatory signals, counter-receptors such as CTLA4, adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1), and cytokines such as GM-CSF; possibly only high avidity T cells can recognise and respond to low levels of cognate antigen expressed by most solid tumour cells. It may be that tumour control by vaccination will require an alternative target where a high avidity repertoire has not already been compromised by preceding antigen exposure [271].

6.4.3. Anti-CTLA4: failure to modulate vaccine therapy or specific immune response

Investigation of anti-CTLA4 blockade in combination with m5T4 heterologous prime boost vaccination showed no efficacy in the therapeutic model used. In contrast, anti-CTLA4 blockade alone showed a small therapeutic benefit, both in terms of slower mean tumour growth, and a 4 day survival benefit. No m5T4 specific responses were seen by IFN-γ ELISPOT, but increased ex vivo IFN-γ was detected in all animals treated with immunotherapy. Alternative IL-2 ELISA again demonstrated increased ex vivo cytokine responses in animals treated with immunotherapy, and modest responses to peptide 35 alone which were also seen in naive and tumour-bearing untreated animals and may reflect pre-existing or non-m5T4 specific responses. These results raise two important questions:
1. Why was there no therapeutic benefit to combination vaccine and anti-CTLA4 blockade?

2. Why were there no convincing specific IFN-γ immune responses?

The first issue to consider is one of design. In retrospect, two additional control treatment groups of animals would have allowed firmer conclusions to be drawn from the combination immunotherapy experiment: control MVA.LacZ/ pAd.GFP vaccination, and control hamster antibody. The choice of vaccination schedule could have been investigated more comprehensively in further preliminary experiments, but there was no strong evidence from earlier work within the group to support the use of an alternative schedule [239]. In retrospect, given that m5T4 specific IFN-γ responses were only ever demonstrated after pAd.m5T4 vaccine, it would have been logical to investigate single and homologous boost pAd.m5T4 in the therapeutic model. In protection assays, this vaccine prevented B16.m5T4 tumour development in m5T4 knockout but not wild type C57BL6 animals [268].

The choice of anti-CTLA4 antibody dose and schedule was based, in the absence of demonstrable effect on specific m5T4 responses after vaccination in non-tumour bearing animals, on available evidence from the literature [135]. Efficacy of anti-CTLA4 blockade is generally enhanced by early dosing after tumour challenge [138, 272], but conflictingly some evidence favours later dosing [130] or later peri-boost anti-CTLA4 blockade in a homologous vaccination schedule [134]. Given the rapid lethality of the model, a peri-prime anti-CTLA4 antibody schedule was chosen. This may not have been ideal, but testing all the permutations of combined treatment in vivo would have required larger numbers of animals than seemed justified given the
lack of supporting evidence for likely benefit from the preliminary experiments. Despite these considerations, the results achieved were not out of keeping with the preceding experiments.

The second issue is one of execution. All of the components of this experiment were titred and assayed as described in the methods, and pAd.m5T4 was utilised contemporaneously within the group in a number of transfection experiments [53]. However, in an autologous vaccination schedule, small differences in vaccine dose delivery or site, despite best attempts at standardising vaccine production, consistent titre measurement, and injections, may be sufficient to alter demonstrable immune responses and effects between animals or (more likely) between experiments which are seen in the variability of presented m5T4 antibody and peptide ELISPOT results. In the combination immunotherapy experiment, unlike the earlier investigation of the vaccine schedule (see 5.2.2.), no therapeutic benefit was seen from vaccine alone. Two differences in design may in part explain this: the control groups were different (tumour alone rather than control vaccination), and the number of animals used was smaller (8 instead of 10) which will reduce the detection of small differences, such as the 2 day survival advantage (p=0.042) seen in the earlier experiment, but should be sufficient to detect a larger useful therapeutic benefit.

The IFN-γ ELISPOT assay has multiple stages and is conducted over several days, making it vulnerable to technical errors, but was very familiar after preliminary work assessing responses in non tumour-bearing animals. High responses to polyclonal stimulant concanavalin A were seen with all animals, but inclusion of non tumour-bearing animal treated with pAd.m5T4 alone would have served as a useful internal
m5T4 peptide response positive control. Replicates within this assay were close and the negative result seen in combination therapy animal was confirmed with concentration titration. The absence of m5T4 specific responses in tumour-bearing animals is consistent with earlier experiments where antibody responses are attenuated. There is strong evidence of ex-vivo non-specific activation in the therapeutic model system following immunotherapy as detected using IFN-\( \gamma \) ELISPOT and IL-2 ELISA, and no convincing evidence of m5T4 specific responses. The strongest effect is seen after combination of vaccine and anti-CTLA4 blockade in keeping with the results described for non tumour-bearing animals. With additional time, it would have been useful to assess the presence and time course of non-specific activation in vivo which could be done by flow cytometry assessment of a panel of activation markers and intracellular cytokines. This would have allowed reassessment of alternative schedules and allowed inclusion of additional controls such as control vaccine to assess effect of viral vector on the demonstrated responses. Considerable effort was taken to minimise the presence and effect of contaminating endotoxin on the model which may have resulted in immune activation. However, the hamster anti-CTLA4 antibodies are known to be immunogenic in the mouse, and there is published evidence of non-specific immune activation after combination with xenogeneic DNA vaccines as demonstrated by enhanced responses to a control chicken ova peptide, as measured by IFN-\( \gamma \) ELISPOT although this does not match or obliterate the high avidity responses to target gp100 [134]. These authors used a bead purified population of CD8\(^+\) lymphocytes isolated from spleens and lymph nodes of treated animals in the ELISPOT assay, with peptides presented by irradiated naive splenocytes; in contrast this work used unselected splenocytes from treated animals. Isolation of CD8\(^+\) lymphocytes would have resulted in an increased frequency of demonstrable responses
in the assay, and it is possible that this would have mitigated any effect of anti-CTLA4 blockade on other lymphocyte (or monocyte) populations seen within the assay.

Perhaps surprisingly, in such a rapidly growing tumour model, anti-CTLA4 blockade alone showed a small therapeutic benefit in the CT26.wt model, with a 4 day improvement in median survival ($p=0.012$). Looking at the growth curves of animals treated by CTLA4 blockade alone, a further cohort of animals treated with further anti-CTLA4 antibody may have shown further delay or regression of tumours and additional survival benefit, although repeated dosing with the potentially immunogenic hamster antibody may have been problematic. The result needs to be interpreted cautiously as it was not possible to repeat the experiment in the time available, but is supported by recently published work: using a mouse monoclonal anti-CTLA4 antibody (9D9), given intravenously at 100µg dose 3, 6, and 9 days after CT26 tumour challenge, Mitsui et al report similar efficacy of monotherapy with delayed tumour growth but no complete tumour rejection [272]. The absence of synergy with the vaccination schedule in terms of tumour growth, survival, and demonstrable immune responses is disappointing but not unexpected given the difficulties of investigating an autologous antigen in a rapidly lethal tumour model.

It is difficult to compare an unsuccessful strategy with the literature as publication tends to favour positive results. Nonetheless, it is clear that anti-CTLA4 blockade alone is not effective therapy in poorly immunogenic tumour models such as B16 and the mammary tumour SM1 as cited by Allison’s group [138]. Whilst several groups report enhanced efficacy in combination with vaccination schedules, this has sometimes required additional adjuncts which enhance the immunogenicity of the
vaccine, such as the cytokine GM-CSF [138]. Also, for example, Davila et al showed that vaccination with a synthetic peptide corresponding to an immunodominant CTL epitope derived from melanoma antigen tyrosinase-related protein 2 (TRP2\textsubscript{180-188}) was only therapeutic in combination with both synthetic oligodeoxynucleotide (ODN) adjuvants and anti-CTLA4 antibody [135]. In addition, and in contrast to their work on a chicken ovalbumin (ova) epitope where adjuvant and peptide vaccine alone were effective as prevention and therapy [273], despite the efficient induction of measurable anti-tumour CD8\textsuperscript{+} responses after single vaccination with adjuvant ± anti-CTLA4 antibody in tumour-free animals, there was no preventative benefit from the autologous regimen. The authors speculate this may be due to the down-regulation of responses in tumour-bearing mice, perhaps due to the absence of CD4\textsuperscript{+} tumour-reactive T cells which were only induced by therapeutic vaccination. It is possible that these caveats also applied to the m5T4 autologous model, but given the low frequency of induced anti-tumour responses it was not possible to characterise these by lymphocyte subtype. The importance of synthetic oligodeoxynucleotide adjuvant which stimulates DC by mimicking bacterial DNA in their model is probably due to the poor immunogeneic properties of peptide vaccines alone; in the m5T4 vaccination schedule the modified viral vectors should serve a similar purpose, but it is possible that in the relatively germ-free environment of the mouse colony the animals have insufficiently activated immune systems to generate sufficient activated DC to stimulate cytotoxic T lymphocytes. In contrast to the viral vector, the adjuvant could be given for five days prior to vaccination.

Even in a model with efficacy for combined vaccination and anti-CTLA4 blockade in both prevention and therapy, the mechanism for tumour rejection varied.
Combination of anti-CTLA4 blockade and a GM-CSF producing irradiated tumour cell vaccine resulted in 100% tumour rejection when used as prophylaxis and 80% tumour eradication when used as therapy against B16 melanoma [138, 274]. Efficacy was CD8+ and to a lesser extent NK1.1+ cell dependent in therapy, and resulted in around two thirds of animals developing autoimmune depigmentation; in contrast, full protection was achieved even in the absence of CD8+ T cells, although none of the CD8+ depleted animals developed depigmentation. Despite the use of a whole cell vaccine approach, post-treatment responses were only demonstrated to one (TRP2180) of a panel of relevant differentiation TAA with no evidence of determinant spreading.

Scheduling of anti-CTLA4 blockade with respect to vaccination can also affect efficacy. Combination of anti-CTLA4 blockade and human xenogeneic DNA vaccines to melanoma differentiation antigens TRP2 and gp100 enhanced B16 tumour rejection in immunised mice, but was less effective at prime than boost vaccination, and was short-lived, showing no additional efficacy in animals challenged with tumour 12 days after completing vaccination despite persistence of demonstrable enhancement of IFN-γ ELISPOT responses to syngeneic mgp100 [134]. Combination of anti-CTLA4 antibody with xenogeneic prostate-specific membrane antigen (PSMA) showed no evidence of effect when given with prime vaccination, and did not result in any autoreactive T cells.

In vivo work is labour intensive and time consuming. The m5T4 TAA model was used in an attempt to create a relevant system in which to investigate the modulation of T lymphocyte regulation against an autologous target, a scenario relevant in the
therapeutic treatment of adult solid tumours in man. Whilst creditable this proved difficult, and with hindsight it may have been wise to utilise a parallel heterologous model to further validate some of the techniques, and to elucidate the differences between immune responses to self and foreign antigen in the context of anti-CTLA4 blockade. The two relevant heterologous models would have been the use of h5T4 transfected tumour cells and parallel vaccines as published [202, 207], or to have used C57BL6 m5T4 knock out animals. This should have allowed easier characterisation of the immune response to m5T4 vaccination, and therefore more time could have been spent concentrating on in vivo immune effects of anti-CTLA4 blockade in the animal model in parallel with the in vivo work from the phase II clinical trial in patients with oesophageal and gastric adenocarcinomas. This would have allowed characterisation of T cell activation and regulatory phenotypes in the mouse model, and perhaps expansion to consider antigen presenting cell phenotypes. The human in vivo work showed CTLA4+ monocytes by intracellular flow cytometry; it would have been interesting to find and further characterise these cells, with a possible role in regulating T cell activation, in the mouse model.

Confirming that, even with a heterologous prime boost schedule, m5T4 vaccination alone or in combination with anti-CTLA4 blockade, shows no significant therapeutic benefit in the autologous model, in contrast to benefit seen using similar approaches in the heterologous h5T4 model, is believable, and problematic if we believe this is a truer parallel of the clinical situation. Despite decades of effective therapeutic cancer vaccines in animal models few have progressed beyond early phase clinical trials, and despite frequently low toxicity, none are used in routine clinical practice in the UK.
The next section will discuss the emergence of immunotherapy which may yet become available in the clinic.

6.5: Immunotherapy: next steps

6.5.1. Sipuleucel-T

The US Food and Drug Administration approved the active cellular immunotherapy sipuleucel-T for the treatment of men with hormone-refractory prostate cancer on 29th April 2010. Sipuleucel-T is a therapeutic cancer vaccine consisting of autologous PBMC activated ex vivo with recombinant fusion protein (PA2024) made by fusing the prostate antigen prostatic acid phosphatase and granulocyte-macrophage colony-stimulating factor (GM-CSF). A large randomised placebo-controlled phase III study showed a significant reduction in HR for death with treatment compared to control (HR 0.78, 95% CI 0.61-0.98, p=0.03) which corresponded to an increase in median overall survival of 4.1 months (25.8 vs. 21.7 months), despite the absence of evidence of delayed progression free survival (3.7 vs. 3.6 months), significant objective CT responses (1 PR in 330 patients), or reduction in serum PSA (8 of 311 patients had a greater than 50% fall from baseline PSA) [78]. This consolidated the results of two smaller randomised placebo-controlled studies which had investigated progression free survival as a primary end-point but discovered an effect on overall survival: one showed a trend towards improved survival [275] whilst the other had shown a significant survival advantage with treatment (HR for death 0.59, 95% CI 0.39-0.88) despite crossover of 34 of 45 placebo-treated patients to the vaccine in an open-label study [79].
Immune responses were assessed in a sub-group of patients by serial measurement of antibodies against the immunising fusion protein PA2024 and component antigen prostate acid phosphatase at weeks 6, 14 and 26, and by week six T lymphocyte proliferative responses to both antigens. Antibody titres exceeding 400 were found to the fusion protein and component antigen in 66% and 29% of treated patients compared to 2.9% and 1.4% of controls; moreover the patients with such titres had a significant survival advantage over those without. In contrast, whilst T lymphocyte proliferative responses were seen with similar frequencies to both the fusion protein and component antigen after treatment (73 and 27% respectively), they were more frequently found in the control treated patients (12 and 8% respectively) and did not correlate with survival [78].

Critique of these results has focused on concern about the absence of objective evidence of anti-tumour efficacy, traditionally demonstrated by regression of measurable lesions or reduction in validated serum tumour markers such as PSA in the case of prostate cancer. The mechanism of action remains obscure; in vitro assay of predicted effector T lymphocytes by proliferative response occur in a proportion of control patients, and have no correlation with survival. It is unclear to what extent the survival benefit results from the use of the prostate cancer specific fusion protein; in the absence of active control treatment of autologous PBMC using GM-CSF alone it is possible that such treatment would also demonstrate benefit [276].

Emerging phase II data suggests that heterologous vaccination with recombinant viral vectors (modified vaccinia followed by fowlpox) encoding transgenes for PSA may also confer a survival advantage in hormone-refractory prostate cancer without
evidence of improved progression free survival [277]. If this result is replicated in phase III trials it will provide supporting evidence that survival benefit can be seen without classical anti-tumour responses, but such a shift in paradigm raises the question of assessment of efficacy in the absence of any generally accepted and reproducible biomarkers. Interestingly, GM-CSF was again used as an immunomodulator within this vaccine regime; necessary further evaluation may allow assessment of its single-agent efficacy.

Perhaps of more practical concern, individualised treatment with sipuleucel-T has been estimated to cost in the region of $93,000 per patient. Even in the US there are concerns about the high cost of such therapy in a palliative setting; in the more constrained financial climate of the current NHS it seems unlikely that such treatment will be available to patients outside a clinical research programme.

6.5.2. PD-1 results

Recently published phase I data has investigated the use of a fully humanised IgG4 monoclonal antibody (MDX-1106) targeting the inhibitory lymphocyte receptor PD-1 in a mixed group of patients with advanced solid tumours [278]. Cohorts received escalating single doses of between 0.3 and 10 mg/kg. Standard pharmacokinetic measurement of serum antibody concentrations estimated serum half-life (t½) at 12-20 days, with maximum concentration and area under the curve (AUC) directly related to dose; in contrast, flow cytometric analysis of PD-1 occupancy at the cell surface of hours after treatment, and mean plateau occupancy of 72% persisting for at least 57 days.
Toxicity was relatively mild compared to anti-CTLA4 blockade: 6 of 39 patients experienced grade 2 or higher fatigue and musculoskeletal side effects including two polyarticular arthropathies which were treated with steroids. Single patients developed presumed immune-related adverse events of hypothyroidism (grade 2) and inflammatory colitis (grade 3) which responded to hormone replacement and immunosuppression with steroids and infliximab respectively. These results were in keeping with predicted effect from animal models of PD-1 knock out phenotype which demonstrate a more benign adult-onset spectrum of strain and organ specific autoimmune pathology than parallel models of CTLA4 deficient animals [279-280].

Three patients had durable objective responses to MDX-1106 after treatment with 3 or 10 mg/kg: 1 CR (21+ months) in a patient with metastatic colorectal cancer, and 2 PR (16+ and 3+ months) in patients with metastatic renal cancer and melanoma who had first demonstrated mixed responses of 4 and 20 months respectively. Two additional patients, with melanoma and non-small cell lung cancer had evidence of mixed responses. These preliminary results suggest similar response kinetics and rate (3 of 27; 11%) to that seen with anti-CTLA4 blockade, and demonstrate some benefit in solid tumours not classically thought to be immunoresponsive.

Like CTLA4, PD-1 opposes CD28-mediated activation of T lymphocytes following antigen recognition by the T cell receptor. The interaction of PD-1 with its ligands PD-L2 and the more ubiquitous PD-L1 which is also found on non-haematopoietic cells such as epithelial cells, hepatocytes, and astrocytes have roles in maintaining peripheral T cell tolerance and preventing organ autoimmunity [281]. As a target for cancer immunotherapy, PD-1 has a less established role in suppression of anti-tumour
immunity than CTLA4, but blockade may result in a more favourable toxicity profile. Whilst these early results are encouraging, it seems likely that further drug development will share some of the difficulties experienced with anti-CTLA4 antibodies, including the possibility of unfamiliar toxicity and a similar pattern of slow but potentially durable responses for which established criteria for looking at CT scans are poorly suited. From these preliminary results responses seem relatively infrequent and occurred in a range of tumour types; targeting potential immune responders remains a critical goal for this therapeutic approach. Tumour samples were available for a small number of patients in this trial of anti-PD-1. The presence of ligand PD-L1 in tumour appeared to correlate with response: clinical benefit was seen in 3 of 4 patients with upregulation of PD-L1 compared to 0 of 5 without [278]. This result identifies a potential predictive biomarker and will require prospective validation, but may provide a rational basis for identifying potential responders. Upregulation of PD-L1 has been studied in NSCLC where it appears to correlate with poor prognosis [282].

6.5.3. Phase III ipilimumab data

Ipilimumab has recently demonstrated a survival advantage for patients with metastatic melanoma and is under licence review by the FDA and European Medicines Agency. In a large randomised phase III study ipilimumab was administered with or without a glycoprotein 100 (gp100) peptide vaccine to 676 patients with metastatic melanoma who had received previous therapy with IL-2 or cytotoxic chemotherapy [283]. Treatment was randomised 3:1:1 to ipilimumab plus active vaccine, ipilimumab plus placebo vaccine or placebo infusion plus active vaccine. The original primary end-point of best overall response was amended to overall survival
after completion of recruitment but whilst the data were still blinded. This was done in
view of the emerging evidence from phase II studies about the low frequency and
difficulties surrounding the objective assessment of responses due to the unusual
kinetics of clinical benefit. Median overall survival was 10.1 months (95% CI 8.0 to
13.8) after ipilimumab alone compared to 6.4 months (95% CI 5.5 to 8.7) for patients
who received vaccine alone (hazard ratio for death 0.66, p=0.003); there was no
benefit or detriment for those treated with ipilimumab who also received vaccine
(median OS 10.0 months; p=0.76).

The objective response rate was highest in patients treated with ipilimumab alone
(10.9%; 95% CI 6.3-17.4; p=0.001), being significantly lower in those who also
received vaccine (5.7%; 95% CI 3.7-8.4) and rare in those who only received vaccine
(2PR; 1.5%). Delayed response kinetics were seen: a minority of patients (12) showed
improvement in objective response beyond six months without further therapy, and
21 of 31 patients who qualified for reinduction therapy (i.e. had demonstrated 12
weeks of at least disease stabilisation prior to progression) had further benefit from
treatment. Durable responses (≥ 2 years) were seen in a majority of patients with
objective responses to ipilimumab alone, and 17% of those with objective responses
to ipilimumab plus gp100 peptide vaccine.

Toxicity was in keeping with data from phase II trials. Immune-related adverse events
(IRAE) were common and occurred in around 60% of patients treated with
ipilimumab compared to 32% treated with vaccine alone. Severe IRAE (grade 3 and 4)
occurred in 10 to 15% of patients treated with ipilimumab and 3% of those treated
with vaccine alone. The commonest IRAE was diarrhoea: 28-30% patients treated
with ipilimumab developed immune related diarrhoea, which was severe in around 4%. For most patients, diarrhoea resolved by median 2 weeks, but there were five deaths associated with this toxicity. Rash and itch were also common (18-24%) but less serious, and a full range of infrequent immune-related endocrine abnormalities were also recorded. Two further deaths were thought to be due to IRAE in patients who developed liver failure and Guillain-Barré syndrome, an acute inflammatory demyelinating polyneuropathy which can cause paralysis of the respiratory muscles and autonomic dysfunction.

This trial can be criticised for the lack of a true placebo arm, particularly in second-line treatment of melanoma where there is no proven benefit from any therapy. The inclusion of HLA-A*0201-restricted gp100 peptide vaccine has not helped the interpretation of the results as it appears to attenuate the objective response rate when given in combination with ipilimumab although this does not translate into detrimental overall survival. There was a rationale for inclusion of this vaccine which had demonstrated induction of immune responses but limited anti-tumour efficacy as a monotherapy [284-285] but targets a widely expressed melanoma differentiation antigen and may improve efficacy of high-dose IL-2 treatment [286]. A true placebo would have ruled out any potentially detrimental effect on survival of the vaccine.

The trial again illustrates the difficulties of assessing responses to immunotherapy given the available objective scanning criteria and the absence of any validated immunological or imaging biomarkers and the variable kinetics of clinical responses. Not all patients with clinical benefit from ipilimumab demonstrate objective responses: whilst only 10.9% had an objective response, 17.5% had stable disease
compared to 9.6% of those treated with vaccine alone, and 23.5% of patients survived more than 2 years.

In contrast, tremelimumab failed to demonstrate any improvement in overall survival as first line treatment for patients with metastatic melanoma in early analysis of a large randomised controlled trial against standard chemotherapy with either dacarbazine or temozolamide. Median overall survival was 11.8 months (95% CI 10.4-13.9) for tremelimumab versus 10.7 months (95% CI 9.3-12.0) for chemotherapy (hazard ratio 1.04). Planned interim analysis after 340 deaths resulted in the trial being stopped for futility; to date, results have been published in abstract only which limits analysis [287]. This negative result in contrast to that reported for ipilimumab may reflect differences in efficacy between the two antibodies, or selection of a less intensive regimen for tremelimumab (15mg/kg every 90 days) compared to ipilimumab (induction 4 cycles 3mg/kg every 3 weeks), or reflect differences between pre-treated and untreated patient groups and the use of a more robust comparison arm. As objective responses, though potential durable, occur in a minority of around 7-10% of patients, this negative result highlights the fact that benefit does not translate to the whole patient group. Future analysis of 2 and even 5 year survival may yet show differences between the treatments, but the early failure of this study emphasises the importance of improved understanding of CTLA4 modulation of the human immune system and rational selection of patients likely to benefit from such treatment [288].

Despite these difficulties, the anti-CTLA4 antibody ipilimumab is the first treatment to demonstrate a survival benefit for patients with metastatic melanoma [289] although recent early phase clinical trial data suggests a novel inhibitor of the serine-threonine
kinase BRAF is highly active for the roughly 40% of patients with activating V600E mutations [290]. Further assessment of the clinical utility of anti-CTLA4 blockade in melanoma awaits the results of the phase III study of ipilimumab as first line treatment of metastatic melanoma.

6.5.4. Envoi: First line Phase III ipilimumab data

Positive results of the first major phase III trial of ipilimumab as first-line treatment for metastatic melanoma were presented this summer (2011). 502 patients with untreated metastatic melanoma were randomised 1:1 to receive dacarbazine (850mg/m2) with ipilimumab (10mg/kg) or placebo for 4 cycles of three weeks followed by dacarbazine alone for a further 4 cycles [291]. Combination treatment improved overall survival from median 9.1 to 11.2 months with higher survival at 1 year (47 vs. 36%) maintained out to 3 years (21 vs. 12%; HR for death 0.72; p<0.001). Whilst there was no significant difference in objective response rates- assessed every twelve weeks using modified WHO criteria (15 vs. 10%; p=0.09) nor progression free survival, responses to combination treatment were strikingly durable (median 19.3 vs. 8.1 months; p=0.03) in keeping with the available phase II data [226].

Combination treatment was toxic: twice as many patients experienced any grade 3 toxicity (40 vs. 18%). Immune-related adverse events were common (any 78 vs. 38%) and serious (grade 3 32 vs. 3%; grade 4 10 vs. 3%) but the pattern varied from previous studies with increased rates of transaminitis (17-21%) and reduced rates of diarrhoea (4%) and colitis (2%), and only a solitary case of hypophysistis in a patient given maintenance ipilimumab beyond the duration of the trial. Dacarbazine is known to be hepatotoxic as monotherapy [292]; it appears that combination treatment has
shifted the observed pattern of toxicity for ipilimumab and that subsequent use of glucocorticoid and other immunosuppressants may also have altered toxicity.

It is notable that concomitant administration of anti-CTLA4 blockade with myelosuppressive chemotherapy shows efficacy and does not inhibit all immune-related adverse events; it would be interesting to consider the development of toxicity and responses in the light of lymphocyte phenotype and numbers following such combination treatment. Both immune-related efficacy and toxicity must require presentation of the relevant TA and self antigens and subsequent enhancement of target immune response. Available evidence suggests that not just choice but scheduling of combination therapy will alter the pattern and efficacy of immune response: Davila demonstrated efficacy of anti-CTLA4 blockade after peptide vaccination in both prophylaxis and therapy [135] and Gregor demonstrated enhanced efficacy of anti-CTLA4 blockade after boost rather than prime vaccination in two xenogeneic DNA vaccine models [134]. This model work is supported by differential clinical responses in Hodi’s early study of ipilimumab in previously vaccinated patients [147, 293]. None of the major studies have focused on post-vaccination responses to anti-CTLA4 blockade nor yet investigated this issue of scheduling; concurrent administration of peptide vaccine and anti-CTLA4 antibody may be responsible for the apparent failure of vaccination to enhance anti-tumour responses [283]. In this project, demonstration of specific though low frequency m5T4 peptide responses only in those animals given peri or post vaccination anti-CTLA4 would support future investigation of the latter schedule. It remains feasible that appropriate vaccination prior to anti-CTLA4 blockade could prime tumour-specific responses without the considerable toxicity of concomitant chemo-immunotherapy. Further investigation
seems warranted both in models and in man, though in this model only vaccination before tumour challenge would have allowed time to investigate such potentially enhanced responses.

6.6: Summary and conclusions

This work aimed to explore the modulation of T regulatory activity for cancer therapy, and asked whether the modulation of immune control with anti-CTLA4 blockade led to significant anti-tumour activity. Clinical and laboratory investigation of anti-CTLA4 blockade using the anti-CTLA4 antibody tremelimumab in a phase II trial of second-line therapy in patients with advanced oesophageal and gastric adenocarcinomas showed limited evidence clinical benefit but this was remarkably durable for the single patient with objective evidence of tumour regression. Lymphocyte phenotype changed with treatment: absolute CD4+ and CD4+CD25high lymphocyte subsets increased transiently with treatment, and parallel changes were seen in relative Treg further defined using FoxP3 and intracellular CTLA4, which they constitutively express [124]. Both markers suggest the proportion of Treg rises in a reproducible temporal pattern after tremelimumab. Additional flow cytometry demonstrates these cells do not secrete IL-2, supporting their definition as natural Treg. Suppression assay data confirms the presence of functional suppressor CD4+CD25high Treg even after prolonged treatment with anti-CTLA4 blockade. These results support the idea that CTLA4 blockade is not acting primarily through Treg as was thought likely at the time the project was conceived.

Evidence of the development of TAA specific responses following tremelimumab treatment, as demonstrated by de novo proliferative responses to two relevant TAA,
5T4 and CEA suggests a possible rationale for combining anti-CTLA4 blockade with one of the antigen targeted therapies currently in clinical development, such as CEA targeted vaccination [294], 5T4 superantigen [295] or targeted vaccination [54], or targeted adoptive cell therapy [296]. The failure of the autologous pre-clinical model based on a therapeutic vaccination schedule against m5T4 to demonstrate benefit from the addition of anti-CTLA4 blockade was disappointing but perhaps in keeping with the recently published phase III data in man discussed above, which shows no benefit from the addition of a gp100 peptide vaccine to anti-CTLA4 blockade as second-line treatment in patients with metastatic melanoma [283]. Even in model systems, the addition of anti-CTLA4 blockade does not override the de facto TAA tolerance which is a crucial barrier to effective anti-tumour immunity [297]. In the absence of supportive data from the model system it would not be appropriate to pursue the combination of heterologous 5T4 vaccine with anti-CTLA4 blockade, but in view of the unusual durability of the best response to tremelimumab, and the in vitro evidence of enhanced proliferative responses to relevant tumour associated antigens, further investigation of drug activity may be warranted in the challenging setting of metastatic gastric and oesophageal second-line treatment despite the disappointing objective response rate in the phase II study. Response rates to anti-CTLA4 monotherapy are low even in melanoma where there is evidence of survival benefit with ipilimumab; the ability to identify and selectively treat potential immune responders will require a better understanding of mechanism of anti-CTLA4 blockade and of the interaction of tumour and host immune system but seems necessary for the practical use of these agents in the clinic.

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## Appendix 1

### ECOG performance status (PS)

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
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</thead>
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<td>0</td>
<td>Fully active, able to carry out all normal activity without restriction.</td>
</tr>
<tr>
<td>1</td>
<td>Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, e.g. office work.</td>
</tr>
<tr>
<td>2</td>
<td>Ambulatory and capable of all self care, but unable to work. Up and about more than 50% of waking hours.</td>
</tr>
<tr>
<td>3</td>
<td>Capable of only limited self care. Confined to bed or chair more than 50% of waking hours.</td>
</tr>
<tr>
<td>4</td>
<td>Completely disabled. Cannot carry out any self-care. Totally confined to bed or chair.</td>
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REFERENCES


