NITROREDUCTASE SUICIDE GENE AND IMMUNOTHERAPY IN LOCALLY RELAPSED, CASTRATE RESISTANT PROSTATE CANCER

Richard Philip Charles Viney

A thesis submitted to
The University of Birmingham
For the degree of
DOCTOR OF PHILOSOPHY

The School of Cancer Sciences
College of Medical and Dental Sciences
The University of Birmingham
March 2013

Abstract

In this thesis we validate the efficacy of a new adenoviral construct in prostate cancer cell lines in preparation for a gene and immunotherapy clinical trial in prostate cancer. By demonstrating the constructs ability to infect prostate cancer cells and cause them to die with the introduction of the prodrug, CB1954 as well as releasing a biologically active cytokine, GMCSF we secured GTAC approval to proceed to a phase I/II clinical trial in patients with local relapse after treatment with curative intent for prostate cancer. A tertiary endpoint in the trial is evidence of immune responses relating to treatment. To measure this we have modified an interferon gamma ELISpot assay to measure T cell mediated immune responses. We have then used this assay on 38 patients with suspected or diagnosed prostate cancer. In this study we have found the assay to be acceptable to patients and deliverable within the setting of the clinical trial. We found evidence of strong immune responses in patients with low and intermediate risk prostate cancer based on D'Amico's classification with these responses declining in more advanced patients. We found that some interventions lead to an increase in immune responses and these observations warrant further exploration.

Dedication

I would like to dedicate this work to my family for without their patience, understanding and support, this would not have been possible.

Acknowledgements

I would like to thank my supervisors Dr. Peter Searle and Dr. Emilio Porfiri for their guidance and mentorship through my three years. I would like to thank the rest of the gene therapy group for making those three years so stimulating and fun and for teaching an old dog some new tricks.

Abbreviations

EDTA	ethylenediamine tetra-acetic acid
EBV	Ebstein-Barr virus
DMSO	dimethyl sulphoxide
DRE	Digital rectal examination
FITC	fluorescein isothiocyanate
HLA	Human leukocyte antigen
HSV-TK	Herpes Simplex Virus: Thymidine kinase
kb	kilo-base
kDa	kilo-Dalton
i.u.	international units
M	molar
mg	milligram
ml	millilitre
mM	millimolar
moi	multiplicity of infection

MTT	(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, stock concentration 5mg / ml)
μg	microgram
μM	micromolar
μm	micrometer
ng	nanogram
nM	nanomolar
nm	nanometer
PBS	phosphate buffered saline
PBMC	Peripheral blood mononucleocyte
PBSc (complete)	2 litres PBS + 0.264g $CaCl_2.2H_2O$ and 0.2g $MgCl_2$
pfu	plaque forming unit(s)
PHA	Phytohaemagglutin
PSA	Prostate Specific Antigen measured in ng/ml
rpm	revolutions per minute
SDS	Sodium dodecyl sulphate
SGT	Suicide gene therapy

TAE	242 g of Tris.HCl, 57.1 ml of glacial acetic acid, 18.2 g of disodium EDTA in 1 litre of dH_2O
$T_{10}E_1$	10 mM Tris, 1 mM EDTA, pH 8
$T_{10}E_1N_{100}$	10 mM Tris, 1 mM EDTA, 100 mM NaCl, pH8
TRUS	Trans rectal ultrasound of prostate
TURP	Trans urethral resection of prostate
Tween	Polyoxyethylene-sorbitan monooleate

Contents

1 Introduction

1.1 The Prostate

The prostate is a walnut-sized part glandular, part fibromuscular gland found at the base of the bladder in men. It envelopes the proximal (membranous) urethra, and is an exocrine gland which contributes 10-30% of the total volume of the ejaculate. In the adult male, it may weigh up to 20 grammes. As the male ages, the gland commonly hypertrophies, driven by testosterone and its more active metabolite, dihydrotestosterone. Malignant change is common in the older prostate gland with a histological prevalence of 15-30% of men aged 50-60 years, rising to 60-70% of men by the age of 80 years [Selley et al. 1997; Pienta 1994; Jones 1993].

1.1.1 Embryology and cellular development

The prostate gland is an endodermal structure, which develops from the urogenital sinus in response to testosterone and its more potent metabolite, 5α dihydrotestosterone. Buds from the prostatic part of the urethra grow in to the surrounding mesenchyme, and differentiate into the glandular epithelium of the gland, and this starts when the foetus is 10 weeks old. These buds branch out to form ducts and terminate in acini. Up until puberty, these ducts and acini are lined with multiple layers of immature simple and stratified squamous epithelial cells [Wernert, Seitz, and Achtstatter 1987].

After birth, the gland undergoes a period of growth through the first year, and then enters a quiescent phase until the testosterone surge associated with the onset of puberty. At this point, the glands grows in size and the immature epithelial cells differentiate into a two-layered epithelium, characterised by a peripheral layer of cuboidal basal cells and an inner layer of secretory epithelial cells [Aumüller et al. 1990]. The mature prostate secretes and stores a fluid that makes up about 30% of the seminal fluid volume, and is rich in zinc as well as being slightly alkaline (pH7.29) [Cunha et al. 1987].

The prostate has three main cell types:

Basal – these cells are found at the basement membrane and are relatively undifferentiated. They express low levels of androgen receptors (AR) and do not undertake any secretory activity [Sar et al. 1990].

Secretory luminal – the most ubiquitous cell type, these cells make up the glandular component of the prostate and constitute the exocrine compartment. They secrete prostate acid phosphatase (PAP) and prostate specific antigen (PSA) and express high levels of AR and rely on androgens for their survival [Kyprianou and Isaacs 1988].

Neuroendocrine – these cells are found throughout the prostate, they express serotonin and thyroid-stimulating hormone receptors and do not express AR [Kyprianou and Isaacs 1988].

Since the 1980s, there has been mounting evidence for the existence of a stem cell population in the prostate. Stem cells have the capacity for self renewal and are responsible for maintaining the full repertoire of differentiated cell types within their native structure. They can undergo asymmetric division, which gives rise to a new stem cell as well as a more differentiated transit amplifying daughter cell. These

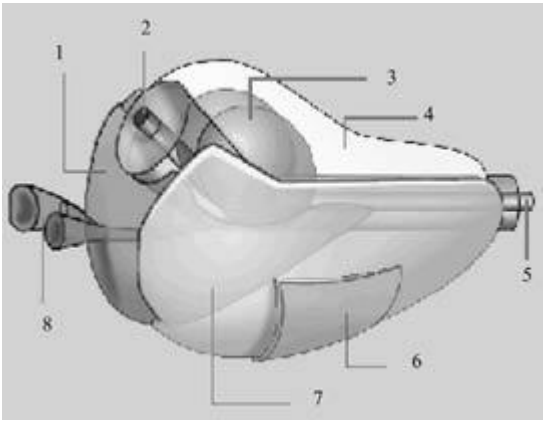
amplifying cells have a limited life, but divide rapidly thus expanding that differentiated cell line [Jones and Watt 1993].

1.1.2 Anatomy

The prostate is located in the true pelvis of the male, enveloping the proximal extra-mural urethra at the base of the bladder. It is an exocrine gland of the male mammalian reproductive system, with 70% of the prostate consisting of tubuloalveolar glands arranged in lobules surrounded by a stroma, and an ill-defined fibromuscular capsule which constitute the other 30% [LeDuc 1939, Brandes 1964].

The stroma is rich in collagen and smooth muscle, and is continuous with the poorly defined capsule. Increased tone in these smooth muscle fibres contributes to obstruction of urine flow through the prostate and reducing this tone by using alpha-adrenergic blocking agents can improve the symptoms of obstruction.

In 1988, McNeal described the anatomy of the prostate as zonal, with each zone being defined by the point at which their ducts drain into the urethra, their differing pathological lesions and their embryological origin [McNeal et al. 1988]. These zones are demonstrated in figure 1.1.



1 – central zone,
2, 5 - urethra,
3 – transitional zone,
4 – fibromuscular zone,
6 – capsule,
7 – peripheral zone,
8 – ductus deferens.

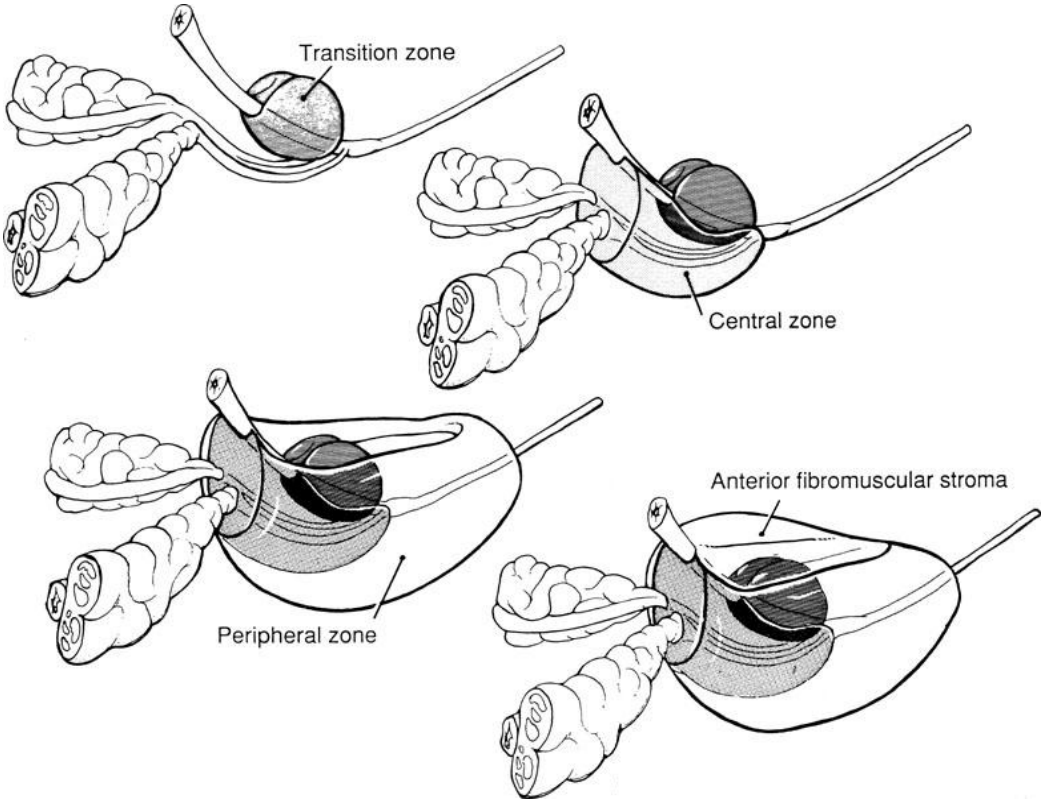


Figure 1.1 – Zonal anatomy of the prostate [McNeal et al. 897-906]

The transitional zone contains 5-10% of the glandular tissue in the prostate, and is the area in which benign prostatic hypertrophy commonly occurs. Up to 20% of prostate cancers originate in this area.

The central zone contains 25% of the glandular tissue in the prostate. It is believed that these glands embryologically originate from the Wolffian duct [McNeal et al. 1988] as they drain around the openings of the ejaculatory ducts. This may explain why cancers are rarely found here (<5%)

The peripheral zone contains 70% of the glandular tissue in the prostate, and these all drain into the prostatic urethra. This area is the origin for around 80% of all prostate cancers.

The anterior fibromuscular stroma has no glands, but can account for up to a third of the weight of the prostate. It is continuous with the capsule and it is rare to find prostate cancer in this area.

1.2 Prostate Cancer

1.2.1 Epidemiology

Prostate cancer is the commonest cancer in men in the UK and North America. In 2010, there were 40,975 new cases of prostate cancer diagnosed in the UK. There were 10,721 deaths from prostate cancer during the same period (http://info.cancerresearchuk.org/cancerstats/types/prostate/). A man has a 16.6% (1:6) lifetime risk of being diagnosed with the disease and a 3.4% lifetime risk of death from the disease. Data from the North American Cancer Registry demonstrates that prostate cancer accounts for 28% of cancers in white men and racial variation was seen from 15% in Korean men to 37% in Afro-American men.

Prostate cancer is the second leading cause of cancer deaths in white and black US men (10% of all cancer deaths among white men and 16% of all cancer deaths among black men).

The incidence of prostate cancer is expected to increase from 12.4% in 2000 to 19.6% in 2030 in the over 65 year-old population, and undergo a four-fold increase over the first half of this century [Lunenfeld 2002]. The overall incidence has increased markedly in recent years due to an increase in PSA testing and prostate biopsying at the end of the last millenium. Figure 1.2 shows that this increase in surveillance may be yielding an increase in incidence, but there is no associated increase in mortality. This is either due to a genuine improvement in treatment or due to the identification of patients with subclinical, non lethal prostate cancer. As such, the role of PSA screening is contentious and has probably contributed to the rapid rise in the prostate cancer incidence in the last twenty years. We know from the European Randomized Study of Screening for Prostate Cancer that 1470 individuals need to be screened, and 48 men treated to prevent one prostate cancer death [Schroder et al. 2009] over 9 years (figure 1.3).

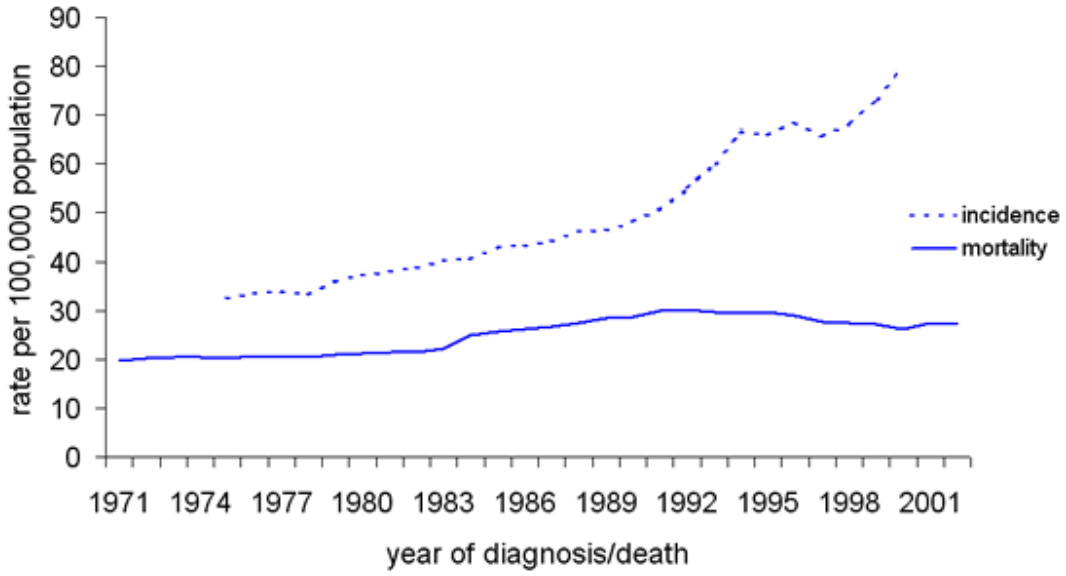


Figure 1.2 – Age standardised (European) incidence and mortality per 100,000 population, prostate cancer, by sex, Great Britain, 1971 – 2002 (http://info.cancerresearchuk.org/cancerstats/types/prostate/incidence/)

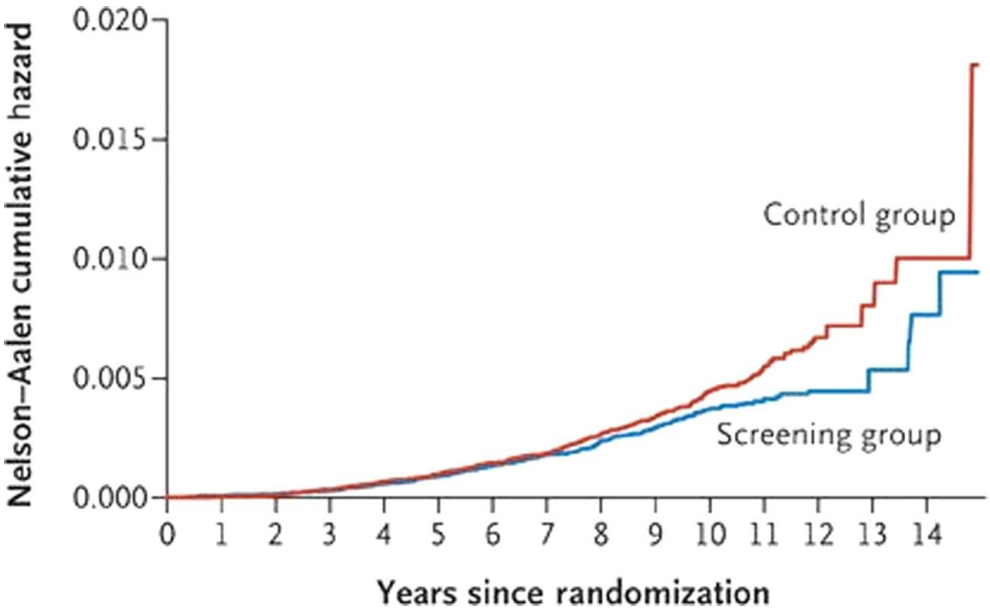


Figure 1.3 - As of December 31, 2006, with an average follow-up time of 8.8 years, there were 214 prostate-cancer deaths in the screening group and 326 in the control group. Deaths that were associated with interventions were categorized as being due to prostate cancer. The adjusted rate ratio for death from prostate cancer in the screening group was 0.80 (95% CI, 0.65 to 0.98; P=0.04). The Nelsen–Aalen method was used for the calculation of cumulative hazard [Schroder et al. 2009].

Figure 1.4 shows data from the Northern Ireland Cancer Registry, the Welsh Cancer Intelligence and Surveillance Unit, the Information and Statistics Division, NHS Scotland and the Office for National Statistics, England to calculate the incidence of prostate cancer by age in the UK population in 2006.

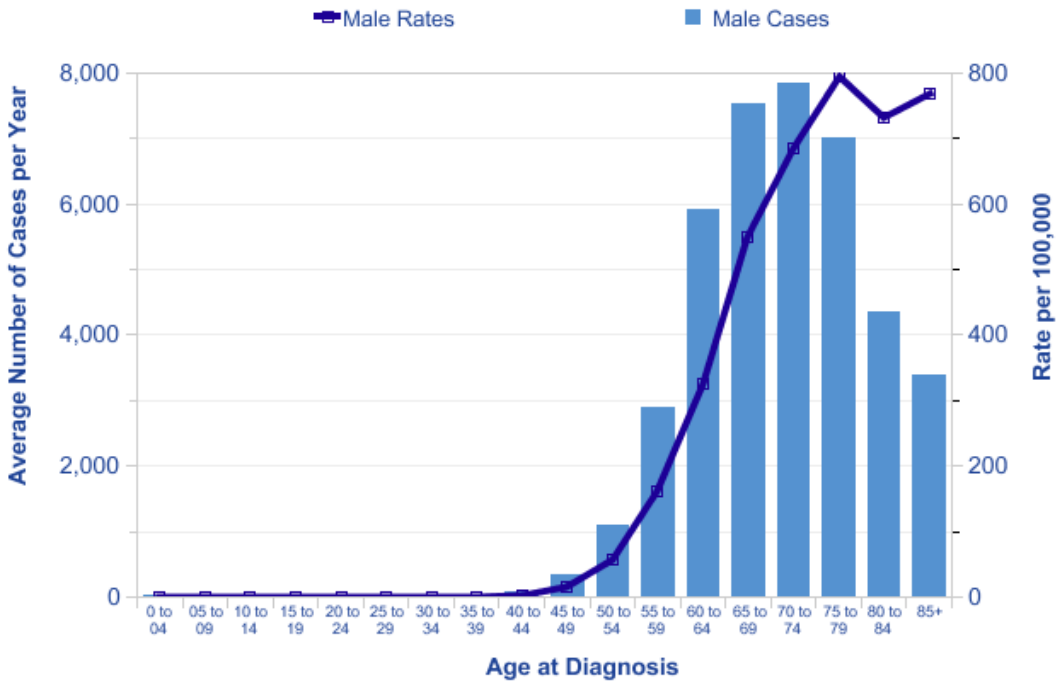


Figure 1.4 – Incidence of prostate cancer by age in the UK population (http://info.cancerresearchuk.org/cancerstats/types/prostate/incidence/).

Prostate cancer risk increases with age (fig 1.4), with 70% of all cases in the United States being diagnosed in men over 65 years of age. Autopsy studies in men who have died from non-prostate related disease suggests prostate cancer is under diagnosed (fig 1.5). It has been estimated that a 50-year-old man has a lifetime risk of 42% for developing histologic evidence of prostate cancer, a 9.5% risk of developing clinical disease, and a 2.9% risk of dying of prostate cancer [Carter, Piantadosi, and Isaacs 1990].

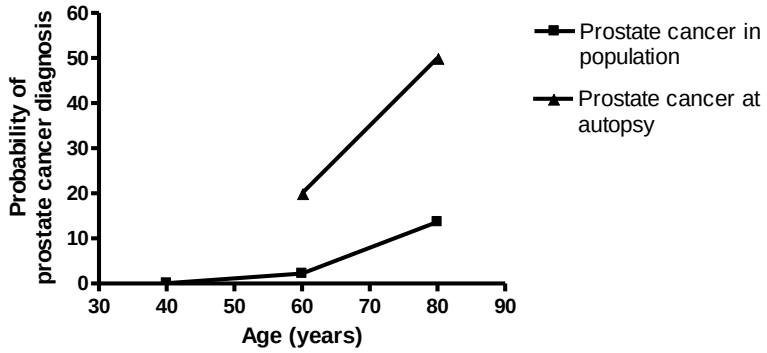


Figure 1.5. The probability of developing prostate cancer with age based on screening the living population and at autopsy in those that have died from heart disease [American Cancer Society 2003].

The natural history of prostate cancer is not entirely understood, however, it is a disease that often progresses from an androgen dependent (AD) to an androgen independent (AI) state. This progression is a multi-step process mediated in part by interactions between the cancer cells and the local stroma. These interactions lead to ongoing activation of the androgen receptor in the absence of androgen. Factors such as stromal insulin-like growth factor-1, epidermal growth factor, forskolin and interleukin-6 have all been shown to stimulate epithelial cancer cells [Culig et al. 1994; Nazareth and Weigel 1996; Ueda et al. 2002].

Prostate tumour growth has been shown to be greater in the presence of prostate stromal cells [Hirschowitz and Kaplan 1979], and fibroblast-derived growth factors from the stroma play an important role in the development of tumours in the prostatic epithelium [Kalluri and Zeisberg 2006].

Early prostate cancer is largely an asymptomatic disease. These tumours commonly arise in the periphery of the gland and have little impact on the flow of urine through the prostatic urethra. Advanced disease can give rise to local symptoms relating to the urinary tract and bowel, or can cause symptoms due to metastatic spread which is commonly to the skeleton.

Early prostate cancer is mostly diagnosed on prostate biopsies undertaken in patients in whom there is a suspicion of prostate cancer. This suspicion may be due to subtle changes found on palpating the gland during a digital rectal examination, an elevated prostate specific antigen blood test, or a strong family history. Early prostate cancer can also be incidentally found in patients having a resection of supposedly benign hypertrophied prostate tissue that is causing symptoms through obstruction.

Advanced prostate cancer is often found in symptomatic patients or those with greatly elevated PSA's with a clinically malignant prostate gland. This diagnosis can be confirmed with a biopsy, although the biopsy will often not influence the management of the patient.

Once a diagnosis is made, the disease should get a histopathological grading and clinical staging.

1.2.2 Histopathological grading

Adenocarcinomas represent 95% of all prostate cancers and 85% of these cancers are multi-focal [Byar and Mostofi 1972]. The histological grading of prostate cancer was devised by the Veterans Administration Cooperative Urological Research Group and was published by Dr Donald F Gleason [Gleason 1966] in the 1960s. Unlike other grading systems that are based on the morphological features of individual cells, the Gleason grading system is based entirely on the architecture and histological pattern of the carcinoma cells in relation to the benign prostatic cell groups in H&E-stained sections. Five basic patterns (scored 1–5) are described. (figure 1.6). To reflect the multifocality of the disease, a histological sum score (summed from scores of two most dominant patterns) is calculated, which can range from 2 (least aggressive) to 10

(most aggressive). Gleason grading of the cancer is the most widely used, and accepted histopathological method for providing information about the prognosis of prostate cancer. Gleason grade in prostate cancer is one of the most significant predictors of patient outcome [Bostwick 1994] and can predict the final pathological stage for patients with clinically localized prostate cancer [Partin et al. 1993].

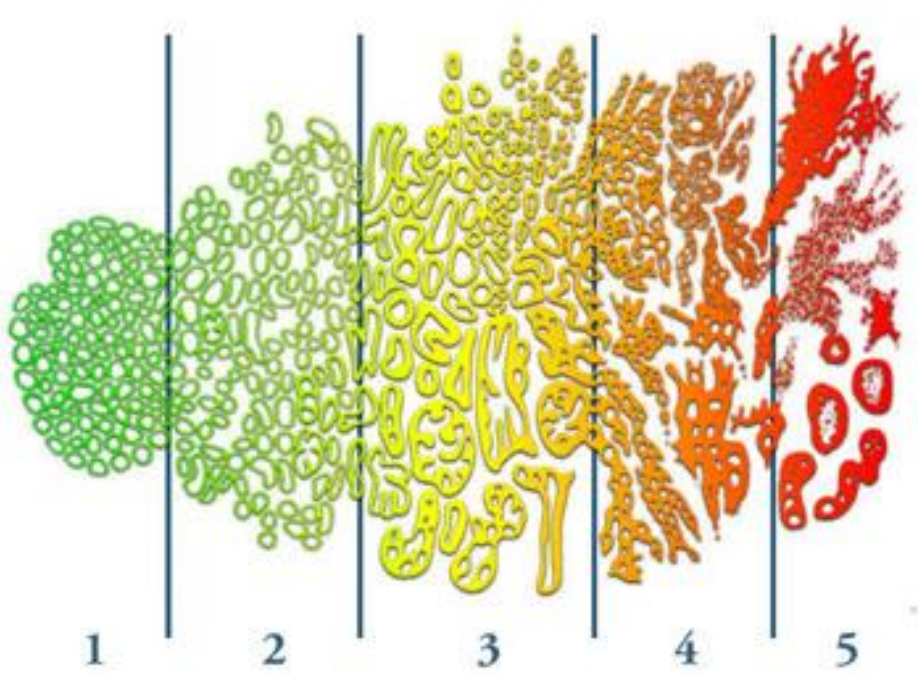


Figure 1.6: Gleason's grading system [Gleason 1966]

Other relevant prognostic variables include PSA, clinical staging and tumour volume. Microscopic evidence of perineural and vascular invasion, as well as capsular and seminal vesicle involvement, are also important prognostic signs.

1.2.3 Staging

Prostate cancers are staged using the TNM system (Urological Tumours) from the American Joint Committee on Cancer (AJCC) 6th edition (2002) and International Union Against Cancer (UICC) 6th edition. as demonstrated in Table 1.1. The incidence data are from the BAUS cancer registry 2008 dataset.

STAGE	SUB-STAGE	DESCRIPTION
T1 Incidence = 0.4%		Clinically unapparent tumour, not detected by DRE nor visible by imaging
	a	Incidental histological finding; <5% of tissue resected during TURP
	b	Incidental histological finding; >5% of tissue resected during TURP
	c	Tumour identified by needle biopsy due to elevated PSA
T2 Incidence = 62.4%		Confined within the prostate (detectable by DRE, not visible on TRUS)
	a	Tumour involves half of the lobe or less
	b	Tumour involves more than one half of one lobe but not both lobes of the prostate
	c	Tumour involves both lobes of the prostate
T3 Incidence = 23.0%		Tumour extends through the prostate capsule but has not spread to other organs
	a	Unilateral extracapsular extension
	b	Bilateral extracapsular extension
	c	Tumour invades seminal vesicle(s)
T4 Incidence = 14.2%		Tumour is fixed or invades adjacent structures other than seminal vesicles
	a	Tumour invades bladder neck and/or external sphincter and/or rectum
	b	Tumour invades levator muscles and/or is fixed to pelvic wall
N0		No lymph node metastasis
N1		Metastasis in single lymph node <2 cm in greatest dimension
N2		Metastasis in single lymph node >2cm but <5 cm in greatest dimension, or multiple lymph nodes, none >5 cm
N3		Metastasis in lymph node >5 cm in greatest dimension
M0		No distant metastasis
M1	a	Non-regional lymph node metastasis
	b	Bone metastasis i) Axial skeleton only ii) Extending to peripheral skeleton
	c	Metastasis at other sites

Table 1.1 - Prostate cancer TNM staging system (Urological Tumours)

1.2.4 Tables and Nomograms

Using pre-treatment PSA, biopsy Gleason's score and TNM (2002) data, a variety of tools are available to predict the outcomes of the treatment options available to the patient. D'Amico demonstrated that pre-operative risk stratification for T1 and T2 disease can be used to predict prostate cancer specific mortality after undergoing radical surgery or radiotherapy [D'Amico et al. 2003] (table 1.2).

Risk	PSA	Gleason score	Stage
Low-risk	< 10 ng/mL	≤ 6	T1c or T2a.
Moderate-risk	10 - 20 ng/mL	= 7	T2b.
High-risk	> 20 ng/mL	≥ 8	T2c

Table 1.2 - D'Amico's risk stratification

Partin demonstrated that these data could be used in the form of Partin's tables to predict the final pathological stage of their prostate cancer after radical surgery [Partin et al. 1993].

1.2.5 Treatment of prostate cancer

1.2.5.1 Organ confined disease (T1a-T2c, N0, M0)

A patient diagnosed with organ confined prostate cancer has a number of treatment options. Which of these options should be favoured depends on a number of variables,

such as the patient's health and life expectancy, and the prognostic characteristics of the prostate cancer itself.

The options include active curative approaches, such as radical surgery or radiotherapy. Non-curative approaches consist of manipulation of the pituitary-gonadal axis either medically (androgen deprivation) or surgically (castration), and conservative approaches such as 'active surveillance' or 'watchful waiting'.

Radical surgery can be performed through a number of routes. A trans-abdominal approach can be used, either trans- or retroperitoneal, and this approach can be made using an open incision or using laparoscopic ('keyhole') techniques. The prostate can also be approach via the perineum in an open fashion. The laparoscopic techniques bring the benefit of quicker recovery from surgery, without compromise to the oncological outcome or potency. The laparoscopic techniques take slightly longer to perform and are harder to master [Roumeguere et al. 2003]. In some series, the laparoscopic techniques resulted in poorer post-operative continence [Touijer et al. 2008]. Those patients with high risk, organ confined prostate cancer may benefit from neoadjuvant taxane based chemotherapy and androgen deprivation therapy [Mellado et al. 2009] although phase III trial data are awaited. There are other developing ablative technologies that are still being researched, such as high intensity focused ultrasound (HIFU) and cryotherapy. Satisfactory long term outcome data relating to oncological efficacy and functional outcomes are still awaited.

Radical radiotherapy can be delivered externally via external beam radiotherapy (which is fractionated to allow delivery of greater levels of radiation whilst minimizing damage to surrounding structures) and from within by implanting radioactive seeds within the prostate (brachytherapy). The oncological outcomes are

similar with evidence to show that patient selection is important in determining the outcome for brachytherapy [Potters et al. 2004].

Whether surgery or radiotherapy is the superior treatment in organ confined prostate cancer is yet to be proved [Welz et al. 2008]. There have been studies showing greater long term survival with surgery [Merglen et al. 2007], but the patient populations are never satisfactorily matched [Barry et al. 2001].

There is little indication to consider non-curative approaches such as androgen deprivation (AD) or surgical castration (SC) in low risk organ confined prostate cancer, as these patients should be considering a curative treatment modality or active surveillance. AD or SC should be offered to patients with intermediate or high risk prostate cancer who are unsuitable for curative treatment modalities.

Recognition of the slow and indolent natural history of some prostate cancers (D'Amico's low risk cancers) has allowed conservative approaches to prostate cancer to evolve. In patients unsuitable for curative therapies, with a life expectancy of less than 10 years, the initial management of low risk, organ confined prostate cancer would be one of, 'watchful waiting'. In this instance the patient's PSA will be monitored on a regular 6-12 monthly basis and treatment instigated in response to signs or symptoms of disease progression. In patients suitable for curative therapies with low risk, organ confined prostate cancer, the initial management would be one of 'active surveillance'. In this instance, the patient is kept under close clinical observation with regular 3 monthly PSA assessments and rectal examinations, as well as regular biopsies of the prostate to identify upgrading of the cancer and a shift into an intermediate or high risk group [Parker 2004].

1.2.5.2 Locally advanced disease (T3-4, Nx/N0, M0 or T1-4, N1,M0)

These patients have fewer options than those with organ confined disease. Their treatment plan relies on the accuracy of their clinical staging, which involves a subjective digital rectal examination which will over-evaluate 20% of T3 tumours. It may also involve the use of an MRI scan of the pelvis, although traditional MRI has little diagnostic superiority over the TRUS biopsy the patient will have undergone [Wefer et al. 2000]. However, developments in MRI techniques such as use of an endoanal coil and gadolinium based contrast agents are showing much promise [Younes 2007].

The gold standard treatment for T3 prostate cancer is the combination of radiotherapy and extended hormone therapy. Surgery is an option to those patients who appear to be N0 on cross sectional imaging and are clinically T3 disease, and this is supported by the European Association of Urology (EAU) guidelines that recommend surgery in patients with cT3a, PSA less than 20ng/ml and a biopsy gleason score less than or equal to 8. Surgery in this group offers 15 year biochemical free survival rates of up to 50% and disease specific survival rates of up to 84%. Functionally, the continence outcomes are similar for patients undergoing surgery for locally advanced disease when compared with organ confined disease but their erectile function is poorer due to a more radical resection being required which will sacrifice the pelvic nerves [Xylinas et al. 2009]. There would appear to be a role for the use of adjuvant or neoadjuvant androgen deprivation therapy [Kumar et al. 2006], although there would be treatment associated morbidity to consider. These patients are often offered salvage

radiotherapy, particularly if there are significant positive margins or a poor post operative PSA nadir. This is the subject of the RADICALS clinical trial (MRC PR10, ISRCTN 40814031) which is due to close to recruitment in 2013.

Around 30% of patients undergoing prostatectomy for suspected organ confined disease are upstaged to T3 disease on pathological assessment of the resected specimen [Viney et al. 2009]. There is good evidence to support the early use of either medical or surgical androgen deprivation [Messing et al. 1999] in these patients. Like those patients undergoing elective prostatectomy for T3 disease, those upstaged post prostatectomy patients with high risk disease would also warrant consideration for adjuvant radiotherapy [Bolla et al. 2005].

These patients can be managed medically with non-steroidal anti androgen agents [Iversen et al. 1998]. The benefit of these agents is that there are fewer libido and skeletal side effects [Iversen et al. 2000], making them an ideal agent for younger, sexually active patients. In patients for whom the above options are unsuitable (such as the elderly and the frail), surgical or medical castration can be offered (for those with high risk disease). Those with low risk disease could be managed with a watchful waiting approach.

1.2.5.3 Metastatic disease (M1)

According to the BAUS cancer registry of 2008, 14.2% of prostate cancer patients present with metastases. The current gold standard in treatment in these cases is AD. This can be achieved either surgically, through bilateral subcapsular orchidectomy, or through pharmacological manipulation of the pituitary-gonadal axis. The pharmacological options include depletion of luteinising hormone (LH) and follicle

stimulating hormone (FSH) from the anterior lobe of the pituitary gland (adenohypophysis) by continuous stimulation of the gonadotrophin releasing hormone (GnRH) receptor by GnRH agonists. Initially there is a flare of LH and FSH secretion that lasts about ten days and thereafter, with continuing stimulation, the LH and FSH secretion stops. This period of flare requires the use of an anti-androgen, usually for 4 weeks, to antagonise the increased circulating levels of androgen caused by the higher levels of circulating LH and FSH. AD in patients with metastatic prostate cancer gives a median survival time of 28 to 53 months [Seidenfeld et al. 1999]. Around 80% of patients with symptomatic disease get relief from AD [Dearnaley 1994]. There are new GnRH antagonists now becoming available. The benefit of using these agents is that there is no flare. This means that an anti-androgen is not needed. GnRH antagonists also induce testosterone and PSA suppression significantly faster than GnRH agonists [Klotz et al. 2008].

After 18 months, many of those managed with AD will start to show signs of biochemical relapse (as evidenced by an increase of the levels of PSA in the serum).

In this instance, an anti-androgen is then added to the management to achieve complete androgen blockade (CAB). CAB offers negligible benefit when used in patients with metatstatic prostate cancer from diagnosis [Seidenfeld et al. 2000; Iversen et al. 1998], but in the event of biochemical relapse, the addition of an anti-androgen can have some benefit [Ryan and Small 2003] which is usually short-lived. Then the anti-androgen can be withdrawn, and this also gives a transient biochemical benefit.

Chemotherapy is currently a second line therapy in metastatic prostate cancer. Prospective randomized phase III clinical trials show a survival benefit of 3 months with associated significant improvement in quality of life in men taking docetaxel and

prednisolone when compared with mitoxantrone [Petrylak et al. 2004; Tannock et al. 2004]. Chemotherapy is currently only indicated in patients who are hormone refractory and symptomatic.

In this patient group, it is also important to manage their symptoms for palliation. The spread of the disease is mainly to the skeleton causing bone pain and pathological fractures, and to local lymph nodes causing ureteric obstruction. Metastatic disease in the skeleton can be treated surgically, in the event of debilitating long bone fracture that will not heal with traditional splinting. In vertebral collapse with associated spinal cord compression, patients are best managed with radiotherapy [Huddart et al. 1997], although surgery is indicated in some instances [Tazi et al. 2003]. Focal treatment to symptomatic non-fractured skeletal metastasis can be managed with external-beam radiotherapy to provide symptom relief.

Multiple symptomatic skeletal metastases can be managed with bisphosphonates. This group of agents interferes with the activity of osteoclasts, the cells responsible for bone reabsorption and demineralisation. When used, bisphosphonates reduce bone pain and skeletal related events in patients with hormone refractory prostate cancer [Saad et al. 2002], although there is no impact on overall survival. There are agents that interact with the receptor activator of nuclear factor kappa-B ligand (RANKL) such as denosumab. In clinical trials denosumab has been shown to delay the development of skeletal metastases in prostate cancer [Smith MR et al. 2009]. Alpha radiation emitters such as alpharadin are showing promise in the treatment of prostate cancer patients with bone metastases.

The roles of both chemotherapy and bisphosphonates as first line therapies in the management of prostate cancer are currently being explored in the STAMPEDE trial [James et al. 2009]. This trial also looked for the potential benefit of combination with

the cyclooxygenase-2 (Cox-2) inhibitor celecoxib, but this arm has closed with poor results. This is in keeping with trial data on Cox-2 inhibitors that show that when they are used alone in prostate cancer, the results have been disappointing [Antonarakis et al. 2009].

New generations of hormone management (e.g. abiraterone and enzalutamide) and chemotherapy (e.g. carbazitaxel) have entered clinical practise. Abiraterone is now approved for metastatic prostate cancer patients who are progressing despite or who are unsuitable for docetaxel. The drug is taken in combination with prednisolone. The role of the new generation treatments within the current management pathways are still being defined through clinical trials with arms appearing in the STAMPEDE trial. Pain associated with advanced cancer of any type is best managed with the input of specialist palliative teams, but is mostly a combination of paracetamol, non-steroidal anti-inflammatory agents and opiates.

1.2.5.4 Recurrent disease after primary curative therapy

Patients undergoing prostatectomy with curative intent for prostate cancer have a PSA-free survival of more than 90% for T2, 80% for T3a, and 70% for T3b prostate cancer [Lein et al. 2006]. In patients undergoing radiotherapeutic treatment modalities, the biochemical treatment failure rate is as high as 40-60% [Stephenson and Eastham 2005]. The poorer outcomes with radiotherapy reflect a higher risk patient demographic and a variety of dosing levels. Outcome data should improve with improvements in dosing regimens and delivery. Treatment failure, as evidenced by a rising PSA after the treatment, may reflect either local recurrence or distant recurrence. Local recurrence is a failure of the primary treatment and means there is

residual disease in the prostatic bed. As such, the patient can still benefit from further curative treatment modalities. In these instances, the cure rates can still be as high as 70% for salvage surgery (but at least a third can expect some treatment related morbidity) [Stephenson and Eastham 2005], and 50% for salvage radiotherapy [Do et al. 1998]. Distal recurrence is a failure in the original staging of the disease, with micro-metastatic spread having already occurred at the time of the primary treatment. These patients will not benefit from further local treatments and will need to be given systemic treatments - the gold standard of which is still AD. Identifying whether recurrence is local or systemic can be difficult. The PSA kinetics after the primary treatment can help profile the likely pattern of recurrence (Table 1.3). Biopsies of the prostatic bed and modern imaging techniques such as indium-111-labeled CYT-356 (ProstaScint) [Fang et al. 2000] can also help.

Parameter	Local recurrence	Distal recurrence
Interval to PSA relapse		
<1 yr	7%	93%
1-2 yr	10%	90%
2-3 yr	61%	39%
3+ yr	74%	26%
PSA doubling time	11.7 months	4.3 months

Table 1.3 Important biochemical parameters predicting local and systemic relapse following radical surgery [Heidenreich et al. 2008]

1.2.6 Unmet therapeutic needs in prostate cancer

Almost half of newly diagnosed prostate cancers are organ confined at presentation. Of those in whom a primary curative therapy is undertaken, 35%-65% will have

evidence of recurrence within 10 years [Garnick and Fair, 1996; Han *et al*, 2001; Jemal *et al*, 2006; Moul, 2000]. These numbers are worse for more advanced disease states, and this means there are some unmet needs in prostate cancer management.

1.2.6.1 Early disease

With improvements in active surveillance protocols the overtreatment of low-risk prostate cancer is diminishing. These protocols still rely on the relatively blunt instrument of blood tests for PSA and the invasive trans-rectal prostate biopsy with the risks that the technique carries. Further work continues in the utility of more accurate serological, histological and radiological techniques to better tailor a patient's management to his disease.

1.2.6.2 Advanced/progressive disease

Like patients with metastatic prostate cancer, patients who have progressed despite local curative therapeutic treatments have a poor prognosis. They were offered treatment with curative intent as they had good life expectancies (10 years or more) and as such are likely to die from their disease. With recurrent and progressing disease, some control can be given with hormone manipulation and other second line therapies, but their median life expectancy with these modalities is 28 to 53 months [Seidenfeld et al. 1999]. It is in these patients that there is the need for further therapeutic options. Within this group, there are patients who are becoming resistant to their hormone therapy as evidenced by a rising PSA but are yet to complain of symptoms. Patients at this point in their treatment journey have no further licensed

treatment options until they become symptomatic from metastatic disease. It is this group that we will be recruiting from in our forthcoming AdGMNR clinical trial.

1.2.6.3 Metastatic disease

With the availability of new treatment modalities (see above), the life expectancy of a patient with metastatic prostate cancer has improved, but many of these patients will ultimately go on to die from their disease. This patient group are in need of further improvements in the efficacy of their systemic treatments and their side-effects.

Immunotherapies and gene therapies may provide novel solutions for some of these unmet needs.

1.3 Immunotherapy for Prostate cancer

1.3.1 Basic immunology

The immune system has two components, innate and adaptive. The innate immune system consists of cells that function through cytokine secretion, antigen presentation and mediating cell lysis. This system includes non-specific cells such as macrophages, mast cells, monocytes, natural killer cells and neutrophils.

The adaptive immune system is made up of B (derived from the bone marrow) and T (derived from the thymus) lymphocytes. The adaptive immune system is designed to respond to specific antigens that are identified as ‘non-self’. The response has the capacity to develop memory of antigens to which it is exposed as well as amplifying its response to antigens. This response is driven by the interaction between B and T cells and their interaction with antigens.

1.3.2 B Cells

B cells are significant constituents of the humoural immune response. Their principal function is to generate antibodies against specific antigens and function as antigen presenting cells. In humans, they are developed in the bone marrow and then migrate as immature B cells to the spleen where they become transitional B cells. As they mature, the antibody they express undergoes a number of changes to generate a unique variable domain in the antibodies generated by that particular B cell. Before exiting the bone marrow, the B cells are screened in the central lymphoid organ in the marrow, and those that bind to self antigens are selected and either induced into apoptosis (programmed cell death), anergy (a state of dormancy) or receptor editing, where the B cell can further alter the conformation of their variable domain to generate a less autoreactive B cell.

Once exposed to a specific antigen, the B cell is activated and undergoes a further change in its phenotype with input from T helper cells, maturing into either an antibody generating plasma cell or cloned memory B cells. The antibodies from a plasma cell can mediate tumoricidal effects through complement-mediated cell lysis or natural killer cell-mediated antibody-dependent cellular cytotoxicity.

1.3.3 T Cells

T cells are significant constituents of cell-mediated immunity. T cells originate from haemopoietic stem cells from the bone marrow which have migrated to the thymus, where they expand in numbers to form immature thymocytes (hence T cell). They are characterized by their expression of T cell receptors (see below). In the thymus, the thymocytes mature into CD4 or CD8 expressing T cells which then enter the

circulation. During this maturation process, 98% of the cells are removed through a process called selection. Selection is where the maturing T cell is presented with self-antigens. Initially, this occurs in the thymic cortex where only those cells that recognise self-antigen survive (positive selection). Those cells that have the capacity to appropriately identify self-antigen then migrate to the thymic medulla where they are presented with more self-antigens. In this setting, those T cells that show too great an affinity for self-antigen are destroyed (negative selection).

T cells consist of subpopulations (T helper cells, cytotoxic T cells, Natural Killer T cells, regulatory T cells, memory T cells and γδ T cells) that are characterised by their behavior and function. They can be profiled by their expression of specific cell-surface molecules called cluster of differentiation (CD) molecules. These molecules are present on all white blood cells, and are often involved in a cell-surface interaction that helps define the role of that particular white blood cell.

Cytotoxic T cells (CTLs) express CD8 on their surface. This is a dimeric glycoprotein that serves as a co-receptor for the T cell receptor. These cells bind to antigen presented on the major histocompatability complex (MHC) class 1 molecule, found on the surface of almost all cells (see below). Antigens from within these cells are expressed on their MHC class 1 and these reflect the health of the cell. Expression of viral or tumour antigens may drive a CTL immune response.

Helper T cells express CD4 on their surface. These cells support the activity of other constituents of the immune system. They are activated when a specific antigen is presented by a MHC class 2 molecule. These are expressed on antigen presenting cells (APCs), and represent antigen products from material ingested and processed by the APC. The interaction with the antigen presenting APC will define the nature of

that differentiation and the cytokines and role that T helper cell will play. This process is poorly understood.

Regulatory T cells (T regs) express CD4, CD25 and FoxP3. They have been categorised into naturally occurring T regs and adaptive T regs and these cells play a key role in immunological tolerance. Immune tolerance is important in switching off a T cell mediated response and to suppress auto-reactive T cells that weren't selected out in the thymus.

1.3.4 Antigen Processing and Presentation

The term antigen is derived from the term **anti**body **gen**eration. In practice, an antigen is a macromolecule, usually a protein, which is capable of driving an adaptive immune response. Antigens will drive an immune response once they have been suitably processed and presented. Antigens are created within a cell through the fragmentation of the antigen, and part of that antigen (the peptide) being bound to MHC molecules and presentation of the peptide-MHC molecules at the cell surface. 'Self' antigens should be tolerated by the immune system. Failure in this leads to autoimmune disorders. 'Non-self' antigens should stimulate an immune response against that antigen. In cancer cells, antigens presented on the class I or class II MHC are called tumour antigens. If these antigens are never expressed by non-malignant cell type, these are called tumour-specific antigens (TSAs). If these antigens are expressed on both the malignant and non-malignant cell type, these are called tumour associated antigens (TAAs).

Once presented at the cell surface the peptide-MHC molecules can bind to a T cell receptor (TCR) on a T cell. The human MHC molecules are coded for on the short

arm of the 6th chromosome. There are two classes of MHC and the pathway leading to the binding of epitopes with MHC molecules differs from class I to class II MHC molecules.

1.3.5 MHC Class 1 Proteins

The class I MHC molecules, of which there are three major (A, B, C) and three minor (E, F, G) types, are found on all nucleated cells in the body and they present peptides from degraded intracellular proteins. These proteins have been broken down in the cytosol by the proteasome, a proteolytic 28 subunit macromolecule, and the resulting smaller, 8 to 10 amino acid peptides are translocated into the endoplasmic reticulum where they can bind to the MHC class 1. The translocation is facilitated by the transporter associated with antigen processing (TAP), and the loading of these peptides onto the MHC class 1 molecule is mediated by tapasin, calreticulin, calnexin and ERP57. MHC Class 1 molecules interact with the TCR found on CD8+ cytotoxic T cells.

MHC Class 1 molecules are heterodimers, consisting of two components of the immunoglobulin (Ig) superfamily, an α chain, which is bound to the cell membrane, and an associated β2-microglobulin. The α chain has three domains named α1, α2, and α3 and a region adjoining α3 that anchors it in the membrane. All these elements are encoded in the MHC by highly polymorphic genes. The β2-microglobulin is not polymorphic or attached to the plasma membrane and is encoded by a gene in the MHC. Peptide binds to MHC class I α1 and α2 domains [Madden et al. 1992].

The MHC class I proteins have a distinct cleft in the upper surface. This is generated by the way the α chain folds. The variable region forms a close-ended cleft with α-

helical sides and a β-pleated sheet floor which will only hold an 8-10 amino acid peptide. The amino acid sequence in the variable region varies from allele to allele, so polymorphism is maximised in the amino acids which contact antigen [Bouvier and Wiley 1994].

The bound peptide lies extended in the long axis of the binding cleft. It is anchored to invariant sites at either end of the cleft by its carboxyl and amino termini. These are not the only points of interaction between the peptide and the binding cleft. Amino acids at two or three other positions along the peptide interact with amino acids in the binding cleft. For a given peptide, the anchor sites of the bound peptide can vary between MHC alleles. Amino acids not in the anchor positions can vary considerably, allowing a wide variety of peptides to be presented by a few MHC class I alleles. These interactions within the cleft present the peptide in a way that can be specifically recognized by the TCR.

The MHC class 1 - peptide complex binds to the complementary TCR on the CTL through the α1 and α2 domains. The CD8 molecule on the CTL binds to the class 1 α3 domain which has a species specific non-variable sequence.

1.3.6 Class 2 MHC Proteins

The class 2 MHC molecules, of which there are three major **(**DP, DQ, & DR) and two minor DM & DO) types, are only found on cells specializing in the presentation of antigens such as B cells, macrophages and dendritic cells. MHC class 2 proteins are also heterodimers but they are made up of an α and β chain which are coded for in the HLA-DP, HLA-DQ, and HLA-DR regions of MHC on chromosome 6 in humans. These two chains each have two domains (α1 and α2 and β1 and β2). Peptides bind

to the class 2 α1 and β1 domains, which are polymorphic from allele to allele, and these are also the domains that bind the TCR of the CD4+ helper T cell [Fremont et al. 1001-04].

They present peptides from extracellular proteins that have been internalized into an intracellular compartment (such as a lysosome) where they are digested, and the peptides bound to the MHC class 2 molecule before its migration to the plasma membrane. The class 2 peptide-binding site also folds to form a cleft but unlike the class I molecule, the cleft is open at either end so that longer, 9 to 30 amino acid epitopes can be bound. These epitopes are not anchored by their amino and carboxyl but interact with binding pockets along the length of the groove, presenting the epitope in a recognizable form for the corresponding TCR [Rammensee 1995]. To prevent MHC class 2 binding endogenous peptides, the peptide-binding cleft is blocked by the invariant chain. The loading of peptide requires that this chain is broken down. This occurs through the action of cathepsin S, resulting in a residual fragment called Class 2-associated invariant chain peptide (CLIP) [Driessen et al. 1999]. This continues to block the cleft until HLA-DM binds to the MHC class 2 and releases it allowing peptide to bind and the MHC class 2 - peptide molecule to be presented at the cell surface [Busch et al. 2005]. Here the MHC class 2 molecule on the APC interacts with the TCR of the CD4+ helper T cell.

1.3.7 The Invariant Chain

The invariant chain is a polypeptide which is complexed with the α and β chains in the endoplasmic reticulum during the synthesis of the class 2 MHC. Its presence in the peptide binding cleft prevents the binding of cellular peptides and endogenous peptides, which would normally be bound to the class 1 MHC. The export of class 2

MHC molecules from the rough endoplasmic reticulum to the golgi apparatus is facilitated by the invariant chain, followed by the subsequent fusion with the late endosome which contains endocytosed degraded proteins. Proteases called cathepsins break down the invariant chain leaving a small fragment which blocks the peptide binding cleft on the MHC molecule. This small fragment is displaced by HLA-DM, an MHC class 2 like structure which facilitates the binding of higher affinity peptides and this more stable class 2 MHC is then presented at the cell surface membrane.

1.3.8 Dendritic cells

Dendritic cells (DCs) are specialist antigen presenting cells (APC) which induce and regulate the adaptive immune response by stimulating T cells and B cells. They are to be found in peripheral tissues and the circulation. The progenitor cells originate from the bone marrow and are CD34+. They circulate in the blood, and subsequently give rise to a number of subsets of phenotypically and functionally distinct DCs which reside in peripheral tissues, such as Langerhans cells in epidermis and dermal DCs in dermis [Banchereau and Steinman 1998; Lipscomb and Masten 2002; Banchereau et al. 2000].

These may have been derived from several different populations of precursor cells such as monocytes [Randolph et al. 1998; Olweus et al. 1997] . The dendritic cell has two phases to its life cycle, immature (an antigen capturing mode) and mature (a T cell stimulating mode).

The immature DC is found in most tissues and in the circulation. They roam the body, seeking antigen to present to T cells. These cells are suited to antigen capture with high levels of expression of antigen capturing Fcγ and Fcγ receptors, but poor at T cell

activation with low expression of stimulatory receptors like; CD40, CD54 and CD86. Once antigen has been identified by an APC it can be taken up by phagocytosis, macropinocytosis or absorptive pinocytosis and within 24 hours, the phenotype of the immature DC changes. The expression of receptors for antigen capture decreases and the expression of receptors associated with T cell stimulation increases. This change in phenotype coincides with migration of the now mature DC to the local lymph nodes and the spleen [Lukas et al. 1996].

The mature DC has the macroscopic appearance that gives rise to the name 'dendritic cell'. They are stellate with multiple fine dendrites and sheet-like processes which extend and retract, increasing the likelihood of interacting with a suitable T cell.

T cells need to have antigen suitably processed and presented to them at the MHC class 2 which are heavily expressed by DCs. The MHC class 2 - peptide complex is recognised by the T cell receptor (TCR) and this leads to the activation of the T cell. This activation is enhanced by the interaction of other costimulatory factors on the DC and the T cell, such as CD40, CD80, CD86 and 4-1BB and their ligands. The DC also expresses adhesive molecules, such as CD11a, CD15s, CD18, CD29, CD44, CD49d, CD50 and CD54 which facilitate the binding of the DC to the T cell [Shortman and Caux 1997; Baggers, Ratzinger, and Young 2000]. It is the expression of these co-stimulatory and adhesive molecules that facilitate and drive an effective immune response. When antigen is presented by 'non professional' APCs, without these molecules, tolerance is induced [Lutz and Schuler 2002].

DCs largely behave in an immunogenic way, although there are some that are tolerogenic. It is believed that those DCs that are derived from the marrow become immunogenic, where as those derived from lymphoid tissue will become tolerogenic

[Lin, Jacek, and Jacek 2006]. Their interactions with the wider immune system are complex and there is still much that is not understood about them, particularly in cancer immunology.

1.3.9 T cell receptors (TCR)

T cells are a constituent of the white cell population known as lymphocytes. They are a key component in cell-mediated immunity. On the surface of these cells are found the membrane-bound T cell receptors. These recognize antigenic epitopes presented on MHC molecules. TCRs are heterodimers composed of two chains, α and β chains. These chains have a membrane anchored constant region and an outward facing variable region, ready to bind to a suitable MHC-peptide complex. The variable region on each chain has three, hypervariable complementarity determining regions (CDR). These CDRs define the antigen-binding specificity. The wide diversity that occurs in the CDRs occurs because they are encoded in gene segments that undergo somatic recombination during T cell development.

Each TCR has a single binding site for its target peptide. Peptides presented on MHC class 1 molecules will be recognized by those T cells with the co-receptor CD8 bound to the TCR. These cells are called cytotoxic T cells [Solheim 1999].

Peptides presented on MHC class 2 molecules will be recognized by those T cells with the co-receptor CD4 bound to the TCR such as T helper cells [Pieters 2000] and regulatory T cells. These co-receptors increase the binding affinity of the T cell for the MHC class 2 - peptide complex and signal the T cell to become activated.

A TCR will recognize a particular MHC-peptide complex, and this TCR will not bind on another MHC allele. This is called MHC restriction. If this specific peptide is

altered, its binding in the MHC binding cleft may change. This can affect the binding of the TCR to the MHC complex - peptide.

1.3.10 Immunology in malignancy

Immunotherapy is an evolving therapeutic field in oncology which can be used as a primary treatment (such as intra-vesical BCG for non-muscle invasive bladder cancer), or can be used to enhance the effectiveness of other conventional treatments (interferon-alfa2b as an adjuvant to surgery for metastatic melanoma). With an increasing understanding of the immune system and its interaction with cancer cells, new therapies are evolving that can mobilize an individual's own immune system to attack and destroy tumour cells. Theoretically, this would be highly specific and thus avoid the systemic complications experienced from relatively unselective treatments such as chemotherapy.

1.3.11 Immune surveillance and Immunoediting

The role of the immune system in protecting the host from cancers by eliminating cancer cells when they arise was proposed in 1957 [Burnet, 1957]. This was called immune surveillance. With growing mouse and human evidence the theory has been further refined and described as Immunoediting.

Immunoediting is the interaction between the immune system and cancer cells and is described as having three phases; elimination, equilibrium and escape [Dunn *et al* 2002]. Newly transformed cells can be initially eliminated by the innate immune system through cells such as natural killer cells. As the tumour progresses, adaptive

immune response can be provoked by tumour antigen-specific T cells to provide the balance seen in the equilibrium phase. After time, immune selection produces tumour cell variants that have reduced class I and II MHC expression and thus decreased tumour antigen presentation. This, combined with tumour-derived soluble factors released in the tumour micro-environment, facilitate the escape from immune attack, allowing progression and metastasis [Kim *et al.* 2006].

1.3.12 Rationale for prostate cancer as an immunotherapeutic target

Ever since William Coley noted the spontaneous regression of some sarcomas in response to local inflammation secondary to local bacterial superinfections in 1891, there has been considerable interest in the relationship between the immune system and cancer.

Immune activity within the prostate is well recognized [Palapattu *et al* 2005; Elkahwaji *et al* 2009]. In men aged 20-40 years old, the most common pathology of the prostate is prostatitis. This is a poorly understood entity with acute and chronic bacterial forms as well as non-bacterial manifestations of the condition. The aetiology may not just be infection [Nelson et al, 2004] but may be due to some autoimmune process [Pontari and Ruggieri, 2004]. What is not clear from the literature, is whether prostatitis is a risk factor for an individual developing prostate cancer. Some studies have shown an increased risk of prostate cancer in men with symptomatic prostatitis [Nakata S 1993]. Other studies have shown that the relative odds of prostate cancer were elevated in men with history of prostatitis [Roberts *et al* 2004], but not with statistical significance. Inflammation is frequently present in prostate biopsies, radical prostatectomy specimens and tissue resected for treatment of benign prostatic

hyperplasia [Platz *et al* 2004]. A possible relationship between inflammation and prostate cancer has led researchers to look at whether anti-inflammatory treatments used to curb prostatitis, might help treat prostate cancer (Pruthi *et al* 2004].

There is a strong relationship between immunological activity within prostate cancer tumour tissue and tumour grade and outcome. There is an inverse relationship between rates of cancer recurrence and number of tumour infiltrating T lymphocytes in prostate cancer, [Vesalainen *et al* 1994]. There is a correlation between macrophage levels within prostate tumours versus the tumours grade [Shimura *et al* 2000]. These observations are consistent with immunoediting and this implies that patient cell–mediated immunity is playing a role in suppressing prostate tumour progression.

When one considers treatments that have a direct tumouricidal effect in prostate cancer, such as androgen deprivation, one finds evidence of profuse infiltration by activated and oligoclonal-specific T cells within the prostate [Mercader *et al* 2001]. As well as enhanced lymphocyte levels and activated antigen specific T-cells in both animals [Roden *et al* 2004] and humans [Drake *et al* 2005].

There is evidence that immune responses against healthy and malignant prostatic tissues can be induced. Considerable work has been done on developing a vaccination system using a variety of proteins or peptide derivatives that are prostate specific such as:

- Six-transmembrane epithelial antigen of the prostate (STEAP) [Hubert *et al* 1999].
- Prostate specific membrane antigen (PSMA) [Murphy et al, 1999; Milowsky et al, 2004; Bander et al, 2005].
- Prostatic acid phosphatase (PAP) [Fong et al, 2001].
- Prostate-specific antigen (PSA) [Pavlenko et al, 2004].

Vaccinations using these peptides have been demonstrated to induce humoural and cellular responses. This approach has lead to the development of the first FDA approved cancer vaccine, Sipuleucel-T, and this is in prostate cancer. This treatment platform involves the extraction of dendritic cells from the patient. These cells are then incubated with a fusion protein known as PA2024 which consists of PAP and GMCSF. These dendritic cells are then re-infused into the patient. There are many other examples of the efficacy of GMCSF as an adjunct to immunotherapy in prostate cancer [James *et al* 2001; Sanda *et al* 1994; Simons *et al* 1999]. There are also other groups exploring similar strategies using APCs that have been modified ex vivo to express prostate tumour associated antigens and cytokines [Barrou *et al* 2004; Zhang *et al* 2003; Rini 2004].

There are other examples of patient's cells being extracted, and modified *ex vivo* as well as *in situ* cells having been modified with gene therapy to generate an immune response in prostate cancer.

In the mouse, TRAMP cells modified with interleukin-12 and B7-1 induced strong protective immune responses in C57BL/6 mice involving CD4+, CD8+ and natural killer cells. The efficacy of B7.1 ligand introduced into prostate cells has also been demonstrated by other authors [Kwon *et al* 1997].

What also makes the prostate an attractive target for immunotherapies, is that in the older male the prostate becomes less important, particularly if he has completed his family. Indeed, in many older males, the gland can become a nuisance, causing urinary symptoms if the gland has enlarged with BPH. As such, any damage caused to non-malignant, healthy prostate tissue will be of little significance. This means that antigen targeting therapies can be used against tumour associated antigens, as well as tumour specific antigens.

1.3.13 Prostate Tumour Associated Antigens (TAAs)

In this section, I will introduce the TAAs that we will be using in our experimental work.

Prostate specific antigen (PSA) - this glandular kallikrein (KLK) was first described in 1979. KLKs are a subfamily of serine proteases that are involved in the post-translational modification of many polypeptides. [Wang *et al* 1979]

The gene encoding for PSA is found on chromosome 19. Transcription is regulated by androgens and it generates a 261 amino-acid PSA precursor. This is then cleaved to form the 237 single chain form that is secreted into the seminal fluid by the ductal columnar epithelial cells. The PSA levels in seminal fluid are one million-fold higher than in serum. The small quantities found in the blood are in free and complexed forms. PSA's physiological function is to liquefy seminal fluid. Clinically, it is

measured in the blood and used as a marker for the diagnosis, staging and monitoring of prostate cancer [Ward, Catto, and Hamdy 2001].

A number of studies suggest that PSA could be a useful antigen for future immunotherapeutic treatments for prostate cancer. Human cytotoxic T lymphocytes (CTLs) respond to peptides derived from PSA [Xue *et al* 1997] and mice immunized with plasmid DNA encoding PSA get potent CTL responses to specific tumour cells [Kim *et al* 1998]. When mRNA from a prostate tumor line was transfected into autologous DCs, antibody responses directed against PSA antigens were seen [Heiser *et al* 2001].

Prostate specific membrane antigen (PSMA) – a type II glycoprotein expressed in both normal and malignant prostate cells. It has a transmembrane and a secreted form [Gregorakis, Holmes, and Murphy 1998]. Its function is not fully understood but the protein has some folate hydrolase activity [Tasch *et al* 2001]. PSMA expression is upregulated in high-grade prostate cancers, metastatic lesions and androgen-independent disease [Wang *et al* 2007]. PSMA is also highly expressed in tumour neovasculature cells making PSMA an attractive target for selective therapies [Gong *et al* 1999]. PSMA specific targeted therapies are being developed in clinical trials [Olson, Heston, and Rajasekaran 2007].

Prostate stem cell antigen (PSCA) - a 123 amino acid glycoprotein which was first identified in 1998 [Reiter *et al* 1998]. It has similarities to stem cell antigen 2 (Sca 2), a cell surface marker in immature thymic lymphocytes, such as being attached to the cell membrane with a glycosylphosphatidylinositol (GPI) anchor [Antica, Wu, and Scollay 1997; Classon and Coverdale 1994].

PSCA mRNA expression was studied in 16 different, healthy tissues but was found principally in prostate and placenta. Using in situ hybridization in normal prostate, the PSCA mRNA expression was found in the basal cell epithelium. This is the putative stem cell compartment of prostatic epithelium, suggesting an association between PSCA and prostate stem cells. PSCA mRNA expression was identified in more than 80% of primary prostate cancers by in situ analysis, and was stronger than in adjacent normal glands. This observation suggests that PSCA may be up-regulated in prostate cancer and therefore may have some cancer specificity. The gene for PSCA is found on chromosome 8q24.2. This region is often affected by mutations in advanced prostate cancers [Cher *et al* 1994]. By using a series of monoclonal antibodies directed against PSCA, the cell surface localization of PSCA has been confirmed. Immunohistochemical analysis of a large series of primary tumours demonstrates that PSCA expression increases in more advanced stages and grades of prostate cancer. In addition, PSCA is expressed strongly in all cases of prostate cancer bone metastases examined [Gu *et al* 2000]. These studies support PSCA as a potential diagnostic tool in prostate cancer.

Prostate acid phosphatase (PAP) – a prostate-specific isoenzyme of acid phosphatase secreted by cells in the prostate was first described in 1936. It is a 100 kDa glycoprotein containing two subunits of approximately 50 kDa each [Gutman *et al* 1936]. It is a major phosphatase and a differentiation marker of epithelial cells in normal, prostate tissue [Yam 1974]. Its use as a marker for prostate cancer and its response to treatment was first described in 1941 [Huggins *et al* 1941] and it was used as such until the establishment of PSA as the new gold standard [Papsidero *et al* 1980]

In prostate epithelial tissue, there are two forms of PAP. There is an intracellular (the cellular) form (cPAP), and the other is a secretory form (sPAP), found in greater concentrations in the seminal fluid. There are some subtle physicochemical differences between the two forms, but the reasons for this are yet to be established. Both forms can hydrolyze a wide variety of organic phosphomonoesters [Vihko 1979; Lin *et al* 1983].

Kallikrein-related peptidase 4 (KLK4) – is the fourth KLK to be described [Stephenson *et al* 1999] and has been shown to only be expressed in prostate cancers that express both progesterone and androgen receptors [Lai *et al* 2009]. KLKs have been shown to play a role in prostate cancer progression. They degrade the extra cellular matrix, activate protease-activated receptors (PAR-1,2,3 and 4) and activate TGF-beta1[Clements *et al* 2004; Takayama *et al* 2001; Ramsay *et al* 2008; Mize, Wang, and Takayama 2008].

In prostate cancer tissue, there is evidence that KLK performs a paracrine function. The cancer epithelium produces KLK4 which activates PAR-1 in the surrounding stroma, driving the releases of stimulatory cytokines such as IL-6 causing cancer cells to proliferate and increase production of further KLKs [Wang *et al* 2009].

1.4 Immunotherapy

Immunotherapy is the treatment of disease by inducing, enhancing, or suppressing an immune response. Those therapies that induce or enhance immune responses are called activation immunotherapies. Those therapies that suppress an immune response are called suppression immunotherapies. In cancer therapy, immunotherapy has become a standard treatment in a number of solid tumours, examples being the use of

interleukin 2 [IL2] in metastatic renal cell carcinoma, monoclonal antibody therapies in breast cancer, and Bacillus Calmette-Guérin [BCG] instillation into the bladders of patients with high grade superficial bladder cancer.

Immunotherapies can also be classified as passive or active immunotherapies.

Passive immunotherapies utilize components of the immune system that are created or modified outside of the individual and then reintroduced, such as monoclonal antibodies.

Active immunotherapies interact with the individuals own immune system, 'actively' stimulating it to deliver a therapeutic result. These include cellular therapies, cancer vaccines and adjuvant therapies.

1.4.1 Passive immunotherapies

1.4.1.1 Monoclonal antibody therapy (mAB)

This is the most widely used cancer immunotherapy. This is a targeted therapy, with the antibody directed at a specific target relating to a cancer cell. This may be a cell surface antigen or receptor. Since 1997, 15 different mABs have been given approval by the Food and Drug Administration (FDA) for direct anticancer therapy. Of these there are 12 'naked' mABs, which work by directly stimulating the host's immune system, and 3 'conjugated' mABs, which are bound to chemotherapy agents, radioactive particles or toxins and this is how they deliver their therapeutic effect.

The outcomes from mAB therapies have been variable with notable successes such as Herceptin in breast cancer and the flu-like side effects are relatively mild when

compared to chemotherapies. In prostate cancer the results have not been as successful as expected. This could be due to prostate tumour heterogeneity, with not all the cells expressing the target antigen.

An agent showing promise and currently in phase 3 trials in prostate cancer is Ipilimumab. This is an anti CTL-associated antigen 4 (CTLA-4) mAB. CTLA-4 is a 'check point' molecule expressed on activated T cells that delivers an inhibitory signal to these T cells. Blockading this molecule augments antitumour activity and has demonstrated efficacy in melanoma for which it now has a license.

There is a possible role for radioisotope labelled mABs in the radiological staging of prostate cancer. The FDA has given approval to (111)In-capromab pendetide (ProstaScint) to be used in identifying small metastatic deposits, although there has been limited success [Elgamal, Troychak, and Murphy 1998; Cutino 1991]. It is best used in assessing those patients in whom there is evidence of biochemical failure with a view to identify their suitability for a salvage therapy.

Where mABs are used therapeutically, they are often used as second or third line therapies. This may reduce their effectiveness, as this is when tumour burden and heterogeneity will be greater, and the patient's immune system may already be significantly weakened.

1.4.2 Active immunotherapies

1.4.2.1 Cellular therapies

These involve taking cells from the patient, modifying them to recognize and kill the patient's tumour and reintroducing them into the patient. Examples of this are lymphocyte activated killer cells and tumour infiltrating lymphocytes with IL-2. Such bespoke therapy is very costly and difficult to deliver commercially. The chosen cell type can be difficult to grow and modify *in vitro* and, if successfully generated, there are hypersensitivity risks associated with the transfusion of large numbers of cells.

1.4.2.2 Cancer Vaccines

Vaccines are a standard tool in prevention of infectious disease. In this setting, it is used in individuals who are free of that particular disease. In the cancer setting vaccines are being used in the same way for treating those strains of human papilloma viruses (HPV) (16 and 18) that are known to be responsible for the development of cervical cancer. In many countries there is now a programme of vaccination for pre-teenage girls to be vaccinated with either Gardasil or Cervarix, two anti-HPV vaccines.

For patients with established cancers, there are therapeutic cancer vaccines. These are designed to stimulate a response in the patient's immune system against the cancer. They will do this through a generalized upregulation of the immune system as well as focusing an immune response against specific tumour antigens. Like the preventative vaccines, a cancer vaccine might consist of part or whole cancer cells alongside adjuvant immune stimulants (e.g., IL-2). These are known as cell-based cancer

vaccines. Other cell based cancer vaccines involve using cellular constituents of the patient's own immune system and modifying them to express tumour antigens and suitable co-stimulatory factors [Heiser *et al* 2002]. Another way of priming the immune system against a specific cancer protein is to use a vector-based vaccine [Eder *et al* 2000]. This approach uses a vector, such as a genetically engineered virus, to introduce specific identifiable components of the tumour to the immune system. Liposomal delivery of cancer associated antigens is another method that has been tried in phase I/II trials [Meidenbauer *et al* 2000]. Immunization has been undertaken using specific tumour associated peptides (TAP) injected intradermally. This technique requires HLA typing of the patient to aid peptide selection. The patient can 'self select' the appropriate peptides by looking for immune responses by patient CTLs *in vitro* against a panel of TAPs. The ones that are recognized by the patients CTLs are then used in the vaccination [Noguchi *et al* 2003]. Naked DNA vaccines using plasmid DNA expressing TAAs have also been used in phase I/II trials with some evidence of efficacy [Mincheff *et al* 2000].

Like mAB therapies, the heterogeneity of tumour cells means that not all tumour cells will express the target antigen. These treatments are also used in the later stages of the disease where disease burden and heterogeneity will be greater and the patient in a poorer physical condition, adversely affecting the efficacy of immune-based treatments. The cells used can be autologous (from the patients themselves) and these will need culturing *in vitro* which will take time, is costly and difficult to deliver commercially. Established cancer cell lines can be used but these can have local hypersensitivity problems. The benefit of this approach is that the cell line can be genetically engineered to express co-stimulation factors or other immune stimulants.

Another problem with this approach is that the tumour cells are often poorly immunogenic.

For prostate cancer there are a number of cancer vaccines in clinical trials with four principal modalities progressing to phase III clinical trials. The first vaccine is the Sipuleucel-T (APC8015, Provenge®; Dendreon Corporation, Seattle, WA) system. This consists of autologous dendritic cells that have been isolated from the patient's peripheral blood mononuclear cells. The DCs are activated and loaded with a recombinant fusion protein of the prostate tumour antigen, PAP, linked to granulocyte-macrophage colony stimulating factor. The patient will get three intravenous infusions of these cells over a total of 6 weeks. In the phase I/II trials, this regime was well tolerated with some rigors and grade 1 fevers reported. T cell responses specifically against the target antigen was seen [Small *et al* 2000]. Two identically designed, randomized, double-blind, placebo-controlled trials (D9901 and D9902A) were conducted in 225 men with hormone refractory prostate cancer (HRPC). The 147 patients randomized to sipuleucel-T demonstrated a 33% reduction in the risk of death (hazard ratio, 1.50; 95% confidence interval, 1.10-2.05; P = .011; log-rank), compared to the 78 patients in the placebo arm. The treatment was well tolerated with around a third of patients experiencing chills, pyrexia, headache, asthenia, dyspnea, vomiting, and tremor. Two studies were undertaken as the first study had disease progression as a primary endpoint, and not overall survival. The second trial addressed the endpoint issues and the data from the two trials were combined [Higano *et al* 2009]. The American Food and Drug Agency have since given Sipuleucel-T a licence for the treatment of advanced, hormone resistant prostate cancer.

The second vaccine based therapy in phase III clinical trials uses the DCVax®-Prostate (Northwest Biotherapeutics, Bothell, WA) system. Once again, autologous dendritic cells are used and they are loaded with recombinant PSMA. This treatment is delivered via an intradermal injection, rather than subcutaneously, allowing the dendritic cells better access to the patients lymph nodes where they can present the PSMA antigen to naïve T cells [Bonnotte *et al* 2003]. The phase I/II data from 32 patients with hormone escaped prostate cancer (12 without evidence of metastatic disease and 20 with evidence of metastases) was promising, but never fully published. A phase III randomized, multi-centre, double blinded clinical trial involving 612 patients was embarked upon. The trial is yet to be completed [Fishman 2009].

The third vaccine based therapy to go to phase III clinical trials used the Prostate GVAX® (Cell Genesys, Inc., San Fransisco, CA) system. This system used two cultured, allogenic, prostate cancer cell lines, PC-3 and LNCaP, which were genetically modified to express GMCSF. The cells were then irradiated, to inactivate them. In phase I/II trials, these cells were injected via an intradermal route using escalating cell numbers and lengthening treatment cycles (from 14 to 28 days). The treatment was well tolerated with no autoimmune reactions or serious adverse events. Efficacy was demonstrated by a downward deflection in the PSA kinetics in 84% of patients, and a significant increase in the median PSA doubling time from 28.7 weeks pre-treatment to 57.1 weeks post-treatment ($p=0.0095$) (Urba et al. 2008). Despite this early promise, the phase III trials were terminated based on futility analysis showing <30% chance of the primary trial of GVAX versus docetaxel and prednisolone (VITAL1) meeting its endpoint. The second trial of combination GVAX and docetaxel versus docetaxel and prednisolone (VITAL2) was stopped by the Independent Data Monitoring Committee. No data were published from these trials.

The fourth vaccine based therapy to get approval for a phase III trial is the ProstVac-VF platform [DiPaola *et al* 2004]. This is a viral vaccine approach produced by combining fowlpox and vaccinia PSA vaccines with a combination of 3 costimulatory molecules (TRICOM), B7-1 (CD80), ICAM-1 (intercellular adhesion molecule 1(CD54)), and LFA-3 (leukocyte function-associated antigen 3 (CD58)). In this phase III study, patients with hormone refractory PSA progression after local therapy will be treated with a prime and boost protocol using the vaccinia and fowlpox-PSA-TRICOM vaccine combined with GM-CSF compared to a placebo control. Since patients will be enrolled without any evidence of metastasis, the primary endpoint of progression to clinically detectable metastasis will represent a firm endpoint independent from PSA. These data will determine the efficacy of this vaccine approach on prostate cancer progression. As yet, there has been no published data from this trial.

1.4.3 Adjuvant immunotherapies

These are any materials that, when delivered alongside another treatment, will enhance the immune response of an individual in relation to that treatment. Adjuvant immunotherapies, such as GMCSF [Fong *et al* 2009], often have their own toxicities and are often limited in the frequency and route through which they can be administered.

1.4.4 Granulocyte Macrophage – Colony Stimulating Factor (GMCSF)

This is the name applied to the cytokine that stimulates the proliferation of granulocyte and macrophage progenitors in semi-solid culture to form maturing colonies of granulocytes and/or macrophages. GMCSF can be detected in all tissues, produced by local macrophages, mast cells, T cells, fibroblasts and epithelial cells. Other cells may also have the capacity to produce GMCSF [Burgess and Metcalf 1980].

GMCSF is a pro-inflammatory cytokine. Expression of GM-CSF is elevated in a number of infections, malignancies, autoimmune and other inflammatory diseases, suggesting a role in the immune processes associated with these diseases [Fleetwood, Cook, and Hamilton 2005; Hamilton 2008; Raivich and Banati 2004]. One of the actions of GMCSF is to drive the differentiation of precursor cells such as monocytes into immature dendritic cells [Steinman *et al* 1988; Witmer-Pack *et al* 1987].

The functional subset of DC created in this way is dependent on the cytokines present in the immediate environment. The presence of IFN-γ or IL-4 will facilitate the development of DCs which will activate T cells [Chen *et al* 2000]. The presence of IL-6 or TNF alpha will facilitate the development of macrophage-like DCs [Chomarat *et al* 2000; Chomarat *et al* 2003]. The presence of IL-10 will facilitate the development of tolerogenic DCs which can anergize T cells [Steinbrink *et al* 1999].

1.4.4.1 Clinical trials using GMCSF

There have been a number of clinical trials using GMCSF in isolation, or as an adjuvant to other therapies. Initially, trials were undertaken with GM-CSF alone. In a two cohort trial, a primary cohort of 22 men were initially treated with 28 day cycles of 250 μg/m^2 of GMCSF daily for 14 days, followed by 14 days off [Small *et al* 1999]. Of these 22 patients, 10 experienced a decline in their PSA at the end of each 14 day treatment cycle only for this to then return to baseline on discontinuation of the GMCSF. In this group the median time to progression was 3.5 months. In the second cohort were 13 men who were also treated initially with 250 μg/m^2 of GMCSF daily for 14 days, only this was followed by thrice weekly maintenance injections of 250 μg/m^2 of GMCSF until disease progression. In this cohort, 12 patients experienced a decline in their PSA but only one of these was sustained and in excess of 50% (PSA of 77ng/ml to 0.1ng/ml). Subsequent work has seen GMCSF used in combination with other therapeutic strategies. These are summarized in table 1.4.

A - Immunotherapy with recombinant GMCSF				
Strategy	Indication	Patients	Toxicity	Reference
S.C. Recombinant GM-CSF	PSA relapse post surgery	30 Patients Decreased PSA doubling time 8.5 to 15 mo 3 Patients >50% PSA decrease	Injection site reaction. Fatigue Flu like syndrome	Rini BI JCO 21;99-105 2003
S.C. Recombinant GM-CSF	PSA relapse M0 – M1	26 Patients Dose dependent PSA decline (>25% in 8 patents)	Injection site reaction Malaise	Schwaab T The Prostate 66:667-74 2006
S.C. Recombinant GM-CSF	PSA relapse M0-M1	16 patients 6 patients 10-15% PSA decrease	Injection site	Dreicer R Investigational new drugs 19:261-65 2001
S.C. Recombinant GM-CSF	PSA relapse HRPC M1 Continuous vs short treatment	36 patients PSA decline more common with continuous (12 out of 13 pts)	Malaise Flu like Injection site	Small EJ Clin Ca Res 5:1738-44 1999
Recombinant GM-CSF + Thalidomide	22 patients HRPC M1	22% PR (PSA) All patients PSA response	Injection site reactions Low blood counts Fatigue	Dreicer R Urol Oncol. 2005 23:82-6

B - Immunotherapy with prostate cancer cells (allogenic or autologous) expressing GMGSF				
Strategy	Indication	Patients	Toxicity	Reference
Allogenic cellular immunotherapy LNCaP	PSA relapse after RRP Hormone naive	21 patients 76% decreased PSA velocity	Injection site reaction Rash, Flu like syndrome	Simons JW Clin Ca Res 12:3394-3401 2006
Allogenic cellular immunotherapy GVAX LNCaP and PC3 expressing GMCSF	55 HRPC 34 M1 21 M0 Low dose vs high dose	55 patients 6/55 PSA decrease >25% Reduction of PSA slope in both groups	Fatigue Flu like syndrome	Small EJ Clin Ca Res 13:3383-3891 2007
Allogenic LNCaP and PC3 expressing GMCSF	M1 HRPC Dose (no. cells) escalation	80 patients PSA decline in 32% of high dose group Decreased osteoclast activity in 62% of tested patients	Not reported	Small E Proc ASCO 2004 Abs. 4565
Autologous vaccination with tumour cells infected with GMCSF expressing retrovirus	M1 at prostatecto my Phase I	8 patients Immune responses to PCA antigens	Injection site reactions	Simons JW Can Res 59:5160-68 1999
GMCSF as an adjuvant to vaccine therapy	Vaccine alone 51 patients Vaccine + GMCSF 44 patients	1 CR, 8 PR with GMCSF 2 CR, 17 PR w/o GMCSF detrimental?	Injection site reactions Pyrexia Fatigue	S.J. Simmons The Prostate 1999 39: 291 - 297
Autologous cancer cells transduced with GM-CSF gene (MFG-GM-CSF)	8 prostate ca pt and 18 kidney ca pt	Immune responses to prostate ca antigens	"Very few side effects"	Nelson WG Cancer Chemother Pharmacol 2000;46 Suppl:S67-72.

C - Clinical trial using GMCSF as an adjuvant to vaccination with prostate antigens				
Strategy	Indication	Patients	Toxicity	Reference
Plasmid pVAX/PSA used for vaccination with GM-CSF and IL2	Phase I HRPC 8 patients Dose escalation	Immune responses to PSA in 2 of the 3 pt treated at high dose Change in PSA slope 2 pt who showed immunity to PSA	Concomitant IL2	Miller AM J Immunother 2005 4:389-95.
PSA146-154 (PSA-peptide) + GM-CSF s.c. + DC primed with peptide	Phase 1 M1 or locally advanced 28 pt	Immune responses to peptide in 50% of patients	Local reaction – skin biopsies	Perambakam S, Cancer Immunol Immunother. 2006 55:1033-42
Plasmid vector pVAX/PSA + GM-CSF + IL2	Phase I HRPC 8 pt	Immune responses to PSA at high doses 2/3 pt decline PSA slope 2 pt who showed immunity to PSA	Concomitant IL2	Pavlenko M, Br J Cancer. 2004 91:688-94
Vaccinia virus expressing (rV-PSA)	Phase I 33 pt with M1 or PSA relapse Dose escalation S.C. GM-CSF in 10 pt	PSA was stable in 14 pt with or w/o GM-CSF	Injection site reactions Flu like syndrome	Eder JP Clin Cancer Res. 2000 6:1632-8

D - Clinical Trials with viral vectors expressing GMCSF				
Strategy	Indication	Patients	Toxicity	Reference
Intralesional OncoVEX	27 patients Single dose Skin mets from various tumours	19 post treatment biopsies had tumour of which 14 also had tissue necrosis.	Local reaction (inflammation, erythema) Febrile response. Nausea fatigue.	JCC Hu Clin Can Research 12:6737-47 2006
Intralesional Vaccinia expressing GMCSF	7 Patients Skin mets from melanoma 2 injections per week for 6/52	4 patients show response in treated and untreated mets. Distant progression. GMCSF mRNA	Flu like syndrome Injection site reactions. Pustula after repeated treatments	MJ MAstrangelo Cancer Gene Ther 6:409-22 1998

Table 1.4 A,B,C,D – Current therapeutic strategies involving the use of GMCSF

Consistent with immunotherapies, there is evidence to suggest better outcomes if GMCSF is used in the earlier stages of prostate cancer with patients experiencing the better long term responses were lower tumour stage, Gleason score, and pre-treatment PSA level. [Dreicer 2005, Klein 2001, Rini 2003, Small 2006].

1.5 Gene therapy

1.5.1 Definition

Gene therapy is the correction of defective genes responsible for disease development. There are several approaches for correcting faulty genes:

- A normal gene may be inserted into a nonspecific location within the genome to replace a non-functional gene
- An abnormal gene could be swapped for a normal gene through homologous recombination
- The abnormal gene could be repaired through selective reverse mutation, which returns the gene to its normal function
- The regulation of a particular gene could be altered (i.e. up regulated or down regulated)

In relation to cancer gene therapies, there are two main approaches - corrective and cytoreductive.

1.5.2 Gene therapy and prostate cancer

Up until 2013 there have been 1701 clinical trials approved in gene therapy and 65% of all gene therapy clinical trials are in cancer. Since gene therapy was first used in prostate cancer in 1994, there have been 108 clinical trials approved of which 78 are

still ongoing as of June 2010 (www.wiley.com/legacy/wileychi/genmed/clinical/, www.gemcris.od.nih.gov/Contents/GC_CT_RPT.asp?advsrch=true).

Prostate cancer is a disease that has a number of characteristics that make it an attractive target for gene therapy as identified in table 1.5.

	PCA-Specific features	Relevance to gene therapy
Anatomy	Localized tumour accessible for procedures such as brachytherapy	Accessibility for localized gene therapy and combined modality therapy in vivo by transurethral/transrectal routes
	Accessory organ	Complete ablation of the normal organ in addition to the tumour is clinically undeleterious
		Cytoreductive transgene expression required only for duration necessary for prostate tissue ablation
Natural history	Long pre-clinical latency	Preventive gene therapy strategies maybe feasible
		Definition of genetic/epigenetic events underlying molecular progression provides rational basis for preventive/corrective strategies
	Propensity for metastatic spread to lymphatics/bone	Targeting of systemic gene therapy for advanced disease must effectively target bone and lymphatics
Current treatment efficacy	Failures even for localized disease	Supports use of novel combined modality treatments to improve local control
	Ineffective for advanced disease	Pressing need for alternative systemic therapies
Biology	Low mitotic rate	Effective vectors for in vivo gene delivery must transduce both non-dividing as well as dividing cells
		Tumours are resistant to S-phase-dependent cytotoxic strategies
	Clinical/biological transitions: Prostatic intraepithelial neoplasia (PIN) to carcinoma	Define gene therapy targets including tumour suppressors, oncogenes etc., for prevention and for treatment of disease at various stages
	Androgen-dependent to androgen-independent tumour growth	
Prostate-specific genes and antigens	~500 or more prostate-specific ESTs in addition to current known markers such as PSA/PSMA	Facilitate prostate specific promoter/antigen targeting and therapeutic monitoring

Table 1.5 – Prostate cancer-specific gene therapy strategies [Mabjeesh et al 2002].

1.5.3 Therapeutic strategies in gene therapy

1.5.3.1 Corrective

This treatment approach involves compensating for the abnormal gene expression that has facilitated malignant change. As such, the gene targets are usually oncogenes or tumour suppressor genes.

1.5.3.1.1 Oncogene suppression

The suppression of oncogenes can be achieved by binding antisense oligonucleotides to the mRNA product of the oncogene, thus preventing its translation. Oncogenes, such as *c-myc* [Buttyan *et al* 1987], have been shown to be over-expressed in prostate cancer and are thus good targets for this therapeutic approach. Tumour suppression human prostate cancer models using a retrovirus expressing an antisense oligonucleotide for *c-myc* has been demonstrated [Steiner *et al* 1998]. An alternative method of disrupting oncogene expression is to use ribozymes to cleave specific sequences on the expressed oncogenic mRNA (Ast 2003).

1.5.3.1.2 Tumour suppressor gene expression

The classic tumour suppressor gene that is down regulated in a variety of cancers, including prostate cancer is *p53* [Effert *et al* 1992]. Studies in murine models have demonstrated anti-tumour efficacy using a recombinant adenoviral *p53* expressing vector [Asgari *et al* 1997]. In small cell cancer of the lung, *p53* replacement using an adenoviral vector has demonstrated some efficacy [Swisher *et al* 1999].

1.5.3.1.3 Difficulties with corrective therapies.

This approach, alone, is limited by the heterogeneous nature of the multiple mutations that will have occurred in the evolution of the cancer. Another limitation lies in the transduction and expression efficiencies of the current gene transfer strategies [Mabjeesh, Zhong, and Simons 2002]. These systems are unlikely to correct enough of the tumour population to be successful. This is reflected by the fact that only 9 of 108 clinical trials relating to gene therapy in prostate cancer involve corrective therapies (table 1.6). To counter this, the corrective approach would need to be multi-faceted, or used in combination with other therapeutic strategies to stand the best chance of success [Lu 2001].

1.5.3.2 Cytoreductive

1.5.3.2.1 Immunotherapies

Immunotherapies are the most popular form of gene therapy platforms, with 74 of 108 clinical trials relating to gene therapy in prostate cancer involving the immune system (table 1.6).

1.5.3.2.2 Enzyme/prodrug therapies

Enzyme/prodrug therapies involve the delivery of a gene expressing a particular enzyme to the targeted tumour cell. When expression of this enzyme is maximal, a prodrug is administered. Prodrugs are selected that are well tolerated by the patients. The enzyme converts the prodrug into a cytotoxic agent (normally poorly tolerated if delivered systemically) within the tumour cells [Gardner *et al* 2002]. An example of this system is the nitroreductase/CB1954 enzyme/prodrug system. This platform we used in our recent clinical trial and modified for the ADUP trial (see below).

1.5.3.2.3 Oncolytic viruses

The use of oncolytic viruses which do not incorporate a therapeutic gene is not, by definition, gene therapy. This approach can be used in combination with gene therapy, and so the use of oncolytic viruses in isolation is worth exploring. Not all oncolytic viruses used in clinical trials are wild-type in nature.

The first trial adopting this approach used CV706, a prostate-specific antigen (PSA)-selective, replication-competent adenovirus that has been shown to selectively kill human prostate cancer xenografts in preclinical models [DeWeese *et al 2001*]. This was a phase 1 study so the primary endpoints were safety and efficacy with dose escalation from 1×10^{11} and 1×10^{13} viral particles delivered by a real-time, transrectal ultrasound-guided transperineal technique using a three-dimensional plan. The authors found that CV706 was safe and was not associated with irreversible grade 3 or any grade 4 toxicity. 5 out of the 20 patients in the trial demonstrated a PSA response. All of the 5 were in the higher adenoviral dose cohorts, suggesting a dose related response. One of the PSA responses lasted 11 months.

Similar responses were seen using a similar oncolytic adenovirus (CG7870) trialed in hormone refractory prostate cancer [Small et al. 2006]. This virus was also under prostate specific promoters but there were also immuno-modulating gene inserts. 5 out of the 23 patients exhibited a 25–49% decline in PSA. Once again, this mostly occurred at the higher dose level.

1.5.3.2.4 Gene-directed radioisotope therapies

This novel approach uses the gene expressing the human sodium iodide symporter. Delivered directly into the target tumour, the transfected cells will express the symporter. The patient is then treated with a radioisotope of iodine, I^{131}. This is used to great effect in thyroid cancer where the cells naturally express the symporter. Those cells expressing the symporter will take up the radioisotope, leading to cell death [Spitzweg et al. 2000].

Category	Gene/promoter	No. of trials
Corrective		
Tumour suppressor gene	P53	3
	P501	2
	P16	1
	Cytochrome P450	1
	RTVP-1	1
Oncogene	c-myc	1
Cytoreductive		
Immunotherapy	PSA	16
	GM-CSF	15
	PSA/ICAM-1/LFA-3/B7.1/GM-CSF	6
	PSMA	5
	IL-2	4
	Il-12	3
	PSA/ICAM-1/LFA-3/B7.1	3
	Human Telomerase reverse transcriptase	2
	MUC-1/IL-2	2
	IL-2/IFN-γ	2
	HPV	2
	PSA/CD80	1
	PAP/GM-CSF	1
	PSMA/CD40	1
	Tumour RNA	1
	TRAIL	1
	IFN- β	1
	CMV/ 1,3 galactosyltransferase	1
	MUC-1/CD40-ligand	1
	Oncofetal antigen 5T4 /GM-CSF	1
	PAP/PSA	1
	PAP	1
	TNF	1
	REIC-Dkk-3	1
Enzyme/prodrug therapy	HSV TK	6
	HSV TK/ cytosine deaminase	6
	ADV TK	2
	Nitroreductase/CB1954	1
	Ad5-yCD/mutTKSR39rep-ADP	1
	FP253/Fludarabine	1
Oncolytic	PSA	4
	Murine osteocalcin	1
	Oncofetal antigen 5T4	1
Gene-directed radioisotope therapy	Sodium/Iodide symporter	2

Table 1.6 details the various clinical trials and therapeutic strategies that have been given approval in prostate cancer. Provided by the Journal of Gene Medicine, Gene Therapy Clinical Trials Worldwide: Update 2012: [http://www.wiley.co.uk/genmed/clinical/] Table includes trials that are open, closed, completed, under review or in submission. Trials which are closed having never been initiated are excluded.

1.5.4 Delivery strategies in gene therapy

1.5.4.1 Viral vectors

The functional unit of gene therapy, is the therapeutic transgene linked to the promoter that will drive the expression of the transgene [Galanis 2001]. This unit needs to be delivered to the target tissue and this is most commonly done using viral vectors. There are a number of characteristics to consider when selecting a viral vector :

The size of the transgene that can be inserted.

The transduction efficiency

Chromosomal insertion

Dividing/non-dividing cells

Titre

Replicating/ conditionally-replicating

Host systemic/local responses to the selected vector

Target specificity

Secondary effects of the wider viral genome on the target cell

Commercial viability

Popular vectors used in prostate cancer include herpes simplex virus and adenovirus. Herpes simplex virus can take a large insert of up to 30 kb and can transfect both replicating and non-replicating cells (useful in prostate cancer). However, the HSV genome can cause insertional mutagenesis and can also cause reactivation of latent

herpes infection. Adenovirus can be generated in far higher titres to GMP standards (10^{11} infectious units per ml compared to 10^{7} infectious units per ml), have a good safety record, can infect replicating and non-replicating cells and the viral genome does not cause insertional mutagenesis [Mulligan, 1993]. Unfortunately, adenovirus can only take inserts of up to 7.5kb and can drive a considerable inflammatory response. In the adenovirus, the vector is usually inserted into the genome at the expense of the genes that are pivotal to replication in the E1 and E3 regions and this is why the insert is limited to 7.5kb. By removing these genes, the vector is rendered replication deficient which has the advantage of limiting the vector to the target tissue.

1.5.4.2 Non-viral vectors

There is a variety of non-viral vectors such as for delivering gene therapy. These vectors are often far easier to manufacture and less immunogenic, making them commercially attractive. There are three main strategies involving naked DNA, complexed DNA and liposome envelopes. The common problem these approaches have is one of low transfection efficiency.

To transfect cells with naked DNA one needs to get the DNA through the cell membrane. The strategies for this are largely physical ones designed at disrupting the membrane to allow the passage of the DNA into the cell. Techniques for this include electroporation, a method of using transient electric fields and cell specific media to temporarily disrupt the membrane and allow the movement of DNA into the cell. These types of technique are impractical *in vivo,* largely limiting their use to the laboratory [Boulaiz *et al* 2005].

To try to get DNA through the cell membrane, attempts have been made to complex the DNA with other molecules or wrap them in a liposome which will bind with the

cell membrane and facilitate uptake of the DNA into the cell. Unfortunately, these approaches have low transfection efficiency in vivo [Nishikawa and Huang, 2001].

1.5.4.3 Tissue specificity

A key consideration in gene therapy is how to limit the effect of the therapy to the target tissue, limiting side effects. The most obvious way of achieving tumour specificity is injecting directly into the tumour. This is easier with radiologically or clinically definable organs/tumours but problematic if trying to treat multiple sites in metastatic disease or anatomically remote sites such as the adrenal gland. There is also a small risk of tumour implantation when undertaking intratumoural injection in any organ (1.1% in hepatocellular carcinomas [Hiroshi *et al* 1998]). Another way of delivering therapy directly to the target organ is to use vectors that are delivered systemically but only taken up by that specific cell type [Lu *et al* 2001].

Another approach is to use a transgene that will only be expressed in the target cell type. This can be achieved by using a transgene which is controlled by regulatory elements specific to the host cell. The weakness in this approach is in poorly differentiated tumours or tumours that are classically heterogeneous, like prostate cancer. In these settings, the regulatory pathways may not be present [Bangma, 2001].

1.5.5 The Birmingham prostate gene therapy clinical trials

1.5.5.1 Background

This forthcoming clinical trial using the AdGMNR virus in prostate cancer is the next step in a programme of research at Birmingham University exploring the utility of the VDEPT platform in the treatment of solid cancers. Initial clinic trials used an E1,E3-deleted replication defective adenovirus type 5 vector expressing bacterial NTR from the CMV immediate early promoter, produced in E1 complementing Per.C6 cells. This virus was named CTL102 and the prodrug used was CB 1954. The nitroreductase is an E. Coli derived enzyme that will convert the harmless prodrug, CB1954 into a DNA alkylating anthracycline (figure 1.7) [Bridgewater *et al* 1995].

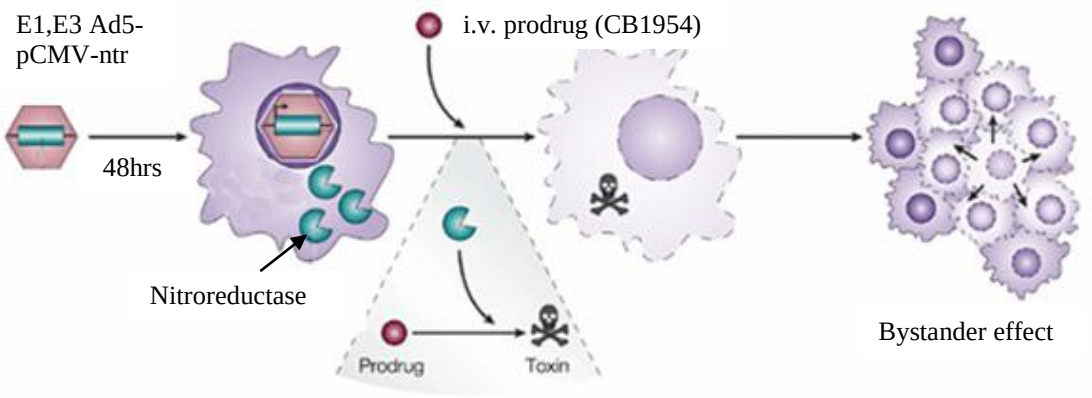


Figure 1.7 The nitroreductase and CB1954 system using an E1/E3 deleted replication deficient adenovirus. This figure adapted from McCormick [McCormick 2001] demonstrates the primary cell death from the activated prodrug and the bystander effect that occurs, killing neighbouring cells from the diffusion of activated prodrug from the dying primary cell.

The diffusion of activated prodrug into the surrounding tissue will cause cell death in neighboring cells, which may not be expressing the therapeutic enzyme. This is called the bystander effect, and this explains the cytotoxicity rates of up to 1000 times those

predicted by gene transfer rates alone. This facet of the enzyme/prodrug system can help to compensate for low gene transfer and expression levels [Kerr *et al* 1997]. The lack of cell cycle specificity, as well as a moderate bystander effect is an advantage in the nitroreductase/CB1964 enzyme/prodrug system [Bridgewater *et al* 1997]. With these enzyme/prodrug systems, there is a risk that non target tissues will be affected. This can be minimized by tumour specific vector targeting or gene regulation.

After encouraging results in cell culture and animal models [Weedon SJ. *et al* 2000], clinical trials were undertaken in cancers of the liver (CTC99020; GTAC Ref No 032). Palmer reported direct intratumoural injection of CTL102 in patients suffering from primary and metastatic liver tumours was feasible and well tolerated [Palmer DH. e*t al* 2003]. Good levels of expression of nitroreductase were achieved in those tumours injected with $1x10^{11}$ virus particles. This system was then used in the prostate cancer trial CTC01010 (GTAC Ref No 055)

1.5.5.2 The CTC01010 trial

The prostate trial was aimed at patients diagnosed with primary and locally recurrent adenocarcinoma of the prostate. The trial consisted of two arms, an initial operable and a subsequent inoperable arm [Patel *et al* 2009].

1.5.5.2.1 "Operable" Arm –

A total of 20 patients who were scheduled for radical prostatectomy received CTL102 alone via intraprostatic injection prior to surgery. Objectives of this phase of the study were safety, tolerability and level of NTR expression in the resected specimen. Immunohistochemistry demonstrated NTR staining in the glandular epithelium of the tumour and of the normal prostate tissue at all dose levels ($5x10^{10}$ to $1x10^{12}$ vps).

Expression of NTR was found in 30%-50% of the tumour specimen slides and seemed to increase when using higher vector doses or multiple injections, however there was no statistically significant relationship between virus dose and extent of NTR expression. The distribution of NTR expression was patchy and mostly localised at the site of injection, hence the rationale for the altered injection technique in the current trial. Gene therapy was well tolerated with only one patient having a possible DLT (transient bilirubin increase) at a dose of $5x10^{10}$ virus particles. This cohort was expanded but there were no other treatment related serious adverse events and dose escalation was resumed. Other adverse events were transient grade 3 lymphopaenia (4 patients) and grade 2 hepatic transaminases increase (3 patients).

1.5.5.2.2 "Inoperable – Therapeutic" Arm –

A total of 19 patients with locally relapsed prostate cancer had escalating doses of CTL102 ($5x10^{10}$ to $1x10^{12}$ vp), followed by iv CB1954. Serious adverse events occurred in 2 of the 5 patients treated with $1x10^{12}$ vps. Based on safety and efficacy data from the first two arms, an expanded cohort of patients was treated with $5x10^{11}$ vps. There was some indication of efficacy in terms of PSA decrease and stabilization. One month after treatment, 2 patients showed PSA decline > 50%, which was maintained for 7 and 10 months respectively. Five patients showed a decline of 10% - 50% and 3 patients had PSA progression with a PSA increase > 10%. The median time to PSA progression was 8.1 months, which was greater than the predicted time to PSA progression, although this difference was not quite statistically significant (see Figure 1.8). Fourteen of the 19 patients received a second cycle of treatment on the basis of indications of efficacy, and 5 of them remained progression free until the end of the study (median time 11.4 months).

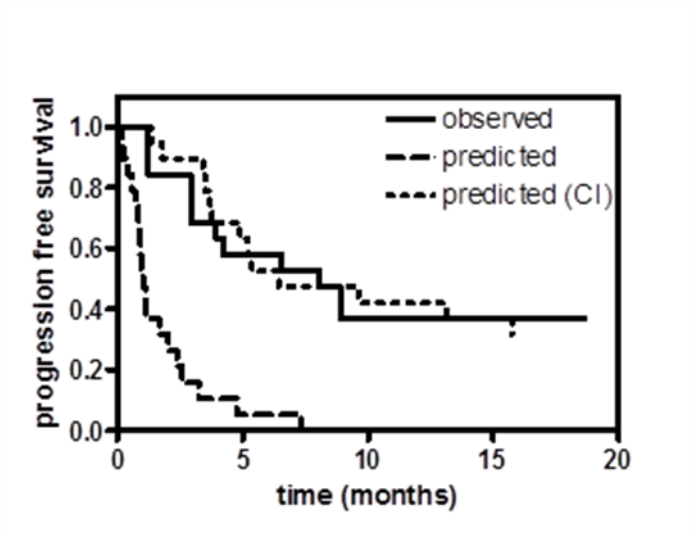


Figure 1.8 – Kaplan-Meier survival curve for patients from the treatment arm of the CTC01010 gene therapy trial with the predicted survival curves for the patients and the 95% confidence interval. The survival advantage demonstrated by the trial survival curve is not quite clinically significant.

1.5.5.2.3 Results

Five patients underwent post treatment prostate biopsies at least one month after the gene therapy. In 4 of these 5 patients the histopathology showed lymphocyte infiltration, although viable tumour cells were found in all 5 biopsies. The tolerability of the CTL102/CB1954 combination was good - the most common side effects were fever, grade 1–2 hepatic toxicity, grade 2 nausea and grade 1-2 diarrhoea. Virus DNA was detectable in the blood immediately after tumour injection and was cleared within 24 hours in most patients. CB1954 pharmacokinetics showed a profile similar to that seen in the trial of CB1954 alone.

Administration of CTL102 plus CB1954 has been well tolerated with no evidence of significant treatment related toxicity to date. Administration of doses up to 5×10^{11} viral particles to patients in the liver study (CTC99020), have shown no indication of toxicity.

Analysis of patient samples 24 hours after viral administration has shown that virus is not present in blood, urine or faeces, and therefore shed virus does not appear to be a significant safety concern. PK analysis of CB1954 has indicated that the prodrug behaves as expected and is rapidly cleared by 2-phase exponential decay, achieving expected peak concentrations of around 7uM

All patients had evidence of pre-existing immunity to adenovirus. Following administration of CTL102 most developed increased levels of Ad specific antibodies. These responses increased up to 3 months post-injection and were generally higher in individuals receiving higher doses of virus. Immune responses were not associated with any significant level of toxicity, although 24% of patients in the prostate trial did experience some pyrexia up to 24 hours post injection.

In the prostate trial, transient lymphopaenia was the most common abnormality post inoculation and occurred during the first 24 hours in 80% of the patients. This was mild and did not result in any opportunistic infections. 41% of the patients experienced a grade 1 transaminitis post inoculation. 33% of the patients experienced grade 1 transient thrombocytopaenia day 4-5 post inoculation.

Overdosing with CB1954 could lead to more severe gastrointestinal toxicity, particularly, nausea, vomiting and diarrhoea. These should be treated appropriately and would be expected to subside rapidly.

In summary, data from the CTC01010 trial showed:

- Intratumoural administration of virus at doses up to 1 x 10^{12} virus particles is not associated with significant treatment-related toxicity. In some cases there was evidence of mild lymphopaenia, but this was transient and only seen at doses >1 x 10^{10} virus particles.

- Subsequent administration of CB1954 is well tolerated although patients do experience some nausea/vomiting and/or diarrhoea, as seen using CB1954 alone.

- Intratumoural injection of CTL102 results in the transduction of tumour cells, as demonstrated by expression of the NTR transgene in liver and prostate tumours.

- Administration of 5×10^{10} virus particles produced sufficient NTR protein for potential therapeutic effect.

- Virus was not detected in the blood, urine or faeces 24 hours after virus administration, indicating that virus shedding is not a safety concern.

- Pharmacodynamics of CB1954 administration were as predicted from the Phase I CB1954 alone study [Chung-Faye G. *et al* 2001].

- In the prostate trial, there was an indication of efficacy, as measured by decrease or stabilisation of serum PSA levels [Patel P. *et al* 2009]

- There was preliminary evidence of immunostimulation as shown by transient T cell responses to prostate tumour antigens PSA and PSMA in some patients.

1.5.6 The ADUP trial

Given the evidence of immune responses of patients in the CTC01010 trial, a gene expressing GMCSF was added to the vector. This was designed to enhance the local immune response in patients undergoing this therapy. This new construct is called AdGMNR (figure 1.9).

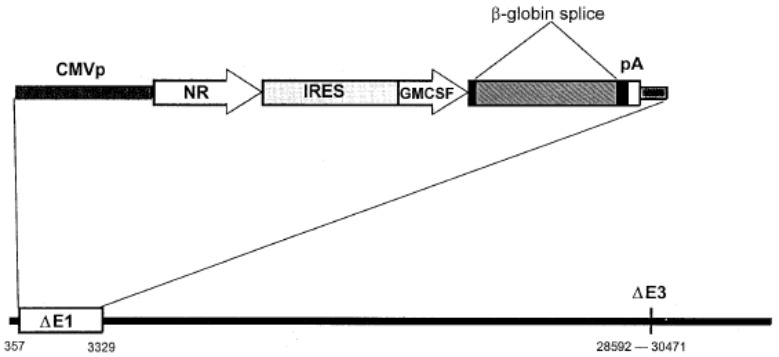


Figure 1.9 – The gene map of AdGMNR, an E1,E3 deleted adenovirus with the CMV promoter/nitroreductase/IRES/GMCSF insert at the E1 locus.

The effects of co-expression of a powerful cytokine (mGMCSF) with NTR in a bicistronic adenoviral vector have been investigated in murine models. Data from these experiments show that the Ad-NTR-mGMCSF vector plus CB1954 has a greater effect than Ad-NTR + CB1954 or Ad-mGMCSF + CB1954, on tumour progression in TRAMP prostate cancer cell xenografts in nude mice (Young *et al* 2008).

The mechanism of cell death in the trial patients will be through apoptosis rather than necrosis and, as such, could be considered to have a weak immunostimulatory impact. Work in mice using monoclonal antibodies targeting anti-TNF-related apoptosis-inducing ligand **(**TRAIL) receptors [Uno *et al* 2006] combined with APC stimulating anti-CD40 and anti-CD137 eradicated established solid tumours. This would suggest

that, with suitable co-stimulation, apoptotic cell death can prove to be immunostimmulatory.

In those prostates removed from patients in the operable arm, patchy NTR expression was demonstrated, suggesting the trans-rectal route has insufficient accuracy to deliver virus to the entire prostate. Work done by DeWeese's group in dogs had shown encouraging results using a transperineal (figure 1.10) route for administration using a brachytherapy template. This technique is used in humans for the accurate placement of brachytherapy seeds in the prostate for the treatment of prostate cancer

[DeWeese TL. *et al* 2003].

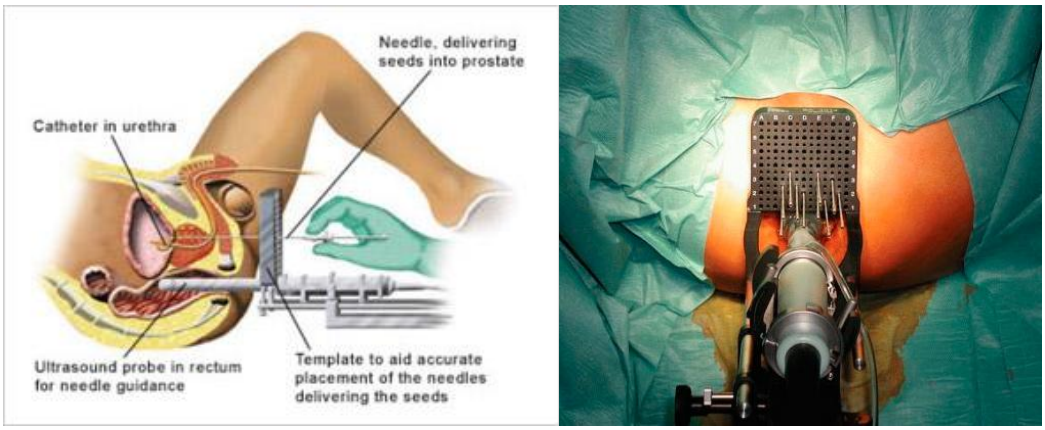


Figure 1.10 – Using a brachytherapy-type template and transrectal ultrasound probe to map the prostate and accurately site needles within the prostate.

A 0.1ml injection of virus solution will mechanically flow through 4mm of the prostate and diffuse a further 1mm (i.e. a radius of 5mm). The introduction of 0.1ml of virus solution will cause the prostate to increase in size and the amount it increases is a direct product of the number of injections. These elements need to be considered when calculating dose planning and placement.

With a better delivery technique and an immunostimulatory addition to the VDEPT

platform a new trial was conceived using the new AdGMNR virus and CB1954. This is the ADUP trial.

1.6 Aims of the project

Building on our units experience from the CTC01010 trial and in preparation for the ADUP trial, the aims of the project were

1. To validate the AdGMNR viral construct for the application to the Gene Therapy Approval Committee (GTAC) for approval to proceed to a phase I/II clinical trial in patients with locally advanced, castrate resistant non-metastatic prostate cancer patients who are not suitable for radiotherapy or surgery.

2. To develop and validate an assay to monitor changes in cellular immune responses in patients undergoing the AdGMNR trial (the tertiary endpoint of the clinical trial) to GCLP standards.

3 To use this assay in patients with prostate cancer at various stages of the disease to document the immune responses in these populations. These data would be helpful in interpreting the results from the trial patients.

2 Materials and Methods

2.1 Introduction

Generally the methods used in the thesis were based on "Molecular Cloning: A Laboratory Manual [Sambrook et al, 1989]" or "Current Protocols in Molecular Biology [Ausubel et al, 1998]" unless otherwise described. All materials purchased from supplier companies were used according to manufacturer's instructions unless otherwise stated.

All experiments with micro-organisms and blood products were performed in specified work places in the School for Cancer Sciences at the University of Birmingham. All experiments involving live bacteria were performed in an ACGM (advisory committee on genetic manipulation) containment level 2 room, and all procedures involving eukaryotic cell culture and viruses were carried out in class 2 biological safety cabinets in the 3rd floor tissue culture room at the School for Cancer Sciences which is also ACGM containment level 2.

2.2 Tissue culture

2.2.1 Maintenance and Passage of Human Cells in Culture

All cells in culture were kept in Galaxy R 37°C humidified incubators with 5% CO_2 (RS Biotech, Irvine, UK).

2.2.2 Maintenance of cell lines

Unless otherwise stated, cell lines were obtained internally, within the School for Cancer Studies (table 2.1). Cells were grown in 25 cm^2, 75 cm^2 or 150 cm^2 tissue culture flasks in appropriate growth media supplemented with foetal calf serum (FCS)

and glutamine (Table 2-1), kept at 37°C in a humidity-controlled incubator with 5% CO_2 and maintained with fresh media twice a week. The media was routinely supplemented with the antibiotics penicillin (100 iu/ml) and streptomycin (100 mg/ml) (pen/strep). Before reaching confluence, cells were passaged into a new flask. This involved carefully removing all the media from the cells with a pipette and gently washing the cells with PBS. 3-5 ml of Trypsin-EDTA solution (0.05% (w/v) Trypsin and 0.02% (w/v) EDTA) was then added before incubating at 37°C and 5% CO2 for 1-3 minutes depending on cell type. Once the cells started to lift into suspension, the flask was tapped briskly with an open palm three to four times to detach the remaining adherent cells. 3-5 ml of suitable medium was added to the cells and these were then aliquoted at a 1:10 dilution into a new flask for routine passage or at a suitable dilution after counting if the cells were required for an experiment.

Cell lines	Cell types	Culture media
PC3 (ATCC #CRL-1435)	Prostate carcinoma clavicle metastsis. Transformed but not immortalized. P53 deleted. [Kaighn et al, 1979]	DME/HEPES with 10% FCS, 2 mM glutamine
22Rv1 (ATCC #CRL-2505)	Prostate carcinoma primari. Transformed but not immortalized. P53 normal. [Sramkoski et al, 1999]	DME/HEPES with 10% FCS, 2 mM glutamine
DU 145 (ATCC #HTB-81)	Prostate carcinoma brain metastasis. Transformed but not immortalized. P53 mutated. [Mickey et al 1980]	DME/HEPES with 10% FCS, 2 mM glutamine

Table 2.1. Cell lines and Culture media. Media, FCS and glutamine were prepared by the kitchen in the Cancer Research UK Institute for Cancer Studies from ingredients supplied by Gibco Life Technologies; DME/HEPES, HEPES buffered Dulbecco's modified Eagle's medium.

In every experiment using cells, cell counting was done by resuspending the cells and then pipetting 20 µl of them onto a chamber of a Hycor KOVA Glasstic® Slide 10 with a grid cell counter (Hycor Biomedical Inc.). The number of cells in a small grid were counted under a light microscope using phase contrast illumination. To estimate the cell concentration, the average number of cells per small grid were multiplied by 10,000 resulting in the number of cells per ml. Cell viability was assessed by staining with x0.4% Trypan blue.

2.2.3 Cryogenic preservation of cells

When cells were to be frozen for storage, they were trypsinised as usual. The trypsinisation was halted by adding 10 ml of growth medium once the cells had detached from the floor of the flask. The suspended cells were transferred to a 15 ml tube and centrifuged at 1,200 rpm (177 x g) for 5 minutes at 4°C (in a Heraeus Megafuge 2.0R). The supernatant was discarded and the cell pellet was resuspended in 1 ml of FCS containing 10% dimethyl sulphoxide (DMSO). The resulting cell suspension was transferred to a labeled cryovial and slowly frozen down to -80°C at a rate of 1°C per minute in a Nalgene^{TM}Cryo 1°C freezing container. After 2hours, the cryovial was placed in a -176°C liquid nitrogen freezer for long-term storage.

To use frozen cells in an experiment, the cells would first be defrosted rapidly in a 37°C water bath. Then 10 mls of medium at 37°C would be added to the cell suspension, and the tube centrifuged in a Heraeus Megafuge 2.0R for 10 minutes at 2000 rpm (492 x g). The supernatant would be discarded and the resulting cell pellet would then be gently resuspended in pre-warmed medium and transferred to a labeled tissue culture flask. The medium would be replaced with fresh medium the next day.

2.3 Adenoviruses

Unless otherwise stated all viruses were obtained from Dr. P.Searle.

2.3.1 Infecting Cells with Adenoviruses

Unless otherwise stated, cells were infected/exposed to the various different Adenoviruses and vectors by the two following methods.

2.3.1.1 Infecting Cells Growing as a Monolayer

Cells were seeded onto tissue culture flasks and plates at least 24 hr prior to infection. To determine the number of cells present per well or flask, replica wells/flasks were harvested by trypsinisation and counted using a Glasstic Slide 10 (Hycor KOVA, Edinburgh, UK). Media was removed from cells growing as monolayers and virus added in the smallest volume of medium possible to completely cover the monolayer. After 90 min incubation (37°C, 5% CO_2) virus was removed from the cells and fresh medium added.

2.3.1.2 Infecting Cells in Suspension

Adherent cells that were to be infected in suspension were removed from flasks either by addition of EDTA and agitation or by scraping the monolayer into medium using a cell scraper so virus receptors were not removed from their surface. Cells were examined microscopically, if aggregates of cells were present then a single cell suspension was produced by rapidly passing the cells through a 0.2 μm diameter needle. Cells were then counted as before. The desired number of cells, either suspended in this manner or from cells that grow in suspension culture, were pelleted by centrifugation (538 g) and supernatant discarded. Cells were re-suspended in a small volume of media containing virus (typically 200 μl for less than $1x10^6$ cells). Cells were incubated with virus for 90 min (37°C, 5% CO_2) before excess volume (typically 10 ml) of medium was added. Cells were pelleted by centrifugation (538 g) and supernatant discarded before the cells were re-suspended in fresh medium.

2.3.2 In vitro cytotoxicity assay (MTT assay)

The MTT assay is a colorimetric assay that measures cell viability. MTT (3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide) is a tetrazolium dye that is reduced by the activity of NAD(P)H-dependent oxidoreductase cellular enzymes to its insoluble formazan, giving a purple color. Selected cells growing in culture flasks were harvested by trypsinisation, resuspended in infection medium and incubated with concentrations of AdGMNR, AdCMV-GMCSF (negative control) and CTL 102 (positive control) of 30 and 300 pfu/cell for 90 minutes. After infection, cells were seeded at 1 x 10^5 cells per well in 150 µl medium on a 96 well flat bottomed plate and allowed to adhere overnight. Medium was removed after 48 hours, and prodrug was added in 180 µl medium to each well in quadruplicates of varying concentration. Prodrug was removed after 16 hour incubation at 37°C and replaced with 180 µl fresh medium. On day five, medium was removed and replaced with 150 µl fresh culture medium containing 0.5 mg/ml MTT, diluted fresh from a 5mg/ml stock solution. Plates were incubated for three hours at 37°C before the MTT solution was aspirated from the wells. Plates were dried for 30 minutes at room temperature before the addition of 150 µl DMSO to each well. Absorbance at 490 nm was read using a Victor plate reader (PerkinElmer, Monza, Italy (Formerly Wallac, Finland)).

2.4 Detection of protein expression in mammalian cells

2.4.1 Preparation of protein samples from cells in tissue culture

Cells were prepared at a density of 1 x 10^5 per ml and 1 ml of cell solution was added to wells on a 24-well plate. These cells were then infected with the desired viruses and harvested at selected time points. Medium was removed and the cells washed with PBS. Cells were then incubated with 125 µl of urea sample buffer (USB) (6M urea,

10% glycerol, 2% SDS, 100 mM DTT and 80 mM Tris pH 6.8). After 3 minutes of incubation the suspension was removed from the well and pipetted into an eppendorf tube. The eppendorf tubes were then placed in a heating block at 100°C for 10 minutes. The protein concentration could now be assayed using the Bradford/Bio-Rad assay (see later). The samples were then stored at -20°C.

2.4.2 Detection of protein expression by Western blotting

2.4.2.1 Seperating the protein bands

Expression of particular proteins in the cellular protein samples generated from the 6M urea lysis of our cells could be detected using Western blotting. Western blotting consisted of three consecutive steps:

- Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE).
- Transfer of proteins to a nitrocellulose membrane.
- Visualisation of the proteins with immunoglobulin antibodies against the specific proteins using an enhanced chemiluminescence system.

Our SDS-PAGE step involved generating a fresh 12% resolving gel and a 5% stacking gel. These gels were made in the gel apparatus rack (Biometra, Florida, USA) using the ingredients listed in table 2.2. The APS was never more than a month old, and the TEMED always added last.

Solution components	12% resolving gel (10 ml)	5% stacking gel (4 ml)
H_20	3.3 ml	2.7 ml
30% acrylamide mix	4 ml	670 µl
1.5 M Tris (pH 8.8)	2.5 ml	-
1.0 M Tris (pH 6.8)	-	500 µl
10% SDS	100 µl	40 µl
10% APS	100 µl	40 µl
TEMED	10 µl	6 µl

Table 2.2. Ingredients of an SDS-PAGE 10% APS = ammonium persulphate (0.1 g in 1ml). Lasts for one month at 4°C once made up. 30% acrylamide mix (37.5:1 acrylamide/bisacrylamide ratio) from BioRad.

The resolving gel solution was placed between the glass plates of the gel apparatus rack up to the level of the green bar holding the plates. The rack was then topped up with dH_2O whilst the gel sets. After 15-30 minutes the polymerisation of the resolving gel was complete. The dH_2O was then removed and the stacking gel was overlaid on the resolving gel to the top of the plates and a comb inserted to create the wells. Once set, the comb was removed, a Tris-glycerine electrophoresis running buffer (25 mM Tris pH 8.3, 250 mM glycerine and 0.1% SDS) was poured into the electrophorator and the wells were loaded with equal amounts of the protein samples, appropriate positive and negative controls and a kaleidoscope standard protein marker (Bio-Rad). 130 volts was applied across the gel for 90 minutes. Once electrophoresis was complete, the proteins that had spread across the acrylamide gel were transferred to a nitrocellulose membrane (Biotrace NT, Gelman Sciences, Pall Corporation). To do this, the gel and membrane were sandwiched between two sheets of 3MM Whatman filter paper and assembled into a blotting rig. The rig was completely submerged in transfer buffer (39 mM glycine, 48 mM Tris pH 8.3, 0.037% SDS and 20% methanol). 100 volts was applied across the transfer buffer for 1-2 hours until the

kaleidoscope markers were seen to have transferred to the nitrocellulose membrane from the gel.

2.4.2.2 Detection and visualisation of the protein bands

Once the protein transfer was complete, the membranes were blocked with a blocking solution (5% non-fat milk made from dried milk powder in PBS/0.1% Tween) for more than 60 minutes at room temperature or agitated overnight at 4°C. This step was repeated with the required primary antibody diluted in blocking solution. The dilutions and antibodies are listed in table 2.3. After adding the primary antibody, the membranes were washed three times with a washing buffer (PBS/0.1% Tween) for 10 minutes on a 3D rocking platform at room temperature. Then the membranes were incubated for further hour at room temperature with the appropriate secondary antibody, and then washed three times with the washing buffer. Once, completed, one final 10 minute wash was undertaken using PBS without 0.1% Tween.

Antibody	Size of target	Information	Dilution used
Actin	45 kDa	Mouse monoclonal	1:20,000
β-tubulin	55 kDa	Mouse monoclonal	1:2000
Anti-mouse	n/a	Peroxidase conjugate	1:1000
Anti-sheep	n/a	Peroxidase conjugate	1:5000
Anti-rabbit	n/a	Peroxidase conjugate	1:2000

Table 2.3. Antibody information and dilutions for Westerns (All antibodies bought from Sigma)

The ECL (enhanced chemiluminescence) detection step was carried out using the manufacturers (Amersham Pharmacia Biotech) protocol. This involved mixing 3 ml of each ECL reagent together and then soaking the blot membranes in it for 60 seconds. Kodak X-OMAT AR 8x10 inch film was exposed for anytime up to 30 minutes, depending on the strength of the signal. The exposed radiographs were then developed in a Kodak X-O-graph developer in a dark room.

2.4.3 Determination of Protein Concentration by the Bradford Assay

The Bradford reagent (BioRad, Basingstoke, UK) was diluted 1 in 5 in dH_2O. This solution was then filtered with an Acrodisc with a 0.45 μm pore size (Pall, Portsmouth, UK). 180 μl of the filtrate was added to wells in a 96 well flat bottom plate. 20 μl of two fold serial dilutions of 1 mg/ml bovine serum albumin (BSA) in the same buffer as the protein being measured was added to the wells. 20 μl of two fold serial dilutions of the target protein was also added. The plate was incubated for 10 min at room temperature and the plate was measured at an absorbance at λ = 595 nm using a plate reader (Bio-Tek Kontron Instruments, Watford, UK). Protein concentration was calculated using linear regression from a standard curve generated from the values of absorbance at λ = 595 nm of known concentrations of BSA dilutions.

2.4.4 Detection of green fluorescence protein (GFP) and red fluorescence protein (RFP) by flow cytometry

To quantify GFP and/or RFP expression in cells mediated by viral vectors bearing the GFP and or RFP gene, fluorescence activated flow cytometry was used. Approximately 3 x 10^5 cells were plated in 24-well plates, infected with the desired moi of viral vectors and harvested at different time points. To harvest, the medium was aspirated and the cells washed with PBS. The PBS was aspirated and 200 μl trypsin/EDTA added. The cells were incubated at 37°C for 10 minutes then 1 ml of PBS + 2% FCS was added. They were transferred to a 2 ml eppendorf tube and spun for 5 minutes at 2000 rpm (492 x g). The supernatant was poured off and another 1 ml of PBS + 2% FCS added to resuspend the cells. The same centrifugation was carried out but with the pellet resuspended in 500 μl 3.7% formaldehyde (diluted in PBS) and transferred to a FACS tube, wrapped in silver foil and kept at 4°C until flow cytometry was performed. They could be kept for one week before analysis using an Epics® XL-MCL Flow Cytometry machine (Beckman Coulter). 20,000 cells were counted per sample and gated for side scatter and forward scatter.

2.5 Flow Cytometry

This section will describe methods for measurement of cell surface antigen and the expression of fluorescent proteins.

2.5.1 Sample Preparation

Adherent cells were selected to be stained with antibody. To avoid the removal of cell surface antigens the cells were removed from flasks by either the addition of EDTA and agitation or by scraping the monolayer into the culture medium using a cell

scraper. The cells were then examined microscopically, and if cell aggregates were present, then a single cell suspension could be produced by passing the cell suspension through a 0.2 μm diameter needle. The cells were then counted as before. A suspension of the desired number of cells was generated and the cells pelleted by centrifugation (538 x g) for 5 minutes. The supernatant was discarded. The cells in the pellet were re-suspended in 4 ml cold FACS buffer (PBS, 2% FCS), pelleted by centrifugation (538 x g, 4°C) and then re-suspended in 50 μl of FACS buffer containing the primary antibody at 4°C. The cells were incubated in this way for 1hr at 4°C before being washed with 3 ml cold FACS buffer and pelleted by centrifugation (538 x g, 4°C) for 5 minutes. If the primary antibody was directly conjugated to a fluorophore then the pellet was re-suspended in 0.3-0.5 ml FACS fix (PBS, 2% FCS), 4% paraformaldehyde (v/v). If the primary antibody was not conjugated to a fluorophore the pellet was re-suspended in 50 μl of FACS buffer with a suitable secondary antibody labelled with a fluorophore. The cells were then incubated for a further hour at 4°C before being washed with 3 ml cold FACS buffer and pelleted by centrifugation (538 g, 4°C) for 5 minutes. This pellet was re-suspended in 0.3-0.5 ml FACS fix. Once these steps were complete, the fixed samples could be stored in the dark for a maximum of 72 hours at 4°C. During this time the analysis by flow cytometry could be undertaken.

For every set of cells stained with a primary antibody (developed in a specific mammalian species) (+fluorophore), a replicate set of cells was stained with an appropriate isotype control antibody (developed in the same mammalian species) , as a negative control. For analysis of GFP expression only, adherent cells could be removed from flasks by trypsinisation. The cell suspensions were washed with 4 ml of cold FACS buffer and pelleted by centrifugation (538 x g) before being re-suspended

in FACS fix (0.3-0.5 ml) and stored in the dark at 4°C until analysis by flow cytometry (within 72 hours).

2.5.2 Antibodies for Flow Cytometry

The following antibodies were used to phenotype T cells and B cell blasts. All antibodies were raised in mice

Antigen	Fluorophore	Isotype	Clone	Source
CD80	RPE-Cy5	IgG_1	2D10	Serotec # MCA2813C
CD88	FITC	IgG_{2a}	P12/1	Serotec # MCA2059F
CD14	RPE-Cy5	IgG_{2a}	TÜK4	Serotec # MCA1568C

Table 2.4 Antibodies for Flow Cytometry.

Antigen	Fluorophore	Isotype	Clone	Source
Rat surface marker	RPE	IgG_1	W3/25	Serotec # MCA928PE
Rat surface marker	FITC	IgG_1	W3/25	Serotec # MCA928F
Rat surface marker	RPE-Cy5	IgG2a	MRC OX-34	Serotec # MCA929C
Rat surface marker	unconjugated	IgG1	W3/25	Serotec # MCA928

Table 2.5 Isotype Control Antibodies for Flow Cytometry.

2.5.3 Analysis of Cells by Flow Cytometry

Cells were analysed using a four colour Beckman Coulter XL flow cytometer using Coulter System II software for data acquisition and WinMDI software for data presentation.

The first samples analysed were the cells stained with isotype control antibodies (table 2.5). Using forward and side scatter properties the desired cell population, stained with the appropriate anti-antigen antibody (table 2.4), could be selected by 'gating' the population with the software. This allows the exclusion of debris and the majority of dead cells. Voltages were typically set so the negative control in each channel generated a peak fluorescence from between 10 and 1. When using multicolour staining, the appropriate colour controls were analysed and compensated for with reference to the other colours being analysed. Once this step was completed, the voltages, gates and compensation were kept constant for all cells subsequently analysed. More than $2x10^4$ events needed to be analysed per sample for the result to be accepted.

Both the percentage positive and mean fluorescence (Mn. Fl.) data were analysed. Percentage positive is the percentage of the gated cells that fluoresce above the level of the negative isotype control (or unstained cells if only GFP expression was being measured). This was measured using a marker set to include only 2% of the negative control cells. Mean fluorescence refers to the mean fluorescence in each channel for all the cells gated on and not just positively fluorescing cells.

2.6 Microscopy

A Zeiss Axiovert 25 inverted microscope was used for all phase-contrast and fluorescence microscopy using 5x, 10, 20, 40x objectives with 21x, 42x, 84x and 168x overall magnification. Pictures were taken using a Spot camera and processed using Spot Advanced software (Diagnostic Instruments, Michigan, USA). The fluorescence pictures shown have the same exposure and gain settings for green colour only with an exposure time of 2 secs and the gain set at 4.

2.7 Experiments on samples from patients and donors.

2.7.1 Samples

Blood and tissue samples throughout the project were obtained following an informed written consent from healthy consenting members of the Institute or patients suffering from prostate cancer in all cases. Local ethical approval was obtained prior to study to carry out this research project (LREC 2002/292; LREC 5689; RRK 1986; and Protocol CTL 102/CB1954/CTC01010: Version VII (last update 09-01-06)). Patients were recruited from outpatient clinics at the University Hospital Birmingham NHS Trust. Blood and tissue collection protocols are outlined in Appendix 1. Control subjects were obtained from healthy volunteers without a history of malignant disease.

2.7.1.1 Separation of Peripheral Blood Mononuclear Cells and Plasma from Whole Blood or Buffy Coats

120mls of whole blood was obtained from consenting donors. 100mls of this was placed into syringes containing sodium heparin (10 U/ml) and processed as soon as possible. The other 20mls was placed into clot activating red top vacutainers for the extraction of the serum. Buffy coats were obtained from West Midlands Blood Transfusion Service (WMBTS). Both were diluted by the addition of an equal volume of RPMI 1640 and layered on top of 15 ml of Lymphoprep (Nycomed, Torshov, Norway) before centrifugation (800 x g) for 30 min. Peripheral blood mononuclear cells (PBMCs) were removed from the sample/medium interface with a Pasteur pipette. PBMCs were washed twice by the addition of RPMI1640 and pelleting cells by centrifugation (538 x g followed by 250 x g) for 5 minutes. Cells were re-suspended in RPMI 1640 medium and counted as above.

For the serum extraction, the vacutainers are stood on the bench for 6 hours at room temperature. The tubes are then centrifuged at 2000 rpm (492 x g) for 10 minutes. Using a pastette, the clear serum extracted. 7 mls of blood produces approximately 4 mls of plasma. The plasma is transferred to a labelled 15ml Falcon tube and placed in a water bath at 56^{o} centigrade for 30 minutes to heat inactivate it (the serum can then be stored in a -20^{o} centigrade freezer)

2.7.1.2 The manufacture of B Cell blasts

Once the PBMCs were extracted using the Lymphoprep density gradient extraction technique The PBMCs were then incubated in media for two hours to allow the monocytes to adhere to the plate. The non-adherent population were then removed and washed. Two thirds of the cells were then put in cryostorage to be used as the 'responder cells', and the other third were used for the manufacture of the B cell blasts.

These cells were incubated on an irradiated (10000 Rads) feeder layer of murine L cells expressing human CD40 ligand for 48 hours in B cell media containing 10 mls of mixed human sera, 100μl of 5μg/ml IL-4 and 66μl of 1mg/ml cyclosporin A in RPMI (to inhibit T cells) in 90ml IMDM with 5ml 200mM glutamine and 5ml penicillin/streptomycin solution. After 48 hours, the B cells are removed from the feeder layer using a pastette and plated out on a fresh feeder layer of irradiated murine CD40 ligand expressing L cells for 48 hours in B cell media contained mixed human sera, IL-4 and cyclosporin A. This passage was repeated a further 4 times until a characteristic clumping was evident in the B cell population. These B cells are designated B cell blasts.

2.7.1.3 B cell preparation using Mega CD40L and autologous sera.

120mls of blood was taken from suitably consenting subjects. 20mls was used to extract serum. The PBMCs were extracted from the remaining 100 mls of blood using the Lymphoprep density gradient extraction technique, as before. The PBMCs are then incubated in media for two hours to allow the monocytes to adhere to the plate. The non-adherent population are then removed and cleaned. Two thirds of the cells are then put in cryostorage, and the other third are used for the manufacture of the B cell blasts.

These cells are incubated for 48 hours in B cell media from a stock consisting of 90mls IMDM, 10mls autologous sera, 100μl 5μg/ml IL-4, 5ml 200mM glutamine, 5ml penicillin/streptomycin solution and 66μl 1mg/ml cyclosporin A (to inhibit T cells) as well as 150ng of soluble human CD40L (MEGA CD40L). After 48 hours, the B cell suspension is centrifuged at 538 x g for 5 minutes. The pellet is washed and then plated out at 48 hours in B cell media.. This passage was repeated every 48 hours for a further 4 times until a characteristic clumping was evident in the B cell population. These B cells are designated B cell blasts.

2.7.1.4 Nucleofection of B cell blasts

The evening before the nucleofection is planned (halfway through the last passage of the B cell blasts) the autologous 'responder cells' were taken out of cryostore, thawed and rested in media overnight.

The B cell blasts were counted, washed and centrifuged. The pellet was reconstituted in a volume of media creating a cell suspension of $1x10^6$/ml cells. 1mls were then placed in a separate 15ml sterile test tube and centrifuged at 1500RPM for 5 minutes. The residual media was aspirated carefully with 200µl Gilson pipette. The pellet is then suspended in premixed nucleofector reagent (Amaxa) specific for human B cells. The suspension was transferred to a nucleofector cuvette. 10µg of the plasmid DNA for the chosen antigen (e.g. BMLF-1) was then added and the cuvette placed in an Amaxa nucleofector set on programme U15 (the programme specific for human B cells). Once the programme was complete, the suspension was carefully transferred into a Bijou containing 1 ml cell culture media.

2.7.1.5 Transfection of the B cell blasts

The PBMCs are counted, washed and centrifuged. The pellet is reconstituted in a volume of media that will create a cell suspension of $1x10^6$/ml cells. 1ml is then placed in a 35mm culture dish. A further 1ml of media is added. A transfection complex is prepared using 97 µl of serum free media, pipetted into a 1.5ml tube. Into the centre of the media (avoiding the sides of the tube), 3 µl of the FuGENE6 Transfection Reagent is pipetted. Flick the tube to mix and leave to stand at room temperature for 5 minutes. 2 µg of the plasmid DNA is added and the tube is flicked to mix. The tube is left to stand for 15-45 minutes at room temperature. The transfection complex is added drop wise to the 35mm plate whilst gently swirling the

media. Expression is maximal at 48 hours and at this point, the cells suspension is centrifuged at 1500RPM for 5 minutes. The pellet is resuspended in 1 ml of cell culture media.

2.7.1.6 IFN-γ ELISpot Assay

The IFN-γ ELISpot was performed using a commercial kit containing both capture and biotinylated detection antibodies and a streptavidin bound alkaline phosphatase (AP) (Mabtech, Nacka Strand, Sweden). MAIP N45 96 well plates (Millipore, (U.K.) LTD, Watford, UK) were coated with 50 µl of anti human IFN-γ monoclonal Ab (1-D1K) diluted to 15 µg/ml in PBS for 3 hrs at room temperature (or overnight at 4°C). The antibody was flicked off and each well was then washed six times with 200 µl of RPMI 1640 and then incubated with 200 µl RPMI 1640, 10%FCS for 1hr at 37°C in an incubator. The medium was flicked off the plate and the subject cells and antigen were added in a total volume of 100 µl of RPMI 1640, 10% autologous serum, 2 mM glutamine, 100 U/ml penicillin and 0.1mg/ml streptomycin. The cells were at a density between $5x10^4$-$5x10^5$ cells/well. The plates were incubated for 16 hr in an incubator at 37°C (5% CO_2) before the cells were removed and wells washed six times with PBS, 0.05% Tween 20. 50 µl of biotinylated anti- IFN-γ Ab (7-B6-1) diluted to 1 µg/ml in PBS was added to each well and incubated for 3-4 hrs at room temperature. The antibody was flicked off and the wells washed six times with PBS, 0.05% Tween 20. Then 50 µl of streptavidin-AP (diluted 1 in 1000 in PBS) was added to each well. The plates were incubated for 1 hr at room temperature. The streptavidin-AP was flicked off and the wells were washed six times with PBS, 0.05% Tween 20. 50 µl of development reagent (1000 fold dilution of AP colour reagent A and B (BioRad) in 1X AP colour development buffer (BioRad) was added to each

well. The plates were incubated for 10-20mins at room temperature or until colour had developed. At this point, the reaction was stopped by washing thoroughly with tap water. Plates were left to air dry overnight before the number of spots formed in individual wells was counted. The spots were counted using an AID automated elispot reader using AID 3.1 software (AID, Strassberg, Germany). The results are represented as the mean (of three replicate wells) number of spot forming cells (SFCs).

2.7.1.7 Protocol for Intracellular Staining With the FIX & PERM® Cell Fixation & Permeabilization Kit

For each sample to be analyzed the appropriate volume of the conjugated anti-HA antibody and the appropriate isotype control is added to an appropriate, labelled 5 ml tube. 1 x 10^6 cells of the nucleofected B cell blasts in 100 µl RPMI 1640 were pipetted into each tube. The tubes were gently mixed using a Vortex, and the tubes are incubated for 15 minutes in the dark at room temperature. We then added 100 µl of Reagent A (Fixation Medium) and incubated for a further 15 minutes in the dark at room temperature.

We then added 3 ml wash medium (PBS + 0.1% NaN3 + 5% FBS) and centrifuged for 5 minutes at 300–350 x g. We then aspirated the supernatant and vortexed to fully resuspend the cell pellet. 100 µl of Reagent B (Permeabilization Medium) and the recommended volume of anti-HA antibodies or the corresponding isotype control were added to the pellets. The samples were then mixed with the Vortex for 1–2 seconds and incubated for 20 minutes in the dark at room temperature. The sample was then washed with 3 ml wash medium and centrifuged for 5 minutes at 300–350xg. The supernatant was then aspirated. The cells were either resuspended in

sheath fluid for immediate analysis, or fixed in 0.5 ml of 0.1% paraformaldehyde and stored at 2–8°C in the dark. Fixed cells should be analyzed within 18 hours.

The cells were analysed using a four colour Beckman Coulter XL flow cytometer using Coulter System II software gated on SSC and FL-3 for data acquisition and WinMDI software for data presentation.

3 Validation of the AdGMNR construct

3.1 Introduction

The CTC 01010 (GTAC Ref No 055) trial demonstrated that a replication deficient adenoviral vector (CTL102) coding for NTR could be injected into human prostate where the transgene is successfully expressed. With the systemic injection of the prodrug, CB1954, there was biochemical evidence of tumour response in some patients as well as evidence of possible immune responses. With a modified virus, AdGMNR, and a modified method of delivery, using a brachytherapy type template transperineal delivery, the new virus required *in vitro* validation before entering it into clinical trial. In this chapter, I am presenting data validating the *in vitro* efficacy of AdGMNR on human prostate cancer cell lines. In these experiments we also explore other variants of NTR obtained by selection and screening in E. coli of libraries of NTR mutants with amino acid substitutions at active site residues identified by X-ray crystallography. With these data, I aim to demonstrate:

- That the human prostate cancer cell lines PC3, DU145 and 22Rv1 can be infected with constructs using an E1, E3-deleted, replication defective Adenovirus type 5 vector.

- That the NTR and GMCSF proteins are expressed in human prostate cancer cell lines infected with AdGMNR.

- That human prostate cancer cell lines infected with AdGMNR are sensitised to CB 1954.

- That AdGMNR is equivalent to CTL102 in its expression of NTR and ability to sensitise cells to CB1954

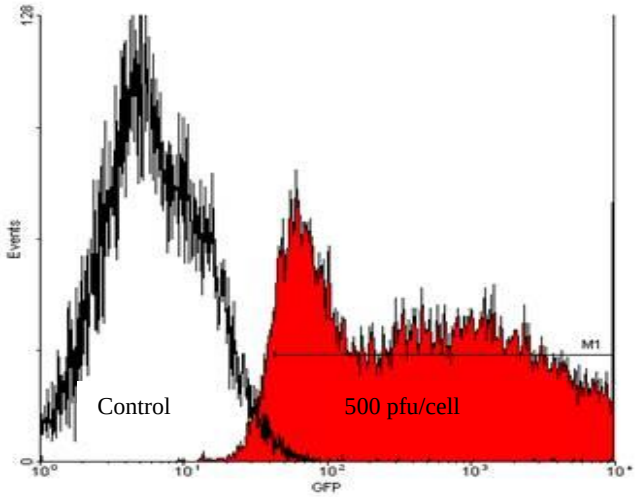
- That the GMCSF produced by human prostate cancer cell lines infected with AdGMNR is biologically active.

The data presented are the best representative data from each experiment which was repeated at least three times. If there was excessive variation further repeats of the experiment was undertaken.

3.2 Susceptibility of PC3, DU145 and 22Rv1 prostate cancer cell lines to infection by adenovirus

The extent of binding and uptake of adenovirus vectors has been found to vary between cell types. In order to investigate the relative infection efficiencies of the cell lines DU145, PC3 and 22Rv1 the following experiments were set up. Susceptibility to infection was assessed by measuring GFP expression following infection with AdGFP, an E1, E3 replication defective adenovirus construct expressing GFP from the CMV IE promoter. The cells were infected with a range of multiplicities of infection (moi), and GFP expression was monitored by flow cytometry 2 days following infection.

Figure 3.1 demonstrates the increasing expression of GFP by DU145 cells infected with increasing moi (pfu/cell) of AdGFP demonstrating moi dependant infectivity. These results demonstrate susceptibility of these prostate cancer cells to infection with adenovirus vectors



moi (pfu/cell)	% expressing GFP	Mean fluorescence
Control	0	67.36
500	94	686.28
5000	95	5316.04

Figure 3.1. Histogram of flow cytometry on DU145 cells infected with AdGFP

When comparing the mean fluorescence in these cell lines after infection, we observed that the primary prostate cancer cell line, 22Rv1 was significantly (P=0.046 in unpaired t-test) more susceptible to infection than another, metastatic prostate cancer cell line, DU145 when comparing moi of 300 pfu/cell as shown in figures 3.2 and 3.3.

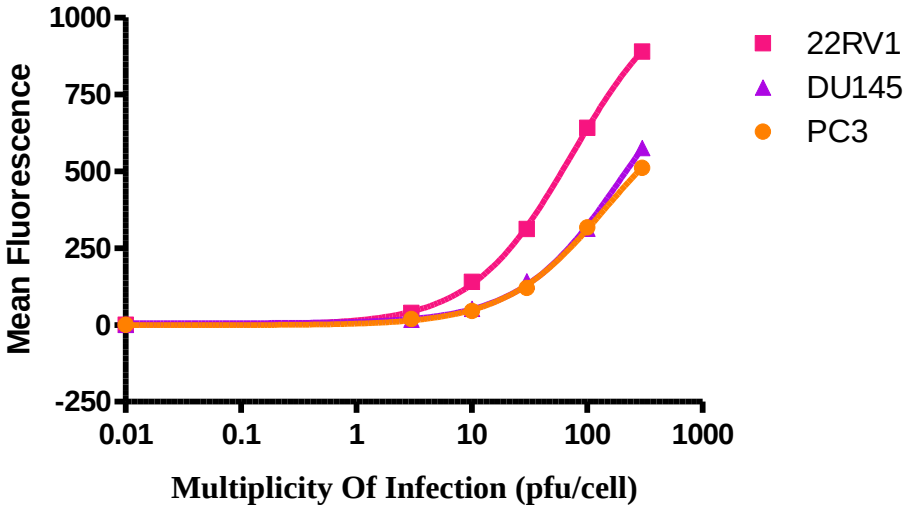


Figure 3.2. Infectability of prostate cancer cell lines with E1 deleted AdGFP at various MOI (pfu/cell)

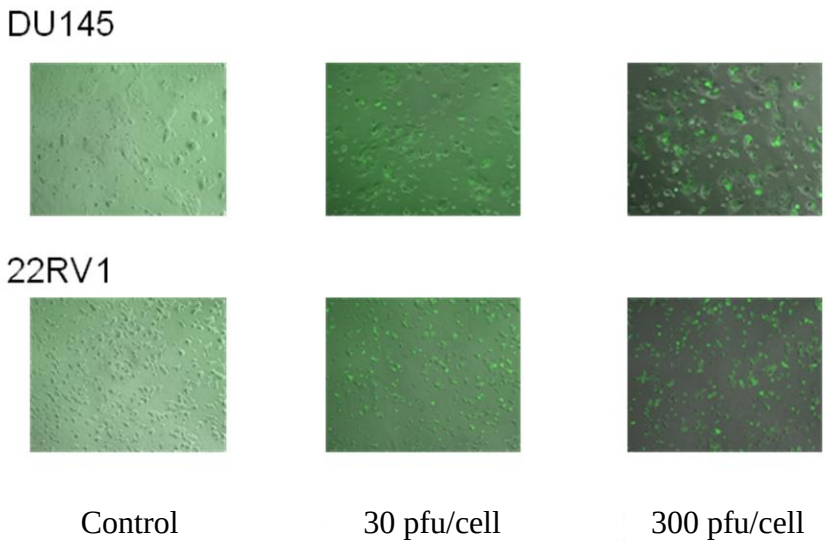


Figure 3.3. Fluorescent microscopic appearance of prostate cancer cell lines infected with various MOI (pfu/cell) of AdGFP.

3.3 NTR and GMCSF proteins are expressed in human prostate cancer cell lines infected with AdGMNR

3.3.1 NTR Expression by PC3 And DU145 cell lines after infection by AdGMNR, AdCMV-GMCSF and CTL102

DU145 and PC3 cells were infected with varying quantities of AdGMNR, AdGMCSF (negative control) and CTL 102 (positive control). The cells were then lysed in urea and NTR expression measured with Western Blotting.

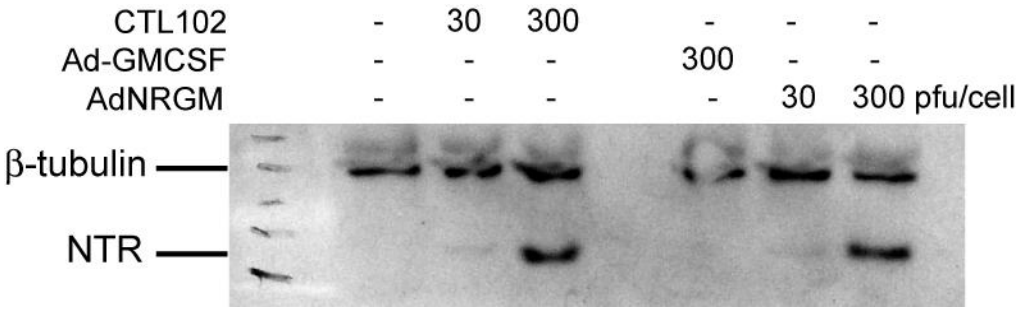


Figure 3.4. Western Blot of NTR expressed by PC3 cells 96 hours after infection by AdGMNR, AdGMCSF and CTL 102

The Western blot demonstrates similar expression levels of NTR by AdGMNR and the positive control (CTL102), in these prostate cancer cell lines when infected by the same concentration of virus particles (fig 3.4). This confirms that the insertion of the IRES and hGMCSF coding sequences into the expression cassette has not resulted in any detectable change in the level of NTR expression from the CMV promoter. As expected, there is no expression of NTR in the control (AdGMCSF) demonstrating that the expression of NTR is due to the presence of the NTR gene in the vector.

3.3.2 GMCSF expression by PC3 and DU145 cell lines after infection by AdGMNR, AdCMV-GMCSF and CTL102

DU145 and PC3 cells were infected with varying quantities of AdGMNR, AdGMCSF) and CTL 102. The expression of GMCSF was assayed using a commercial ELISA kit.

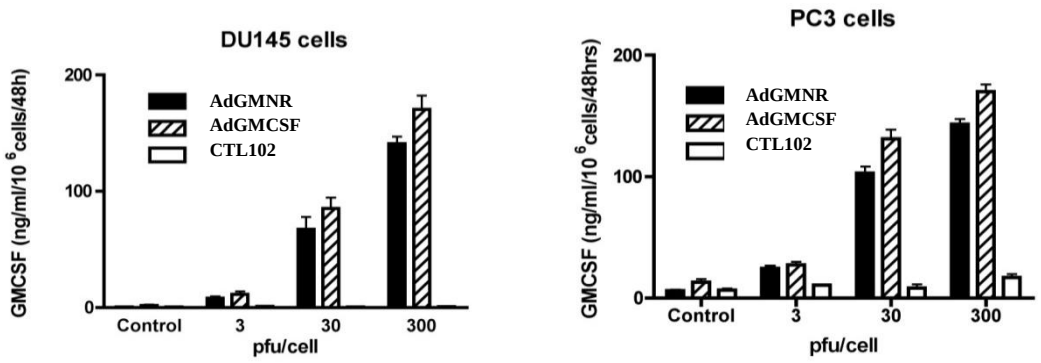


Figure 3.5. GMCSF expression by PC3 and DU145 cells infected by AdGMNR and AdGMCSF, with CTL 102 as the control virus at 48 hours post infection. The controls are cells in media alone. All values represent the mean plus or minus the standard deviation.

The results from the ELISA demonstrate similar levels of GMCSF expression by AdGMNR infected prostate cancer cells and the positive control (AdGMCSF infected prostate cancer cells) when these prostate cancer cell lines are infected by the same number of plaque forming units. There is no expression of GMCSF in the control DU145 cells and the negative controls (CTL102 infected prostate cancer cells) demonstrating that the expression of GMCSF is due to the presence of the GMCSF gene in the vector (Figure 3.5). Uninfected PC3 cells produce a low level of GMCSF (<10ng/ml). This was a consistent feature when the experiment was repeated. This has been noted in the literature (Rokhlin OW. *et al* 1996). Infection with just 3 pfu/cell of

either Ad-NRGM or Ad-GM-CSF resulted in 25 to 27.1 ng/ml/10^6/48hrs of GMCSF in comparison to 6 to 10 ng/ml/10^6/48hrs of GMCSF in CTL102 and uninfected cells. With higher MOI the levels of GMCSF were higher.

The time-dependence of GM-CSF production was also examined. The experiment was repeated with the media being changed every 24 hours and the media being removed was aliquoted and stored at -20°C. Figure 3.6 demonstrates that for those cells infected by AdGMNR, the greatest expression of GMCSF was seen between 48 and 72 hours. In both of these experiments, the levels of GMCSF expression are lower in the AdGMNR infected prostate cancer cells when compared to AdGMCSF infected prostate cancer cells. This observation was consistent, and also evident in the 22RV1 cell line. This may be due to the position of the gene in relation to the internal ribosome entry site.

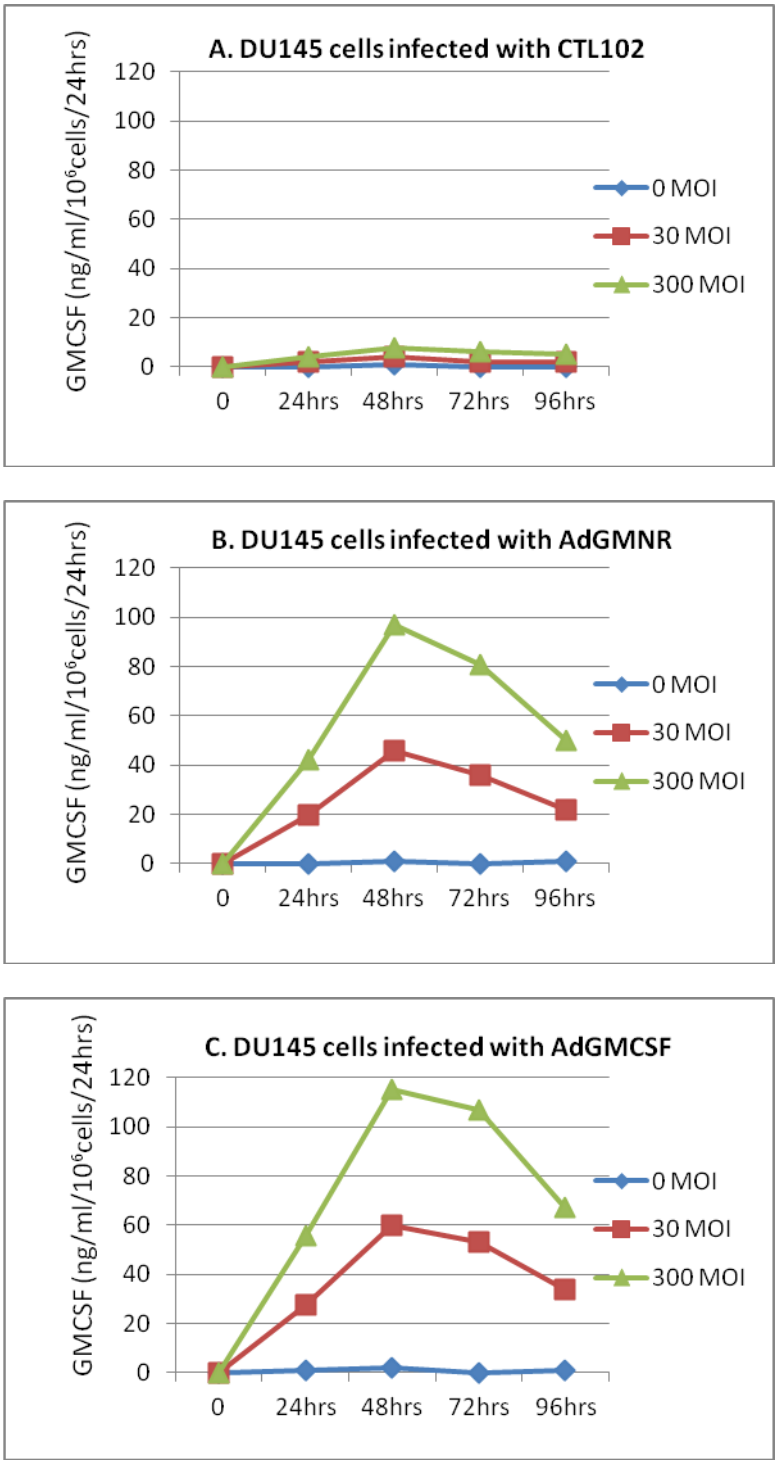


Figure 3.6. GMCSF expression by DU145 cells over time after infection with CTL102 (A), AdGMCSF (B) and AdGMNR (C). Medium was changed every 24 hours and the concentration of GMCSF in the old medium was measured by ELISA

3.4 Human prostate cancer cell lines infected with AdGMNR are sensitised to CB 1954

Expression of E.coli NTR in a range of tumour cell lines, as well as in some normal tissues, has been shown to enable these cells to convert the prodrug CB 1954 into its active cytotoxic form resulting in cell killing at concentrations of CB 1954 which have no effect on non-expressing cells (Bridgewater JA. et al 1995, Green NK. et al 1997). This section summarises studies which demonstrate that NTR expression sensitises cell lines derived from human prostate tumours to CB 1954 when cultured in vitro. These studies show that NTR expression can result in efficient killing of these cells by prodrug applied to the cells in culture.

PC3 and DU145 cells were harvested by trypsinisation, resuspended in infection medium and incubated with varying concentrations of AdGMNR, AdGMCSF (negative control) and CTL 102 (positive control) from 0 to 300 pfu/cell for 90 minutes. The cells were then seeded into micro-wells (1×10^5 cells/well) and incubated for 48 hours to allow full expression of the transgenes. After this expression period, the cells were exposed to a range of concentrations of CB 1954. Cell viability was then determined using an MTT assay. The results for DU145 cells and PC3 cells are shown in figure 3.7. The uninfected cells were susceptible to high concentrations of CB1954. Those cells infected with AdGMCSF had no change in sensitivity to CB1954 from uninfected cells, at either MOI. Those cells infected with CTL102 and AdGMNR had a virus dose-dependent increase in sensitivity to CB1954.

These figures show that the greater the multiplicity of infection with NTR expressing vectors, the more sensitive the prostate cancer cell lines are to the prodrug CB1954.

From the IC50 measures, in both cell lines, the CTL 102 was marginally more cytotoxic than AdGMNR in combination with CB1954 but this was not statistically significant.

Prostate cancer cells infected with non-NTR expressing adenoviral vectors are insensitive to CB1954. The figures also show that this sensitivity is dose dependent and that in high doses CB1954 is toxic to all the prostate cancer cells regardless of infection.

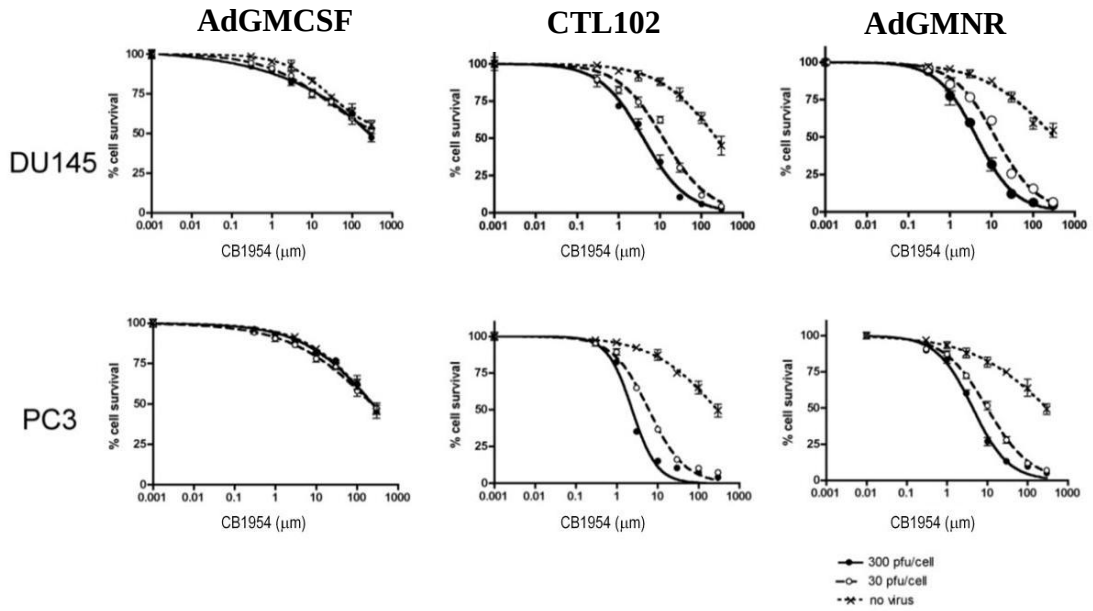


Figure 3.7: Relative survival of prostate cancer cell lines following infection with AdGMCSF, CTL102 or AdGMNR at 0, 30 or 300 pfu/cell and treatment with different concentrations of CB1954.

cells:	DU145			PC3		
virus moi (pfu/cell)	0	30	300	0	30	300
AdGMCSF	362.8	281.6	256.6	238.8	235.5	241.0
CTL102	232.9	12.0	3.9	291.6	6.0	2.4
AdGMNR	323.9	12.3	4.3	305.7	9.8	4.3

Table 3.2: IC50 (CB1954 concentration resulting in 50% reduction in viable cells) following infection with the 3 viruses. IC50 determined from the curves in Figure 3.7.

Infection of the human prostate cancer cell lines with AdGMNR resulted in sensitisation to killing by CB 1954. The results from the negative control (AdGMCSF) demonstrated adenovirus infection per se did not contribute to this cell killing. The results from the positive control (CTL102) demonstrate that the addition of the GMCSF gene has slightly diminished the amount of cell kill in cells infected with AdGMNR and exposed to CB1954.

3.5 Biological activity of GMCSF produced by PC3 and DU145 cell lines after infection by AdGMNR

GMCSF causes mononucleocytes to differentiate into immature dendritic cells. In differentiating, various cell surface proteins change. In this assay, we look for changes in expression of CD14. This is expressed in mononucleocytes but not in immature dendritic cells. If the supernatant taken from infected cells contains biologically active GMCSF then this supernatant will drive mononucleocytes to differentiate in to immature dendritic cells. This can be confirmed by seeing changes in the levels of expression of CD14.

DU145 and PC3 cells suspended in growth medium were mixed with suspensions of varying concentrations of AdGMNR, ps1307 (a virus identical to AdGMNR except that the NTR gene has two amino acid changes in the active site) and CTL 102 (negative control) for 90 minutes and seeded into tissue culture plates with fresh media. The cells were then incubated at 37°C for approximately 96 hours, and the growth medium was removed and replaced with fresh medium every 24 hours. The removed medium was aliquoted and frozen at -20°C.

Freshly isolated PBMCs were plated out at $1x10^7$ cells/ml in medium and incubated for 2 hr (37°C, 5% CO2). Non-adherent cells were removed by two washes with 5 ml of PBMC medium before addition of 2mls PBMC medium and 3mls of the infected cell supernatants. As a positive control, 100ng/ml commercial grade GMCSF at varying concentrations was added to some of the wells. Cells were fed by half medium change at day three. After 5 days, the cells were phenotyped by measuring

cell surface antigen using monoclonal Antibody for CD14 and flow cytometry. Figure 3.8 demonstrates changes in CD14 expression in mononucleocytes after exposure to the supernatants of prostate cancer cell lines infected with AdGMNR, ps1307 and CTL102, as well as cells exposed to GMCSF solutions.

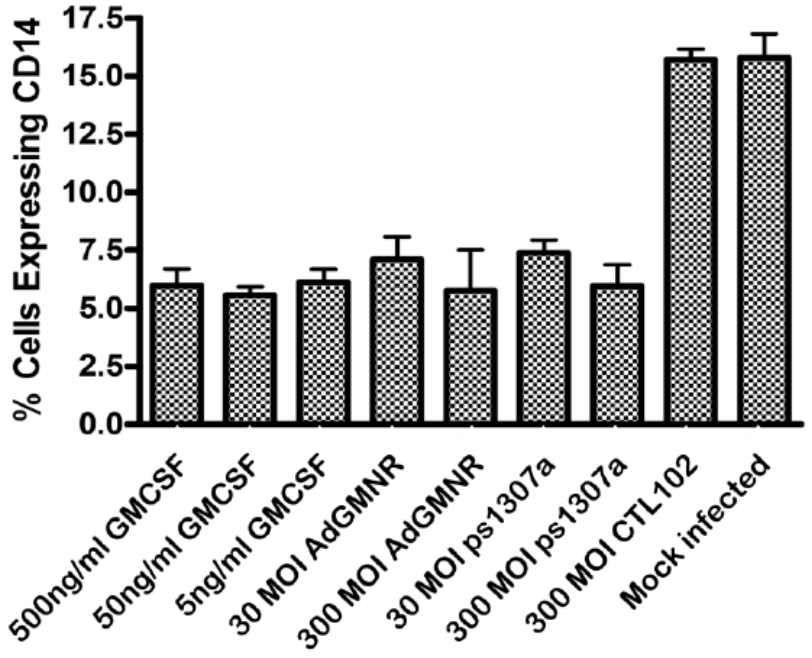


Figure 3.8. CD14 expression in non-adherent PBMCs after exposure to supernatants from DU145 cells infected with GMCSF expressing viruses (AdGMNR and ps1307a) at varying multiplicities of infection or recombinant GMCSF at varying concentrations. All values represent the mean plus or minus the standard deviation.

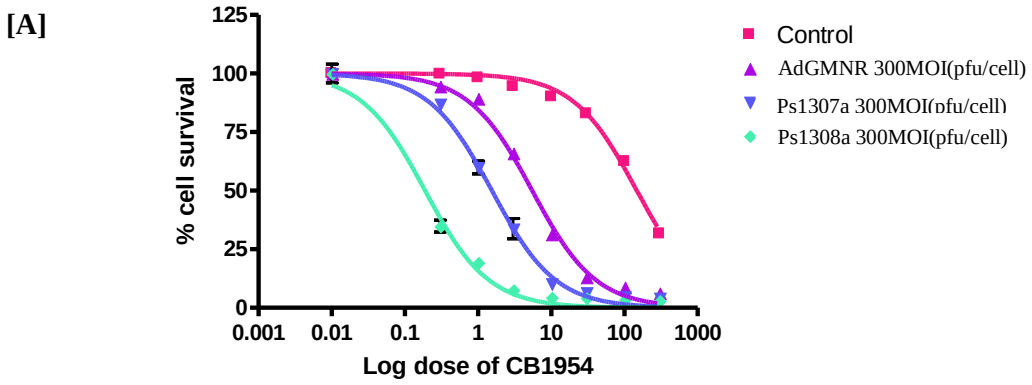
As mononucleocytes differentiate into immature dendritic cells they stop expressing CD14. This experiment demonstrates that the supernatants of prostate cancer cells infected with AdGMNR and positive controls (varying doses of GMCSF), as well as GMCSF solution cause mononucleocytes to lose expression of CD14, suggesting differentiation into immature dendritic cells and indicating the presence of active GMCSF. Mononucleocytes exposed to the negative control (supernatant from prostate cancer cells infected with CTL102) are just as likely to express CD14 as those which are mock infected indicating that it is unlikely that GMCSF is present. These data

suggest that the GMCSF released by prostate cancer cells infected with AdGMNR and the mutant strain ps1307a is biologically active.

3.6 Comparing the efficacy of AdGMNR with similar viruses ps1307 and ps1308 expressing improved mutants of NTR and AdGMNR in PC3 cell lines

During the validation work for AdGMNR, other constructs using modified NTR instead of wildtype were used to see if the efficacy of the system could be improved upon. The data presented in figure 3.9 show that the modified constructs could considerably improve the efficacy of the VDEPT platform with regards to cell kill, without compromising the production of GMCSF.

PC3 cells infected with AdGMNR, ps1307a or ps1308a and treated with CB1954



GMCSF secretion by PC3 cells infected at varying MOI (PFU) of E1/E3 deleted adenoviruses engineered to express NTR +/- GMCSF

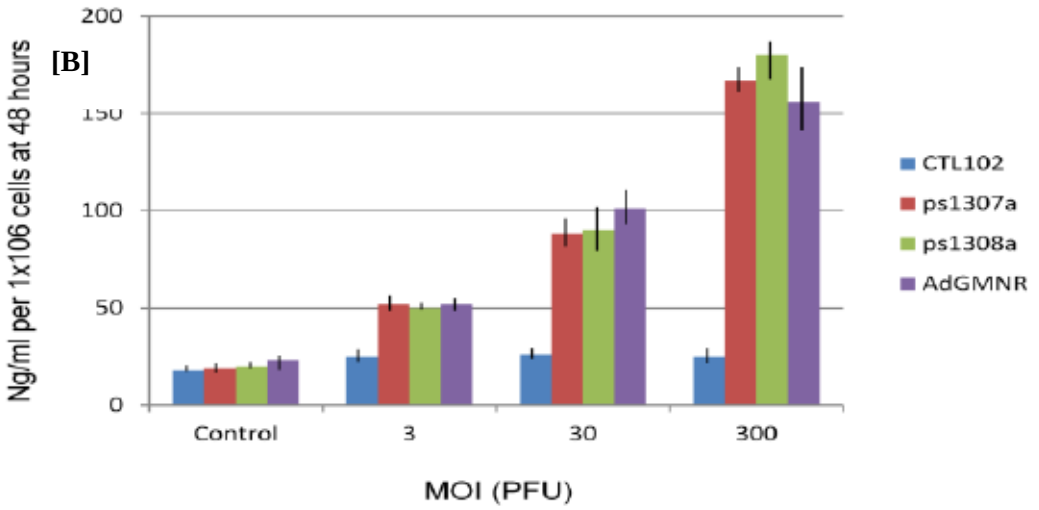


Figure 3.9. PC3 cells are infected with two different E1/E3 deleted adenoviruses (ps1307 and 1308a) expressing GMCSF with various mutations of NTR. CTL102 and AdGMNR are used as positive controls for wildtype NTR and GMCSF. The figures demonstrate that ps1307a and 1308a increasingly sensitize PC3 to CB1954 [A] without impacting on GMCSF secretion [B]. All values represent the mean plus or minus the standard deviation.

3.7 Summary

In this chapter, we have presented data that demonstrate that the E1/E3 depleted adenoviral vector will successfully infect prostate cancer cell lines. We have also demonstrated that, once infected, these cell lines will express both the nitroreductase and the GMCSF gene inserts. The expressed NTR is active and will cause cell death in the infected prostate cancer cell population when those cells are exposed to the inactive prodrug, CB1954, in a dose dependent fashion and equivalent to CTL102. The expressed GMCSF is biologically active, showing a similar effect on PBMCs as commercially available reconstituted GMCSF. Exposure of PBMCs to the supernatant from infected cells causes phenotypic changes with a down-regulation of the expression of CD14, a step in the development of immature dendritic cells. Although we are using wildtype NTR in the trial construct, future trials could benefit from more active mutants of NTR.

4 Development and validation of a reproducible assay of cellular immune responses to common prostate cancer associated antigens

4.1 Introduction

Cancer sciences at Birmingham University will be undertaking their second gene therapy clinical trial, using a virus delivered enzyme-prodrug therapy in prostate cancer. An E1/E3 deleted replication deficient adenovirus will be used expressing wild-type NTR and GMCSF with a CMV promoter. The virus will be delivered using a brachytherapy style technique in patients with locally advanced hormone escaped prostate cancer under general anaesthetic. 48 hours after the injection of the virus, the patient will receive a systemic dose of the harmless prodrug CB1954. This is converted to a DNA cross-linking cytotoxic drug in those cells expressing NTR, causing cell death. The previous trial (CTC01010) used a similar construct but expressing only wild-type NTR which was delivered via an ultrasound guided trans-rectal route under local anaesthetic. Despite patchy expression within the prostate, there was evidence of efficacy as seen with PSA responses in some of the patients. Also noted was some evidence of immune responses in these patients with heavy lymphocyte infiltration noted in those patients who had been biopsied after treatment. These immune responses led to the inclusion of gene expressing the powerful immune-stimulant GMCSF for the second clinical trial. The trial is a phase I/II study so the primary objectives are safety and tolerability. The secondary objectives are tumour responses as measured by PSA and the tertiary objective is a change in immune response.

The aim of the work reported in this chapter is to develop and validate a reproducible assay to identify immune responses in patients with prostate cancer that can be used to measure this tertiary objective.

To observe the impact of treatment in the immune system, we intend to measure T cell responses. This can be done by looking at T cell responses to peptides or whole antigens. The attraction of using peptides is that the assays are easier to perform. The disadvantage is that the peptide epitopes are HLA specific and thus knowledge of the epitopes and HLA types of the subjects are required. Using whole antigen is attractive as the antigen is processed within the autologous APC and this avoids the need for HLA typing and the patient's own cells select their own epitope. The challenge with this approach is getting the whole antigen into the APCs. Initially, we adapted a B cell blast assay developed in the School for Cancer Sciences at Birmingham University by the melanoma group. These B cell blasts are developed from the blood of a subject and used as antigen presenting cells (APCs). These APCs are nucleofected by electroporation with plasmid expressing whole tumour antigen (MART-1 in the melanoma patients). After overnight incubation the cells are mixed with autologous non-adherent PBMCs and any IFN-γ released is assayed for by using an ELISA technique. The melanoma group had tried to use an ELIspot technique but found too much background IFN-γ release in their controls.

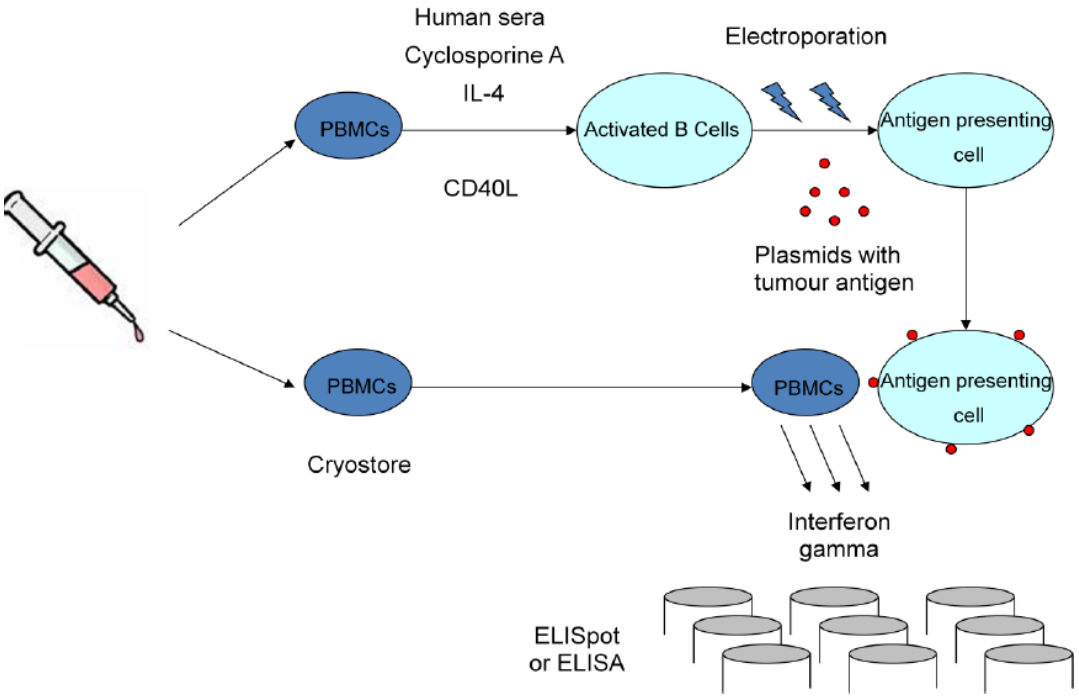


Figure 4.1. Schematic for autologous B cell blasts nucleofected with tumour antigens and IFN-γ ELIspot and ELISA assay

4.2 Initial trial of previously established protocol using autologous B blasts to present nucleofected antigen in an ELIspot assay

The suitably consented subject was venesected and 120mls of heparinized blood was drawn.

The PBMCs were then extracted using a Lymphoprep density gradient extraction technique. Once the monocytes are removed, the remaining cells were washed with two thirds of the cells being frozen and stored in liquid nitrogen. The other third were then used to create B cell blasts. This process involved using four to six 48 hour passages on an irradiated feeder layer of murine L cells expressing human CD40 ligand with mixed human sera, IL-4 and cyclosporin A (to inhibit T cells). Once the cells started to bunch, they were designated B cell blasts.

The autologous ‘responder cells’ were taken out of cryostore, thawed and rested in media overnight. The nucleofected B cell blasts were at a concentration of 1 x 10^6/ml and therefore at the correct concentration to be added to the wells on the plate (100µl/well).

The responder cells were added to the wells at a cell concentration of 5 x 10^6 cells/ml in ELISpot medium. Using PHA as a positive control, PBMCs and nucleofected B blasts on their own as negative controls IFN-γ ELISpot assays were undertaken and wells using a 10 fold dilution of the responder cells were also tested. The results of the ELISpot are shown in fig 4.1. In these experiments, an ELISA for IFN- γ was also undertaken (fig 4.2) using the medium removed from the wells after the overnight incubation.

The data shown in figures 4.1 and 4.2 demonstrate that when nucleofected B cell blasts from an individual with known EBV exposure are nucleofected with the EBV antigen BMLF-1 and then incubated with autologous PBMCs a measurable release of IFN-γ occurs. The ELISA demonstrates that this response increases with the number of PBMCs in the wells.

Unfortunately, there is considerable background release of IFN-γ in the control wells, and this high background could make it impossible to detect small, specific T cell responses. This corroborates the experience of the colleagues who attempted to use this assay to monitor melanoma antigen-specific T cell responses in clinical trial patients. It was therefore decided that the assay was unsuitable in its current form, and we therefore considered possible causes of the high background. We considered that the following features of the protocol were probably contributing causes of the high background:

- The use of a murine feeder cell layer.

  There could potentially be a variety of foreign antigens from the mouse cells, some of which may trigger responses in the assay.

- The use of mixed human sera and foetal calf serum in B cell media. The mixed human sera and the foetal calf serum components in the media may have antigens that could trigger immune responses in the assay.

- The destructive nature of nucleofection.

  The act of nucleofection causes some cell death and the consequent release of antigens may trigger immune responses in the assay.

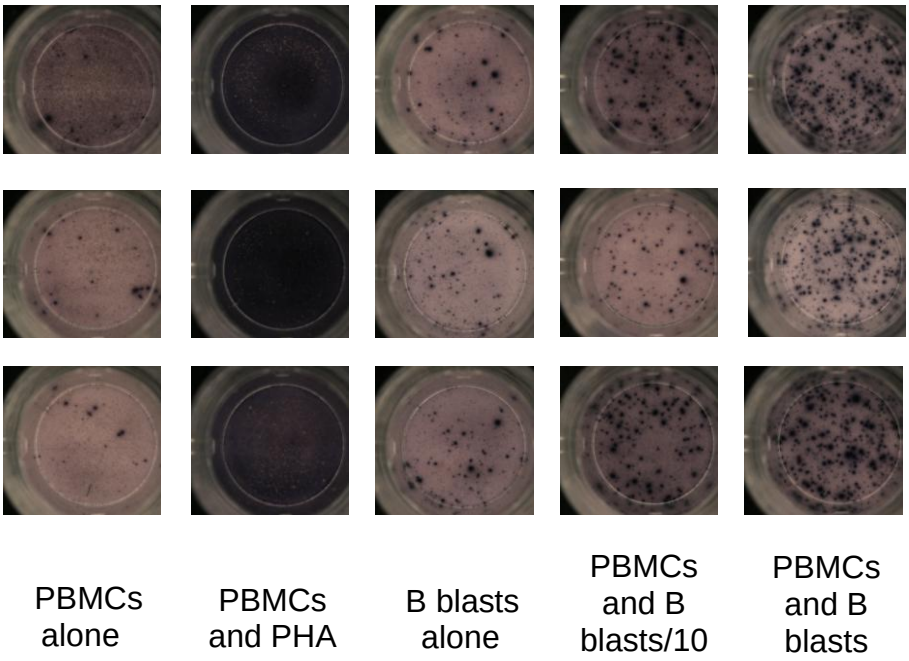


Figure 4.2. Wells in triplicate from an Elispot for IFN-γ release by PBMCs from an A1 B8 B62 C10 C7 HLA typed donor, mixed with autologous B cell blasts nucleofected with the EBV antigen BMLF -1 and controls.

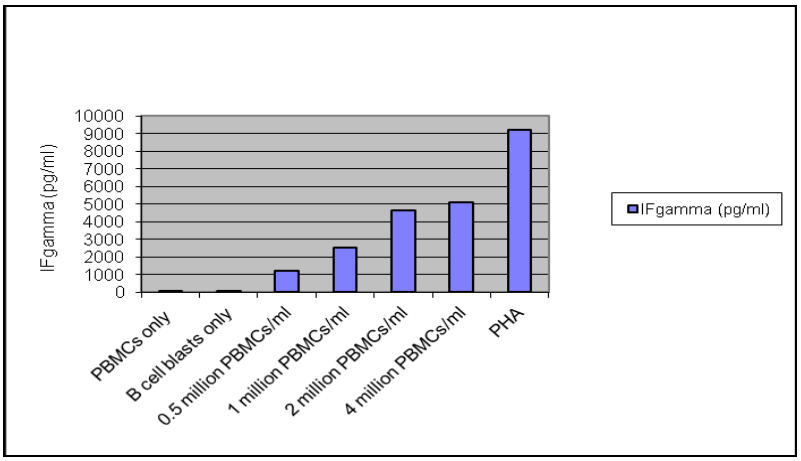


Figure 4.3. IFN-γ release by PBMCs from an A1 B8 B62 C10 C7 HLA typed donor, mixed with B cell blasts nucleofected with the EBV antigen BMLF -1 and controls as assayed by ELISA.

4.3 Assay improvement by avoidance of murine feeder cells and use of autologous serum utilizing both nucleofection and transfection

A suitably consented subject was venesected, and 100mls of heparinized blood drawn. At this time, 20mls of blood was also taken to generate autologous sera. The sera was used instead of mixed human sera. A commercially available soluble human CD40 ligand was used in the B cell manufacture process rather than CD40 ligand expressing murine L cells. The CD40 ligand used was MegaCD40L™, a highly active construct in which two trimeric CD40 ligands which were artificially linked via the collagen domain of ACRP30/adiponectin. This construct very effectively simulates the natural membrane-assisted aggregation of CD40L in vivo.

In parallel, the experiment is undertaken using a commercially available transfection kit (FuGENE) to attempt transfection rather than nucleofection for delivering the plasmid DNA to the B cell blasts to see if this is a better alternative.

We wanted to establish at what point in the B cell blast development, the B cell blasts are best suited to present antigen to stimulate an IFN-□ response measurable by ELIspot. Those cells being passaged to become B cell blasts were sampled at passage (P) 1, 3 and 5. These cells were frozen and put in cryostore. The evening before the nucleofections and transfections were planned (halfway through the last passage of the B cell blasts) the PBMCs and P1 and P3 B cell blasts were taken out of cryostore, thawed and rested in media overnight. The B cell blasts were then nucleofected or transfected and incubated with the PBMCs overnight. When the experiment was complete, they were assayed with ELISpot immediately in the nucleofected cell population. The ELISpot was undertaken at 48 hours in the transfection cell population to allow adequate expression.

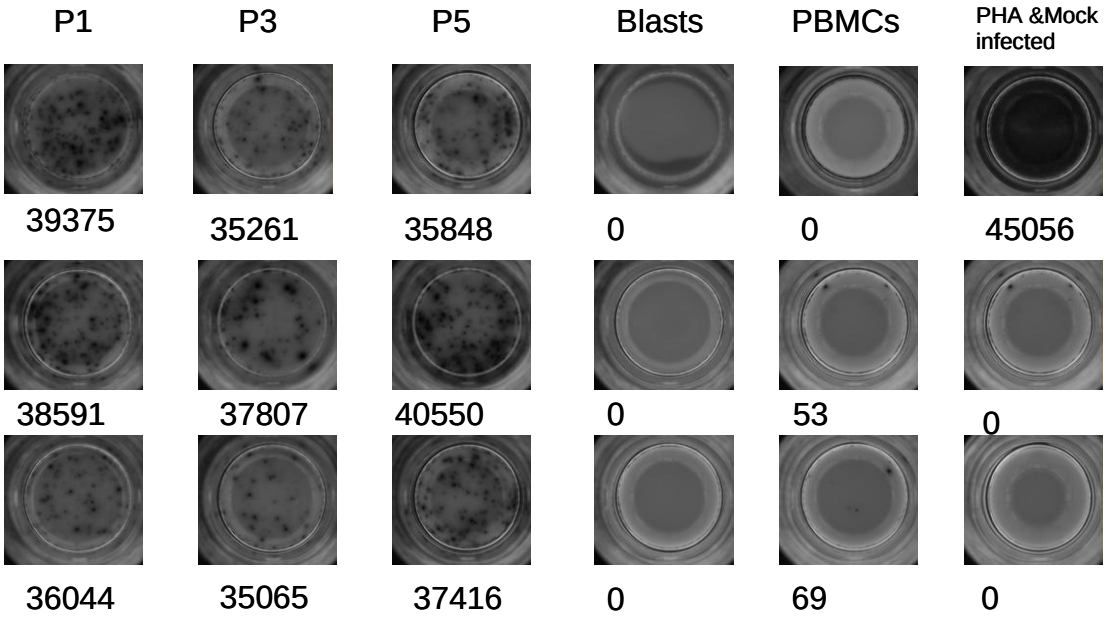


Figure 4.4 - Wells in triplicate from an Elispot for IFN-γ release by PBMCs from an A1 B8 B62 C10 C7 HLA typed donor. The PBMCs were mixed with B cell blasts from various points in passage with megaCD40L and autologous sera and nucleofected with the EBV antigen BMLF -1 and controls. The cytokine activity results are shown as there were too many spots for the reader in some of the wells

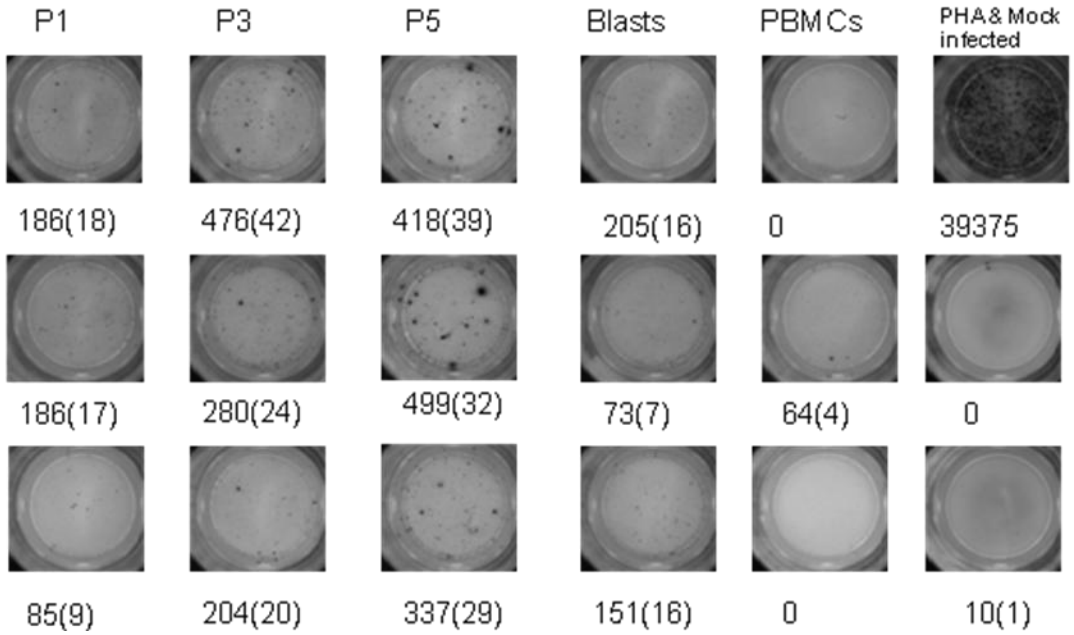


Figure 4.5- Wells in triplicate from an Elispot for IFN-γ release by PBMCs from an A1 B8 B62 C10 C7 HLA typed donor. The PBMCs were mixed with B cell blasts from various points in passage with megaCD40L and autologous sera and transfected with the EBV antigen BMLF -1 and controls. The cytokine activity results are shown with spot counts in brackets.

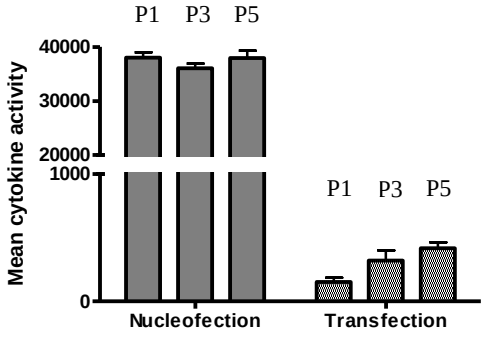


Figure 4.6 Differences in mean cytokine activity with background responses subtracted between nucleofected and transfected cells from an ELISpot for IFN-γ release by PBMCs from an A1 B8 B62 C10 C7 HLA typed donor. The PBMCs were mixed with B cell blasts after first, third and fifth passage with megaCD40L and autologous sera and nucleofected/transfected with the EBV antigen BMLF -1 and controls. All values represent the mean plus or minus the standard deviation.

Figures 4.3 and 4.4 showed that in samples from the same donor nucleofection resulted in a far greater response in the ELISpot than transfection. This is keeping with observations in the literature [Maurise, R. et al 2010] although the differences vary with cell type.

There was considerable improvement in the background response as evidenced in the controls for using megaCD40L and autologous sera. The background in both the nucleofected and transfected cells was similar. Using MegaCD40L allowed the B cell blasts to generate spots from the first passage.

Given these observations we were keen to establish whether B cell blasts generated using a murine L cell feeder layer and mixed human sera are also able to generate spots on an ELISpot after the first passage? We were also keen to establish if the difference in background is due to the differences in B cell blast generation.

4.4 Optimizing B cell generation for a nucleofection based IFN-γ ELISpot assay.

A suitably consented subject was venesected and 100mls of heparinized blood drawn. At this time, 20 mls of blood is also taken to generate autologous sera. On this occasion, some B cells were generated on irradiated murine L cells in monolayer with mixed human sera and some B cells were generated using megaCD40L and autologous sera. B cells were sampled at each passage and frozen. After 4 passages, all the constituent cells are thawed out and nucleofected with BMLF-1. The cell numbers per well were adjusted to $1.5x10^5$ B cell blasts and $7.5x10^5$ PBMCs per well to try to get a countable number of spots per well. At each passage a small sample of the B cells were taken and phenotyped for expression of the co-stimulatory molecules CD80 and CD86.

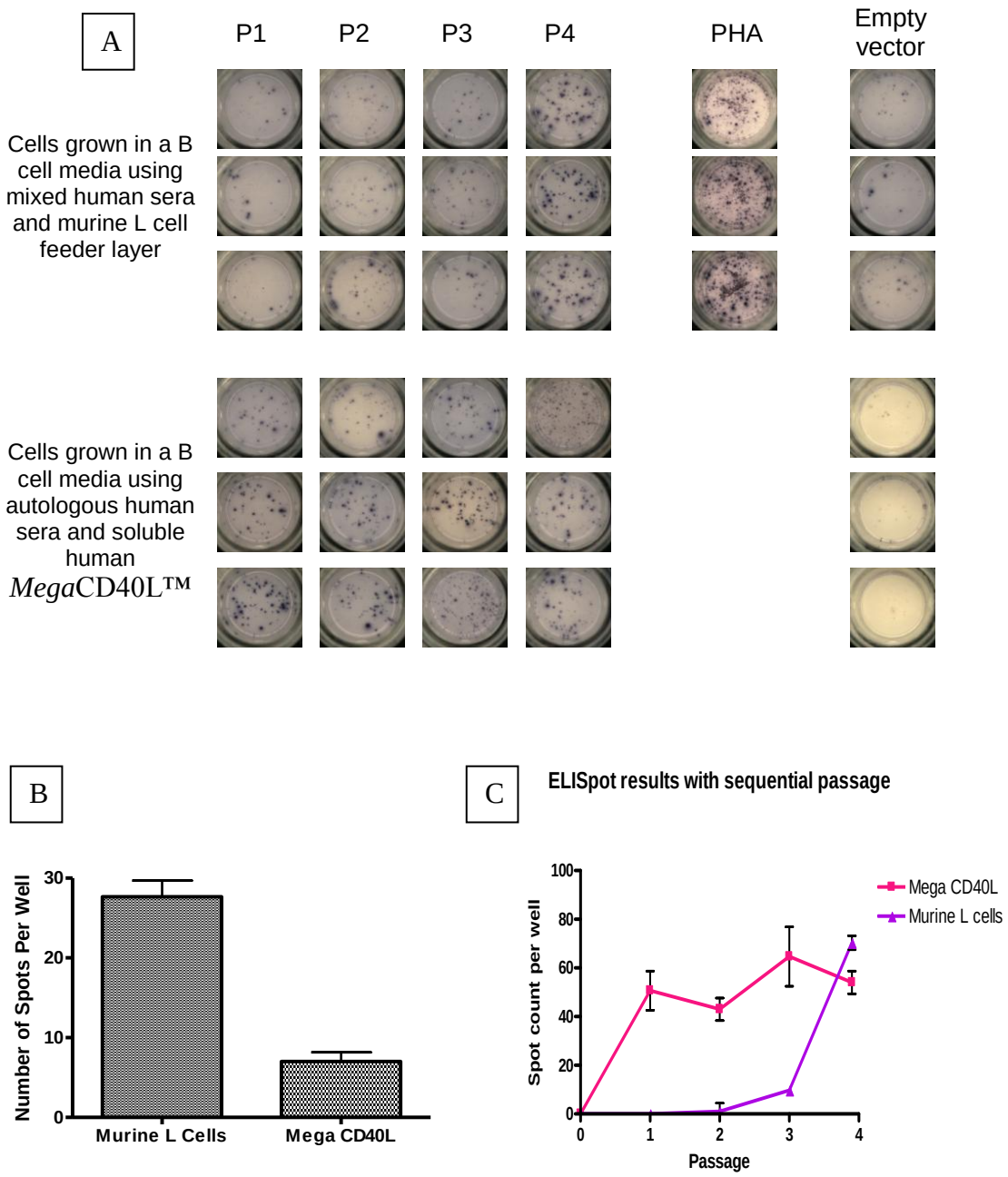


Figure 4.7 - Comparing the IFN-γ ELISpot assay for B cell blasts at passage 1-4 generated by using a murine L cell layer with mixed human sera vs those generated by MegaCD40L and autologous human sera (A). The cells are nucleofected with BMLF-1 plasmid in an A1 B8 B62 C10 C7 HLA typed donor at $7.5x10^5$ PBMCs to $1.5x10^5$ B cell blasts per well. The difference in the background spot counts is demonstrated in (B). The data from the spot counts are represented in the line chart(C). All values represent the mean plus or minus the standard deviation.

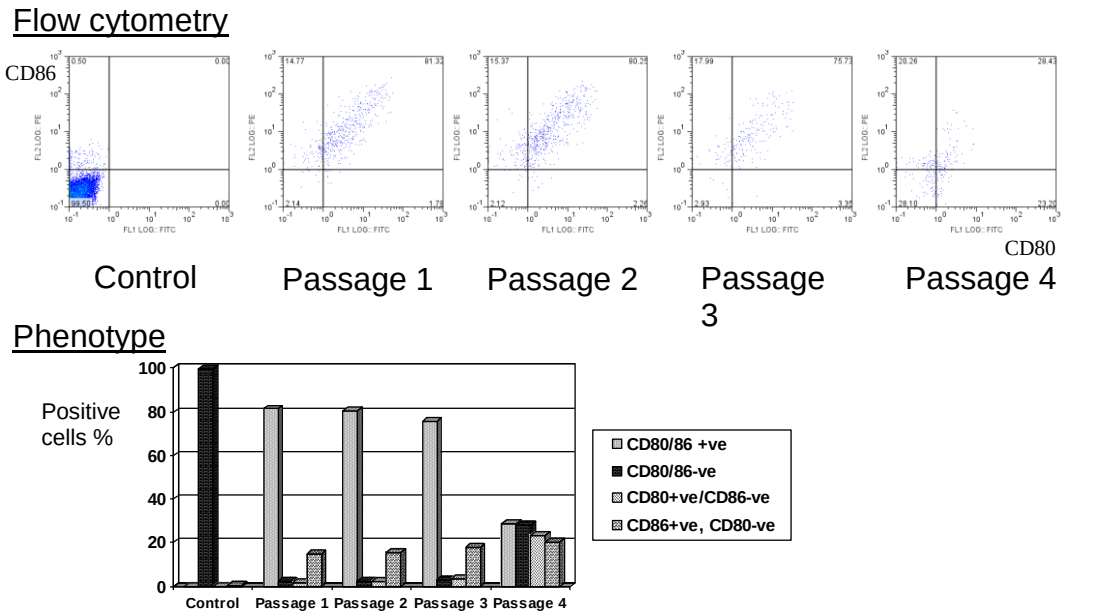


Figure 4.8 – Phenotypic changes of B cell blasts generated with *Mega*CD40L™ and autologous sera with CD86 expression recorded on the y axis and CD80 expression recorded on the x axis

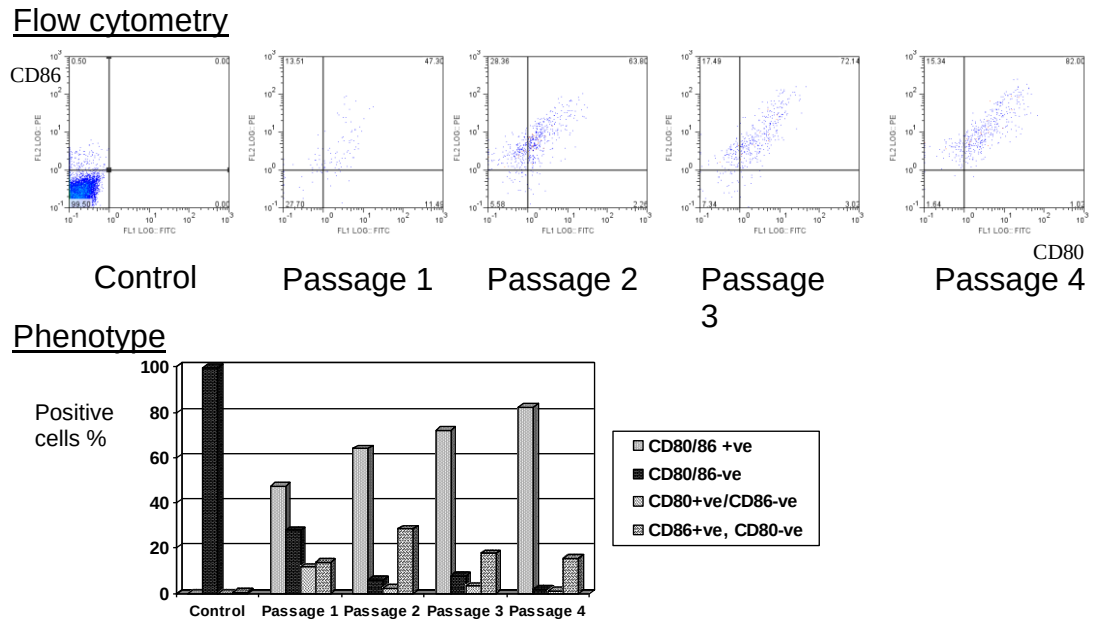


Figure 4.9 – Phenotypic changes of B cell blasts generated with murine L cell layer and mixed human sera with CD86 expression recorded on the y axis and CD80 expression recorded on the x axis

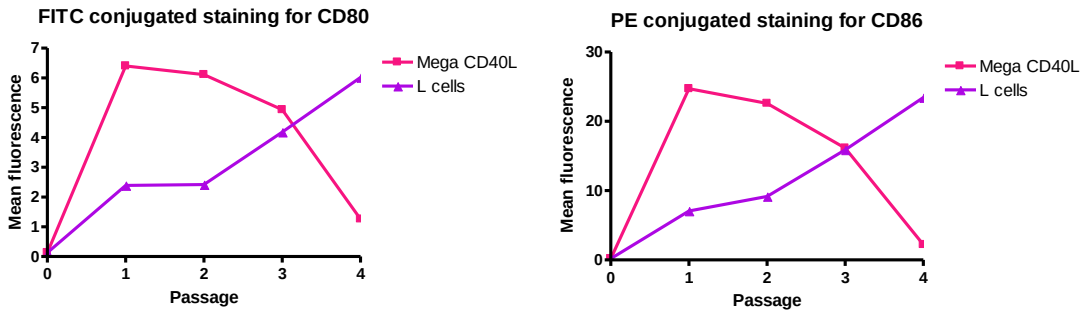


Figure 4.10. Comparing the phenotypic changes of B cell blasts generated with murine L cell layer and mixed human sera vs. Mega CD40L and autologous human sera

The data in figures 4.6 demonstrate that B cell blasts generated with MegaCD40L™ and autologous human sera produced spots in the ELIspot from the first passage. Figure 4.7 showed that over 80% of B cell blasts generated with MegaCD40L™ and autologous human sera expressed co-stimulatory factors CD80 and CD86 from the first passage. Figure 4.6 demonstrated that B cell blasts generated with the murine L cell feeder layer and mixed human sera took three passages before good numbers of antigen-specific spots were found in the ELIspot. Figure 4.9 also showed that CD80 and CD86 expression increased with sequential passage with the murine L cell feeder layer and mixed human sera with 80% of B cell blasts expressing costimulatory factors CD80 and CD86 after three passages. There were fewer background spots in the ELIspot using B cell blasts generated with MegaCD40L™ and autologous human sera (Figure 4.6).

4.5 Handling the PBMCs whilst awaiting the generation of the B cell blasts.

The findings in 4.4 suggest the nucleofection and ELIspot assay using B cell blasts generated with MegaCD40L™ and autologous human sera, can be undertaken after one passage. We wanted to establish the optimum time for the passage and also identify how best to handle the PBMCs during this time.

Once again, blood was taken from an EBV exposed donor, and the PBMCs extracted. Some PBMCs were frozen and some of the PBMCs were placed in cell culture media in a 37^{o} C incubator. Those PBMCs set aside for B cell blast preparation only undertake one passage with MegaCD40L™ and autologous sera. During this passage, small samples of the developing B cell blasts and the incubated PBMCs were taken at 12, 24 and 36 hours and phenotyped for the expression of the co-stimulatory molecules CD80 and CD86 using flow cytometry. The B cell blasts are nucleofected with BMLF-1 after 36 hours incubation and the IFN-γ ELISpot assay was done with $1.5x10^{5}$ B cell blasts per well and $7.5x10^{5}$ PBMCs per well. The results are shown in figures 4.10. This experiment demonstrates the need for careful handling of the PBMC population as they can undergo phenotypic change, which can impact on the background seen in the assay. The experiment was undertaken on two other donors and similar results were seen.

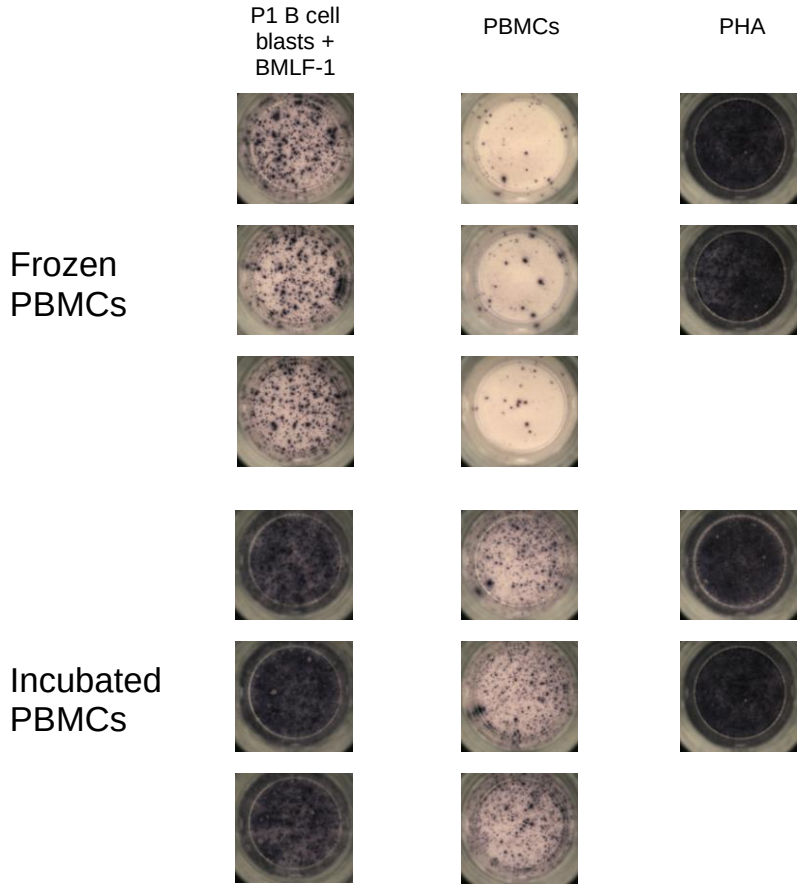


Figure 4.11 - ELIspot assay of interferon gamma release by frozen and incubated PBMCs in response to autologous B cell blasts generated by a single passage *Mega*CD40L™ and autologous human sera which are expressing BMLF-1 after Amaxa nucleofection

4.6 Selecting the best plasmid construct for the assay

We were keen to explore the best plasmid backbone for the nucleofection step. Using PBMCs extracted from Buffy coat samples, CD40 L activated B cells were produced using a murine L cell feeder layer. These cells were nucleofected using 4 different plasmid constructs

- SL 1 (pCMV-TnT-EGFP)
- pPS1098A8
- 4B6 (pPG004B6)
- 4A8 (pPG004A8) containing green fluorescent protein (GFP).

All four constructs use the CMV immediate early promoter to express EGFP, however they differ in the following ways:

pCMVpTNT-EGFP includes a hybrid CMV/beta-globin intron upstream of the EGFP gene, and uses 3' end sequences (for addition of polyA and mRNA termination) from SV40 virus. The plasmid also contains some sequences from bacteriophage f1, and SP6 and T7 polymerase promoters that can be used to transcribe the EGFP in vitro

pPS1098A2 is in a retrovirus vector plasmid (based on pLNCX [Miller, A.D. 1989]), hence contains upstream and downstream LTRs, and the neomycin-resistance gene.

4B6 and 4A8 plasmids contain the Ii invariant chain leader sequence (which may possibly reduce apparent expression of EGFP due to endosomal degradation) and HA-tag. pPG004A8 incorporates the beta-globin 2nd intron, and 3' end signals from the human complement C2 gene (the same combination as in CTL102 and AdNRGM). pPG004B6 has no intron, and uses the 3' end signals from the bovine growth hormone gene (sourced from pCDNA3).

Using flow cytometry and microscopy the most effective construct was selected. By repeating the flow cytometry at 48 and 72 hours post nucleofection, it was demonstrated that GFP expression was greatest at 24 hours post nucleofection (Fig 4.11) and the SL1 construct appeared to perform best in nucleofection. Using this construct, five plasmids were then made replacing the GFP with one of five prostate TSAs (PSA, PSMA, PSCA, PAP and KLK4).

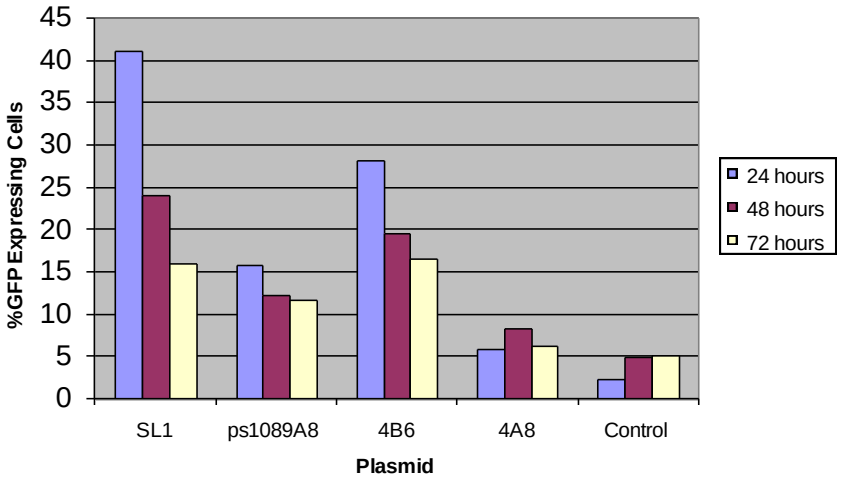


Figure 4.12 Percentage of cells expressing GFP in CD40L activated B cells nucleofected with different GFP expressing plasmid constructs over time

4.7 Multiple antigen nucleofection

When looking at a number of antigens, parallel assays can be run using individual antigens. However, if cell numbers are limited, it could be more efficient to analyze multiple antigens in the same reactions.

We wanted to see if it were possible to use multiple antigens within one assay and assess what implications this may have on the results. CD40L activated B cells were nucleofected with 12 μg of plasmid expressing GFP and 2 μg of plasmid expressing

GFP diluted with plasmids expressing 5 different PSTAs in equal 2 μg quantities (12 μg in total). The expression of GFP was measured using flow cytometry. There was a small decrease in the percentage of GFP expressing cells (figure 4.12) and the mean fluorescent intensity was reduced by 7%

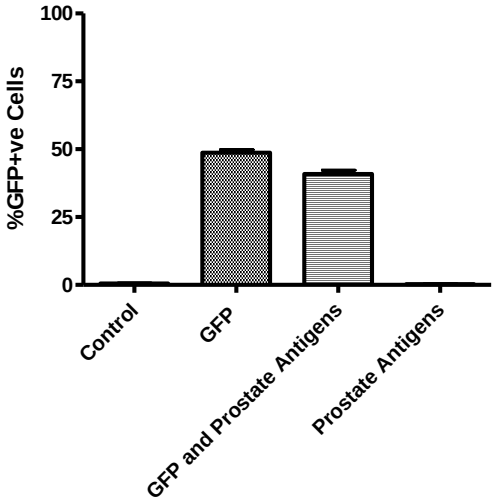


Figure 4.13 Dilutional effects of multiple antigen nucleofection on their expression. CD40L activated B cells were nucleofected with 2 μg of SL-1 plasmid expressing GFP and 2 μg each of SL-1 plasmid expressing the 5 prostate tumour antigens and 2 μg each of SL-1 plasmid expressing GFP and the 5 prostate tumour antigens. The expression of the GFP was measured with flow cytometry.

These data were unable to tell us if the GFP +ve cells were co-expressing any of the PSTAs. To look at co-expression in multi-antigen nucleofection, CD40L activated B cells were nucleofected with a plasmid expressing GFP and a plasmid expressing red fluorescent protein (RFP). The percentage of nucleofected cells expressing these proteins was measured using flow cytometry. 32% of cells expressed both RFP and GFP. 16% were positive for GFP alone and 10% were positive for RFP alone (figure 4.13).

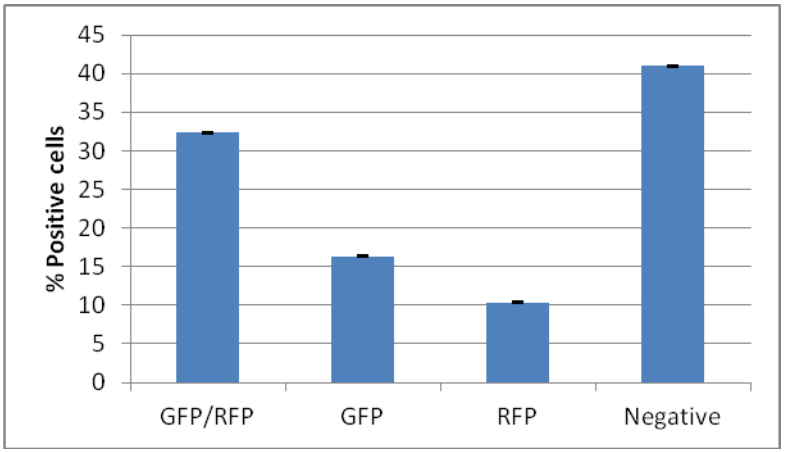


Figure 4.14 Dilutional effects of multiple antigen nucleofection on their expression. CD40L activated B cells were nucleofected with 2 μg GFP and 2 μg of RFP. The expression of the GFP and RFP was measured with flow cytometry. 32% of the cells expressed both GFP and RFP with 41% expressing neither. 17% expressed GFP alone and 10% expressed RFP alone.

The experiments described above indicated that, although co-nucleofection of multiple plasmids could lead to co-expression of multiple antigens in some cells (GFP and RFP co-expression), not all the nucleofected cells would necessarily express all the antigens, and there could be some reduction in the level of expression of any one antigen. To investigate the possible impact of this in the ELIspot assay, CD40L activated B cells from a healthy EBV exposed donor were nucleofected with BMLF-1 plasmid alone and the BMLF-1 plasmid mixed with 5 PSTAs plasmids. In this experiment, we are assuming that the donor will not mount a significant response to the PSTAs. As shown in Figure 4.14, there was no significant difference in the ELISpot counts.

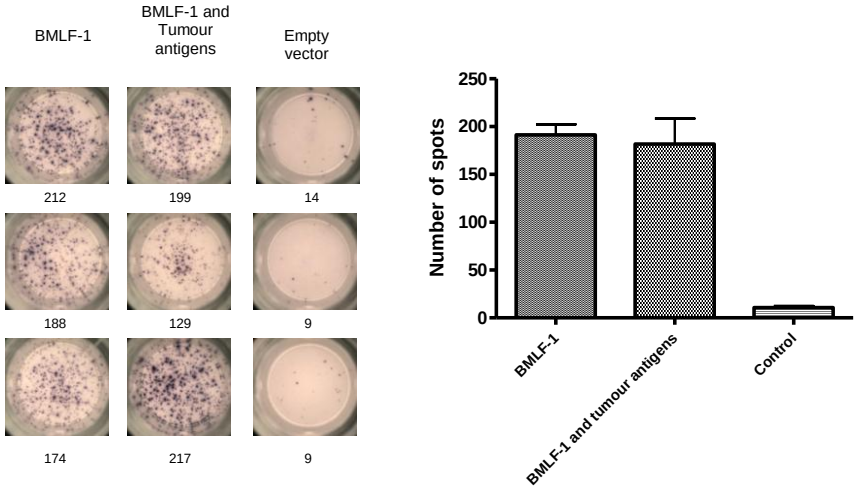


Figure 4.15 Dilutional effects of multiple antigen nucleofection on the IFN-γ ELISpot assay. CD40L activated B cells from a donor with known EBV exposure were nucleofected with 2 μg BMLF-1 plasmid and 2 μg each of the 5 prostate tumour antigens. Further CD40L activated B cells from the same donor were nucleofected with 2 μg of BMLF-1. The nucleofected cells were then mixed with PBMCs from the same donor and an ELISpot performed. All values represent the mean plus or minus the standard deviation.

These data suggest that multiple antigen nucleofection will lead to a variety of single and multiple antigen expression in the target cells and that this will not significantly dilute the response to any one of these antigens.

4.8 Adding the invariant chain to utilize the MHC class II pathway

Using nucleofection to create antigen expressing cells, the endogenously expressed antigens will be presented via the MHC class I pathway. We wanted to see if we could use the MHC class II pathway as well to enhance the assay further. The additional presentation of antigen via this route would allow the detection of CD4 T cell mediated responses as well. To do this, PSTAs with invariant chain (IC) were developed. CD40L activated B cells from a prostate cancer patient were nucleofected

with either PSTAs or PSTAs with IC. The ELISpot assay and an IFN-γ ELISA were undertaken. There was a greater spot count and IFN-γ release seen in the assays using the invariant chain (figure 4.15). This was the consistent finding in patients with stronger responses. In patients with weaker responses, there were no significant differences between PSTAs with IC and PSTAs without IC.

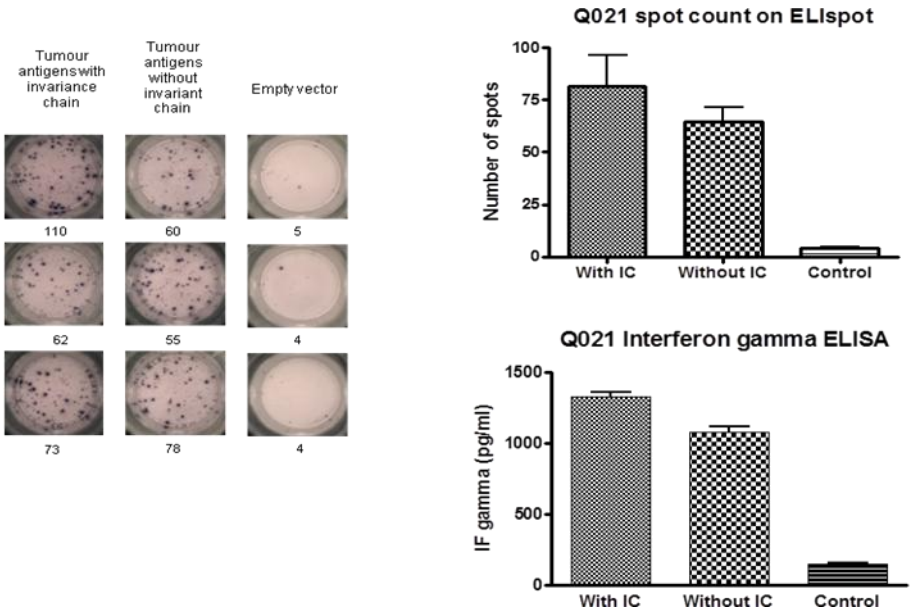


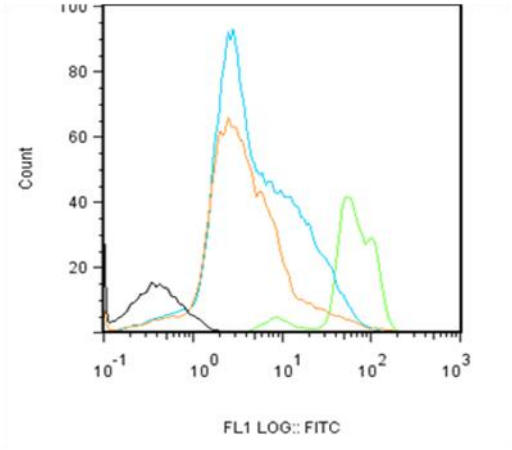
Figure 4.16 Effects on the IFN-γ ELISpot of the addition of the invariant chain to the PSTAs. CD40L activated B cells from a patient with Gleason 3+4=7 prostate cancer on active surveillance were nucleofected with 5 PSTAs with IC, 5 PSTAs without IC and an empty vector as control. The nucleofected cells were then mixed with PBMCs from the same donor and an ELISpot performed. All values represent the mean plus or minus the standard deviation.

4.9 Nucleofection and the impact on cells

The process of nucleofection can be harmful to cells and we wanted to know what impact it had on cell survival. To measure this we used a propidium iodide assay. We found that the number of cells that died during nucleofection with 5 PSTAs varied from 9 to 25% across eight samples (mean 17.9 SD 5.67) compared with 6% of cells nucleofected with no antigen.

4.10 Measuring nucleofection efficiency

All the PSTAs used had a haemagglutin (HA) tag attached to them. The purpose of this was to allow us to measure the efficiency of nucleofection. This would be measured as the percentage of the cells that were expressing HA after nucleofection. Assaying for this proved difficult at first as the HA was intracytoplasmic. A reproducible assay was possible using the FIX & PERM® kit from Caltag Med-systems. This cell fixation and cell permeabilization kit achieves mild fixation and permeabilization of cells that leaves their morphological flow cytometric scatter characteristics intact. This enables the accurate identification of intracellular markers, such as HA. We used a FITC labelled anti-HA antibody and figure 4.16 shows a typical result. A commercially generated E. coli positive control whole cell lysate (ab5395) and mock nucleofected CD40L activated B cells were used as the negative control.



	Sample	%+ve	Mean Fluorescence
	-ve control	3.40	0.45
	+ve control	88.04	66.23
	HA with invariant chain	87.59	7.31
	HA without invariant chain	82.53	9.14

Figure 4.17. Nucleofector efficiency as measured in CD40L activated B cells nucleofected with 5 PSTAs with HA tag using the Fix and Perm kit with FITC labelled anti HA antibodies. We found that nucleofection efficiency in our assay was consistently between 25 and 45%.

4.11 Optimizing the number of antigen expressing cells

In optimizing the number of antigen expressing cells in the assay we needed to see what impact the number of antigen expressing cells has on the spot count. To measure this, sequential dilutions of antigen expressing cells were used in the IFN-γ ELISpot assays. The results demonstrated that any number above 10,000 cells per well appeared adequate (figure 4.17, an example with nucleofector efficiency of 42.7% therefore around 4,270 antigen expressing cells per well). Increasing the cell numbers beyond 10,000 has no significant impact on the assay.

The HA tag was useful in ensuring adequate nucleofection and is a tool to allow correction for variability in the nucleofection efficiency. Provided 10,000 nucleofected cells are used, variations in nucleofection efficiency from 20% or more have no significant impact on the spot counts in our assays.

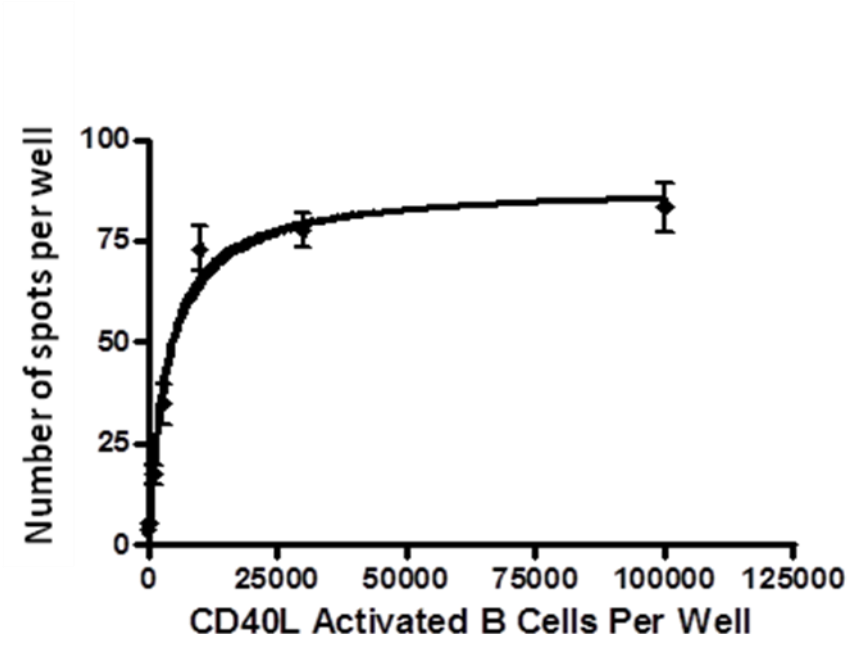


Figure 4.18. Effects of sequential dilution of CD40L activated B cells nucleofected to express BMLF-1 (nucleofection efficiency 42.7%) from an EBV exposed donor on the ELISpot assay. All values represent the mean plus or minus the standard deviation.

4.12 Summary

The results in this chapter have illustrated the development of a reproducible assay for quantifying cellular immune responses to prostate tumour antigens by measuring the number of IFN-γ releasing cells in blood samples from patients. A variety of approaches were used to generate autologous cells suitable for presenting a panel of prostate cancer tumour antigens. The initial assay had unacceptably high background, but this was reduced by modifying the process for generation of the antigen presenting cells (B-blasts) from PBMC. Optimal cell handling was identified to ensure a protocol that could be reproduced in a clinical trial setting.

We explored the implications of multiple antigen nucleofection and found this was practical, improving the sensitivity and the cost efficiency of the assay. Our data has demonstrated that using an invariant chain with the plasmid insert further improves the sensitivity of the assay. Attaching an HA tag to the plasmid being inserted through electroporation nucleofection allows one to measure the efficiency of the nucleofection. This allows one to monitor the efficiency of the nucleofection step in the assay.

5 Results from the Immune Responses in Adenocarcinoma Prostate (IRAP). Study REC reference number 09/H1202/1 and CRCTU protocol number RG_08-098

5.1 Introduction

The primary endpoints of the forthcoming ADUP study are safety and adverse events with a secondary endpoint of anti-tumour activity as measured by serum prostate specific antigen (PSA). The trial has a tertiary endpoint of evidence of immune responses. To measure this outcome we will be using the CD40 ligand activated B cell / IFN-γ ELISpot immune assay described and validated in chapter 4. Before using this assay on trial patient samples, the Immune Responses in Adenocarcinoma of the Prostate (IRAP) study was conceived.

The main purpose of this study is to validate assays for measuring cell-mediated immune responses in patients with prostate cancer and the objectives are summarised in table 5.1. We wanted to explore the immune responses in patients with prostate cancer to a pool of 5 prostate specific tumour antigens (PSTAs):

- Prostate Specific Antigen (PSA)
- Prostate Specific Membrane Antigen (PSMA)
- Prostate Stem Cell Antigen (PSCA)
- Prostate Acid Phosphatase (PAP)
- Kallikrein-related peptidase 4 (KLK4)

For the purposes of this study we did not seek to undertake the assays in healthy donors as we were unable to counsel them suitably as to how to interpret the results and whether there may be implications for the donor with regards to his prostate

5.2 Study design

Primary objective:	• To optimize and validate assays for measuring immune responses to prostate cancer • To determine if immune responses to prostate and prostate cancer antigens can be detected and quantified in prostate cancer patients.
Secondary objectives:	• To generate baseline data to help interpret immune response data from a forthcoming gene therapy clinical trial.
Tertiary (Exploratory) Objectives:	• To observe any changes in the immune response with treatment • To identify any patient, treatment, tumour characteristics that may influence the immune response. • To assess if there is any relationship between the level of immune response and the patients response to treatment, as assessed by the prostate cancer tumour marker PSA in the blood.

Table 5.1 - IRAP study objectives

We aimed to recruit up to 50 patients [table 5.2], who were to be undergoing surgical, radiotherapeutic, medical (hormonal) or conservative treatment for their prostate cancer to the study. We aimed to recruit the majority of patients when they were newly diagnosed with prostate cancer, and include longitudinal observations upon diagnosis and subsequent therapy. We aimed to recruit about 10 patients from each treatment group.

We aimed to further develop and validate laboratory-based assays that have been described in chapter 4. As shown in table 5.1, the study aims to generate a body of baseline data on the immune responses to the PSTAs in newly diagnosed patients, any changes following primary treatment, as well as in patients at later stages of the disease and in healthy donors. The information gained will be valuable background

for our planned gene and immunotherapy, and for the planning of other immunotherapies or more detailed cohort studies.

Eligible patients were identified amongst those diagnosed with prostate cancer at the Queen Elizabeth Hospital, Birmingham. Staff at the Queen Elizabeth Hospital and at the Institute for Cancer Studies at the University of Birmingham were eligible for recruitment as healthy donors (see table 5.2).

	Inclusion criteria	Exclusion criteria
Patients	• Histologically proven adenocarcinoma of the prostate • Informed consent • Age greater than 18 • Normal haemoglobin	• Evidence of active pathologies or undergoing treatments that potentially affect the outcome measurements • Inability to give informed consent • Advanced disease state with a life expectancy of less than three months
Donors	• Informed consent • Age greater than 18 • Normal haemoglobin	• No personal history or family history of adenocarcinoma of the prostate • No urinary tract infection (including prostatitis) within the last year • Not taking any prostate related medication (such as 5 alpha-reductase inhibitors) • Evidence of active pathologies potentially affecting the outcome measurements • Actively involved in this study

Table 5.2 - IRAP recruitment criteria

Ethical approval for the IRAP study was obtained from the South Birmingham Ethics Committee (reference 09/H1202/1). Recruitment started in February 2009 and over the following 14 months, 38 patients were recruited.

5.3 Study patients and methods

Study ref no.	History/histology	Treatment	Risk stratification (D'Amico)	PSA (ng/ml) at diagnosis
P001	Organ confined prostate cancer Gl 3+4=7	HIFU	Low	6.5
Q001	Organ confined prostate cancer Gl 4+5=9	Naive	High	58
Q002	Organ confined prostate cancer Gl 3+4=7	Naive	High	8.9
Q003	Locally advanced prostate cancer Gl 4+3=7	Naive	Medium	12.5
Q004	Locally advanced prostate cancer Gl 4+4=8	Post TURP but naive	Medium	204
Q005	Organ confined prostate cancer Gl 3+3=6	Naive	Low	6.2
Q006	Organ confined prostate cancer Gl 3+3=6	Naive	Low	9.9
Q007	Locally advanced prostate cancer Gl 4+4=8	Hormone refractory	Medium	745
Q008	Locally advanced prostate cancer Gl 5+4=9	Naive	High	116
Q009	Organ confined prostate cancer Gl 4+4=8	Observation	Medium	12.6

Q010	Locally advanced prostate cancer Gl 4+5=9	Naive	High	21.4
Q011	Organ confined prostate cancer Gl 4+4=8	Naive	Medium	8.7
Q012	Organ confined prostate cancer Gl 3+3=6	Observation	Low	3.4
Q013	Locally advanced prostate cancer Gl 5+5=10	Naive	High	166
Q014	Locally advanced prostate cancer Gl 4+5=9	Naive	High	59.2
Q015	Locally advanced prostate cancer Gl 4+3=7	Naive	Medium	503.5
Q016	BPH on biopsy	Naive	N/A	2.9
Q017	BPH	TURP/naive	N/A	39.8
Q018	Locally advanced prostate cancer Gl 4+5=9	Naive	High	11.4
Q019	Organ confined prostate cancer Gl 3+3=6	Naive	Low	6.7
Q020	Biopsies and TURP for ? locally advanced prostate cancer but BPH	Naive / recent retention and infection	N/A	860
Q021	Organ confined prostate cancer Gl 3+4=7	Observation	Low	3
Q022	Prostatitis	Naive	N/A	84

Q023	Locally advanced prostate cancer Gl 4+3=7	Hormone refractory	Medium	23
Q024	Locally advanced prostate cancer Gl 4+5=9	Naive	High	14.9
Q025	Locally advanced prostate cancer Gl 4+5=9	Post TURP Treatment naive	High	95
Q026	Organ confined prostate cancer Gl 3+3=6	Observation	Medium	12.3
Q027	Organ confined prostate cancer Gl 3+4=7	Observation	Medium	12
Q028	Locally advanced prostate cancer Gl 4+5=9	Hormone refractory	High	72
Q029	Locally advanced prostate cancer Gl 4+5=9	Hormone refractory	High	254
Q030	Locally advanced prostate cancer Gl 4+5=9	Hormone refractory	High	12
Q031	BPH on TURP	Observation	N/A	24
Q032	Locally advanced prostate cancer Gl 4+4=8	Hormone refractory	Medium	21
Q033	Organ confined prostate cancer Gl 3+4=7	Naive	Low	15
Q034	Organ confined prostate cancer Gl 3+3=6	Observation	Low	23

Q035	Locally advanced prostate cancer Gl 4+5=9	Hormone refractory	High	21
Q036	Organ confined prostate cancer Gl 3+3=6	Naive	Low	15
Q037	Locally advanced prostate cancer Gl 5+5=10	Hormone refractory	High	35

Table 5.3 IRAP patients disease state (observation includes watchful waiting and active surveillance)

Of the 38 patients recruited to the study so far, we have data on all 38 patients but we only have follow up data on 10 patients. All the blood samples taken were immediately transferred to the laboratory where the PBMCs were removed, aliquoted and labelled and put into cryostorage. The immune response assays were done in batches, adhering to the SOP developed in chapter 4 (see appendix 1). No issues with the SOP became apparent during the course of the IRAP study.

5.4 Study results

IFN-γ ELISpot responses to the five tumour antigens were seen in all the patients tested. Reassuringly, the assay seemed robust and repeatable with a relatively consistent background spot count (mean 11.3, SD 8.2). The IFN-γ ELISpot responses to PSTAs varied with disease stage and treatment (see figure 5.1 for typical PSA profiles and IFN-γ ELISpot data for each patient group; IFN-γ ELISpot data for all patients are summarised in Figure 5.2).

In our subjects, the viability of the nucleofected B cell blasts was measured using PI staining and flow cytometry. The mean survival was 34.9% of nucleofected cells (SD

5.42). The nucleofection efficiency was also measured and the mean efficiency was 32.4% (SD 10.0). Reassuringly, all the nucleofections were greater than 20%.

Figure 5.2A demonstrates that there are minimal cellular immune responses to the 5 common prostate tumour antigens in patients with benign complaints of the prostate. These 3 patients entered the study as they were presumed to have prostate cancer. On further investigation, after recruitment into the study, they were found to have benign complaints of the prostate. These data demonstrated a trend toward stronger immune responses in patients with treatment naïve prostate cancer when compared to patients with treatment naïve benign prostatic diseases ($p=0.138$). The treatment naive patients represent prostate cancer patients with disease at a variety of grades and stages in progression. This is consistent with the wide spread of results we see in figure 5.2A.

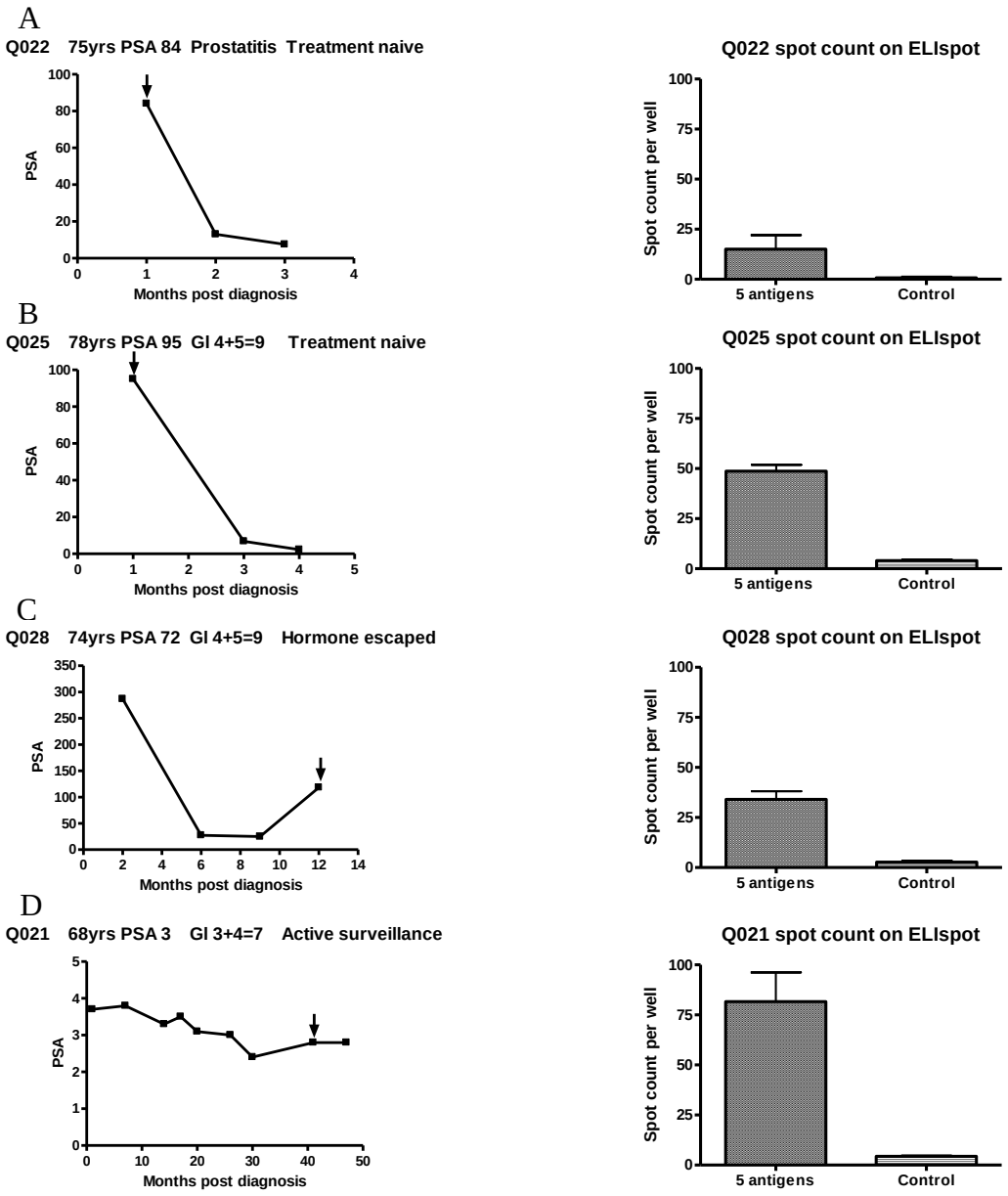


Figure 5.1. The PSA kinetics and IFN-γ ELIspot of selected patients from the IRAP study. (A) The PSA kinetics and IFN-γ ELIspot demonstrating the immune response to 5 PSTAs in a patient with prostatitis. (B) The PSA kinetics and IFN-γ ELIspot demonstrating the immune response to 5 PSTAs in a treatment naïve patient with high risk prostate cancer. (C) The PSA kinetics and IFN-γ ELIspot demonstrating the immune response to 5 PSTAs in a treatment failing patient with high risk prostate cancer. (D) The PSA kinetics and IFN-γ ELIspot demonstrating the immune response to 5 PSTAs in a patient with low risk prostate cancer who has not been undergoing any treatment (active surveillance). The arrows on the PSA kinetic graphs show the point at which the IFN-γ ELIspot was undertaken. The IFN-γ ELISpot results are the number of IFN-γ spots per 5 x 10^5 PBMCs. PSA is measured in ng/ml. All values represent the mean plus or minus the standard deviation.

The active surveillance group have strong immune responses to PSTAs when compared with the wider treatment naive group. These patients will have undergone at least one biopsy over the previous 18 months and will have been carrying an indolent malignancy in their prostate for probably much more than 18 months. When applying the unpaired student *t* test to the data, the hormone escaped group in figure 5.2A have significantly weaker immune responses when compared to the wider treatment naive group ($p=0.014$) and those on active surveillance ($p<0.0001$). Their responses are comparable to patients with benign disease.

When the treatment naïve patients are divided into high, intermediate and low risk groups (figure 5.2B) using D'Amico's classification, the high risk group had significantly weaker immune responses than those patients with intermediate ($p=0.016$) and low risk prostate cancer ($p=0.031$).

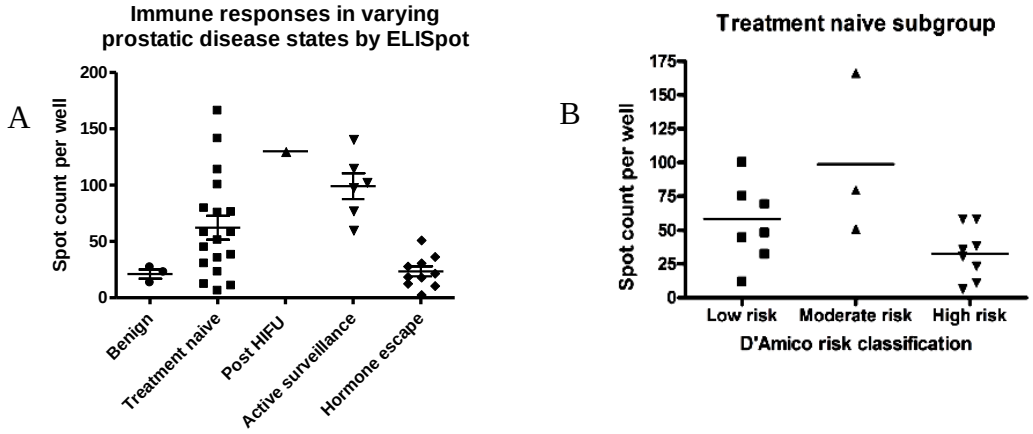


Figure 5.2. This figure shows the level of T cell response to PSTAs, monitored via IFN-γ ELISpot, for patients with benign and malignant prostate disease. (A) The spot counts have been grouped by the point at which they have reached in their treatment. All values represent the mean plus or minus the standard deviation. (B) The results for the treatment naïve patients has been grouped by their D'Amico risk classification.

During the period of the study, we were able to capture a second sample from 10 patients at a subsequent routine hospital appointment. The follow-up data for the 10 patients in the study is shown in figure 5.3B. In this group, one patient had undergone HIFU and demonstrated a significant increase in immune response. Six other patients were being managed with hormone deprivation therapies of which two were diagnosed hormone refractory at their second visit, two were stable on Active surveillance and one had undergone a transurethral resection of his prostate gland. These patients demonstrated a variety of changes in their immune responses. Figure 5.4 identifies those patients who have undergone an intervention for their prostate and in all these patients there had been an increase in immune responses over time. Figure 5.5 identifies those patients in whom there had been no new intervention between samples. In 5 of the 6 patients, there was a decline in immune responses over time.

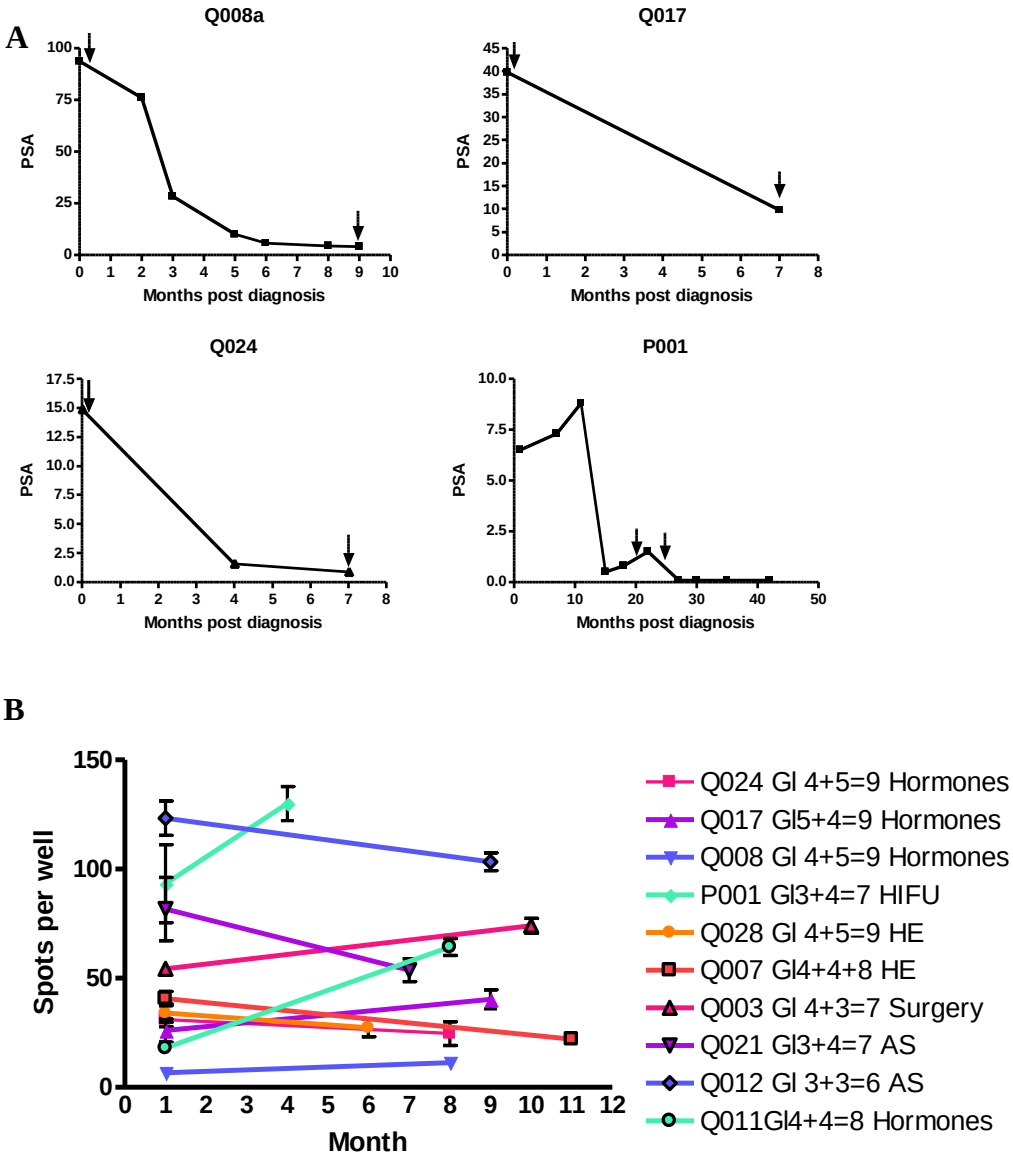


Figure 5.3. The PSA kinetics and IFN-γ ELISpot demonstrating the immune response to 5 PSTAs in 10 patients for whom repeat samples were available. (A) The PSA kinetics of 4 of the patients is demonstrated and the points at which the IFN-γ ELISpots were undertaken are shown by arrows. Q008a, Q017and Q024 have high grade disease and are being managed with hormones. P001 has low grade disease and has been managed with HIFU. (B) The changes in immune responses with time in 10 patients on whom we have data are demonstrated. All values represent the mean plus or minus the standard deviation.

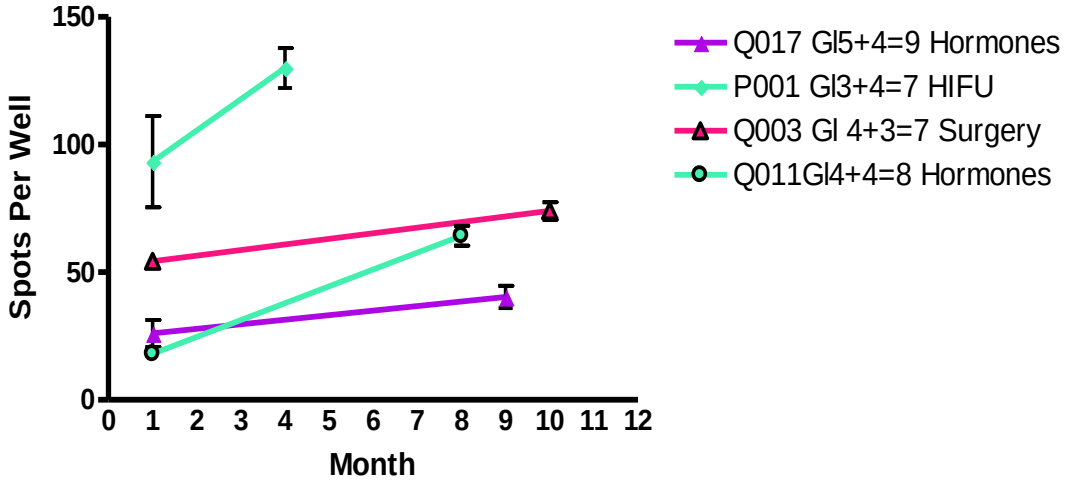


Fig 5.4. Changes in immune responses with time after intervention. Patient Q003 was treatment naive at the time of the first sample and then underwent prostatectomy. Q011 was treatment naïve when the first sample was taken and was then commenced on LHRH analogue hormone manipulation. Q017 was on LHRH analogue hormone manipulation at the time of the first sample with the second sample being taken after the patient had undergone TURP. P001 had been treated with HIFU prior to his first sample. The second sample was taken after the patient had received a second HIFU treatment. All values represent the mean plus or minus the standard deviation.

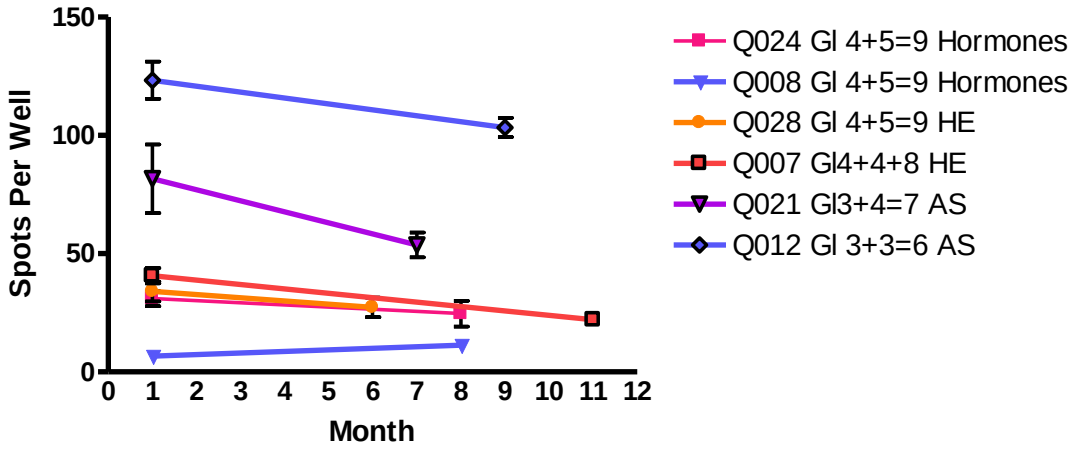


Fig 5.5 Changes in immune responses with time without intervention. The immune responses decreased over time in five of the six patients undergoing therapy or active surveillance, in whom there had been no change in their treatment. All values represent the mean plus or minus the standard deviation.

5.5 Summary

The use of a CD40L activated B cell based IFN-γ assay of peripheral blood in patients undergoing treatment for prostate cancer can be done. Despite the modifications to the assay that are discussed in chapter 4, the assay still detects a persistent and consistent low level of background which was never more than 20% of the overall signal seen when the PSTAs were used. This assay can be used to measure changes in the cellular immune response that might occur through the course of treatment and will make a suitable experimental tool for the forthcoming gene and immunotherapy trial. We have yet to define the ideal timing of the post treatment assay, but expect to have this identified before the trial begins. The small repeat sample size of 10 makes it difficult to interpret whether the variation in repeat samples is due to variability of the assay, or actual changes in the immune response being measured. Further work is required to validate these observations.

These observations have been made on a small sample size (38 patients) with smaller subsets making robust statistical evaluation difficult. These data do suggest some trends which can support some hypotheses in regards to cellular immune responses in prostate cancer.

The change in immune response noted in the single patients treated with HIFU (a thermo-therapeutic strategy) is particularly interesting (figure 5.3). Measurable changes in inflammatory cytokines have been described in the published literature [Li LY. *et al* 2009] and one might expect this treatment to be quite immunostimulatory. Low levels of immune response are noted in the hormone escaped patients. This is also noted in those treatment naïve patients with high grade disease (Fig 3B). These observations can generate the two hypotheses; that failing immune surveillance

facilitates disease grade progression in prostate cancer, or that disease grade progression inhibits immune surveillance in prostate cancer.

Changes in the tumour [Blades RA. *et al* 1995], tumour infiltrating lymphocytes (TIL) [Sfanos KS. *et al* 2008] and the tumour microenvironment [Shafer-Weaver KA. *et al* 2009, Joel B. *et al* 1997] have been associated with local failure of immune responses (immunoediting). With ethics in place to look at TILs, there is an opportunity to look at any possible relationship between TIL phenotype and the level of peripheral immune responses to PSTAs. By capturing data on a larger population of prostate cancer patients we can start to understand more about the natural history of immune surveillance in prostate cancer. This may help us identify which treatments and at which points in the disease pathway that immunotherapies might be used.

6 Discussion

6.1 AdGMNR validation

The CTC01010 gene therapy trial in prostate cancer demonstrated that the VDEPT platform using an E1/E3 deleted adenovirus via a trans-rectal delivery route and the CB1954 prodrug was safe with virus doses of up to $1x10^{12}$. The PSA kinetics of the patients in the trial demonstrated efficacy with a mean decline in PSA of 23.2% after treatment with the mean time to the PSA nadir being 2.4 months and 9 of the 19 patients still having a negative PSA velocity at 6 months after their primary treatment.

The trial highlighted the limitations of using a transrectal route for virus delivery with immunostaining demonstrating less than 50% of prostates treated with the largest viral dose expressing NTR. The trial also demonstrated evidence of cellular immune responses with lymphocytic infiltration in post treatment biopsies in some of the patients.

Given these findings, the ADUP trial was proposed to address virus delivery and to try and enhance the observed immune responses. For delivery, the virus is to be delivered via a transperineal route using a brachytherapy template. To harness any immune responses, the cytokine GMCSF has been added to the virus construct, called AdGMNR.

In the first results chapter, we present the results from the validation of AdGMNR. The construct could infect a variety of prostate cancer cell lines effectively. We selected the metastatic prostate cancer cell lines, PC3 (from an androgen resistant, non PSA secreting clavicular bone metastasis), DU145 (from an androgen resistant, non PSA secreting brain metastasis) and 22RV1 (a cell line developed from an androgen

sensitive, PSA secreting xenograft, CWR22). It is interesting to note that the better differentiated 22RV1 cell line displayed much greater infectability with regards to our Ad5 construct. In the ADUP trial, our patients with have androgen insensitive disease which we have shown is infected by the construct but poses the question of whether this platform would have greater efficacy in androgen sensitive disease.

Whether the construct is coding for GFP, NTR or GMCSF, infected cells will express the gene insert. This expression occurs in a dose dependant way but there are variations in expression between the cell lines. When the construct is encoding for both NTR and GMCSF with an IRES, the upstream gene expression is no different because of the addition of the downstream gene and IRES. The expression of GMCSF, which is the downstream gene, is reduced slightly when compared to its expression without the NTR and IRES upstream. Expression of both genes varied over time with the greatest expression occurring between 48 and 72 hours. This is to be expected and validates the timing of the prodrug in the ADUP trial at 48 hours.

The cell culture work demonstrated that prostate cancer cell lines infected by AdGMNR are sensitised to the prodrug CB1954 and the cells will die in a dose dependent way when the prodrug is applied 48 hours after infection. The sensitivity of the infected cell line is also dose dependent with the amount of prodrug required to kill 50% of the cells decreasing if more AdGMNR is used at the time of infection.

Testing the supernatant from prostate cancer cell lines infected with AdGMNR we were able to explore whether GMCSF was present and then explore whether the supernatant containing the expressed GMCSF was biologically active. We know that PBMCs express CD14 on their surface. If these cells are exposed to GMCSF, some will differentiate into immature dendritic cells and in doing so, stop expressing CD14. We demonstrated that the supernatants from prostate cancer cells infected with

AdGMNR and the same construct expressing only GMCSF reduced the number of cells expressing CD14 by over 50%. This was the same as the changes seen in PBMCs exposed to various dilutions of commercially available recombinant human GMCSF. Even at very high dilutions, these changes were observed reflecting the potency of GMCSF as a cytokine. Assuming that $1cm^3$ of prostate tissue contains 1.2 x 10^9 prostate cells (based on HELA cell models), the local dose of GMCSF could be as high as 100 mg/cm^3 of prostate per day in the prostates of patients in the ADUP trial. In clinical trials there are systemic doses ranging from 125-500 mg/m2 delivered in a variety of schedules ranging from daily to 3 times a week.

The data demonstrate that the levels of GMCSF vary between cell lines, and this was a consistent feature. The greater expression in PC3 cells compared to DU145 cells cannot be explained by differences in infectability. The AdGFP work had demonstrated that DU145 cells were slighter more susceptible to infection with our E1, E3 deleted Ad5 platform. Some prostate cancer cell lines, such as PC3, autonomously secrete GMCSF when stressed and this may help account for the differences in the levels of GMCSF secretion in the cell lines. With the heterogeneity of prostate cancers we would expect to see similar variability in the responses of patients in the ADUP trial.

We have shown that the expression of GMCSF reaches a peak at 48 hours. It is at this time that a patient treated with the AdGMNR virus would be receiving their dose of CB1954. As such, cell death would be occurring within the subsequent 24 hours. This means that the patient will have received a short but continuous neoadjuvant local dosing with GMCSF from the infected prostate cells. There are a few similar clinical trials in the literature, such as the recombinant vaccinia virus encoding human prostate-specific antigen (rV-PSA). In this trial, 10 of the 33 patients received a

subcutaneous injection of 250mcg of GMCSF on treatment days -1, 0, 1, 2 at the site of the subcutaneous injection of the rV-PSA [Eder J, P. *et al* 2000]. The rationale for this dosing regimen came from a rat model [Disis et al 1996]. A more recent study in a murine model not only confirmed that this dose schedule was optimal, but also demonstrated that it enhanced the CEA-specific T-cell responses when a recombinant vaccinia virus was used as an immunogen [Kass et al 2000]. Those patients receiving the GMCSF had a slightly greater incidence of low grade toxicity, but there was a non-significant trend to greater efficacy as measured by PSA response.

Aside from improving the delivery strategy and introducing immunostimulation to the platform, much work had been done in the laboratory on improving the efficacy of the NTR. A number of mutant strains of NTR had been developed and when comparing them with wild-type their performance was dramatically enhanced as measured by cell death with the administration of CB1954. These mutants demonstrated comparable levels of GMCSF generated. The benefits of these mutant strains could include the need for less virus which would have cost benefits and the need for less prodrug. The mutant strains might also generate a greater bystander effect which could benefit the efficacy of the treatment.

With the introduction of a transperineal injection method and the addition of the immunostimulant GMCSF it was felt further modifications to the trial design would make it harder to identify which modification was responsible for any changes observed in outcome. Pending the outcome of the ADUP trial, the mutant NTR strains as well as enhanced prodrugs may form the basis of future clinical trials.

The target patient population lends itself well to this trial. The patients will have biochemical and histological evidence of local recurrence. The patient will have evidence of evolving castrate resistant disease with a climbing PSA despite

biochemical evidence of castrate levels of testosterone. In current guidelines, these patients are not eligible for modifications to their treatment until they become symptomatic. This can take many months and the lack of an active therapy for these patients is an unmet need. As the disease burden is still relatively low, it makes it an attractive target for an immunotherapy. Many immunotherapy trials occur in patients with advanced, highly heterogeneous, burdensome disease in which poor outcomes are likely. Best results with immunotherapies are seen in early, low volume disease. The ideal population for this platform would be patients with newly diagnosed, organ confined disease with smaller prostate and low risk disease, the group usually opting for traditional brachytherapy.

One of the differences between the ADUP trial and CTC01010 trial is that we are unable to deliver a boost dose. This is due to the differences in virus delivery between the two studies. The transrectal approach under local anaesthetic that was used in CTC01010 is much easier to repeat than using the transperineal approach under a general anaesthetic. The value of the boost doses was unclear from the CTC01010 trial given the small numbers. The question over whether boost doses will improve the efficacy of the platform would need to be addressed in a separate trial.

It will be interesting to see the impact the GMCSF has on the success of this platform. There are a number of further immunomodifying treatments that might also be used to enhance the immune elements of the platform such as checkpoint inhibitors like ipilimumab or cyclophosphamide to inhibit the action of T reg cells.

6.2 Development of the immune assay

For the forthcoming ADUP trial, a reliable, validated immune assay was required to identify immune responses in patients with prostate cancer. This assay could then be used to measure the immune responses in ADUP trial patients, the tertiary objective of the study. Within the University, there had been a recent immunotherapy trial in melanoma for which an IFN-γ based ELISpot assay had been developed. This assay involved the use of a whole protein (MART-1) being inserted into an antigen presenting cell (a B cell blast in this instance) using nucleofection. Subsequently the B cell blast is allowed to present the appropriate antigen to autologous T cells. The attraction of using whole peptide in this approach is that it avoided the need for HLA typing patients and finding suitable HLA specific antigens. Avoiding patient selection on the grounds of HLA type would make recruitment to the trial much easier.

Our initial experiments using the melanoma group's assay demonstrated that the assay worked when using the BMLF -1 protein (an early lytic EBV protein) on samples from EBV exposed donors. The assay demonstrated strong responses from EBV exposed patients, when compared with empty vector controls. The problems with the assay were the lengthy and variable manner in which the B cell blasts were developed and the level of background spots seen in the control wells of the ELISpot. The Melanoma group had experienced this problem themselves and had settled for using ELISA data instead. Our early experiments had demonstrated that ELISA was very straightforward but gave quite variable results when repeated in the same donor so we elected to attempt to refine the B cell blast assay.

We felt the possible steps in the assay that could be implicated in the high background readings are:

- Use of the irradiated murine L-cell monolayer as a source of CD40L
- Use of commercially sourced human serum in the B cell media
- Use of nucleofection in protein insertion
- Storage and handling of the evolving B cell blasts
- Storage and handling of the PBMCs

6.2.1 Reducing the background readings

In the early experimental work with the assay, we changed from using the commercially available mixed human sera to simply taking an extra 20 mls of non-heparinized blood from the subject. This sample was used to generate enough autologous serum for the generation of the incubation media for the evolving B cell blasts. We also stopped using the irradiated murine L-cell feeder monolayer and incubated out evolving B cell blasts in media containing a commercially available CD40L called *Mega*CD40L™. This is a highly active construct in which two trimeric CD40 ligands are artificially linked via the collagen domain of ACRP30/adiponectin. This construct very effectively simulates the natural membrane-assisted aggregation of CD40L *in vivo*.

The results from this approach were very encouraging, with a great improvement in the level of background activity that was seen in the control wells. We were keen to see if the nucleofection step might be contributing to the background readings that we

were seeing in the assay. It was evident using a PI cell viability assay that there was considerable cell death occurring after nucleofection. We explored the possibility of using commercially available transfection systems instead of using nucleofection. The attraction of this approach was that it would be cheaper and there would be much less cell death in the B cell blast population. This would have the benefit of leaving us a greater number of B cell blasts for use in the experiments, increasing the number of replications in each experiment as well as possibly reducing the level of background activity in the controls. We were also keen to identify the technique that would give the most consistent expression levels. We initially identified the most effective available transfection kit by comparing the JETPEI and FUGENE systems. Transfecting PC3 cells with GFP and measuring expression with flow cytometry demonstrated that the FUGENE system was superior. To establish if transfection with the FUGENE system could be used as an alternative to nucleofection, the autologous serum and MegaCD40L immune responses assay was run in parallel with cells from the same EBV exposed donor. The experiment repeatedly demonstrated no meaningful difference in the background responses seen in the ELISpot between the two protein insertion methods. There was a significant difference between the ELISpot results with the nucleofected cells generating many more spots per well. These data would suggest that the cell death caused by nucleofection does not cause a strong response as measured by the assay and that it is more effective at delivering antigen to the B cell blast. Nucleofection was the favoured method of plasmid insertion.

Within this experiment we also aimed to identify the point at which the evolving CD40L stimulated B cells would deliver the best results in the assay. Developing CD40L stimulated B cells were sampled at the 1^{st}, 3^{rd} and 5^{th} passage. In the

nucleofected cells, there were strong responses after the first passage and these responses remained consistent through the third and fifth passage.

To investigate this further we took blood from an EBV exposed donor and developed CD40L stimulated B cells using both the murine L cell feeder layer and the MegaCD40L techniques. Cells were sampled at the 1^{st}, 2^{nd}, 3^{rd} and 4^{th} passage, nucleofected with BMLF-1 and the IFN-γ ELISpot assay undertaken. Those cells prepared with the MegaCD40L were demonstrating strong responses after the first passage but those cells prepared using the murine L cell feeder layer didn't start to generate meaningful responses until the fourth passage. The empty vector controls in the murine L cell feeder layer consistently had a much greater background spot count than those from the MegaCD40L controls.

6.2.2 Optimizing the standard operating procedure

During the B cell blast manufacture, we looked for phenotypic changes in the cells that could enable us to better understand and measure their development. We measured expression of the co-stimulatory molecules, CD80 and CD86 in the cell population during the sequential passages and found that B cell blasts grown with MegaCD40L expressed CD80 and CD86 much earlier than those B cell blasts grown using the murine monolayer. The changes in the expression of these co-stimulatory molecules mirrored the spot counts seen in the ELISpot assays. These data suggest a possible utility for the expression of CD80 and CD86 as markers for B cell blast development.

The accelerated development of B cell blasts with *Mega*CD40L caused us to look at how best to handle the PBMCs during the B cell blast manufacture process. The original assay involved the freezing of PBMCs and their subsequent thawing. To optimize the assay, we needed to characterize any phenotypic changes that were occurring in the PBMC population that might be affecting the ELISpot. Using blood from an EBV exposed donor and the BMLF-1 plasmid we compared the outcomes of PBMCs that had been frozen with PBMCs that we maintained in culture media. We found that PBMCs maintained in culture media had a higher background in the ELISpot than the frozen PBMCs. When we characterised the phenotype of the PBMCs, we found that there was an increase in expression of CD80 and CD86. Initially, the expression of these co-stimulatory markers was the same in frozen and the non-frozen PBMCs but after 24 hours, the increase became apparent. We therefore still recommend the freezing of the PBMCs.

Having established that the B cell blasts could be generated with a single passage in *Mega*CD40L containing media with autologous serum to help minimize the background readings, we wanted to identify the best insertion plasmid to use in the experiment. Four different plasmid constructs (SL1, ps1089A8, 4B6, 4A8) were generated, containing green fluorescent protein (GFP). This allowed us to identify which construct gave the greatest expression of GFP after nucleofection and at what time point after nucleofection was the expression greatest. The SL1 plasmid consistently generated the greatest expression of GFP and in all the plasmids the expression of GFP was greatest at 24 hours after nucleofection. These data confirmed that the ELISpot was best undertaken within 24 hours after nucleofection.

THE SL1 plasmid was then used to construct five new plasmids, replacing the GFP with one of five PSTAs (PSA, PSMA, PSCA, PAP and KLK4). These PSTAs are

commonly associated with prostate tumours and we wanted to explore immune responses to all 5 of the antigens as we are not going to know which, if any of these antigens were being expressed by the tumours of the trial subjects. Ways of approaching this experimentally included undertaking five separate nucleofections per subject on one set of B cell blasts, divide the subjects B cell blasts into 5 and nucleofect each cohort with a different plasmid or undertake a single nucleofection using all five plasmids in one set of B cell blasts per subject.

To test the impact on expression with multiple plasmid nucleofection in one go, we nucleofected B cell blasts from a single donor with the GFP expressing plasmid and compared GFP expression with B cell blasts nucleofected with the GFP expressing plasmid diluted down with even concentrations of plasmids expressing the 5 PSTAs. We found that there was a reduction in the percentage of GFP expressing cells from 49% to 41%. Repeating the experiment with plasmid expressing GFP and RFP rather than the plasmids expressing PSTAs allowed us to demonstrate that variably 29-36% of surviving B cell blasts nucleofected with both plasmids expressed both fluorescent proteins with 14-17% expressing GFP alone and 10-11% expressing RFP alone. Around 40% of the nucleofected cells did not express either of the fluorescent proteins. These data gave us confidence that our 5 PSTA nucleofected B cell blast population would have cells expressing one or more of the PSTAs.

We then undertook ELISpots on EBV exposed patients using B cell blasts nucleofected with BMLF-1 alone or BMLF-1 diluted with equal concentrations of plasmids expressing the 5 PSTAs so the overall quantity of plasmid used was the same. We found that the dilution of the BMLF-1 consistently yielded no significant difference in the spot counts on the ELISpot. In our experiments we found that multiple antigen nucleofection led to a variable expression of single and multiple

antigens in the target cells but this did not appear to impact on the ELISpot responses to any individual antigen. This could be explained by the fact that there are probably only a small number of T cells specific for the antigen being presented by the nucleofected B cell blast and so the sensitivity of the assay only deteriorates when the density of B cell blasts expressing that antigen is very low.

Using nucleofection in this way we would expect the expression of the nucleofected antigen to occur through the MHC 1 pathway. By adding the invariant chain to the antigen, we wanted to see if we could utilize the MHC II pathway.

The ELISpot assay was undertaken using blood samples from prostate cancer patients, nucleofecting with plasmids expressing the five PSTAs or the five PSTAs with invariant chain. Significant increases in the number of spots were consistently seen in those patients in which a strong response was seen. In those patients with weak responses, the difference was much less and was mostly not significant. As a consequence, the preferred assay would use the plasmids expressing PSTAs with the invariant chain.

Using a propidium iodide assay, we found that nucleofection with the 5 PSTAs was harmful to the B cell blasts with 9 to 25% of cells dying. We also identified that the effectiveness of nucleofection varied within the same cell population nucleofected with the same plasmids with expression varying from 25% - 48%. The impact of this variability on the validity of the assay needed exploring. By using a HA tag in the plasmids the nucleofected cells could be probed for the intracytoplasmic expression of HA using the commercial ‘fix and perm’ assay. This allowed the results to be normalized for nucleofection efficiency, as calculated from the percentage of the cells expressing of intracytoplasmic HA. By looking at the ELISpot results from sequentially diluted nucleofected B cell blasts, it was evident that the percentage of B

cell blasts expressing antigen had little impact on the number of spots seen in the assay. This would suggest that the prostate antigen specific T-cells are highly sensitive and able to identify prostate antigen expressing B cells even when in low concentrations. Provided the nucleofection efficiency is greater than 20% the assay is a reliable measure of prostate antigen specific T-cells.

6.3 Measuring immune responses in prostate cancer patients

Having demonstrated that multiple antigen nucleofection and subsequent ELISpot could be done with a protocol that minimized background responses, we wanted to apply the assay to prostate cancer patients. To explore this, we undertook a study titled, 'immune responses in adenocarcinoma of the prostate (IRAP). The aim of this study was to apply the assay in a clinical setting, allowing us to further refine the assay in preparation for the ADUP clinical trial. It would also give us an opportunity to catalogue immune responses in prostate cancer patients to help us interpret the results from the ADUP trial. By sampling patients with various disease grades and stages, the data would also help hypothesis formation for future studies. After suitable ethical and research and development approval, prostate patients were recruited from prostate clinic and from the ward.

It was reassuring to find that patient recruitment to the study was straightforward. Only 5 patients (12%) declined to participate demonstrating the acceptability of the tests to this patient population. There were no difficulties encountered with the retrieval of blood samples from theatres and outpatients and their transfer to the laboratories in the School for Cancer Sciences. The cellular yields from the samples were satisfactory for the completion of the assays with cells to spare. It was noted that

the yields from the theatre patients were slightly higher, and that could reflect their relative dehydration (as the patient has been starved for a general anaesthetic) or that they were a younger cohort of patients. In the forthcoming ADUP trial, the patients will have their primary sample taken in theatre and I expect the cellular yields to also be high.

The assay performed well in this patient population with consistently low background readings and consistent spot counts. We found evidence of immune responses to prostate tumour antigens in both patients with prostate cancer, and patients without prostate cancer. The levels of response were much less in the non-cancer patients but within the cancer patient cohort we found a wide variety of responses. In patients with low volume early stage disease the responses were brisk, as were the responses in patients on active surveillance. These findings are consistent with immuno-editing theory but, in the active surveillance patients, these findings could be attributed to the fact the patients are undergoing regular prostate biopsies. In those patients with advanced disease, the immune responses were far less evident. This could be due to exhaustion of the T cell populations targeting our panel of prostate specific tumour antigens and this may be contributing to the progression of the disease. It may be because of progressive loss of MHC expression in these tumours, their increasing heterogeneity or mechanisms within the progressive tumour microenvironment that there is suppression of this T cell population. It is possible that these patients have always had poor responses, and that has contributed to their disease progression.

It was interesting to observe a trend towards an increase in immune responses from low risk to intermediate risk patients before the drop in immune responses seen in high risk patients. Initially, this increase could be due to increase in disease volume without a major shift in cellular morphology driving greater immune responses. As

the disease progresses and the cellular morphology becomes more poorly differentiated the immune responses coincidently drop away. There will usually be greater disease bulk in these patients making it difficult to identify why the immune responses decrease. It could be morphology, tumour volume or both that is driving the exhaustion of the T cell response. It could also be that failure in the immune response facilitates the progression of prostate cancer in these patients.

This raises interesting considerations for the timing of immune–related therapies in prostate cancer. Clinical trials using the autologous dendritic cell based vaccine therapy, Sipuleucel T were undertaken on patients with advanced disease. If our observations are a genuine reflection of the patterns of immune responses in prostate cancer patients then these patients might be expected to benefit least from this type of treatment. If it were practical, it would be interesting to see how efficacious this treatment would be in patients with earlier staged disease. The ADUP trial will be recruiting patients at an earlier stage in their disease pathway than those in the Sipuleucel T trials and the disease bulk will be much less. The immune stimulating element of the treatment should benefit from this and it will be interesting to monitor the immune responses in these patients as their treatment progresses.

In the study, we were fortunate enough to get sequential samples on 10 patients. In those patients in whom there were no treatment modifications, the immune responses appear to be gradually declining. Those patients in whom a direct insult to the prostate is offered, there appears to be an upward trend in the immune responses. The most remarkable change in immune responses is seen in the patient who underwent HIFU. This observation is consistent with observations made in the literature relating to this treatment in the prostate and other organs. Given the coagulative necrosis caused by the HIFU, and the rim of thermally stressed but surviving tissues, there would be an

expectation to see immune responses mounted to antigens released within the treatment environment. It would be worth further study to see if this is a consistent feature for this treatment and if similar responses are seen in other thermotherapeutic modalities such as cryotherapy. If this is a consistent observation, it would be worth investigating whether neoadjuvant use of immune stimulants alongside thermotherapies can be used to enhance their efficacy, or even see them used in advanced disease to try and offer local disease control whilst delivering a systemic immune benefit.

If the assay were used serially in a patient it would be interesting to see if it could have a role in predicting outcome, particularly in those patients who would ordinarily select active surveillance. We have seen the gradual decline over time in patients on stable treatment, and this kind of assay could serve as an indicator for contemplating a treatment change or escalation. The limitations with the assay, would be in the cost and logisitics involved in delivering it in mainstream healthcare and, consequently, it is likely to remain a research tool.

6.4 Summary

In this thesis I have demonstrated the modified virus AdGMNR will infect prostate cancer cells and the NTR and GMCSF genes are expressed. Those cancer cells expressing NTR and subsequently exposed to CB1954 will die with neighbouring cells also dying, due to the bystander effect. The GMCSF produced by infected prostate cancer cells will induce morphological changes in PBMCs consistent differentiation into immature dendritic cells.

I have adapted an ELISpot assay that gave high background readings. Modifying the assay has minimized those background readings. The platform allows us to test for responses to a panel of 5 prostate specific cancer antigens. I expect this to increase the sensitivity of the assay.

I have used the assay on a number of prostate cancer patients at various disease stages and have been able to measure immune responses in a satisfactory way. The observed immune responses show consistencies with immune editing theory, and generates some good background data against which we can compare the results from the forthcoming ADUP trial as well as helping to formulate possible hypotheses to be tested in future studies.

6.5 Future work

We have started to recruit to the ADUP trial, and will start to generate data on the immune responses in these patients as they undergo treatment. I am keen to explore the immune responses in a larger cohort of prostate cancer patients undergoing treatment with a particular emphasis on thermotherapies. I would like to identify which treatments appear most immuno-stimulatory and would therefore lend themselves best to augmentation with immuno-modulating agents. These data would help us select treatments to take forward into clinical trials with neo-adjuvant immunotherapies.

7 References

American Cancer Society: "Cancer Facts & Figures 2003." Atlanta, GA: American Cancer Society, 2003.

Antica, M., L. Wu, and R. Scollay. "Stem cell antigen 2 expression in adult and developing mice." Immunol.Lett. 55.1 (1997): 47-51.

Antonarakis, E. S. et al. "Phase II, randomized, placebo-controlled trial of neoadjuvant celecoxib in men with clinically localized prostate cancer: evaluation of drug-specific biomarkers." J.Clin.Oncol. 27.30 (2009): 4986-93.

Asgari, K. et al. "Inhibition of the growth of pre-established subcutaneous tumor nodules of human prostate cancer cells by single injection of the recombinant adenovirus p53 expression vector." Int.J.Cancer 71.3 (1997): 377-82.

Ast, G. "Drug-targeting strategies for prostate cancer." Curr.Pharm.Des 9.6 (2003): 455-66.

Aumüller G, Groos S, Renneberg H, et al. Embryology and postnatal development of the prostate. In Pathology of the Prostate, Foster C , Bostwick D (eds). W. B. Saunders: New York, 1990; 1-17.

Ausubel, F. et al. Current Protocols in Molecular Biology, Volume 4. Wiley (1998)

Baggers, J., G. Ratzinger, and J. W. Young. "Dendritic cells as immunologic adjuvants for the treatment of cancer." J.Clin.Oncol. 18.23 (2000): 3879-82.

Banchereau, J. et al. "Immunobiology of dendritic cells." Annu.Rev.Immunol. 18 (2000): 767-811.

Banchereau, J. and R. M. Steinman. "Dendritic cells and the control of immunity." Nature 392.6673 (1998): 245-52.

Barry, M. J. et al. "Outcomes for men with clinically nonmetastatic prostate carcinoma managed with radical prostactectomy, external beam radiotherapy, or expectant management: a retrospective analysis." Cancer 91.12 (2001): 2302-14.

Bolla, M. et al. "Postoperative radiotherapy after radical prostatectomy: a randomised controlled trial (EORTC trial 22911)." Lancet 366.9485 (2005): 572-78.

Bonnotte, B. et al. "Intradermal injection, as opposed to subcutaneous injection, enhances immunogenicity and suppresses tumorigenicity of tumor cells." Cancer Res. 63.9 (2003): 2145-49.

Bostwick, D. G. "Grading prostate cancer." Am J.Clin Pathol. 102.4 Suppl 1 (1994): S38-S56.

Bouvier, M. and D. C. Wiley. "Importance of peptide amino and carboxyl termini to the stability of MHC class I molecules." Science 265.5170 (1994): 398-402.

Brandes, D., D. Kirchheim, and W. W. Scott. "Ultrastructure of the human prostate: Normal and neoplastic." Lab Invest 13 (1964): 1541-60.

Burgess, A. W. and D. Metcalf. "The nature and action of granulocyte-macrophage colony stimulating factors." Blood 56.6 (1980): 947-58.

Burnet, M. (1957). "Cancer—A Biological Approach: I. The Processes Of Control. II. The Significance of Somatic Mutation". Brit. Med. Jour. 1 (5022): 779–786.

Busch, R. et al. "Achieving stability through editing and chaperoning: regulation of MHC class II peptide binding and expression." Immunol.Rev. 207 (2005): 242-60.

Buttyan, R. et al. "Enhanced expression of the c-myc protooncogene in high-grade human prostate cancers." Prostate 11.4 (1987): 327-37.

Byar, D. P. and F. K. Mostofi. "Carcinoma of the prostate: prognostic evaluation of certain pathologic features in 208 radical prostatectomies. Examined by the step-section technique." Cancer 30.1 (1972): 5-13.

Carter, H. B., S. Piantadosi, and J. T. Isaacs. "Clinical evidence for and implications of the multistep development of prostate cancer." J.Urol. 143.4 (1990): 742-46.

Chen, X. et al. "Interferon alpha in combination with GM-CSF induces the differentiation of leukaemic antigen-presenting cells that have the capacity to stimulate a specific anti-leukaemic cytotoxic T-cell response from patients with chronic myeloid leukaemia." Br.J.Haematol. 111.2 (2000): 596-607.

Cher, M. L. et al. "Comparative genomic hybridization, allelic imbalance, and fluorescence in situ hybridization on chromosome 8 in prostate cancer." Genes Chromosomes.Cancer 11.3 (1994): 153-62.

Chomarat, P. et al. "IL-6 switches the differentiation of monocytes from dendritic cells to macrophages." Nat.Immunol. 1.6 (2000): 510-14.

Chomarat, P. et al. "TNF skews monocyte differentiation from macrophages to dendritic cells." J.Immunol. 171.5 (2003): 2262-69.

Chung-Faye, G. et al. Virus-directed, enzyme prodrug therapy with nitroimidazole reductase: a phase I and pharmacokinetic study of its prodrug, CB1954. Clin Cancer Res 7 (2001): 2662–2668

Classon, B. J. and L. Coverdale. "Mouse stem cell antigen Sca-2 is a member of the Ly-6 family of cell surface proteins." Proc.Natl.Acad.Sci.U.S.A 91.12 (1994): 5296-300.

Clements, J. A. et al. "The tissue kallikrein family of serine proteases: functional roles in human disease and potential as clinical biomarkers." Crit Rev.Clin.Lab Sci. 41.3 (2004): 265-312.

Culig, Z. et al. "Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor." Cancer Res. 54.20 (1994): 5474-78.

Cunha, G. R. et al. "The endocrinology and developmental biology of the prostate." Endocr.Rev. 8.3 (1987): 338-62.

Cutino, A. "Adaptation of the slit lamp for patients with large breasts." Ophthalmic Surg. 22.10 (1991): 623.

D'Amico, A. V. et al. "Cancer-specific mortality after surgery or radiation for patients with clinically localized prostate cancer managed during the prostate-specific antigen era." J.Clin Oncol 21.11 (2003): 2163-72.

Dearnaley, D. P. "Cancer of the prostate." BMJ 308.6931 (1994): 780-84.

Disis, M. L. “Granulocyte-Macrophage Colony-Stimulating Factor: An Effective Adjuvant.” Blood 88 **(**1996): 202-210.

for Protein and Peptide-Based Vaccines

DiPaola et al. Proceedings of the American Association Cancer Research 45: 1035 Abs # 4485, (2004)

Do, T. et al. "Salvage radiotherapy for biochemical and clinical failures following radical prostatectomy." Cancer J.Sci.Am. 4.5 (1998): 324-30.

Driessen, C. et al. "Cathepsin S controls the trafficking and maturation of MHC class II molecules in dendritic cells." J.Cell Biol. 147.4 (1999): 775-90.

Dunn, G. P. ey al.). "Cancer immunoediting: from immunosurveillance to tumor escape." Nature Immunology 3 (11): 991–998

Eder, J. P. et al. "A phase I trial of a recombinant vaccinia virus expressing prostate-specific antigen in advanced prostate cancer." Clin.Cancer Res. 6.5 (2000): 1632-38.

Effert, P. J. et al. "Alterations of the P53 gene are associated with the progression of a human prostate carcinoma." J.Urol. 147.3 Pt 2 (1992): 789-93.

Elgamal, A. A., M. J. Troychak, and G. P. Murphy. "ProstaScint scan may enhance identification of prostate cancer recurrences after prostatectomy, radiation, or hormone therapy: analysis of 136 scans of 100 patients." Prostate 37.4 (1998): 261-69.

Fang, D. X. et al. "Use of radioimmunoscintigraphy with indium-111-labeled CYT-356 (ProstaScint) scan for evaluation of patients for salvage brachytherapy." Tech.Urol. 6.2 (2000): 146-50.

Fishman, M. "A changing world for DCvax: a PSMA loaded autologous dendritic cell vaccine for prostate cancer." Expert.Opin.Biol.Ther. 9.12 (2009): 1565-75.

Fleetwood, A. J., A. D. Cook, and J. A. Hamilton. "Functions of granulocyte-macrophage colony-stimulating factor." Crit Rev.Immunol. 25.5 (2005): 405-28.

Fong, L. et al. "Potentiating endogenous antitumor immunity to prostate cancer through combination immunotherapy with CTLA4 blockade and GM-CSF." Cancer Res. 69.2 (2009): 609-15.

Fremont, D. H. et al. "Structures of an MHC class II molecule with covalently bound single peptides." Science 272.5264 (1996): 1001-04.

Gleason, D. F. "Classification of prostatic carcinomas." Cancer Chemother.Rep. 50.3 (1966): 125-28.

Gong, M. C. et al. "Prostate-specific membrane antigen (PSMA)-specific monoclonal antibodies in the treatment of prostate and other cancers." Cancer Metastasis Rev. 18.4 (1999): 483-90.

Gregorakis, A. K., E. H. Holmes, and G. P. Murphy. "Prostate-specific membrane antigen: current and future utility." Semin.Urol.Oncol. 16.1 (1998): 2-12.

Gu, Z. et al. "Prostate stem cell antigen (PSCA) expression increases with high gleason score, advanced stage and bone metastasis in prostate cancer." Oncogene 19.10 (2000): 1288-96.

Guinan, P. et al. "Adjuvant immunotherapy with bacillus Calmette-Guerin in prostatic cancer." Urology 14.6 (1979): 561-65.

Guinan, P. D. et al. "Adjuvant immunotherapy (BCG) in stage D prostate cancer." Am.J.Clin.Oncol. 5.1 (1982): 65-68.

Gutman EB, Sproul EE & Gutman AB 1936 Significance of increased phosphatase activity at the site of osteoplastic metastases secondary to carcinoma of the prostate gland. *American Journal of Cancer* **28** 485–495.

Hamilton, J. A. "Colony-stimulating factors in inflammation and autoimmunity." Nat.Rev.Immunol. 8.7 (2008): 533-44.

Heidenreich, A. et al. "EAU guidelines on prostate cancer." Eur.Urol. 53.1 (2008): 68-80.

Heiser, A. et al. "Autologous dendritic cells transfected with prostate-specific antigen RNA stimulate CTL responses against metastatic prostate tumors." J.Clin.Invest 109.3 (2002): 409-17.

Heiser, A. et al. "Induction of polyclonal prostate cancer-specific CTL using dendritic cells transfected with amplified tumor RNA." J.Immunol. 166.5 (2001): 2953-60.

Higano, C. S. et al. "Integrated data from 2 randomized, double-blind, placebo-controlled, phase 3 trials of active cellular immunotherapy with sipuleucel-T in advanced prostate cancer." Cancer 115.16 (2009): 3670-79.

Hirschowitz, A. S. and S. Kaplan. "Minimal dosage ketamine in paediatric dentistry." J.Dent.Assoc.S.Afr. 34.6 (1979): 321-24.

Hubert, R. S. et al. "STEAP: a prostate-specific cell-surface antigen highly expressed in human prostate tumors." Proc.Natl.Acad.Sci.U.S.A 96.25 (1999): 14523-28.

Huddart, R. A. et al. "Spinal cord compression in prostate cancer: treatment outcome and prognostic factors." Radiother.Oncol 44.3 (1997): 229-36.

Huggins C & Hodges CV 1941 Studies on prostatic cancer: the effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. *Cancer Research* **1** 293–297

Iversen, P. et al. "Casodex (bicalutamide) 150-mg monotherapy compared with castration in patients with previously untreated nonmetastatic prostate cancer: results from two multicenter randomized trials at a median follow-up of 4 years." Urology 51.3 (1998): 389-96.

Iversen, P. et al. "Casodex (bicalutamide) 150-mg monotherapy compared with castration in patients with previously untreated nonmetastatic prostate cancer: results from two multicenter randomized trials at a median follow-up of 4 years." Urology 51.3 (1998): 389-96.

Iversen, P. et al. "Bicalutamide monotherapy compared with castration in patients with nonmetastatic locally advanced prostate cancer: 6.3 years of followup." J.Urol. 164.5 (2000): 1579-82.

James, N. D. et al. "Systemic therapy for advancing or metastatic prostate cancer (STAMPEDE): a multi-arm, multistage randomized controlled trial." BJU.Int. 103.4 (2009): 464-69.

Jones, G. W. "Prostate cancer. Magnitude of the problem. American College of Surgeons database and epidemiologic study." Cancer 71.3 Suppl (1993): 887-90.

Jones, P. H. and F. M. Watt. "Separation of human epidermal stem cells from transit amplifying cells on the basis of differences in integrin function and expression." Cell 73.4 (1993): 713-24.

Kass, E. Et al. “Comparative studies of the effects of recombinant GM-CSF and GM-CSF administered via a poxvirus to enhance the concentration of antigen-presenting cells in regional lymph nodes.” Cytokine 12(7) (2000):960-71

Kaighn, M.E et al. “Establishment and characterization of a human prostatic carcinoma cell line (PC-3).” Invest Urol. 1979 Jul;17(1):16-23.

Kalluri, R. and M. Zeisberg. "Fibroblasts in cancer." Nat.Rev.Cancer 6.5 (2006): 392-401.

Kim, J. J. et al. "Molecular and immunological analysis of genetic prostate specific antigen (PSA) vaccine." Oncogene 17.24 (1998): 3125-35.

Kim, R. “Tumor-driven evolution of immunosuppressive networks during malignant progression.: Cancer Res 2006 Jun 1;66(11):5527-36.

Klotz, L. et al. "The efficacy and safety of degarelix: a 12-month, comparative, randomized, open-label, parallel-group phase III study in patients with prostate cancer." BJU.Int. 102.11 (2008): 1531-38.

Kumar, S. et al. "Neo-adjuvant and adjuvant hormone therapy for localised and locally advanced prostate cancer." Cochrane.Database.Syst.Rev.4 (2006): CD006019.

Kyprianou, N. and J. T. Isaacs. "Activation of programmed cell death in the rat ventral prostate after castration." Endocrinology 122.2 (1988): 552-62.

Lai, J. et al. "Direct progesterone receptor and indirect androgen receptor interactions with the kallikrein-related peptidase 4 gene promoter in breast and prostate cancer." Mol.Cancer Res. 7.1 (2009): 129-41.

LeDuc, I.E., The anatomy of the prostate and the pathology of early benign hypertrophy, *J. Urol*., **42**,1217,1939.

Lein, M. et al. "Complications, urinary continence, and oncologic outcome of 1000 laparoscopic transperitoneal radical prostatectomies-experience at the Charite Hospital Berlin, Campus Mitte." Eur.Urol. 50.6 (2006): 1278-82.

Lin, K. W., T. Jacek, and R. Jacek. "Dendritic cells heterogeneity and its role in cancer immunity." J.Cancer Res.Ther. 2.2 (2006): 35-40.

Lin, M. F. et al. "Purification and characterization of a new human prostatic acid phosphatase isoenzyme." Biochemistry 22.5 (1983): 1055-62.

Lipscomb, M. F. and B. J. Masten. "Dendritic cells: immune regulators in health and disease." Physiol Rev. 82.1 (2002): 97-130.

Lu, Y. "Viral based gene therapy for prostate cancer." Curr.Gene Ther. 1.2 (2001): 183-200.

Lukas, M. et al. "Human cutaneous dendritic cells migrate through dermal lymphatic vessels in a skin organ culture model." J.Invest Dermatol. 106.6 (1996): 1293-99.

Lunenfeld, B. "The ageing male: demographics and challenges." World J.Urol. 20.1 (2002): 11-16.

Lutz, M. B. and G. Schuler. "Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity?" Trends Immunol. 23.9 (2002): 445-49.

Maurisse, R. et al. ‘Comparative transfection of DNA into primary and transformed mammalian cells form different lineages.’ BMC Biotch 2010, 10:9 doi:10.1186/1472-6750-10-9

Mabjeesh, N. J., H. Zhong, and J. W. Simons. "Gene therapy of prostate cancer: current and future directions." Endocr.Relat Cancer 9.2 (2002): 115-39.

Madden, D. R. et al. "The three-dimensional structure of HLA-B27 at 2.1 A resolution suggests a general mechanism for tight peptide binding to MHC." Cell 70.6 (1992): 1035-48.

McNeal, J. E. et al. "Zonal distribution of prostatic adenocarcinoma. Correlation with histologic pattern and direction of spread." Am.J.Surg.Pathol. 12.12 (1988): 897-906.

McNeal, J. E. et al. "Zonal distribution of prostatic adenocarcinoma. Correlation with histologic pattern and direction of spread." Am.J.Surg.Pathol. 12.12 (1988): 897-906.

Meidenbauer, N. et al. "Generation of PSA-reactive effector cells after vaccination with a PSA-based vaccine in patients with prostate cancer." Prostate 43.2 (2000): 88-100.

Mellado, B. et al. "Phase II trial of short-term neoadjuvant docetaxel and complete androgen blockade in high-risk prostate cancer." Br.J.Cancer 101.8 (2009): 1248-52.

Merglen, A. et al. "Short- and long-term mortality with localized prostate cancer." Arch.Intern.Med. 167.18 (2007): 1944-50.

Messing, E. M. et al. "Immediate hormonal therapy compared with observation after radical prostatectomy and pelvic lymphadenectomy in men with node-positive prostate cancer." N.Engl.J.Med. 341.24 (1999): 1781-88.

Mickey, D. D. et al. “Characterization of a human prostate adenocarcinoma cell line (DU 145) as a monolayer culture and as a solid tumor in athymic mice.” Prog Clin Biol Res. 1980;37:67-84.

Miller AD, Rosman GJ. Improved retroviral vectors for gene transfer and expression. *BioTechniques* 1989;7:980-88

Mincheff, M. et al. "Naked DNA and adenoviral immunizations for immunotherapy of prostate cancer: a phase I/II clinical trial." Eur.Urol. 38.2 (2000): 208-17.

Mize, G. J., W. Wang, and T. K. Takayama. "Prostate-specific kallikreins-2 and -4 enhance the proliferation of DU-145 prostate cancer cells through protease-activated receptors-1 and -2." Mol.Cancer Res. 6.6 (2008): 1043-51.

Nakamura, T. M. et al. "Telomerase catalytic subunit homologs from fission yeast and human." Science 277.5328 (1997): 955-59.

Nazareth, L. V. and N. L. Weigel. "Activation of the human androgen receptor through a protein kinase A signaling pathway." J.Biol.Chem. 271.33 (1996): 19900-07.

Neeley, Y. C. et al. "Partially circumventing peripheral tolerance for oncogene-specific prostate cancer immunotherapy." Prostate 68.7 (2008): 715-27.

Noguchi, M. et al. "Induction of cellular and humoural immune responses to tumor cells and peptides in HLA-A24 positive hormone-refractory prostate cancer patients by peptide vaccination." Prostate 57.1 (2003): 80-92.

Olson, W. C., W. D. Heston, and A. K. Rajasekaran. "Clinical trials of cancer therapies targeting prostate-specific membrane antigen." Rev.Recent Clin.Trials 2.3 (2007): 182-90.

Olweus, J. et al. "Dendritic cell ontogeny: a human dendritic cell lineage of myeloid origin." Proc.Natl.Acad.Sci.U.S.A 94.23 (1997): 12551-56.

Papsidero, L. D. et al. "Isolation of prostatic acid phosphatase-binding immunoglobulin G from human sera and its potential for use as a tumor-localizing reagent." Cancer Res. 40.8 Pt 2 (1980): 3032-35.

Parker, C. "Active surveillance: towards a new paradigm in the management of early prostate cancer." Lancet Oncol. 5.2 (2004): 101-06.

Partin, A. W. et al. "The use of prostate specific antigen, clinical stage and Gleason score to predict pathological stage in men with localized prostate cancer." J.Urol. 150.1 (1993): 110-14.

Partin, A. W. et al. "The use of prostate specific antigen, clinical stage and Gleason score to predict pathological stage in men with localized prostate cancer." J.Urol. 150.1 (1993): 110-14.

Patel, P. et al. A phase I/II clinical trial in localized prostate cancer of an adenovirus expressing nitroreductase with CB1954 [correction of CB1984].Mol Ther. 17(7) (2009): 1292-9.

Petrylak, D. P. et al. "Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer." N.Engl.J.Med. 351.15 (2004): 1513-20.

Pienta, K. J. "The epidemiology of prostate cancer: clues for chemoprevention." In Vivo 8.3 (1994): 419-22.

Pieters, J. "MHC class II-restricted antigen processing and presentation." Adv.Immunol. 75 (2000): 159-208.

Potters, L. et al. "Monotherapy for stage T1-T2 prostate cancer: radical prostatectomy, external beam radiotherapy, or permanent seed implantation." Radiother.Oncol. 71.1 (2004): 29-33.

Potters, L. et al. "Monotherapy for stage T1-T2 prostate cancer: radical prostatectomy, external beam radiotherapy, or permanent seed implantation." Radiother.Oncol. 71.1 (2004): 29-33.

Raivich, G. and R. Banati. "Brain microglia and blood-derived macrophages: molecular profiles and functional roles in multiple sclerosis and animal models of autoimmune demyelinating disease." Brain Res.Brain Res.Rev. 46.3 (2004): 261-81.

Rammensee, H. G. "Chemistry of peptides associated with MHC class I and class II molecules." Curr.Opin.Immunol. 7.1 (1995): 85-96.

Ramsay, A. J. et al. "Kallikrein-related peptidase 4 (KLK4) initiates intracellular signaling via protease-activated receptors (PARs). KLK4 and PAR-2 are co-expressed during prostate cancer progression." J.Biol.Chem. 283.18 (2008): 12293-304.

Randolph, G. J. et al. "Differentiation of monocytes into dendritic cells in a model of transendothelial trafficking." Science 282.5388 (1998): 480-83.

Reiter, R. E. et al. "Prostate stem cell antigen: a cell surface marker overexpressed in prostate cancer." Proc.Natl.Acad.Sci.U.S.A 95.4 (1998): 1735-40.

Ross, S. et al. "Prostate stem cell antigen as therapy target: tissue expression and in vivo efficacy of an immunoconjugate." Cancer Res. 62.9 (2002): 2546-53.

Roumeguere, T. et al. "Radical prostatectomy: a prospective comparison of oncological and functional results between open and laparoscopic approaches." World J.Urol. 20.6 (2003): 360-66.

Ryan, C. J. and E. J. Small. "Role of secondary hormonal therapy in the management of recurrent prostate cancer." Urology 62 Suppl 1 (2003): 87-94.

Saad, F. et al. "A randomized, placebo-controlled trial of zoledronic acid in patients with hormone-refractory metastatic prostate carcinoma." J.Natl.Cancer Inst. 94.19 (2002): 1458-68.

Salgaller M. " Prostate cancer immunotherapy at the dawn of the new millennium." Expert Opin Investig Drugs 9 (2000):1217–1229

Sambrook et al. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press (1989)

Sar, M. et al. "Immunohistochemical localization of the androgen receptor in rat and human tissues." Endocrinology 127.6 (1990): 3180-86.

Schroder, F. H. et al. "Screening and prostate-cancer mortality in a randomized European study." N.Engl.J.Med. 360.13 (2009): 1320-28.

Seidenfeld, J. et al. "Relative effectiveness and cost-effectiveness of methods of androgen suppression in the treatment of advanced prostate cancer." Evid.Rep.Technol.Assess.(Summ.).4 (1999): i-246, I1.

Seidenfeld, J. et al. "Single-therapy androgen suppression in men with advanced prostate cancer: a systematic review and meta-analysis." Ann.Intern.Med. 132.7 (2000): 566-77.

Seliger, B. et al. "Association of HLA class I antigen abnormalities with disease progression and early recurrence in prostate cancer." Cancer Immunol.Immunother. (2009).

Selley, S. et al. "Diagnosis, management and screening of early localised prostate cancer." Health Technol.Assess. 1.2 (1997): i, 1-i,96.

Shortman, K. and C. Caux. "Dendritic cell development: multiple pathways to nature's adjuvants." Stem Cells 15.6 (1997): 409-19.

Small, E. J. et al. "Immunotherapy of hormone-refractory prostate cancer with antigen-loaded dendritic cells." J.Clin.Oncol. 18.23 (2000): 3894-903.

Small, E. J. et al. "Therapy of advanced prostate cancer with granulocyte macrophage colony-stimulating factor." Clin.Cancer Res. 5.7 (1999): 1738-44.

Sobin, L. H. "Urological Tumours." 6th ed. Hoboken, New Jersey: John Wiley & Sons, 2002.

Solheim, J. C. "Class I MHC molecules: assembly and antigen presentation." Immunol.Rev. 172 (1999): 11-19.

Sramkoski, R.M. et al. "A new human prostate carcinoma cell line, 22Rv1." In Vitro Cellular & Developmental Biology - Animal. July–August 1999, Volume 35, Issue 7, pp 403-409

Steinbrink, K. et al. "Interleukin-10-treated human dendritic cells induce a melanoma-antigen-specific anergy in CD8(+) T cells resulting in a failure to lyse tumor cells." Blood 93.5 (1999): 1634-42.

Steiner, M. S. et al. "Antisense c-myc retroviral vector suppresses established human prostate cancer." Hum.Gene Ther. 9.5 (1998): 747-55.

Steinman, R. M. et al. "The sensitization phase of T-cell-mediated immunity." Ann.N.Y.Acad.Sci. 546 (1988): 80-90.

Stephenson, A. J. and J. A. Eastham. "Role of salvage radical prostatectomy for recurrent prostate cancer after radiation therapy." J.Clin.Oncol. 23.32 (2005): 8198-203.

Stephenson, A. J. and J. A. Eastham. "Role of salvage radical prostatectomy for recurrent prostate cancer after radiation therapy." J.Clin.Oncol. 23.32 (2005): 8198-203.

Stephenson, S. A. et al. "Localization of a new prostate-specific antigen-related serine protease gene, KLK4, is evidence for an expanded human kallikrein gene family cluster on chromosome 19q13.3-13.4." J.Biol.Chem. 274.33 (1999): 23210-14.

Swisher, S. G. et al. "Adenovirus-mediated p53 gene transfer in advanced non-small-cell lung cancer." J.Natl.Cancer Inst. 91.9 (1999): 763-71.

Takayama, T. K. et al. "Characterization of hK4 (prostase), a prostate-specific serine protease: activation of the precursor of prostate specific antigen (pro-PSA) and single-chain urokinase-type plasminogen activator and degradation of prostatic acid phosphatase." Biochemistry 40.50 (2001): 15341-48.

Takayama, T. K. et al. "Characterization of hK4 (prostase), a prostate-specific serine protease: activation of the precursor of prostate specific antigen (pro-PSA) and single-chain urokinase-type plasminogen activator and degradation of prostatic acid phosphatase." Biochemistry 40.50 (2001): 15341-48.

Tannock, I. F. et al. "Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer." N.Engl.J.Med. 351.15 (2004): 1502-12.

Tasch, J. et al. "A unique folate hydrolase, prostate-specific membrane antigen (PSMA): a target for immunotherapy?" Crit Rev.Immunol. 21.1-3 (2001): 249-61.

Tazi, H. et al. "Spinal cord compression in metastatic prostate cancer." Eur.Urol 44.5 (2003): 527-32.

Touijer, K. et al. "Comprehensive prospective comparative analysis of outcomes between open and laparoscopic radical prostatectomy conducted in 2003 to 2005." J.Urol. 179.5 (2008): 1811-17.

Ueda, T. et al. "Ligand-independent activation of the androgen receptor by interleukin-6 and the role of steroid receptor coactivator-1 in prostate cancer cells." J.Biol.Chem. 277.41 (2002): 38087-94.

Uno, T. et al. Eradication of established tumors in mice by a combination antibody-based therapy.Nat Med. 12(6) (2006): 693-8.

Urba, W. J. et al. "Treatment of biochemical recurrence of prostate cancer with granulocyte-macrophage colony-stimulating factor secreting, allogeneic, cellular immunotherapy." J.Urol. 180.5 (2008): 2011-17.

Vihko, P. "Human prostatic acid phosphatases: purification of a minor enzyme and comparisons of the enzymes." Invest Urol. 16.5 (1979): 349-52.

Viney, R. et al. "Ultrasensitive prostate specific antigen assay following laparoscopic radical prostatectomy--an outcome measure for defining the learning curve." Ann.R.Coll.Surg.Engl. 91.5 (2009): 399-403.

Wang, M. C. et al. "Purification of a human prostate specific antigen." Invest Urol. 17.2 (1979): 159-63.

Wang, W. et al. "Kallikrein-related peptidase-4 initiates tumor-stroma interactions in prostate cancer through protease-activated receptor-1." Int.J.Cancer (2009).

Wang, X. et al. "Targeted treatment of prostate cancer." J.Cell Biochem. 102.3 (2007): 571-79.

Ward, A. M., J. W. Catto, and F. C. Hamdy. "Prostate specific antigen: biology, biochemistry and available commercial assays." Ann.Clin.Biochem. 38.Pt 6 (2001): 633-51.

Wefer, A. E. et al. "Sextant localization of prostate cancer: comparison of sextant biopsy, magnetic resonance imaging and magnetic resonance spectroscopic imaging with step section histology." J.Urol. 164.2 (2000): 400-04.

Welz, S. et al. "Surgery vs. radiotherapy in localized prostate cancer. Which is best?" Radiat.Oncol. 3 (2008): 23.

Wernert, N., G. Seitz, and T. Achtstatter. "Immunohistochemical investigation of different cytokeratins and vimentin in the prostate from the fetal period up to adulthood and in prostate carcinoma." Pathol.Res.Pract. 182.5 (1987): 617-26.

Witmer-Pack, M. D. et al. "Granulocyte/macrophage colony-stimulating factor is essential for the viability and function of cultured murine epidermal Langerhans cells." J.Exp.Med. 166.5 (1987): 1484-98.

Xu, J. et al. "Identification and characterization of prostein, a novel prostate-specific protein." Cancer Res. 61.4 (2001): 1563-68.

Xue, B. H. et al. "Induction of human cytotoxic T lymphocytes specific for prostate-specific antigen." Prostate 30.2 (1997): 73-78.

Xylinas, E. et al. "[Oncologic and functional outcomes after radical prostatectomy in T3 prostate cancer]." Prog.Urol. 19.5 (2009): 285-90.

Yam, L. T. "Clinical significance of the human acid phosphatases: a review." Am.J.Med. 56.5 (1974): 604-16.

Younes, P. "[MRI of prostate cancer]." Presse Med. 36.12 Pt 3 (2007): 1935-46.

Young, J. G.et al. Combining gene and immunotherapy for prostate cancer. Prostate Cancer and Prostatic Diseases 11(2008):187-193

Zhou, Y. et al. "Serological cloning of PARIS-1: a new TBC domain-containing, immunogenic tumor antigen from a prostate cancer cell line." Biochem.Biophys.Res.Commun. 290.2 (2002): 830-38.