Profiling Tumour Infiltrating Lymphocytes in Prostate Cancer

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Abstract

The purpose of this study was to understand the T cell processes that take place in prostate pathology. The subset of T cells and their functional and activational status was determined using flow cytometry and immunohistochemistry. Furthermore the use of androgen deprivation therapy (ADT) on the impact on T cells was also determined. The three main conditions of prostate pathology investigated were benign prostatic hyperplasia (BPH), chronic abacterial prostatitis (CAP) and prostate cancer (PCa).

Patients with BPH were used as the control group. The results in CAP showed a trend of an active immune process, where there was an influx of CD4+ T lymphocytes cells (9.45%, p 0.1407) in prostate tissue and a reduction in T regulatory (Treg) cells (2.32%, p 0.0675) in the tissue and the blood (3.13%, p 0.0042). In PCa tissue there was an influx of both CD4+ T lymphocytes (9.63%, p 0.0296) and CD8+ lymphocytes (12.81%, p 0.0063). Correspondingly a trend was seen with an increase in Treg cells in prostate tissue (2.12%, p 0.2480). This resulted in the ratios between CD4:CD8, CD4:Treg and CD8:Treg in PCa to be similar to BPH indicating a net homeostasis in immune action. The use of Treg cells as a prognostic indicator was compared to current pathological markers, where a higher number of Treg cells was seen in patients with extraprostatic spread (3.52%, p 0.0371), seminal vesicle extension (10.74%, p 0.0249), PSA relapse (4.39%, p 0.0162) and higher Gleason score (3.89%, p 0.0376).

Despite showing a homeostatic picture in PCa, the functional status of CD8+ T lymphocytes was assessed. There were lower proportions of naïve T cells (9.86%, p 0.0068) and central memory T cells (8.60%, p 0.0258) in PCa tissue. While there was an increase in terminal effector T cells (12.06%, p 0.0019) and effector memory T cells (8.63%, p 0.0253). This implied that the tumour cells

had been recognized and a shift in the dynamics towards a local cytotoxic environment. These cytotoxic T lymphocytes were activated as demonstrated by increased expression of CD69 (13.35%, p 0.0083). Despite showing activation, the effectiveness of these cells in the implementation of an anti-tumour response was assessed by evaluating the distribution of cytokines IFNγ, TGFβ and IL-10. However no clear pattern was seen, but it will require further investigation with a larger sample population.

The use of ADT has been shown to reverse thymic involution and restore peripheral T cell population. However from this study there was no difference seen in the subset numbers, functional and activational status of T lymphocytes.

From this study, the profile of prostatic pathology is better understood. The T cell profile especially in the in the local tumour environment of PCa shows a dynamic process between cytotoxic and immunosuppressive cells. This will allow for the development of further studies to understand the immune interaction with PCa.

Declaration

This is to certify that:

i) the thesis comprises only my original work towards the degree of doctorate of Medicine

ii) due acknowledgement has been made in the text to all other material used

iii) the thesis is fewer than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Signature:

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Nieroshan Rajarubendra

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Abbreviations

3D-CRT	Three-dimensional conformal radiation therapy
γδT	Gamma delta T cell
ACT	α1-antichymotrypsin
ADT	Androgen deprivation therapy
AICD	Activation induced cell death
APC	Antigen presenting cell
AR	Androgen receptor
A2M	α2-macroglobulin
BPH	Benign prostatic hyperplasia
CAP	Chronic abacterial prostatitis
CCR7	CC-chemokine receptor 7
CD62L	L-selectin
COX-2	Cyclooxygenase-2
CMV	Cytomegalovirus
CT	Computed tomography
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
DC	Dendritic cell
DHT	Dihydrotestosterone
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
DRE	Digital rectal examination
EBV	Epstein-Barr virus
ER	Oestrogen receptor
ERSPC	European randomised study of screening in prostate cancer
FCS	Foetal calf serum
FoxP3	Forkhead-winged helix transcription factor
FSC	Forward scatter
FSH	Follicle-stimulating hormone
fTh	Follicular T helper cell
GITR	Glucocorticoid-induced tumour necrosis factor family-related gene
GM-CSF	Granulocyte macrophage-colony stimulating factor
Gy	Gray
H&E	Haematoxylin and Eosin
HLA	Human leukocyte antigen
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HSV	Human herpes virus
IDO	Indoleamine 2,3-dioxygenase
IFNγ	Interferon γ
IHC	Immunohistochemistry
IGRT	Image-guided radiotherapy
IL	Interleukin

IMRT	Intensity modulated radiation therapy
IPEX	Immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
IPSS	International Prostate Symptoms Score
LAG-3	Lymphocyte activation gene-3
LAMP-1	Lysosomal-associated membrane protein-1
LH	Luteinizing hormone
LHRH	Luteinizing hormone-releasing hormone
MAIT	Mucosal-associated invariant T cell
MCH	Major-histocompatibility complex
MDSC	Myeloid-derived suppressor cells
MQ	Milli-Q water
MR1	Monomorphic MHC class I like related molecule
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
NIH	National institute of health
NK	Natural killer cell
NKT	Natural killer T cell
NO	Nitric oxide
NOS	Nitric oxide synthase
NSAID	Non-steroidal anti-inflammatory drugs
PAP	Prostatic acid phosphatase
PBMC	Peripheral blood mononuclear cell

PBS	Phosphate-buffered saline
PCa	Prostate cancer
PD1	Programmed death-1
PET	Positron emission tomography
PIA	Proliferative inflammatory atrophy
PIN	Prostatic intraepithelial neoplasia
PLCO	Prostate, lung, colorectal and ovarian cancer screening trial
PPMT	Pre-post-massage test
PSA	Prostate specific antigen
Q_{max}	Peak flow rate
RNase L	Ribonuclease L
RPMI	Roswell Park Memorial Institute medium
SSC	Side scatter
TCR	T cell receptor
TGFβ	Transforming growth factor β
Th1	Type 1 helper cell
Th2	Type 2 helper cell
Th17	T helper 17 cell
TIL	Tumour infiltrating lymphocyte
TNF	Tumour necrosis factor
TNM	Tissue, Node and Metastasis
TRAMP	Transgenic adenocarcinoma of the mouse prostate
Treg	T regulatory cell

TRUS	Transrectal ultrasound
VEGF	Vascular endothelial growth factor
XLAAD	X-linked autoimmune-allergic dysregulation
XMRV	Xenotropic murine leukaemia virus
XRT	External beam radiotherapy

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Dedication

To Gopika and my family

Thank you for believing in me.

1.0 Literature Review

Prostate cancer (PCa) is a prevalent condition that affects both the individual and the community at large. Despite increasing awareness, there is much to learn and understand about this disease. A major component is the role of the immune system and its interaction with the cancer cells. This study assesses the influence of T lymphocytes in the setting of prostate pathology.

1.1 The prostate

The prostate is a gland that is located at the junction between the bladder and urethra of males. Its normal volume is approximately 30g and resembles the shape of a walnut (refer to figure 1.1).

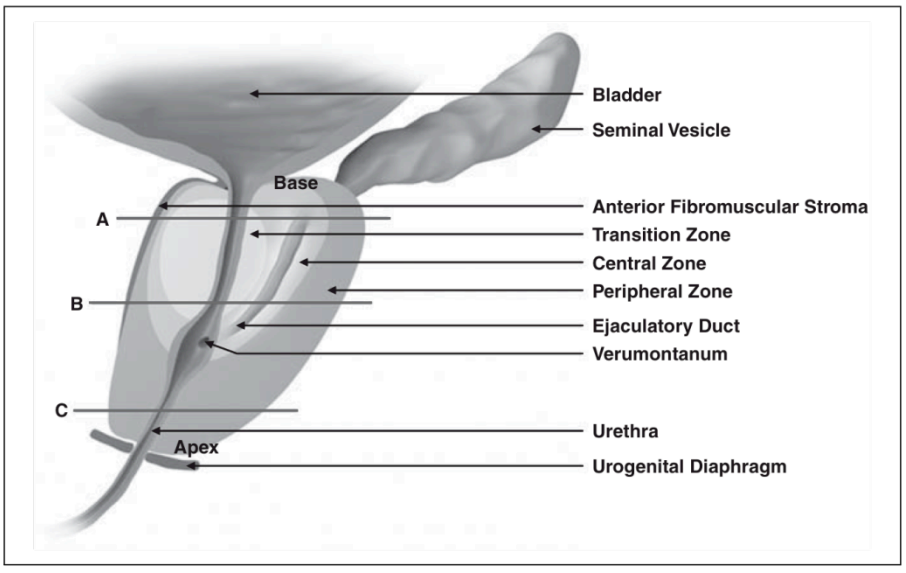


Figure 1.1 Anatomy of the prostate. The prostate gland located between the bladder and the urethra [1].

The prostate can be considered to have three general sections; base, middle and apex. It encompasses the urethra and this length of urethra is called the prostatic urethra. The base of the prostate is adjoined to the bladder and the apex is continuous with the penile urethra. The seminal vesicles store

spermatozoa and are located posteriorly adjoining the ejaculatory ducts. The vas deferens joins the ejaculatory ducts and delivers spermatozoa from the testicles. The prostatic urethra amalgamates with the two ejaculatory ducts at the verumontanum. As a result the prostatic urethra has both urine and semen passing through it. The ejaculate is expelled by the contraction of the pelvic floor muscles that surround the prostate.

The prostate is further divided into three zones which are peripheral, central and transitional zones (refer to figure 1.2). The peripheral zone is the largest zone comprising about 60 to 70% of the prostate and it is located posteriorly and encompasses the distal urethra. The central zone occupies 25% of the gland and it surrounds the ejaculatory ducts. The transitional zone is approximately 5% of the gland it located around the proximal urethra. The majority of PCa occurs in the peripheral zones. It occurs less frequently in the transitional zones and seldom in the central zones [2].

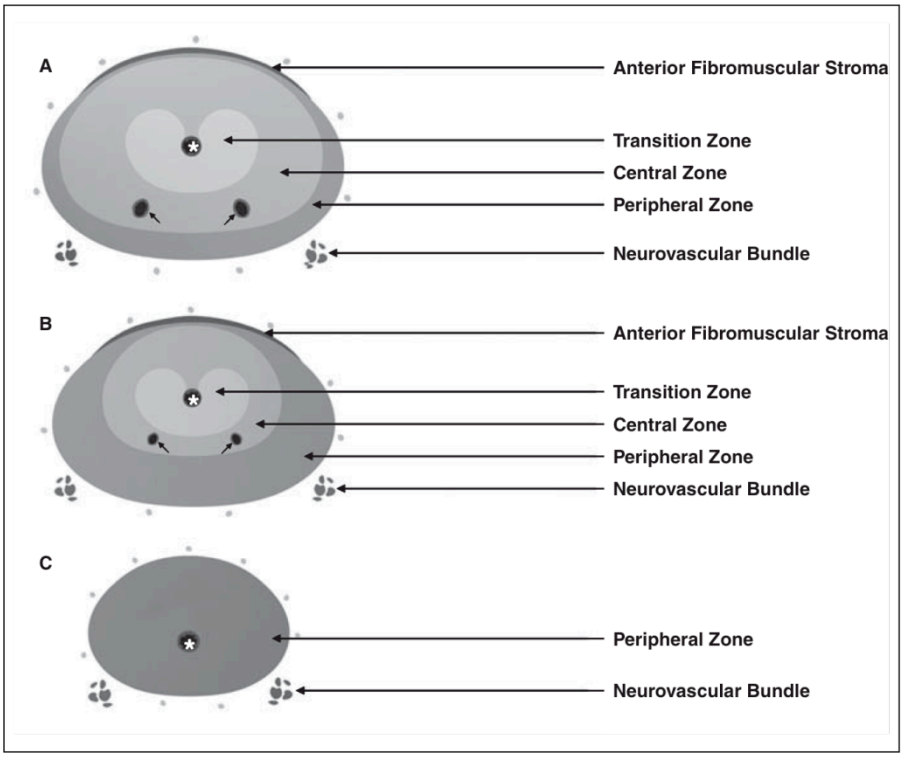


Figure 1.2 Zones of the prostate. Cross-sections depicting zones of the prostate at different levels. Level A is at the base, level B is at the middle section and level C is at the apex [1]. The base is close to the bladder neck while the apex continues with the penile urethra.

The prostate is a tubuloalveolar exocrine gland. It is composed of branched tubulo-acinar glands embedded in fibromuscular stroma. The epithelium of the prostate contains a double layer of cells consisting of tall columnar secretary cells with basal nuclei. The second layer of cells are flattened basal cells, which function to replace the dead secretory cells [3].

The function of the prostate is to provide secretions for the nourishment of sperm and assists in liquefaction of semen. The volume of fluid secreted accounts for approximately 25-30% of the seminal fluid. The purpose of producing the alkaline fluid is to neutralize the acidic environment in the vaginal tract enabling the spermatozoa to have a longer time to survive. The majority of the alkaline fluid is produced in the seminal vesicles and this fluid along with the majority of the spermatozoa is expelled first during ejaculation. However most of the prostatic fluid is secreted last with a few spermatozoa with better motility and longer lifespan for improving the chance of conception.

The prostate is therefore an important accessory sex organ with an anatomical and physiological role in reproduction. However there are three main diseases that may affect the prostate that result in significant morbidity or mortality. These conditions are benign prostatic hyperplasia (BPH), chronic abacterial prostatitis (CAP) and PCa.

1.2 Benign Prostatic Hyperplasia

Benign prostatic hyperplasia is a condition leading to enlargement of the prostate gland. Here there is hyperplasia of the stromal cells which are the connective tissue and the epithelial cells which are the cells that line the lumen of ducts that are located in the periurethral (transition zone) region in a nodular pattern, results in the compression of the urethra. The human prostate is unique in that it has a capsule. Hyperplasia of prostatic tissue in this confined

capsule results in increased obstruction of the urethra. This narrowing leads to the characteristic obstructive lower urinary tract symptoms.

The incidence of BPH is very common as the age of men increases. Histological evidence has shown that it affects 10% of men in their 30s, 20% in their 40s, 50% to 60% in their 60s, 80% in their 70s and 90% of men in their 80s [4]. Clinically the number of men who are symptomatic is less. For instance 40% of men in their 60's have symptoms secondary to BPH [5]. Apart from increasing age, other risk factors for the development of BPH are normal testosterone levels, race and genetic and environmental factors such as dietary intake [6].

Androgens are important in the growth of the prostate. Men who are castrated before puberty or have impaired androgen production due to genetic conditions such as Klinefelter syndrome, testicular damage such as trauma, chemo/radiotherapy and pituitary disorders do not develop BPH [7]. Testosterone is converted to the more powerful dihydrotestosterone (DHT) by two isozymes of 5α-reductase (type I and type II). Type I 5α–reductase is mainly found in extraprostatic tissues such as skin and liver whereas type II 5α–reductase is predominantly in the prostate and it is inhibited by 5α–reductase inhibitors.

There is emerging evidence of an important role of oestrogen in normal prostate tissue homeostasis. Two oestrogen receptors (ER), alpha and beta have been identified to influence prostate growth. Activation of ER-alpha leads for proliferation and inflammation while activation of ER-beta leads to antiproliferative effects to counter the actions of androgens on the epithelium [8, 9].

More men develop symptoms from BPH as their age increases [10-13]. For example, a survey conducted in Minnesota of men from 40 to 79 years of age found that symptoms increased from 13% to 28% [4]. The symptoms men experience with BPH are weak urinary stream, hesitancy, urgency, frequency, nocturia, incomplete bladder emptying, incontinence, dysuria and post-void dribbling. A validated index that is used to assess the severity of symptoms in patients with voiding dysfunction is the International Prostate Symptoms Score (IPSS). This questionnaire defines the level of hindrance the symptoms are causing the patient and allows treatment response to be monitored.

The symptoms are a result of poor bladder emptying due bladder outflow obstruction by the prostate. This leads to urine stasis which further predisposes the patient to urinary tract infections, bladder stone formation and acute urinary retention.

The European Urology Association guidelines recommend the clinical history, physical examination, and the use of IPSS to assist in making the diagnosis of BPH. A digital rectal examination (DRE) is performed to assess the size and texture of the prostate gland and a Prostate Specific Antigen (PSA) blood test is performed to assess the risk of prostate cancer. Urinalysis is performed to ensure no urinary tract infection is present. Diaries can be used to document frequency and volume of urine passed. In patients with long-standing urinary dysfunction, serum creatinine should be checked to assess for renal impairment. Imaging modalities such as renal tract ultrasound are used to rule out anatomical anomalies. Functional studies such as uroflowmetry, post-void residual volume and urodynamics provide information on the mechanics of the outflow tract.

The management for men with low to moderate symptoms is by modifying their lifestyle and monitoring their problems periodically. Lifestyle changes such as reducing fluid intake especially before bed and moderating caffeine. Also

reviewing medications or altering their timing, for example, diuretics, can relieve symptoms.

The medical management of BPH is mainly by two types of agents. These are 5α–reductase inhibitors and α-blockers. 5α–reductase inhibitors reduce the amount of DHT in the prostate and as a result inhibit the growth of the prostate. Treatment with 5α–reductase inhibitors has been shown to reduce the prostate size by 20-30% resulting in improved urinary flow and improve symptom score by 15% [14-17]. However the full effect of these inhibitors is seen six months after commencement of therapy. Alpha-blockers function by relaxing the smooth muscle in the prostate and bladder neck resulting in reduced resistance to urine flow. The effects are faster than the use of 5α–reductase inhibitors but have larger side effect profiles most notably orthostatic hypotension.

Surgical intervention is recommended for men with moderate to severe symptoms that have not responded to medical management. The two main surgical treatments for BPH are transurethral prostatectomy and open prostatectomy. TURP is the current gold standard of surgical care. Sections of the prostate are removed by electrocautery or laser via the urethra. An open prostatectomy may be performed on large prostates that are greater than 100cc. The success rate of surgical intervention in relieving the lower urinary tract symptoms is 70% [18].

1.3 Chronic abacterial prostatitis

Chronic abacterial prostatitis or chronic pelvic pain syndrome is one of the subsets of prostatitis. The National Institute of Health (NIH) in 1999 released a consensus on the classification of prostatitis (refer to table 1.1) [19]. CAP was described as chronic pelvic pain and urinary symptoms without the presence of a urinary tract infection.

Category	Symptoms
I. Acute bacterial prostatitis	Associated with severe symptoms of prostatitis, systemic infection, and acute bacterial urinary tract infection
II. Chronic bacterial prostatitis	Caused by chronic bacterial infection of the prostate with or without symptoms of prostatitis and usually with recurrent urinary tract infections caused by the same bacterial strain
III. CAP or Chronic pelvic pain syndrome Subdivided into: • IIIa Inflammatory • IIIb non-inflammatory	Characterized by symptoms of chronic pelvic pain and possibly symptoms on voiding in the absence of urinary tract infection
IV. Asymptomatic inflammatory prostatitis	Characterized by evidence of inflammation of the prostate in the absence of genitourinary tract symptoms; an incidental finding during evaluation for other conditions, such as infertility or elevated serum prostate-specific antigen levels

Table 1.1 The NIH classification of prostatitis [19].

Of the four subtypes of prostatitis, CAP accounts for 90-95% of cases [20]. In the United States, 8% of urologist visits and 1% of primary care physician visits are for ailments relating to CAP [21]. It can be present in men of any age but is most prevalent in men between the ages of 36 to 50 [22].

A diagnosis of CAP requires the duration of symptoms to be at least three months. The main symptom of CAP is pain, commonly located in the lower back, tip of the penis, suprapubic region or perineal region. Men can also suffer from painful ejaculation as well as dysuria. Obstructive symptoms such as

weak urinary stream, frequency and incomplete bladder emptying are also associated.

The symptoms of CAP can severe enough to disrupt quality of life. The debilitating effects have been comparable to those who suffer from congestive cardiac failure, diabetes mellitus, angina and Crohn's disease [23, 24].

There is no generally accepted known aetiology of the pathogenesis of CAP. There are many hypotheses for its cause and they are:

- High pressure voiding resulting in pain and lower urinary tract symptoms are due to obstruction of the outflow tact. This is thought to be due to bladder neck dyssynergia, detrusor sphincter dysfunction or urethral strictures resulting in high pressure voiding
- Intraprostatic ductal reflux due to anatomical abnormalities distal to the prostate resulting in high-pressure turbulent voiding. The urinary reflux in the prostate precipitates a sterile inflammatory response [25-27]
- Fastidious microbes that are not identified using standard isolation methods
- Immunological reaction to an unrecognized pathogen or an autoimmune response [28, 29]
- A neuromuscular cause where prostatic symptoms are due to a type of reflex sympathetic dystrophy [30]
- Physiological factors relating to neurosis, psychosomatization, depression and sexual dysfunction have also been implicated [20]

The employment of validated severity index questionnaires such as the NIH Prostatitis Symptom Index or the IPSS will provide a subjective evaluation so

that repeated tests can be performed allowing the physician to monitor the effectiveness of therapies applied [31].

Physical examination including a DRE is aimed to assess for other urological pathology such as bladder outlet obstruction or prostatic malignancy. However some men experience prostatic pain on DRE especially those with acute prostatitis.

Urinalysis is used to assess for the possibility of a urinary tract infection. The 4-glass test is a method used to assist in the diagnosis of CAP. This is a method where four urine samples are obtained. First sample is the initial void, second is the mid stream sample, the third sample is obtained after prostatic massage and the final sample is the volume at the end of the void. It is aimed to localize bacteria from the prostatic secretion. Diagnosis of CAP is made when there are less than 10,000 colony-forming urogenital bacteria in the expressed prostatic secretion and little or no leucocytes or bacteria are present in the ejaculate [32]. The 4-glass test is quite uncomfortable for the patient and is not favoured or implemented by many urologists. As a result a modified method such as the two-glass test or pre-post-massage test (PPMT) is used. The use of PPMT has been shown to accurately diagnose 96% of patients with CAP [33]. Urodynamic studies as well as cystoscopy are indicated where men report a history of lower urinary tract symptoms.

As the cause of CAP cannot be clearly defined at present, the management of the illness is anecdotal. There are many treatment options but the three main choices are alpha-blockers, antibiotics and non-steroidal anti-inflammatory drugs (NSAID).

A number of randomised control trials have shown that alpha-blockers such as tamsulosin, terazosin and doxazosin improve urinary flow and reduce

symptoms such as prostatic pain related to CAP [34-36]. The treatment duration to assess therapeutic effectiveness should be a minimum of three to six months. A meta-analysis conducted by Yang et al, has shown that the use of alpha-blockers was effective in controlling symptoms according to the patient self-assessment severity index forms but was not effective with pain management [37].

Antimicrobial therapy, especially the use of quinolones such as ciprofloxacin has some encouraging results in relieving symptoms but the level of evidence to support their use is low. The duration of therapy for patients who are responding to therapy is at least four to six weeks. However men who do not respond to antibiotic treatment should try a different therapeutic modality.

The rationale for the use of NSAIDs is on the basis that an inflammatory process modulates CAP. The only randomized control trial used rofecoxib, which is no longer on the market, and showed some benefit to patients [38]. However NSAIDs should be used with caution and monitored due to the risk of side effects.

1.4 Prostate Cancer

Prostate cancer is the most frequently diagnosed non-skin malignancy in the community. In 2007, there were 19403 new cases of PCa diagnosed in Australia. In the same report compiled by the Australian Institute of Health and Welfare there were 2938 deaths in the same year [39], making PCa the third commonest cause of male cancer death after lung and bowel cancer. Generally, PCa is a disease of older men. The median age of diagnosis of PCa was 68 and the median age of death was 80 [40]. Table 1.2 describes the incidence of PCa in different age groups.

All ages	% Diagnosis	% Death
35-44	0.6	0.1
45-54	8.6	1.4
55-64	28	6.9
65-74	36.1	20.4
75-84	22	41.5
85+	4.7	29.7
All	100	100

Table 1.2 The Incidence of PCa with reference to age [40].

The major risk factor for developing PCa is a positive family history. If a first-degree relative has PCa the risk is double that of the general community but if two or more first-degree relatives have the disease the risk is increased five to ten fold [41, 42]. There is higher incidence of PCa in men in the United States and Europe in comparison to men in Southeast Asia, indicating an ethnic and environmental risk [43]. Also, environmental factors play an important role in predisposition towards PCa. Japanese men migrating from Japan to the United States show an increased incidence of development of PCa more consistent with men born in the United States [44]. Dietary modification such as reduced animal fat and increased fruits and vegetables reduce the risk [45-47].

1.4.1 Grading

Ninety five percent of primary PCa are lobular adenocarcinoma; they develop from prostatic duct acini [1]. Refer to figure 1.3 for appearance of adenocarcinoma of the prostate. The other forms of primary prostate cancers

are sarcoma, transitional cell and squamous, mucinous, ductal and small cell carcinoma.

Neuroendocrine differentiation has also been seen in adenocarcinoma of the prostate. The presence of these cells seen on immunohistochemistry (IHC) is associated with poor prognosis and disease progression. They increasingly occur in the setting of castrate resistant PCa. There are several molecular mechanisms by which this is achieved. The cells evade androgen deprivation by being androgen receptor negative and are present in increasing numbers once androgen deprivation therapy has been initiated [48-50].

Neuroendocrine cells also produce hormones and growth factors such as chromogranin A and bombesin to stimulate proliferation. They can also inhibit apoptosis by secreting survivin which inhibits caspase activation. Angiogenesis is also accelerated by the production of vascular endothelial growth factor (VEGF) [51]. With increasing understanding of the mechanism of evasion, new therapeutic approaches can be developed.

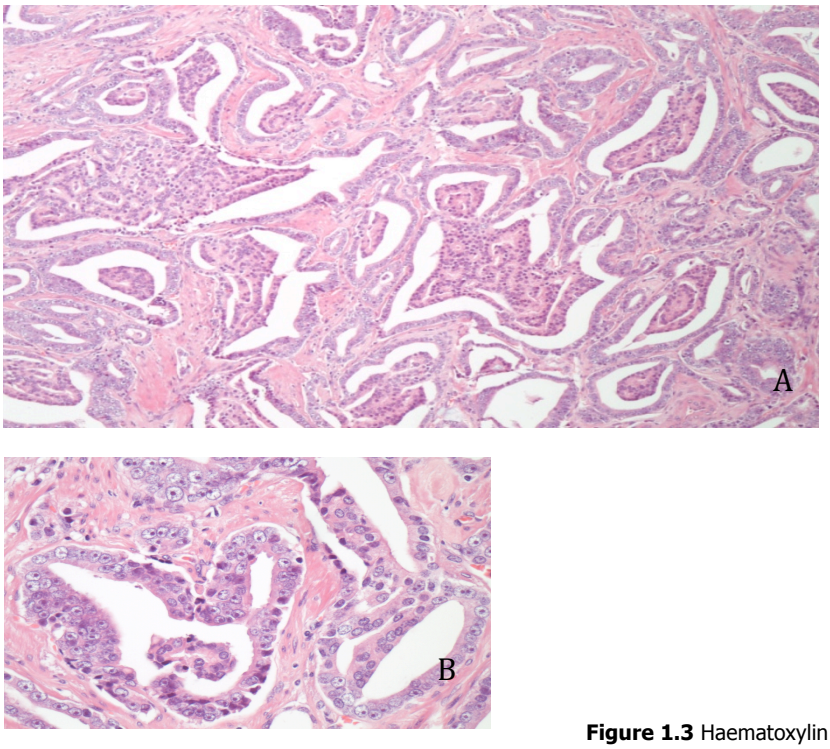


Figure 1.3 Haematoxylin and eosin (H&E) staining of prostate tissue showing adenocarcinoma. Image A is at 100x magnification while image B is at 400x magnification.

The grading method used for adenocarcinoma of the prostate is the Gleason scoring system. Dr Donald Gleason developed this grading system with his colleagues in the Veterans Administration Cooperative Urological Research Group in the 1960s [52, 53]. It was based on the observation that well differentiated cancer progress slowly while poorly differentiated cancers progress rapidly. From their studies, five main histological patterns were identified (refer to figure 1.4). The grades range from one to five and they ascend from well differentiated to poorly differentiated where grade one being the least aggressive and five being the most aggressive. However grades one and two are rarely seen and there has been a shift by pathologists away from reporting low Gleason grades [54].

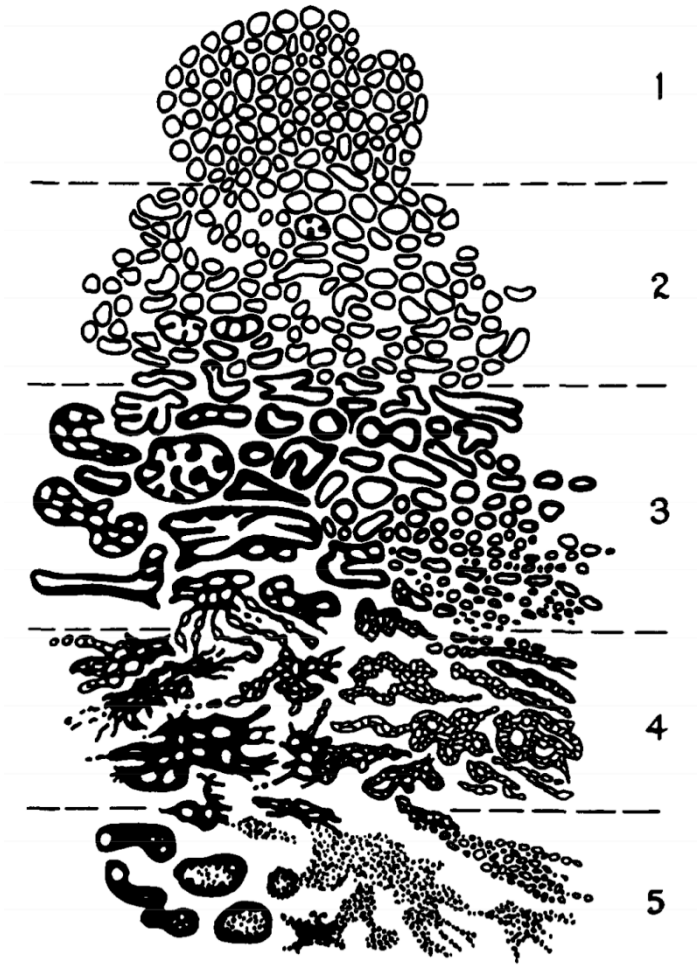


Figure 1.4 The Gleason grades. Descending from well differentiated down to poorly differentiated [53].

The Gleason score is derived by the sum of the two most prevalent histological grades also known as the primary grade with the second most prevalent pattern the secondary grade. The commonest grade has to be of 50% or more of the cancer observed and the secondary grade is less than 50% but greater than 5%. For example if the most common grade seen in a tumour is 4 and second most prevalent pattern is 3 then the Gleason score will be 4 + 3 = 7. As a result the Gleason score can range from 2 to 10 where 10 is the most aggressive form of cancer. It is also important to note that there are variations in the aggressiveness of PCa in an individual Gleason score. For instance a Gleason score of 7, the sum 4 + 3 is more aggressive in comparison to a cancer with the sum of 3 + 4. Of recent times, a tertiary grade that is usually an aggressive pattern, even if seen in small volume (less than 5%), should be reported in biopsy samples. For tertiary scores, the recommendation from the 2005

international Society of Urological Pathology consensus conference was to report the two highest grades on transrectal ultrasound (TRUS) biopsy but on radical prostatectomy samples to report the two most prevalent scores and comment on the tertiary pattern [55].

This grading system has been validated and has been shown to correlate with survival rates [56, 57]. With this information, nomograms such as those developed by the Memorial Sloan-Kettering Cancer Centre have been devised for clinicians to assist in the management of patients with PCa [58].

1.4.2 Screening

Screening involves the assessment of a population of men who are at an increased risk of PCa for the presence of the disease. The main modalities of screening are by the use of the serum PSA and DRE. The Urological Society of Australia and New Zealand, the Australian Prostate Cancer Collaboration and the Prostate Cancer Foundation of Australia recommend the commencement of PCa screening from the age of 50 but those with a strong family history to start earlier, from the age of 40 [59]. The Royal Australian College of General Practitioners do not advocate for the routine screening for PCa. They recommend that the patient should decide if they want to undertake PSA testing after understanding the risks and benefits of PCa screening [60].

Of recent times there has been a lot of publicity on the validity of population screening for PCa. The initial two large studies, one from the United States and the other from Europe, showed contradictory results for the screening process.

The Prostate, Lung, Colorectal and Ovarian (PLCO) cancer screening trial on prostate cancer mortality study was conducted in the United States. In this study with a sample population of 76,693 men were randomized to two groups.

One group was screened every two to four years with PSA and DRE and the other was the usual care as the control group. These men were then followed up from seven to ten years documenting the development of cancers and cause of death. The result of the study showed that the incidence of PCa was higher in the screened group in comparison to the control group. However the mortality in the screened group was almost identical to the control group [61], leading to the conclusion that screening did not affect mortality.

The flaw with this study was that the control arm was contaminated. In the six-year time period 52% if the men had a PSA check and 46% had a DRE. Also 44% of men were screened prior to randomisation resulting in selection bias. It was interesting to see these men had a 25% lower PCa mortality than those men who were not pre-screened prior to enrolment. This supports the positive effects of screening and need for longer follow up.

This study also assigns a PSA of 4ng/ml as an indication for biopsy in all age groups. However there have been studies showing aggressive PCa with lower PSA levels [62].

The second study called the European Randomised Study of Screening in Prostate Cancer (ERSPC), assessed the effect of PSA screening and the deaths associated with PCa [63]. This was a much larger trial with a study sample of 182,000 men between the ages of 55 to 69. These men were randomized into two groups, one the screening group, where men had PSA checks every two to four years while the second, control group, had no such investigation. The primary outcome of this study was the death rate related to PCa. This study showed that 1410 men needed to be screened and 48 additional cases of PCa would need to be treated to prevent one death from PCa. The findings of the study showed that the adjusted rate ratio for death from PCa was 0.80 in the screened group [63]. They concluded that a high

number of men were over diagnosed with PCa who would not have a cancer related death.

The problem with this paper is that it is a combination of studies from different with variable indications for biopsies and screening intervals. This makes it difficult to determine and develop an effective screening regimen.

A criticism of this study is the high number needed to treat to prevent one PCa death. This results in significant treatment costs and impaired to quality of life. However as the study continues for a longer duration, the cancer specific mortality will fall, resulting in a lower number of men needed to be treated. Also limiting treatment to men with intermediate to high risk cancers, and the use of active surveillance for low risk cancers, the number needed to treat will also decline.

The 12-year follow up of the ERSPC study has shown that screening had an overall risk reduction of 31% in metastatic disease [64]**.** This further supports the use of screening for PCa. Screening is a complex issue, and effects of a positive result will have physical, psychological and financial ramification on the patient, their family and the community. Prior to embarking on the screening process a lengthy discussion between the medical practitioner and patient is needed to inform of the potential paths that may be taken if a positive result is encountered.

1.4.3 Diagnosis

The diagnosis of prostate cancer is made by the combination of DRE, PSA and a histological diagnosis via TRUS guided biopsy of the prostate.

1.4.3.1 Digital Rectal Exam

Digital rectal examination was the primary and extensively used screening tool for PCa prior to the PSA era. It is the process of manually palpating the prostate through the rectum. The aim of the examination is to ascertain the shape, size and smoothness of the prostate to determine if there is evidence of palpable PCa.

The incidence of detecting an abnormal prostate gland has been associated with detecting an high grade PCa [65]. However one of the limitations of this examination is the inability to reproduce the exact findings by different examiners despite their level of experience [66].

1.4.3.2 Prostate Specific Antigen

Prostate specific antigen is a single-chain glycoprotein, which is a membrane of the kallikrein family of serine proteases. It is produced by the prostatic epithelium and periurethral glands secreted into the seminal plasma at concentration of 0.5-2.0g/L. The function of PSA is to liquefy the seminal coagulum by proteolysis with release of entrapped spermatozoa. Also it has been linked with a bioactive role in fertilization [67].

PSA was first identified in human prostatic tissue in 1970 and then it was purified and characterized in 1979 before being detected in serum in 1980. The serum PSA test was introduced in 1988. It is the only biomarker for the prostate

however its downfall is that it is not cancer specific. The limitation of PSA as a tumour marker is that it can be elevated due to urinary tract infections, prostatitis, BPH, PCa or any other process affecting the prostate. Other factors confounding interpretation are post DRE, ejaculation, prostate biopsy, TURP, cystoscopy and even exercise such as cycling [68]. These factors may elevate PSA transiently and not clinically significantly.

The "gold standard" assay used for PSA detection in serum is the Hybritech Tandem-R assay [68]. For PCa, there is no agreed universal cut-off for its detection but the reading >4ng/mL has been used in many studies. There is a close relation with normal PSA and age and prostate volume. As a result the age related PSA reference ranges have been established in order to reduce unnecessary biopsy in elderly men and to avoid missing early cancers in younger men. Refer to table 1.3 for the age related normal PSA levels. With the development of these cut-offs, unnecessary prostate biopsies have reduced by 44% [69]. Despite having these figures to detect PCa, the development of clinical significant PCa is very difficult to determine.

Age	PSA range (ng/mL)
40-49	0.0-2.5
50-59	0.0-3.5
60-69	0.0-4.5
70-79	0.0-6.5

Table 1.3 Age specific ranges for normal PSA [70].

Other adaptations of the serum PSA test have been made to improve the specificity of detecting PCa. PSA density is determined by dividing PSA by the prostate volume. A greater positive predictive value for PCa is obtained when PSA density is greater than 0.15 [71]. The limitation of this method is that

prostatic volume is needed to calculate the density which is measured using an ultrasound.

PSA is present in free form and as a complexed molecule in circulation. Approximately 70 to 90% is in complex form, bound to endogenous protease inhibitors mainly with α1-antichymotrypsin (ACT) and also with α2-macroglobulin (A2M). A small but variable proportion of total PSA is present in circulation in its free form. The measure of free/total PSA ratio less than 25% increases the sensitivity to 95%. This method of PSA analysis is more commonly used when reassessing elevated PSA after negative biopsies [72].

Another modification that is commonly used is the measure of rate of change of PSA over time. This is termed PSA velocity and a rate greater than 0.75 ng/mL per year increased the specificity of detecting PCa by 90% [73]. The limitation of this result is that long intervals, usually one year, between repeated PSA tests are required to obtain a reliable trend to assess PSA velocity. This however has the risk of potentially delaying the diagnosis of PCa at an early and potentially curable state.

The use of PSA is not only for diagnosis of PCa; it is an important tool in the assessment of the effectiveness of treatment. For instance after radical prostatectomy, radiotherapy or even androgen ablation therapy, PSA is used routinely to monitor disease progress or determine biochemical recurrence.

1.4.3.3 Transurethral Ultrasonography guided biopsy

The histological diagnosis for PCa is assisted by the use of TRUS to obtain tissue samples. The ultrasound probe was developed in the 1960s and it wasn't until 1981 when a diagnostic procedure for PCa was developed [74, 75].

This procedure has two roles. One is to visualize cancerous lesions and the second is to guide the trajectory of the biopsy needle. The classical image of PCa seen on TRUS is a hypoechoic lesion usually in the peripheral zone as seen in figure 1.5.

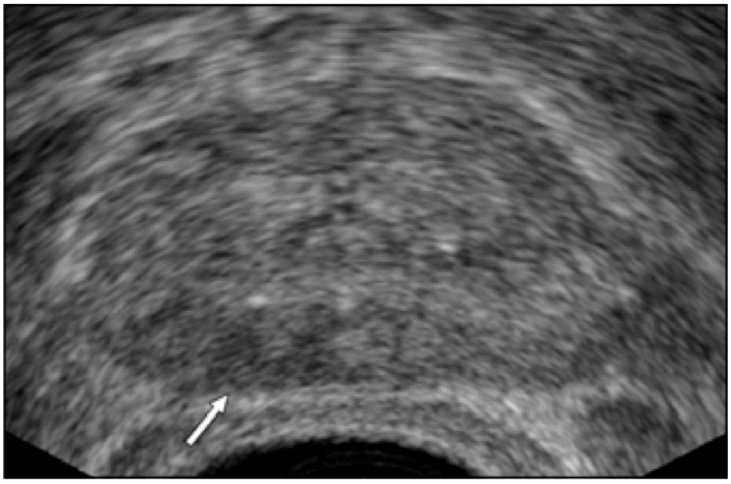


Figure 1.5 TRUS image of the prostate. Arrow indicating the presence of a cancerous nodule present as a hypoechoic lesion [1].

The appearance of PCa as a hypoechoic area is not always the case. The incidence of detecting PCa in a hypoechoic lesion has been reported from 20% to 37.6% [76, 77]. The main function of TRUS is to provide images to allow the clinician perform systematic sampling of the gland.

Performing systematic biopsies ranging from 8 to 12 samples from the apex, mid-gland and base of the prostate has superseded sextant biopsies [78-82]. With this procedure it has been reported 15 to 35% increase in PCa detection [72, 83]. The main reason for this increased yield is due to the sampling of lateral peripheral zones [80]. However the downside of this sampling scheme is the increased risk of side effects. Repeat biopsy after an initial negative biopsy is carried out when there is still a high suspicion of PCa. There have been studies showing detection rates with repeat biopsies of up to 21% [84, 85]. An increased yield in detecting PCa with is seen with a saturation biopsy which

involves taking 24 samples [86]. Morbidity from repeated biopsies of the prostate is not increased dramatically [87, 88].

The complications with this procedure are rare but less than 1% suffer from septicaemia requiring hospital admission for intravenous antibiotics. Usually a three-day course of oral antibiotics is used to reduce the incidence of urosepsis [78]. Patients also may have rectal bleeding, haematuria and haematospermia that persist for a few days. Also TRUS biopsy can precipitate voiding dysfunction and can lead to urinary retention [89].

The interpretation of the TRUS biopsy results is crucial in managing a patient who is suspected of having PCa. It is important to be aware that a negative TRUS biopsy result does not mean that the patient does not have PCa and that the results could be attributed to sampling error. Also a positive biopsy does not necessitate immediate radical treatment.

1.4.4 Staging

The Tissue, Node and Metastasis (TNM) classification system is used for PCa. It describes the extent of the cancer in the body. "T" portrays the extent of the tumour locally, "N" depicts the extent of lymph node involvement and "M" indicates the presence of distant metastases. The TNM classification is maintained by the International Union Against Cancer and is a universally accepted classifying system. This information will allow the clinician to decide and plan their intention of treatment.

1.4.4.1 T-stage

T-stage involves the assessment of local spread of cancer. The main modality used in determining T-stage is DRE, however imaging can also provide some assistance. DRE as mentioned earlier, is subjective and cannot be reliably

reproduced. The sensitivity of DRE in finding organ confined PCa is about 50% [90]. However a patient with PCa who has an elevated PSA but a nonpalpable cancer on DRE is said to have T1c disease. A patient with palpable disease confined to the capsule has T2 disease. When the cancer is palpated beyond the capsule T3 disease is ascertained. Finally T4 is the presence of cancer extending to surrounding structures such as the bladder.

The use of radiological imaging such as computed tomography (CT), magnetic resonance imaging (MRI) and positron emission tomography (PET) have been of some assistance. However there is not evidence to implement them as a routine cost effective diagnostic tool[91, 92]. There have been studies looking at the presence of extraprostatic spread of PCa preoperatively so that negative surgical margins can be achieved. Specifically with MRI, studies have reported sensitivity of 42% and specificity of 95% but due its cost and availability it is not routinely used [93]. The aim of T-staging is to distinguish between intracapsular (T1-T2) with extracapsular (T3-T4) disease.

The most accurate method of examining localized PCa is with pathological sections of the prostate after a radical prostatectomy. With this, the pathologist is able to identify extracapsular spread, surgical margins, seminal vesicle involvement and neurovascular invasion.

1.4.4.2 N-staging

The assessment of nodal involvement in PCa is very important, as it is a strong indicator of metastatic disease [94, 95]. The implication of positive spread to lymph nodes is associated with poor prognosis, with a 5-year survival of 17% [96].

The spread of PCa to lymph nodes occurs to obturator, internal iliac, common

iliac and presacral regions [96]. The “gold standard” of determining lymph node involvement is to perform a pelvic node dissection at the time of radical prostatectomy. This usually involves the dissection of the obturator packet of lymph nodes.

The two main methods used to detect lymph node involvement are surgical resections of the draining lymph nodes and CT scans. The limitation with CT scans is that it is unable to detect early spread because 75% of metastatic deposits are smaller than 8 to 10mm [72]. A systematic review comparing CT scan results with pathological results from a lymphadenectomy showed positive detection rates of 2.5% and 15.3% respectively [97].

Obturator and external iliac lymph node dissection could also be performed at the time of surgery to provide information on staging in patients suspected of spread for patients with intermediate and high risk PCa [98]. The complications of performing lymph node dissection include lymphocele, lymphoedema, deep venous thrombosis and pulmonary embolus [99, 100]. However careful consideration is needed before embarking on this dissection, as the benefit from the information obtained for more accurate staging will need to outweigh the risks. There is no role or added benefit from doing an extended pelvic lymph node dissection in PCa.

The recommendation of the European Association of Urology is to not to perform staging CT for patients with stages T2 or less, a PSA of less than 20 ng/mL and a Gleason score that is less than or equal to 6 [101]. The likelihood of metastatic spread in this demographic of patients is less than 10% [98].

1.4.4.3 M-staging

PCa commonly spreads via the haematogenous route to the lumbar spine,

pelvis, ribs and femoral heads. An effective method of determining spread to bone is by the use of bone scintigraphy [102]. Refer to figure 1.6 for example of bone scintigraphy. The disadvantage of this modality is the rate of false positive results and the need for other imaging modalities to examine the suspicious site. The use of bone scintigraphy is recommended in asymptomatic patients if the PSA is greater than 20 ng/mL or the Gleason score equal to or greater than 8 [97], as these patients are more likely to have detectable metastatic disease.

The use of plain films as a sole imaging modality is not effective in determining bony metastasis as they require at least 50% of the bone density to be replaced by tumour before being able to be seen [90]. A degree of clinical suspicion is needed before targeted imaging is instituted.

The spread of PCa to non-bony regions is uncommon but can involve distant lymph nodes, lung, liver, adrenal gland and other viscera.

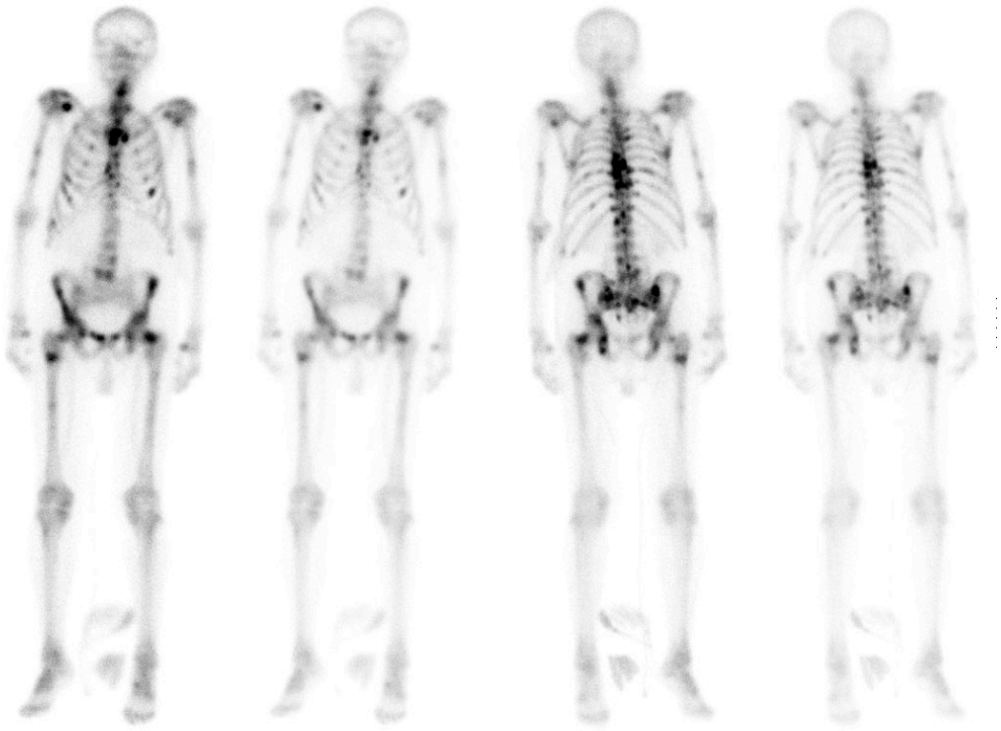


Figure 1.6 Bone scintigraphy. The scan shows evidence of tracer uptake throughout the vertebrae of an 82-year-old male with metastatic PCa [102].

1.4.5 Treatment Options

1.4.5.1 Active surveillance

This is a process of carefully monitoring a patient who has been diagnosed with PCa whether it is with repeated PSA or biopsy until signs of disease progression is seen so that treatment options are implemented. This modality is not only reserved for patients who are treated on a palliative course until symptoms occur but also for young patients who have a very low grade tumour and curative treatment is delayed until increased tumour activity is seen such as rapidly rising PSA or increasing Gleason scores on repeated TRUS biopsy. Undertaking active surveillance involves selecting the correct patient, as they must be well informed of the risks and benefits and also tolerate possible anxiety.

The difference between active surveillance and watchful waiting is that in active surveillance the patients are suitable for curative treatment if biochemical or pathological progression is demonstrated. Whereas in watchful waiting the development of symptoms guide the mode for treatment offered with no radical intent.

The recommendation by the European Urology Association with active surveillance is indicated for very young patients with a life expectancy of greater than ten years and who have a presumed organ confined tumour. They can undergo this method if they are very closely followed with repeat PSA and TRUS biopsies.

1.4.5.2 Radical prostatectomy

In most cases, performing a radical prostatectomy on a patient with PCa is for curative intent. The procedure is commonly performed as an open procedure where the entire prostate and the seminal vesicles are removed and the bladder is anastomosed with the urethra. Over the last decade the use of laparoscopic and robotic surgery have become increasingly popular due to the their less invasive nature. However data has shown that laparoscopic and robotic radical prostatectomy were effective in reducing blood loss in comparison to open radical prostatectomy but the evidence was not significant in proving functional and oncological outcome superiority [98].

The main complications associated with radical prostatectomy are urinary incontinence, bladder neck stenosis, impotence, lymphocele and rectal injury [98]. The individual surgeon's experience, patient demographic and centre expertise influence the rates of the complications.

In general, radical prostatectomy is indicated as a curative procedure for patients with low to intermediate risk where they have the following characteristics:

- Life expectancy greater than 10 years
- cT1a-T2b and
- Gleason score 2-7 and
- PSA less than 20
- Life expectancy greater than ten years

1.4.5.3 Radiotherapy

Radiotherapy is the use of ionizing radiation to eradicate cancerous cells. It can be used with both curative and palliative intent. As a curative therapy, it is used as primary treatment or as adjuvant or as salvage therapy after radical prostatectomy.

The Deoxyribonucleic acid (DNA) in cancerous cells is damaged by directly by the radiation or by the creation of free radicals. Here the breaks in the double stranded DNA results in cell apoptosis. Cancerous cells have a reduced ability to repair damaged cells when compared to normal well-differentiated cells. As a result the damaged cancerous cell continue to undergo cell division with their acquired DNA defects ultimately ending in cell death or reduced reproduction. The amount of radiation is measured in gray (Gy). For most solid organ tumours the dosage varies from 60Gy to 80Gy. In adjuvant radiotherapy, the doses are usually between 45-60Gy.

The most common form of radiotherapy used in prostate cancer is external beam radiotherapy (XRT) or brachytherapy. XRT is a process that has been present for many years. Early on conventional XRT had limitations in the level

of radiation that could be given due to the high level of toxicity. The prostate gland was targeted by a process of elimination whereby the bladder and rectum were filled with contrast and an assumption was made on the location of the prostate. As a result normal tissue was also included in the radiation field. Surrounding organs such as the bladder, intestines and the rectum were unnecessarily radiated. Due to the inaccuracy of targeting the prostate, radiotherapists were unable to escalate the dose of treatment due to the adverse effects incurred to surrounding tissue.

However over the years advancements in software and hardware have allowed this modality to be more precise and effective. An example of this is the linking of CT and radiotherapy, allowing accurate identification of the prostate gland and surrounding normal tissue so that the radiation therapy can be targeted. This modality is called three dimensional conformal radiation therapy (3D-CRT). With 3D-CRT, higher doses of radiation are able to be delivered with steep drop-off of radiation intensity to surrounding organs. The incidence of radiation related side effects are lower in comparison to conventional XRT [103].

More recently the development of intensity modulated radiation therapy (IMRT) allows the physician to control the amount of radiation delivered in each beamlet so that the recommended dose can be delivered to the specific target. Refer to figure 1.7 for localization of prostate gland. This method has allowed physicians to treat localized prostate cancer with higher doses of radiation, up to 84 gray. A series performed at Memorial Sloan-Kettering Cancer Center, where 772 men were treated with dose escalation, showed at least comparable PSA control rates with reduced early and late rectal toxicity in comparison to 3D-CTR [104].

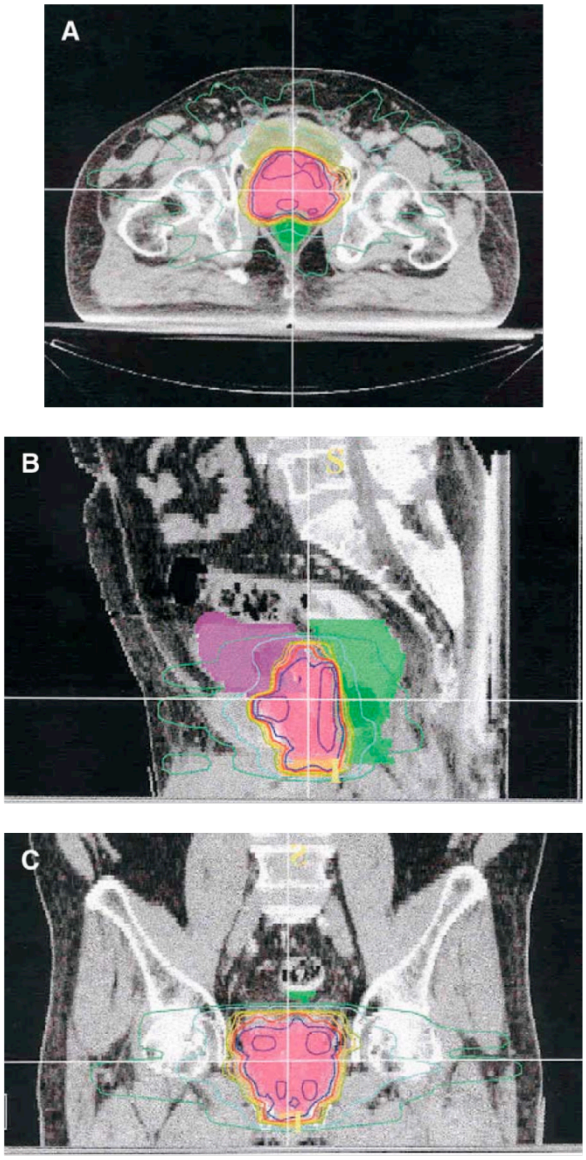


Figure 1.7 Intensity modulated radiation therapy. Images show the use of IMRT to localize the prostate gland and deliver high dose of radiation to the prostate (pink) and avoid the surrounding structures bladder (purple) and rectum (green). A – axial section, B – sagittal section and C- coronal section [105].

The side effects with XRT can be seen in the early and late phases of treatment. The early manifestation is seen at the site where XRT is focused. This predominantly involves the skin which presents as erythema, swelling, pain and alopecia. The late side effects include cystitis, haematuria, urinary stricture, urinary incontinence, erectile dysfunction, proctitis, small bowel obstruction and leg oedema [105, 106]. These however are reduced in the new modalities. Prostatic motion due to changes in rectal volume can alter the target field and results in increased adverse effects on surrounding tissue. The

use of image-guided radiotherapy (IGRT) reassesses the location of the prostate before dose delivery to reduce the error in margins of the field [107-109].

The NIH guideline indicates that external beam radiation therapy has the same long-term survival outcome as surgery while providing the same level of quality of life [98]. The European urological association guidelines recommend the use for XRT in patients with:

- Localized PCa who do not want surgical resection.
- Intermediate-risk patients treated with dose escalation.
- High-risk patients treated with a short term with hormone therapy prior to radiation therapy. This has been shown to increase overall survival.
- Immediate post radical prostatectomy where the pathological staging show T3 disease. This improves both biochemical and clinical disease free survival.
- Locally advanced PCa with adjuvant hormone therapy for 2 – 3 years.

The other main form of radiotherapy used for the treatment of localized prostate cancer is transperineal brachytherapy. This is in the form of low dose and high dose rate brachytherapy. Low dose rate brachytherapy is the process of implanting radioactive seeds in the prostate using a closed transperineal approach. The seeds are guided into the appropriate location with the combination of a template and high-resolution transrectal ultrasound. For this modality, specific patient characteristics are required. The indications for brachytherapy are listed in table 1.4. Brachytherapy is recommended for patients who have a life expectancy of more than five years. Also patients who have had TURP or patients who may need a TURP in the future which can be

assessed with poor IPSS scores or poor urine flow rates (Q_{max}) have a high incidence of urinary incontinence after brachytherapy.

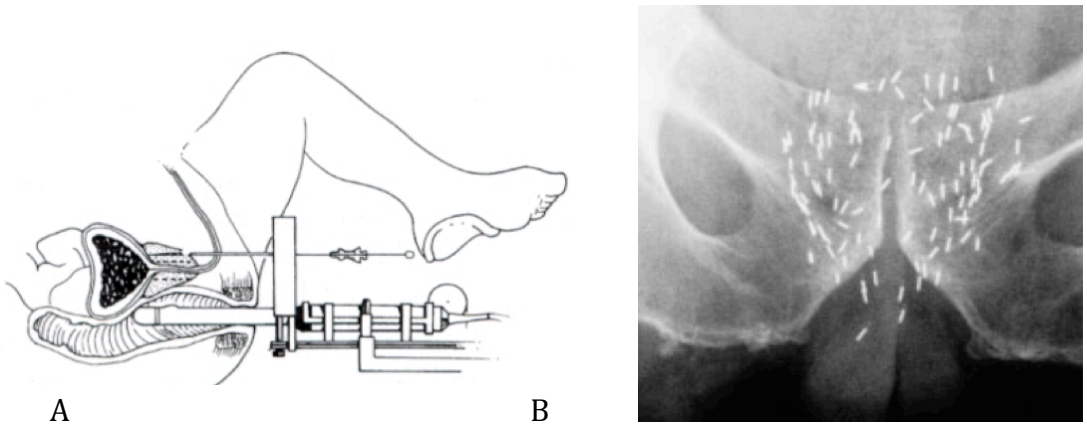
Criteria	Respond well	Respond moderately	Respond poorly
PSA (ng/mL)	<10	10-20	>20
Gleason score	5-6	7	8-10
Stage	T1c-T2a	T2b-T2c	T3
IPSS*	0-8	9-19	>20
Prostate volume (g)	<40	40-60	>60
Q_{max} (mL/s)**	>15	15-10	<10

Table 1.4 The indications for brachytherapy[110].

* International Prostate Symptom Score (IPSS)

** Peak flow rate (Q_{max}) – measure of highest voiding velocity.

Approximately 80 to 120 iodine-125 seeds are implanted into the prostate with guidance of the template and TRUS (refer to figure 1.8). The procedure takes one hour to complete and the patient usually can be discharged home the same day. The seeds that are inserted are left in permanently and they have a radiation half-life of 60 days [111].



A B

Figure 1.8 Brachytherapy seed implantation. A. Demonstrates the TRUS and guidance plate positioning for insertion of radioactive seeds. B. X-ray showing the radio-opaque seeds in the prostate [112].

The adverse effects of this procedure are listed in table 1.5. Nevertheless the use of brachytherapy for the treatment of low risk local prostate cancer is gaining popularity.

Acute adverse effects (<12 months)	Percentage
Minor irritative urinary symptoms	46-54
Urinary retention	1-14
Proctitis	1-2
Chronic adverse effects (>12 months)	**Percentage**
Moderate irritative urinary symptoms	29% at 12months 14% at 24 months
Incontinence	5-6%
Incontinence after TURP	13%
Haematuria	1-2%
Stricture	1-2%
Proctitis	1-3%
Impotence	4-14%

Table 1.5 Commonly encountered adverse effects of brachytherapy[112].

High dose rate brachytherapy is used in combination with external beam radiotherapy or as monotherapy. With this method, dose delivery is optimised to shape around the prostate to avoid affecting surrounding normal tissue. This varies from low dose rate brachytherapy radiobiologically and dosimetrically. It is delivered in two or more fractions of 5 to 10 Gy combined with 40-50 Gy external beam [113, 114].

High dose rate brachytherapy as a monotherapy is used in low-risk disease. Combined high dose rate brachytherapy and external beam radiotherapy is effective in bulky local disease (stage T2b-T3) or intermediate to high-grade cancers [115, 116].

The procedure is conducted in a similar fashion as low dose rate brachytherapy. A transperineal approach is used and 12-24 catheters are placed into the prostate using TRUS and a template. Treatment delivery is via these temporary catheters [117].

The side effect profile is similar to the low dose rate brachytherapy. However from the radiobiology point of view, high dose rate brachytherapy treatment delivery is over a few minutes rather than over weeks and months. Also the patient is not radioactive when they return home after treatment [118].

1.5.5.4 Androgen deprivation therapy

Huggins and Hodges developed the use of hormone therapy also known as androgen deprivation therapy (ADT) to treat prostate cancer in 1941 [119, 120]. It was their study on castration of patients or administration of oestrogen (stilbestrol) that showed the benefits of androgen ablation in metastatic prostate cancer. The effects were measured by the changes in prostate size and levels of serum acid-phosphatase. This discovery was so substantial Huggins received the Nobel Prize for his work in 1966 [121].

The androgen, testosterone is essential in the growth of PCa cells. It provides stimulation, growth and proliferation of the tumour cells. The main production of testosterone is from the testicles. However 5 to 10% of testosterone in the form of androstenedione, DHT and dehydroepiandrosterone sulphate is from the adrenal glands.

The control of production of testosterone is from the hypothalamic-pituitary-gonadal axis (refer to figure 1.9a). Androgens bind to the androgen receptors in the hypothalamus to release luteinizing hormone-releasing hormone (LHRH) which then travels to the anterior pituitary gland and activates the LHRH receptor. This results in the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH travels to the testicle where it interacts with the LH receptor in the Leydig cells. Here testosterone is produced from cholesterol. The secreted testosterone then enters the prostate where it is converted to DHT by the enzyme 5α-reductase. DHT attaches to the androgen receptor in the cytoplasm and it translocates to the nucleus which allows it to activate the genes involved in cell growth and survival. DHT is ten times more potent than testosterone [122]. Elevated circulating testosterone levels have a negative feedback control on the hypothalamus and result in reduced production of LHRH and LH.

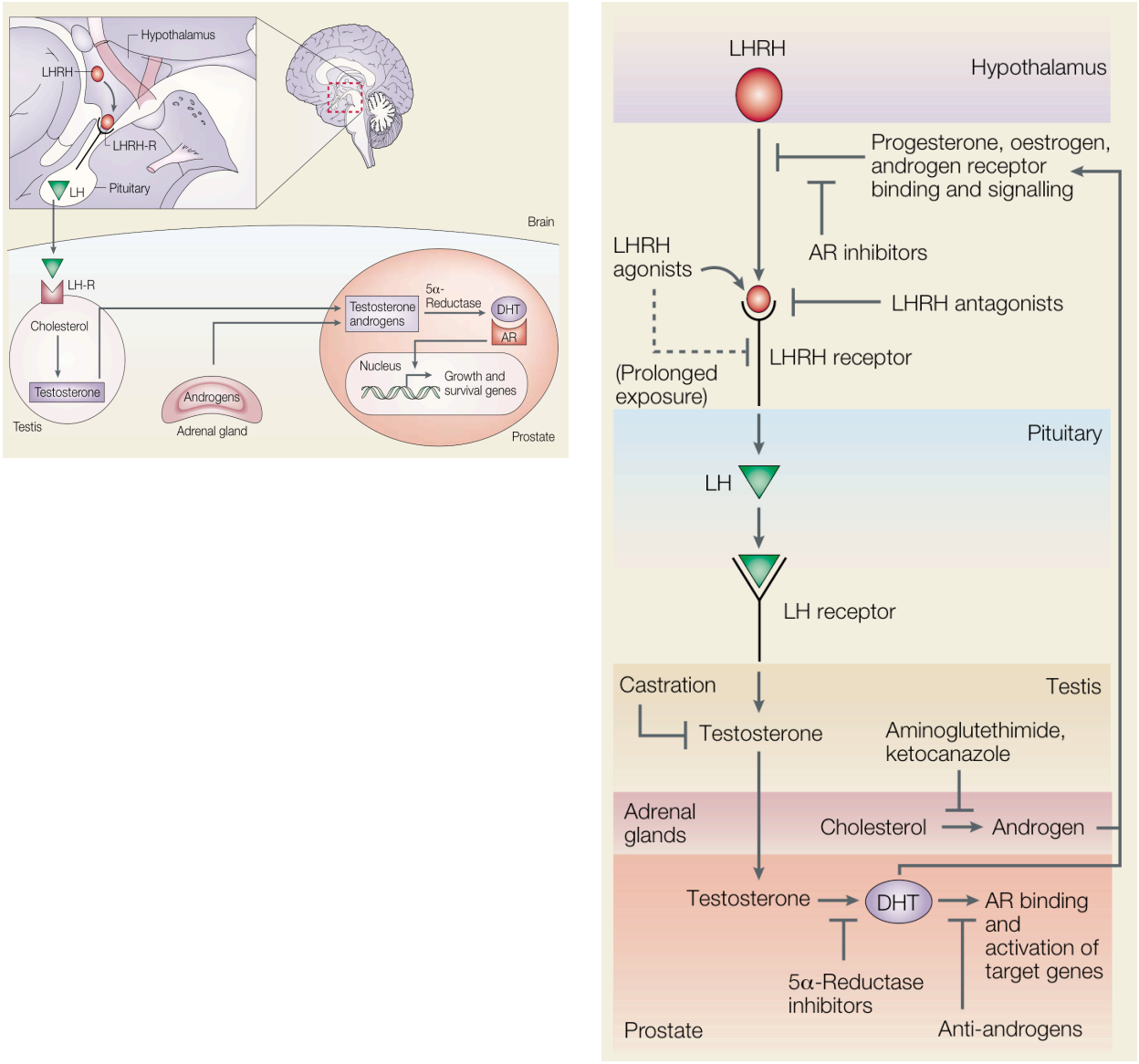


Figure 1.9 Androgen deprivation therapy pathways **a)** Demonstrates the hypothalamic-pituitary-gonadal axis. **b)** Depicts the regions where LHRH agonists and antagonists act [121].

It was Andrew Schally who was the first to discover the hypothalamic hormone LHRH [123]. He was also able to manipulate this axis by the development and use of a LHRH agonist. When a LHRH agonist is administered there is an initial rise of testosterone known as the "testosterone flare". This is however transient and can result in increased bony pain and urinary outlet obstruction. The administration of LHRH agonist for a prolonged period, greater than two weeks, results in the negative feedback resulting in down regulation of LHRH receptors

in the pituitary gland (refer to figure 1.9b). This has a flow on effect in that there will be reduced LH produced and this will lead to lowered testosterone levels. The testosterone levels are equivalent to the levels seen after bilateral orchidectomy [124].

The clinical benefits of this treatment will result in reduction of tumour growth as shown by studies in rats as well as prostate cancer cell apoptosis. It provides symptomatic benefit such as reduced bony pain. The advantage of this form of therapy is the delivery via a depot form.

The adverse effects of this androgen deprivation therapy include loss of libido, erectile dysfunction, hot flushes, fatigue, gynecomastia, increased body fat, muscle wasting and decreased bone mineral density [125-130].

Another common method of androgen deprivation is by the use of LHRH antagonist. The antagonists competitively bind to the LHRH receptors in the pituitary gland and inhibit the production of LH. As a result, it reduces the production of testosterone and the advantage of this is that it eliminates the "testosterone flare" effect. There is no long-lasting depot form of administration as yet such as those offered by the LHRH agonists.

The use of androgen deprivation therapy is mainly used in the palliative setting where it is effective in delaying the progression of PCa [131]. It is not curative. It is however used in conjunction with other treatment modalities such as radiotherapy.

Over time a large proportion of men develop androgen independent PCa. This is when despite undertaking ADT, the cancer progresses. There are two main

pathways by which this happens, one related to androgen receptors (AR) and the other bypasses the receptors.

Hormone refractory PCa develops via the AR pathway by many mechanisms such as increasing receptor numbers, receptor mutation, deregulation of growth factors or cytokines and alteration of coactivators [132, 133]. Amplification of AR gene resulting in increased AR numbers allows for low levels of androgens to stimulate a response [134, 135]. The pathways by which the AR is bypassed are by deregulation of the apoptotic gene and by neuroendocrine differentiation of prostate cancer cells [136-138]. Outlaw or androgen independent pathway is another mechanism of androgen independence. Certain growth factors such as insulin-like growth-factor-1, keratinocyte growth factor and epidermal growth factor can activate the AR in absence of androgen [139].

1.5 The immune system

The immune system is a collection of cells and molecules that function together to protect the body from invading pathogens and monitor for abnormal cell proliferation. There are two main defence mechanisms involved with the immune system. One is the innate response which is the body's natural response. The other mechanism is the adaptive response that involves acquired memory responses.

The organs of the immune system are lymphoid organs. They are divided into primary lymphoid organs where lymphocytes are generated and these are mainly the thymus and bone marrow. The secondary lymphoid organs are where the adaptive immune response is commenced. The secondary lymphoid organs also known as the peripheral lymphoid organs consist of the lymph nodes, spleen, tonsils, adenoids and Peyer's patches of the small intestines.

1.5.1 Innate immune system

The physical external barrier such as skin and mucous membranes are part of the innate immune system. The external barrier includes the creation of unfavourable environment such as gastric acid and enzymatic secretions to restrict pathogens from entering the circulation.

The innate immune response is a rapid natural response that lacks memory and therefore is limited to detecting common characteristics associated with many pathogens. As a result, despite repeated infection by a certain pathogen, the innate response is same on each occasion. This type of response is developed early in evolution.

The main functions of the innate immune system are to recruit immune cells to sites of infection by the method of producing chemical mediators such as cytokines. The complement cascade is also activated to identify pathogens, cell activation and removal of dead cells and antibody complexes by phagocytosis. Finally, the innate immune system facilitates antigen presenting to activate the adaptive immune system.

There are two main types of cellular components of the innate response. One is the local tissue residing cells such as macrophages and dendritic cells (DC); however DC are involved in both the innate and adaptive responses. The other cells are in circulation such as neutrophils, eosinophils, monocytes and natural killer (NK) cells. These cells are in circulation and are recruited quickly to regions of invading pathogens or cellular abnormalities.

The recognition of potential pathogens is crucial before the innate response can be instituted. A number of germ-line encoded receptors also known as pattern recognition receptors are involved in the innate system to initiate the response.

These receptors recognize pathogen-derived molecules which are generally distinct for a certain class of pathogen, for example gram positive cocci. One of the most important pattern recognition receptors is the Toll-like receptor which is made up of a family of molecules. These receptors are capable of recognizing a wide variety of pathogens from viruses to parasites [140]. The innate immune system is also able to detect infectious events through detecting cellular damage of dying cells.

With recognition of the foreign pathogen, the locally residing cells such as endothelial cells and macrophages secrete chemokines such as CCL5 which is a chemotactic for T cells. These are small soluble proteins which enhance cellular migration of particularly phagocytes from the blood to the area of the tissue infection and damage. The activated local cells and the phagocytes produce cytokines which are a large family of low-molecular weight soluble proteins such as tumour necrosis factor (TNF) and interleukins (IL) involved in regulating cellular activity. This results in increased vascular permeability allowing the transition of cells from blood to the tissue and upregulated phagocytic activity. This will lead to inflammation which appears as swelling, redness, pain and heat. This process of inflammation not only has anti-microbial activity but also has healing properties for damaged tissues.

Macrophages are large phagocytic leukocytes, which originate from blood-borne monocytes. They are capable of migrating outside of the vessels into tissue to detect invading pathogens. The foreign molecules as well as the body's own dead or dying tissue undergo the process of phagocytosis once they have been detected by their receptors. Within the phagocyte, the digested material are subjected to toxic material such as superoxide anion, hydroxyl radicals, hypochlorous acid, nitric oxide (NO), antimicrobial cationic proteins and peptides and lysozymes [141]. Also the activated macrophage produces chemokines to recruit other cells to the inflammatory site.

The dendritic cell is present locally in tissue and is in contact with the external environment, namely in regions such as skin and mucosal lining of the gastrointestinal and respiratory tracts. They have phagocytic properties by means of engulfing extracellular antigens. They also behave like antigen presenting cells where they detect pathogen-associated molecular patterns on the surface of microorganisms via their pattern recognition receptors on the surface [142]. These cells are also activated by endogenous danger signals such as interferon-α, heat-shock proteins and tumour necrosis factor α which are released at the sight of infection [143].

Dendritic cells serve as a one of the links between the innate and adaptive immune system. Once the dendritic cell is activated, there is increased expression of surface B7 co-stimulatory molecules. These molecules facilitate lymphocyte activation in addition to the stimulation provided by the antigen receptor. The T lymphocytes are activated by the presentation of the antigen by the dendritic cell after it has migrated to the local lymph node (refer to figure 1.10). In the dendritic cell, the antigen is processed intracellularly by proteolytic cleavage before being presented on its surface presenting molecules encoded by the major-histocompatibility complex (MHC).

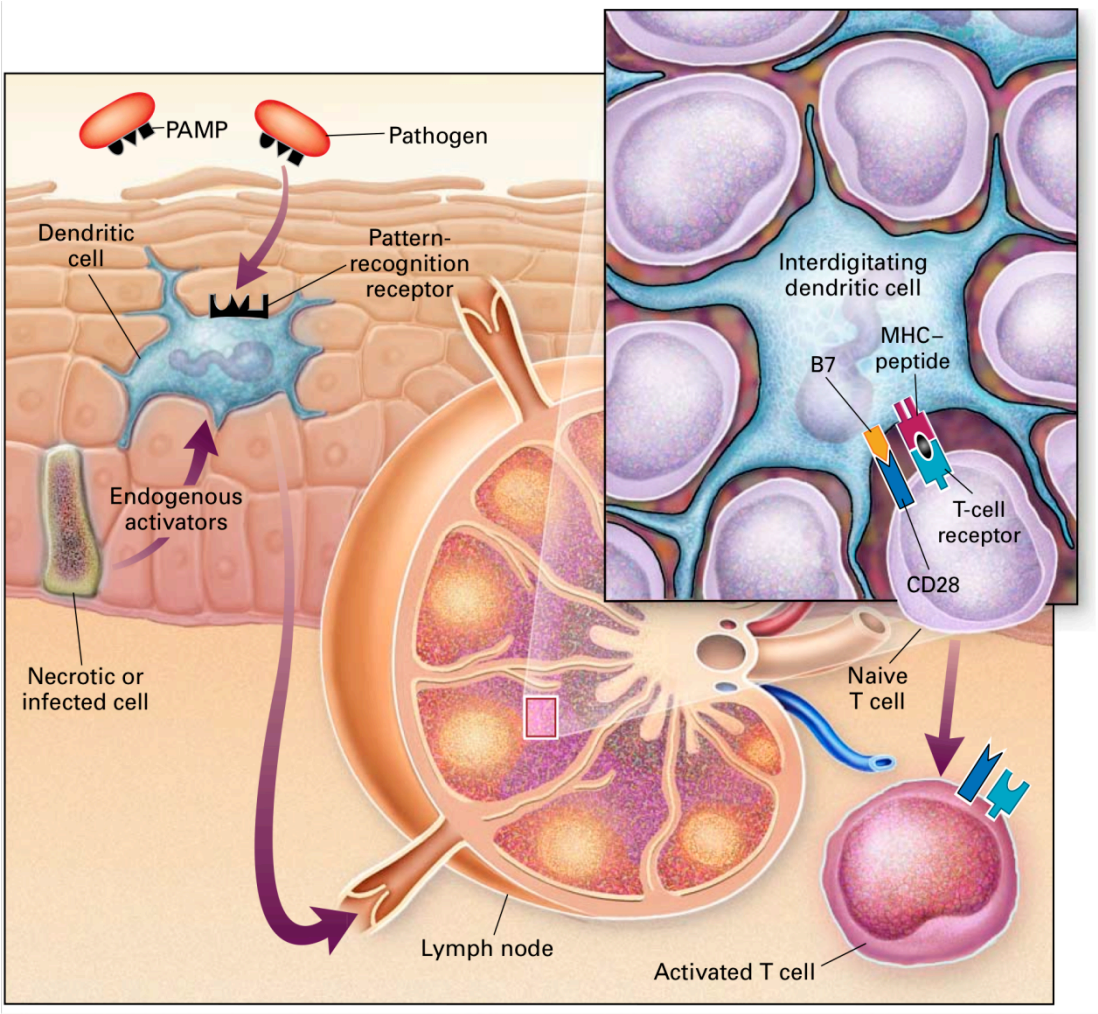


Figure 1.10 Dendritic cell activation. The dendritic cell in the tissue is activated via endogenous activators or via the pattern recognition receptor. It then travels to the local lymph node where after processing the antigen behaves like an antigen presenting cell. Within the lymph node, the dendritic cell actives the T lymphocyte [141].

There are two distinct families of MHC molecules in humans, also known as Human leukocyte antigen (HLA), named as class I and class II. Class I molecules are present on the majority of nucleated cells except antigen presenting cells such as dendritic cells, macrophages and B cells. Class II molecules are present mainly on antigen-presenting cells such as dendritic cells, macrophages and B cells. The structure of the molecule consists of two chains that are folded together to create a cleft for the antigen peptide to sit. MHC I molecules are classified as a trimer because they are composed of a polypeptide that is folded to create three separate domains. While MHC II molecules are heterodimers which consists of two homogenous peptides, α and β chains. There are three main isoforms of the class I molecule and they are

HLA-A, B and C. There are also three main isoforms of class II molecules and they are HLA-DP, DQ and DR.

Nucleated cells present self-peptides loaded onto the MHC I molecule on the cell surface. It is obtained from protein turnover and defective ribosomal products during viral infections, intracellular microorganism infections and cancers. While with antigen presenting cells, the proteins are cleaved into peptides and incorporated with the MHC II molecule and externalized on the cell surface [144].

Another important cell of the innate immune system is the NK cell. Their name was derived from their ability to destroy cells which were infected or had cancerous change without having to be activated. They are able to detect suspicious cells by identifying abnormalities in those cells' MHC molecules.

1.5.2 Adaptive Immune system

The innate immune system is limited to the variations of pathogens it can detect. As a result the adaptive immune system is required to detect pathogens that have high number of mutations and also the ability to replicate intracellularly. The major components of the adaptive immune system are lymphocytes. They originate from bone marrow derived precursors and differentiate peripherally into mature effector cells.

There are two main lymphocytes involved with the adaptive immune response and they are B cells and T cells. B cells produce antibodies also known as immunoglobulins, which are involved in the humoral activity while T cells function by cell-mediated immunity.

More than 10^{12} variations of antibodies are capable of being produced by B cells. Antibodies consist of two identical heavy chains and two identical light chains which are held together by disulfide bonds [145]. There are two types of light chains (κ and λ) and five types of heavy chains (α, γ, δ, ε and μ). The C terminal of the heavy and light chain is the constant region of the antibody. The amino acid sequence of the heavy chain determines the five classes (IgG, IgA, IgM, IgD and IgE) of the immunoglobulins. The variable domain is located on the N terminal and it is here where binding to the antigen occurs.

Once a B cell encounters an antigen by interacting with its receptor and with the assistance of supportive cells and signals, it divides into plasma cells [146]. The majority of plasma cells return to the bone marrow, where the B cell originated, to produce large quantities of specific antibodies into the blood. This process is known as the humoral response and does not require the plasma cell to be present at the site of the antigen, allowing for the generation of a response from a distance. The plasma cells have a short life, about two to three days. A small proportion of plasma cells become long-term antigen specific memory cells. These cells are primed with antibodies and are primed to produce an acute response upon re-infection of the same pathogen.

Antibodies attach to their specific antigen and are able to exert many effects on the pathogen. Some may block and neutralize the pathogen from binding to specific cellular targets or receptors required for replication. They can form antibody-antigen complex facilitating the recruitment of effector cells and allow the pathogen to undergo phagocytosis by the innate immune response. Also they can activate the complement cascade resulting in cell lysis.

1.5.2.1 T Lymphocytes

T lymphocytes or T cells play an important role in cell-mediated immunity. These cells are capable of detecting intracellular pathogens by using the

nucleated cells ability to externalize peptide fragments onto the surface via the MHC molecule. In order to interact with these molecules, T cells have antigen-specific receptors on the surface called T cell receptors (TCR).

Like B cell antibodies, there are similar numbers of TCRs for specific antigens. The diversity of TCR allows interaction with a specific peptide/MHC combination. The internal pathogen of a cell can be detected on the surface allowing for the cells integrity to be maintained. The structure of the TCR is composed of α/β or γ/δ heterodimers which contain both variable and constant domains. This leads to the development of a variety of TCR which has the potential to form autoreactive receptors to self. As a result these receptors are screened and eliminated in clonal selection. T cells originate as stem cells from the bone marrow and migrate to the thymus where they develop into the individual subsets [147]. In the thymus T cells are positively selected if the express a TCR that is specific for a MHC complex. For this cell, the spontaneous apoptosis signal is switched off and the cell is sent into circulation. However about 95% of developing T cells are not positively selected and die within the thymus. However there are T cells which are induced to go into apoptosis in the thymus as part of the negative selection process. They undergo this process because they express TCR that have high affinity to self-peptide or self-MHC on dendritic cells and macrophages.

Within the thymus, T cells undergo a process of education where their function is defined by the switching on or off certain surface molecules. These surface molecules are important in the immune response and were characterized according to their interaction with panels of monoclonal antibodies. CD3 is expressed by all T lymphocytes, CD4 binds to MHC calls II molecules while CD8 binds to MHC class I molecules. During the early development of T cells they initially express both CD4 and CD8 within the thymus. As these immature T cells mature, they lose one of the CD molecules and become positive for either CD4 or CD8.

There are subsets of T lymphocytes which have different functions. The main T cell subpopulations are: T helper, cytotoxic, memory, regulatory and gamma delta T cells. These cells express different CD markers on their surface and are shown in table 1.6.

Name	CD marker
Total lymphocyte count	CD45
T cells	CD3
B cells	CD19
Cytotoxic T lymphocyte	CD3, CD8
T helper cell	CD3, CD4
Memory T cell	CD3, CD45RO
Treg cell	CD3, CD4, CD25

Table 1.6 CD markers for specific lymphocyte population. However additional non-CD markers are required to define specific T cells [148].

1.5.2.2 Cytotoxic T Lymphocytes

Cytotoxic T lymphocytes (CTL) are also known as effector T cells. They are CD8 positive lymphocytes and a have high affinity for MHC class I molecules. Their function is to kill infected or damaged cells such as tumour cells. When the TCR of a naïve CD8+ CTL encounters a specific MHC I and antigen complex, it binds strongly to this complex depending on its affinity. Once the CTL has been activated, it undergoes clonal expansion and rapidly produces activated CTLs to travel through the body in search of its cognate MHC I and antigen complex.

When the CTLs encounter these infected or dysfunctional cells, they will secrete perforins which are pore forming proteins. This will facilitate the influx of water and ions into the cell resulting in the cell bursting or lysing. Also CTLs secrete proteases such as granzymes intracellularly through the cell pores resulting in the activation of the caspase enzymes that initiates apoptosis of the antigen-expressing cell [149]. CTLs also have the capability to inhibit viral replication without damaging the host cells, which is important in cells such as neurons. The CTLs have another mechanism by which they activate the caspase enzymes to induce apoptosis and this is via the activation of the Fas ligand on the target cell [150].

CTLs have the capability to produce cytokines such as tissue necrosis factor α, lymphotoxin and interferon γ. These cytokines have the ability to inhibit intracellular viral and cancer cell replication.

When the acute phase of the infection is finished, majority of the CTLs will die and be disposed of by phagocytes. There is a small population of these cells who become memory cells. These cells will undergo rapid expansion and initiate an effector response more timely when the antigen in re-encountered in the future.

1.5.2.3 T helper cells

T helper cells are also known as CD4 lymphocytes due to the expression of CD4 on their surface. These cells function by assisting other cells such as CTLs with the adaptive immune response. There is emerging evidence of the presence of a small proportion of CD4+ T lymphocytes with cytolytic properties. These cells have been found in persistent viral infections such as Epstein-Barr virus (EBV), hepatitis C virus and Human immunodeficiency virus (HIV) [151-153].

These lymphocytes' TCRs are activated by interacting with MHC II and antigen complex. Also helper cells are activated by dendritic cells which migrate to lymphoid organs and induce differentiation of naïve T cells to helper cells by delivering antigen and co-stimulatory signals.

There are four main types of T helper lymphocytes. Type 1 (Th1) helper cells secrete IL-2, TNFα and interferon γ (IFNγ) that assist with increasing the expression of MHC molecules and effective virus eradication. Also it promotes CTL replication and activity as well as phagocytosis. Th1 is associated with supporting an immune response against intracellular pathogens.

Type 2 (Th2) helper cells secrete interleukin-4, 5, 6, 10 and 13. These cells are associated with the activation of eosinophils and mastocytes that are involved in killing large extracellular bacteria, parasites and toxins [154].

Another group of helper T cells known as the follicular T helper cells (fTh), was initially associated with Th2 group. It is promotes the activation of B cells in follicular lymphoid organs especially after vaccination. They were differentiated for the Th2 group as they expressed a separate genetic makeup that did not produce interleukin-4 and 13 [155].

Some of these T helper cells secrete cytokines interleukin-17 and 22 and are referred to as T helper 17 cell (Th17). They are involved with the assistance of the immune system against the selected bacterial and fungal pathogens [156]. Th17 are also involved with governance of the immune system in the gastrointestinal and respiratory tract.

Like other lymphocytes, T helper lymphocytes die after an immune response referred to as activation induced cell death (AICD). However a small

percentage of these cells remain as memory cells, primed for the re-encounter with the same pathogen.

1.5.2.4 Gamma delta T cells

Gamma delta T cells (γδT) possess a variant of TCR that does not interact with the conventional MHC antigen complex. They are capable of recognizing molecules such as glycolipids.

1.5.2.5 Mucosal-associated invariant T cells

Constitute 10% of the peripheral T cell population [157]. A range of microorganisms such as bacteria and yeasts activates mucosal-associated invariant T (MAIT) cells. This is achieved by their TCR similar to that seen in NKT cells. MAIT TCRs are restricted to monomorphic MHC class I like related molecule (MR1).

1.5.2.6 Memory T cells

The major characteristic of the adaptive immune system is the ability for it to have memory. This is carried out by a set of antigen specific T cells that expand and mount an optimal immune response on re-exposure of a previously encountered antigen. On the recurrent encounter of the same pathogen, they rapidly expand and institute an immune response.

The development of memory T cells occurs after an acute infection or immunization. After encountering an acute encounter with a pathogen, the CTL undergo massive expansion known as clonal expansion. Here the antigen specific CTL increases 50,000 fold in comparison to its precursor population [158, 159]. Once the pathogen has been cleared, the CTL undergo the contraction phase where approximately 90-95% of the cells undergo apoptosis [160, 161]. The remaining cells are antigen-specific memory cells which

provide long-lived protection from reinfection [162, 163]. These memory cells can survive for periods greater than 50 years without the presence of encountering the same antigen [164, 165].

Two main processes preserve the pool of memory cells. One is the process of constant cell turnover and the second is the survival in a resting state. Cytokines such as interleukin-15 (IL-15), IL-7 and IL-21 govern the maintenance of this memory cell population [166-169]. Interleukin-15 controls proliferation while IL-7 directs survival by increasing antiapoptotic molecules such as Bcl-2 and Bcl-XL, however their functions do overlap [170, 171]. IL-21 can promoted expansion of CD8+ T cells is valuable in Ig production by B cells, inhibit dendritic cells and be pro-apoptotic for B and NK cells [172-174].

There is phenotypic heterogeneity in the memory cell population. Memory cells consist of two main subtypes: central memory T cells and effector memory T cells. This division is based on the expression of lymphoid homing receptors CC-chemokine receptor 7 (CCR7) and L-selectin (CD62L) [161, 175, 176]. The central memory T cells are $CCR7^+$ and $CD62L^+$ while effector memory T cells are $CCR7^-$ and $CD62L^-$ [166, 177]. The migration and function of these cells are varied. Central memory T cells circulate through secondary lymphoid tissues, such as liver, spleen and bone marrow, where they produce IL2 and small proportion of IFNγ and no perforins. The effector memory T cells on the other hand circulate to non-lymphoid tissue in the periphery such as the lungs, gut and the skin, where they secrete IFNγ, perforins, IL-4 and to a lesser degree of IL-2 [176, 178, 179].

There is controversy on the derivation of this subset of memory cells. One theory is that after an acute infection, where there is an initial high population effector memory T cells, there is gradual transformation of these cells to central memory T cells [180]. There have been other theories put forward that effector memory and central memory T cells are derived from different precursor cells

[161, 181]. However it has been proposed that the central memory T cells undergo proliferation in the lymphoid organs and are responsible for replenishing the memory T cell population. In the periphery, effector memory T cells function to eliminate antigen and mediate effector function.

Upon the second encounter on a certain pathogen, the memory cells in the periphery initiate an immediate effector function. This results in inhibiting early pathogen infected cells from replication [165, 177, 182]. Also another function carried out simultaneously by the peripheral memory cells is to secrete pro-inflammatory cytokines and chemokines which instigate the recruitment of circulating memory cells to the site of inflammation [183, 184]. Of the increased infiltration of memory cells to the site, the proportion of the antigen specific memory cells is only a fraction. The recruited antigen specific memory cells undergo clonal expansion in the periphery and amplify the immune response. This results in rapid clearance of the reoffending pathogen.

1.5.2.7 Regulatory T cells

In the 1970's, Greshon et al first described the presence of suppressor cells in the immune system where they inhibit T cell function and mediate immunological tolerance and self/non-self discrimination [185, 186]. This initial research was disputed and eventually abandoned in the 1980's due to the lack of markers to characterize these cells [187, 188]. However it was in the mid-1990's where a subpopulation of CD4 positive cells which had suppressive function was characterized as Regulatory T (Treg) cells. As a result there are two main lineages of CD4 T cells, which are T helper cells, and Treg cells.

Treg cells are a group of T cells that police the immune system. They are mainly primary mediators of peripheral tolerance where they are involved in preventing autoimmune diseases such as type I diabetes mellitus, asthma and inflammatory bowel disease [189-192]. However they also have a negative

effect in that they have the potential to block beneficial responses. Here they inhibit sterilizing immunity to certain pathogens or limit antitumour immunity.

The identification of Treg cells is made by the use of multiple markers. They express cell surface markers such as CD25, the α-chain of IL-2 receptor. Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) is a negative regulator of T cell activation and is upregulated on CD4 and CD8 cells a few days after activation. Glucocorticoid-induced tumour necrosis factor family-related gene (GITR) and lymphocyte activation gene-3 (LAG-3) are similarly expressed in activated T cells. Treg cells express low numbers of CD127, the α-chain of the IL-7 receptor in comparison to the higher amounts expressed on T helper cells. The most sensitive marker that is currently available for detecting Treg cells is the Forkhead-winged helix transcription factor (FoxP3) [193, 194]. This marker is mainly restricted to CD4 T cells but has also been found to be positive in some CD8 cells [195].

In 2003 FoxP3 was described as the specific marker distinguishing Treg cells from other T cells [193, 194]. It is mostly present in lymphoid tissues and is highly restricted to $\alpha\beta$ T cells, and almost undetectable in B cells, $\gamma\delta$T cells, NK cells, macrophages and DC [194-196].

FoxP3 expression programs T cells to have their regulatory function. There are many studies showing the importance of FoxP3 as a transcriptional regulator. One such study showed that CD4 and CD8 cells that were initially FoxP3 negative but were reprogrammed to be suppressor cells by gene transfer with FoxP3 [197]. This was shown in a mouse model where autoimmune gastritis and colitis were resolved with transfer of CD4+ T cells with transfected FoxP3 [197].

The significance of FoxP3 in Treg cell function was realized in two severe multiorgan autoimmune syndromes in humans where FoxP3 is absent. These two syndromes are XLAAD (X-linked autoimmune-allergic dysregulation syndrome) and IPEX (Immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome). With IPEX, boys are affected and often die in infancy or up to the age of two. They exhibit autoimmune disorders of multiple organs resulting in insulin-dependent diabetes mellitus, thyroid autoimmunity, haemolytic anaemia, inflammatory skin lesions and diarrhoea [198]. Their organs are infiltrated by large quantities of lymphocytes that are autoreactive, which are not regulated and suppressed by Treg cells. The treatment for this syndrome is bone marrow transplantation which results in restoration of Treg cell population.

A similar autoimmune disease is found in mice called scurfy mice. It was in 1949 in Oak Ridge National Laboratory where the gene scurfin, was isolated and was found to be mutated. This resulted in the male mice to exhibit an almost a graft-versus-host like disease where lymphocyte infiltration was found in multiple organs and the mice succumbing after three to four weeks [199]. The scurfin gene was shown to be a member of FoxP3 [200]. Reconstituting normal CD4+CD25+ T cells could also treat these mice. Conversely specific ablation of FoxP3 in normal mice resulted in lymphoproliferative autoimmune syndrome similar to that seen in scurfy mice [195]. Therefore FoxP3 is an essential component of Treg function.

There are four main mechanisms by which Treg cells exert their suppressive activity by: inhibitory cytokines, cytolysis of effector T cells, metabolic disruption and targeting DC (refer to figure 1.11).

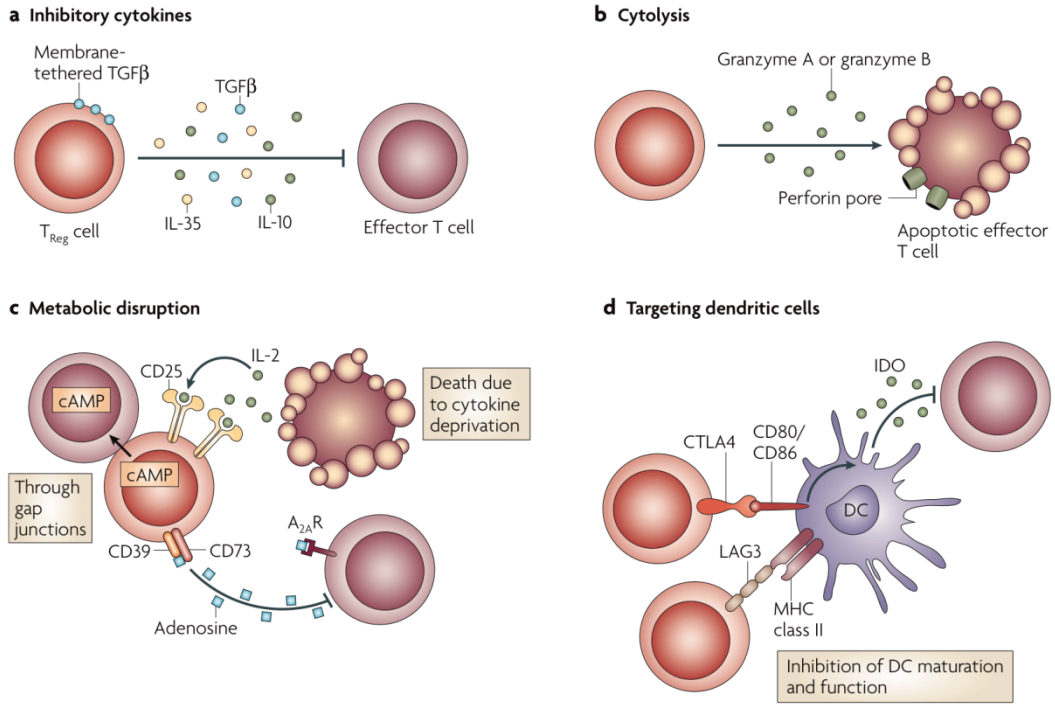


Figure 1.11 The four modalities of immunosuppression exerted by Treg cells [201].

The two main inhibitory cytokines that are well established and are used by Treg cells are transforming growth factor β (TGFβ) and IL-10. These cytokines are directly secreted by Treg cells at the site of inflammation to affect the activity of effector T cells and antigen presenting cells (APC). When TGFβ-1 is secreted, it inhibits and reduces the amount of cytokines produced by activated T cells without disrupting their capability to expand or inducing apoptosis [202, 203]. Membrane bound TGFβ can also facilitate suppression by Treg cells in a cell-to-cell contact dependent manner [204]. This mechanism has been shown to delay the development of diabetes where membrane bound TGFβ of Treg cells inhibit the infiltration of CD8 cells into pancreatic islet cells [205].

Another function of TGFβ is to induce the production of its co-inhibitory cytokine IL-10 from Th1 cells [203]. One of IL-10 function is to inhibit CTL production of cytokines [206]. It also makes T cells more susceptible to the effects of TGFβ by means of TGF-receptor expression [203].

The secretion of IL-10 by Treg cells also inhibits the activity of APC. This is achieved by downregulating the production of the cytokine IL-12 by APC which is required to activate other APC [207]. As a result this indirectly inhibits the production of IL-2 which is produced when T cells interact with activated APC and hence reducing the magnitude of the immune reaction [208].

The second mechanism is cytolysis of effector T cells. This is achieved with the secretion of granzymes or perforins by the Treg cells [209]. Initially thought to be products of natural killer T (NKT) cells and CTLs, Tregs have been shown to posses this mechanism as part of its arsenal. Both granzyme A and B have been shown to be involved with Treg cells induced cytolysis of effector T cells [209-211].

The mechanism of T cell suppression by metabolic disruption describes the inhibition of T cell proliferation and cytokine production [212, 213]. Depletion of IL-2 by Treg cells reduces the survival of actively dividing effector T cells [212]. This is achieved by either inhibition of IL-2 production or increased consumption [214, 215]. These two methods are implemented once the TCR is activated.

Two other methods have also been proposed as part of metabolic disruption employed by Treg cells. The expression of ectoenzymes CD39 and CD73, releases adenosine nucleosides which suppress effector T cells by activating the adenosine receptor 2A [216-218]. This not only inhibits effector T cell function but also inhibits IL-6 expression and promotes the production of TGFβ [219]. Interleukin-6 functions by inhibiting Treg cells proliferation and enhances the immune response by supporting Th17 cell maturation [210, 211, 220]. The other method by which Treg cells act is by transferring messenger cyclic AMP in the effector T cell via membrane gap junctions resulting in its inhibition [221].

The final method employed by Treg cells to suppress effector function is by inhibiting DC. The evidence to support this theory is limited but the effect on DC maturation and function is important as it affects effector T cell activation. Treg cells have the capacity to downregulate the ability of DC from activating effector T cells. This is achieved by lowering the expression of co-stimulatory molecules such as CD80 and CD86 [222]. Treg cells also condition DC to express indoleamine 2,3-dioxygenase (IDO). This is a molecule involved with promoting and producing pro-apoptotic metabolites that result in effector T cell suppression via the mechanism dependent on interaction between CTLA4 and CD80 and/or CD86 [223, 224]. Another mechanism used by Treg cells is the utilization of LAG-3 also known as CD223. This has been implicated in blocking DC maturation and its capacity to stimulate the immune system [225].

1.5.2.8 Tumour infiltrating lymphocytes

Tumour infiltrating lymphocytes (TILs) have been thought as a manifestation of the host's immune system in combating a cancer. The influx of T cells into the tumour environment in order to exerts its antitumour effector function. There are numerous studies correlating the presence of TILs and prognosis. Most of these results do show an improved outcome in patients who exhibit a higher number or TILs.

An example of a study is by Zhang et al in 2003 who examined the relationship between TILs with epithelial ovarian cancer recurrence and survival [226]. The study had enrolled 186 patients and showed that 60% had the presence of TILs. There was a positive correlation between the presence of TILs and improved survival, where there was a 38% five-year survival in comparison to 4.5% in patients who did not have TILs. Also 40% of the sample population had a debulking procedure and platinum based tumour therapy. In this group 74% of patient who had TILs had complete clinical response while only 12% of patients with no TILs showed a similar effect. Similar results have been shown

in other tumours such colorectal, non small cell lung cancer, hepatocellular carcinoma and melanoma [227].

The interpretation of TILs should be conducted carefully as the true picture of the local tumour environment may be distorted. Infiltration by TILs may not always be indicative of a positive prognosis. The type of T cells present and their quantity are important in predicting prognosis [228]. There are reports showing the presence of high number of TILs with poor prognosis. This is attributed to the TILs being defective, incompletely activated and to the presence of high numbers of Treg cells [229-232]. For instance high numbers of Tregs in melanoma have been shown to be associated with tumour recurrence [233].

The preferred method of analysing the TILs is by assessing the CD4:CD8 ratio. This provides a more accurate prognostic factor for patient survival [234]. Also patients with high number of intraepithelial CD8 T cell infiltration had a better prognosis [235, 236]. Finally a high CD8:Treg ratio has also been shown to have good prognostic outcome in cancers such as ovarian, hepatocellular and cervical cancer [235, 237, 238]. There is still considerable debate on the assessment of TILs as a prognostic marker. Until there are large trials showing the prognostic value of TILs, this method remains in the research realm rather than in common clinical practice.

1.6 The link between inflammation and carcinogenesis

Inflammation in the prostate could arise after infection, epithelial cellular injury due to urinary reflux resulting in chemical or physical injury, or dietary factors. High reactive chemical compounds such as superoxides, hydrogen peroxide, singlet oxygen and NO are released from activated phagocytic inflammatory

cells of the innate immune system and this can result in oxidative and nitrosative damage to DNA in epithelial cells or initiate the free radical chain reaction [239]. When these epithelial cells undergo DNA synthesis in the setting of free radicals or DNA damage, they are prone to mutation and tumourigenesis. Cytokines secreted by activated inflammatory cells are capable of enhancing cell proliferation and facilitating angiogenesis for tumour growth.

Histological sections of prostate tissue often show evidence of inflammatory infiltration and proliferative inflammatory atrophy (PIA). PIA is a term used to describe tissue with epithelial atrophy, low apoptotic index and increased proliferative index [240]. PIA develops due to the above causes of inflammation and in its high proliferative state, may accrue a number of mutations, such as GSTP1, to progress to high-grade prostatic intraepithelial neoplasia (PIN) and eventually cancer [241]. Refer to figure 1.12 showing the passage of events from chronic inflammation to the development of tumour.

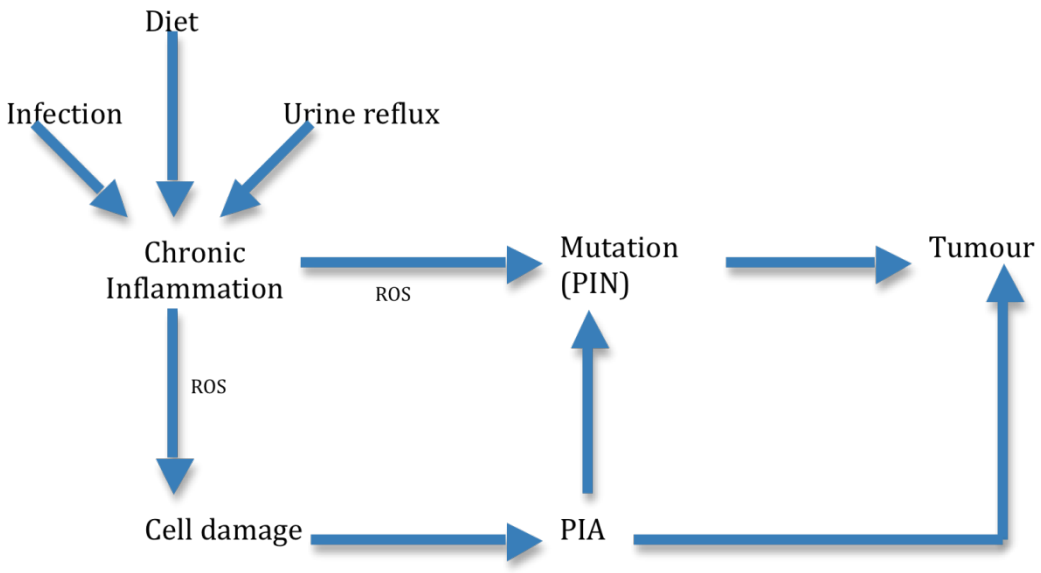


Figure 1.12 Progression from chronic inflammation to tumourigenesis. ROS - Reactive oxygen species. Modified from [242].

Inflammation, including CAP, is associated with increased risk of PCa development, the use of anti-inflammatory medications such as NSAIDs have been studied. In some small prospective studies a 15-20% reduction has been shown in patients who regularly use aspirin or NSAIDs [243-245]. However this result was disputed by larger studies which have shown that there were no protective effects [246].

The precise cause of prostate inflammation is not certain however there are potential causes that result in inflammation. Some of these are infection, urine reflux resulting in chemical or physical trauma, dietary and environmental factors. These possible inflammatory causes can occur individually or take place in combination.

The American Cancer Society estimates that 20% of all cancers develop after the onset of infections [239, 247]. For example cancers such as gastric cancers arise due to previous Helicobacter pylori infections. Cervical cancers are almost always related to Human papillomavirus (HPV) infection, EBV is associated with the development of Hodgkin's and Burkitt's lymphoma while hepatocellular carcinoma can develop after chronic Hepatitis B or C infections [242].

A meta-analysis conducted on case-control studies from 1966 to 2000 demonstrated an association between a history of prostatitis and the future risk of developing PCa (odds ratio 1.8, 95% confidence interval 1.1 to 3.0) [248]. It was also observed that there was an increased relative risk, in men with a history of sexually transmitted infections such as syphilis (odds ratio 2.3) and gonorrhoea (odds ratio 1.3). This study is limited by recall bias however it supports a plausible association between prostatic inflammation and PCa.

Although viruses are often present in prostate tissue, their mechanism of causing an inflammatory response is not fully understood [249]. Viruses such as HPV, cytomegalovirus (CMV) and human herpes simplex virus (HSV)(type 2 and 8) have been detected in the prostate. In 2006, Xenotropic murine leukaemia virus (XMRV), a gammaretrovirus previously found in animals, has been isolated from human PCa tissue. One study showed that it was present in 40% of patients who had homozygous mutation of ribonuclease L (RNase L) especially the R462Q variant [250]. RNase L is an endoribonuclease that is important in the antiviral defence of the host, suggesting that impaired immunity against this pathogen may have been associated with the risk of developing malignancy. Another study which looked at 334 PCa specimens found 23% of the samples to have XMRV protein expression in high grade tumour [249]. However in this study the XMRV infection and the development of cancer were independent of RNase L gene mutation. In contrast, a study conducted in Germany which examined more than 500 tissue samples showed no evidence of infection [251]. This was initially thought to be due to a variable geographic distribution.

However of recent times, the link between and XMRV and PCa development has been disproven. There have been many negative studies showing no link and studies demonstrating contamination of samples and reagents leading to a false positive result [252-257].

Physical or chemical damage to the prostate epithelial cells can be attributed to urine reflux. The main chemical in the urine that is most risk to the tissue is uric acid [25]. However urine reflux in conjunction with infection can exacerbate the magnitude of inflammation. Also spermatozoa have been seen in prostate tissue as a result of retrograde movement. They have been localized in areas of inflammation and PIA [258].

1.7 Role of immunity in control of disease (Immunoediting)

Inflammation may either promote or inhibit tumour growth, depending on context. In the 1950s, Burnet and Thomas put forward the 'immune surveillance' hypothesis, where the immune system has the ability to recognise and destroy nascent transformed cells [259]. However, this hypothesis subsequently fell into disrepute. The broader term "cancer immunoediting" was more appropriate to describe the host-protecting and tumour-sculpting actions of the immune system [260]. In cancer immunoediting, there are three proposed phases: (1) elimination; (2) equilibrium; and (3) escape (refer to figure 1.13). These are commonly referred to as the three Es of cancer immunoediting.

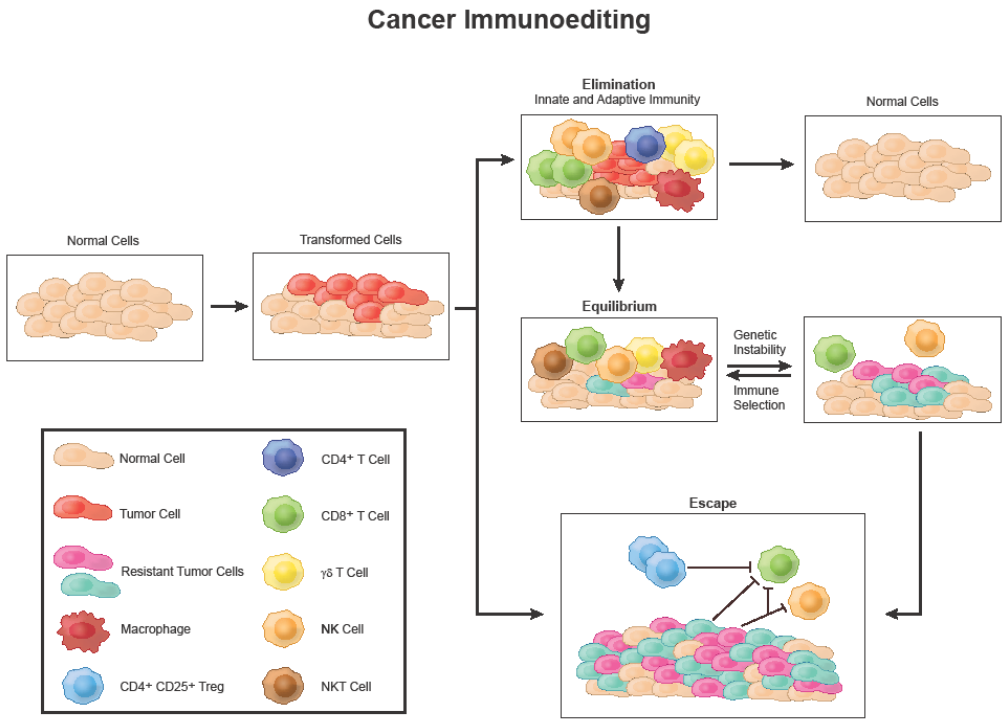


Figure 1.13 The three phases of immunoediting: Elimination-the process of identifying and eradicating tumour cells; Equilibrium- where the proliferation and destruction of the tumour cells is at a balance; Escape- where the tumour growth outweighs its elimination [261].

1.7.1 Elimination

The elimination phase encompasses the original cancer immune surveillance hypothesis in which there is successful eradication of the developing tumour. The process of elimination includes innate and adaptive immune responses to the tumour cells. Important components of the immune system are T lymphocytes and NK cells discussed earlier.

There are four phases in the elimination process. The first phase is where cells of the innate immune system are recruited to the tumour site via pro-inflammatory signals. T cells and NK cells produce IFN-γ. In the second phase IFN-γ and other cytokines, chemokines and inflammatory molecules induce the production of chemokines from tumour cells as well as the surrounding normal tissue. These chemokines cause tumour death by anti-angiogenic effects and NK and macrophage recruitment. In the third phase, the recruited tumour-infiltrating NK and macrophages kill more tumour cells by activating cytotoxic mechanisms such as tumour necrosis factor-related apoptosis-inducing ligand, perforin and reactive oxygen and nitrogen intermediates. Within local lymph nodes, DC that have migrated from the inflamed region induce tumour-specific CD4+ T helper cells, which in turn facilitate the development of tumour-specific CD8+ T cells. During the fourth phase, the tumour-specific CD4+ and CD8+ T cells travel to the tumour site. Here the cytotoxic T lymphocytes eradicate the remaining tumour cells which have been exposed to local IFN-γ.

1.7.2 Equilibrium

The equilibrium stage is where the tumour cells that have survived the immune surveillance and the host immune system are in balance. There may be ongoing destruction of tumour cells by lymphocytes but this immune pressure in turn leads to selection and “sculpting” of the antigenic profile of the tumour cells, leading to variant mutations able to resist the immune attack. This phase may

be the longest of the three phases and is thought to often proceed over many years.

1.7.3 Escape

Finally during the escape phase, the tumour cells have evaded detection and elimination by the host immune system either by genetic or epigenetic transformation. These cells proliferate uncontrollably resulting in dissemination of the tumour variant. If this process is unhindered, the outcome will be the demise of the host.

Mechanisms by which PCa can escape the host's immunogenic defences include defective antigen presentation, expression of immunosuppressive molecules, TCR dysfunction, and active immune down-modulation through mechanisms such as Treg cells or myeloid-derived suppressor cells.

1.7.3.1 Defective antigen presentation

T cells recognize antigenic peptides presented on HLA complexes [262]. DC are APC that lead to specific immune responses, as they are able to activate antigen-specific T cells. Tumour cells can evade recognition and eradication from CTL if there is defective antigen presentation, such as down-regulation of HLA class I expression, defective antigen processing, or simply loss of antigen expression. HLA class I antigen expression has been shown often to be reduced in PCa. This implies that this mechanism of immune evasion is feasible in PCa, and that selective pressure has occurred in vivo in order to select for cancer clones with low levels of expression [263]. In PCa tissue, DC have been found to be reduced in number in comparison to normal prostate tissue [264]. They were also found to have reduced activity. However the DC in peripheral blood were found to be fully functional and able to be targeted with immunotherapeutic treatments [265].

1.7.3.2 Immunosuppressive substances

Immunosuppressive cytokines such as interleukins have been found to be present in higher levels in serum of PCa patients in comparison to normal controls. The presence of high levels of IL-6 has been shown to be a poor prognostic factor and also to have direct effects on tumour growth and T cell dysfunction [266]. IL-6 and its receptors are expressed in the epithelium and stroma of prostate tissue. As a result, cytokines may regulate PCa growth in an autocrine and paracrine manner.

Another immunosuppressive substance is TGFβ. It is a cytokine that controls proliferation, and plays a role in many immune processes such as homing, cellular adhesion, chemotaxis and T cell activation, differentiation and apoptosis. It is also involved in PCa cell growth whereby stromal cells deficient in TGF-β responsiveness were prone to tumourigenesis via an oncogenic signalling pathway using Wnt3a proteins.

L-arginine is an amino acid; it is metabolised by nitric oxide synthase (NOS) to produce free radicals such as NO and L-ornithine. The increased metabolism of L-arginine within tumours has been suggested as resulting in tumour growth, angiogenesis, metastasis and tumour-related immunosuppression [267]. Furthermore, studies have shown that high levels of NOS is related to poor survival in PCa [268].

Finally, cyclooxygenase-2 (COX-2) is an enzyme that converts arachidonic acid to prostaglandins. It is often overexpressed and activated in inflammation and cancers [269]. In PCa, a high COX-2 expression has been associated with a high Gleason score, local chronic inflammation and tumour neovascularisation [270]. Immunosuppression and pro-tumourigenic effects such as inhibition of

apoptosis, angiogenesis and enhanced cell invasiveness have all been linked with COX-2 actions.

1.7.3.3 T cell receptor dysfunction

Antigens are recognised by T cells via the TCR molecule. Diminished or irregular expression of TCR has been implicated in many tumours and is seen to be a mechanism of tumour escape. Also co-inhibitory signals such as CTLA-4, programmed death-1 (PD-1), SHP1 can inhibit TCR signalling and down regulate the immune activity [271].

Viruses such as hepatitis C virus are able to substitute viral amino acid resulting in decreased MHC binding and impaired antigen processing. It results in reduced antigen density on the cell surface allowing for evasion of T cell responses [272]. This substitution in viral epitopes alters the TCR contact residues and results in escape [272].

1.7.3.4 Active immune down-modulation

T regulatory cells are a subset of T cells which express CD25, the ∝–chain of the IL-2 receptor and FoxP3 a transcription factor. They play a role ongoing active suppression of immune responses to normal self-antigens, thereby protecting against clinical autoimmune diseases such as inflammatory bowel diseases [273]. There has been evidence showing a high frequency of Treg cells in PCa tissue allowing for the tumour cells to escape the inhibitory effects of the immune system [274].

Myeloid-derived suppressor cells (MDSC), have suppressive properties in cancer where they inhibit the activation and proliferation of tumour specific T cells and also induce apoptosis. They originate from haematopoietic progenitor cells and are released into circulation to localise in the tumour

microenvironment and lymph nodes. Their mode of action is by secretion of factors such as reactive oxygen species, NO, IL-10 and TGFβ [275].

1.8 Use of immune strategies to treat cancer

Facets of the immune system can be harvested and activated to combat tumour cells.

1.8.1 Immunotherapy

The role of anti-tumour immunotherapy is to generate a T cell response to recognise and destroy tumour cells [276]. Immunotherapy functions by inducing T cells against a specific tumour antigen and it can be delivered in the form of a vaccine.

One approach tested recently in castrate resistant PCa is the GVAX vaccine. This preparation comprises cells from PCa cell lines; LNCaP and PC3, which are genetically modified to produce granulocyte macrophage-colony stimulating factor (GM-CSF). The GVAX vaccine is recognised as a source of foreign antigens and antigen presentation is enhanced by the presence of the GM-CSF. Antigen-loaded APC travel to lymph nodes and stimulate and activate CD4+ and CD8+ T cells resulting in an immune reaction against the antigen. The initial phase I and II studies of GVAX were promising however phase III studies named VITAL-1 and VITAL-2 had to be terminated due to a higher death rate in the GVAX arm, with futility analysis showed less than 30% chance of obtaining a survival benefit. As a result the future use of this vaccine is not clear.

In contrast, the most recent immunotherapeutic agent showing promise in PCa is Sipuleucel-T, also known as APC8015 or Provenge® (Dendreon). This

treatment involves central processing and preparation of the therapeutic product, comprising autologous APC primed with a fusion protein of prostatic acid phosphatase (PAP), a molecule that is commonly expressed on PCa cells. The PAP antigen is linked to GM-CSF, an immune cell activator. Activated and loaded APCs present these antigens to T cells and stimulate and expand effector T cells. The IMPACT trial, a multi-centre, randomised, double blinded, placebo-controlled study treated men with asymptomatic metastatic androgen-independent PCa with the immunotherapeutic agent Sipuleucel-T. When compared to the placebo, Sipuleucel-T improved median survival by 4.1 months, improved three-year survival by 38% and reduced the overall risk of death by 22.5% while having a modest toxicity profile [277]. The result of this therapy is promising and it has now been FDA approved for clinical use.

Other immunotherapy methods used in PCa, block the immune checkpoints. One of them blocks CTLA-4 which is an inhibitory regulator of T cell responses and is able to downregulate immune response against tumour cells. The blockade of CTLA-4 has been studied extensively in melanoma using the agent Ipilimumab [278]. In PCa there have been trials which have shown a decline in PSA and tumour size regression [279]. However the use of this agent is associated with immune associated toxicities such as colitis, dermatitis, pneumonitis, hypothyroidism and neutropenia [278, 279].

Another immune checkpoint inhibitor that has been developed to results in an antitumour response targets PD-1. The interaction between PD-1 and its ligand results in inhibition of T cell function and by blocking this process, an antitumour response is achieved. An anti-PD-1 antibody, MDX-1106, was examined in a phase 1 trial of patients with solid organ malignancies including metastatic castrate resistant PCa [280]. The study had lower toxicity profile than CTLA-4 blockade but no significant result was seen. This was due to the only small study population of 17 however further larger studies are proposed to assess the potential benefits [281].

1.8.2 Androgen deprivation therapy and immune function

As mentioned earlier, the role of ADT in PCa is mainly to suppress the growth of the cancerous cells. However an additional role is emerging for the use of ADT in reconstituting T cell populations. The group headed by R. Boyd has conducted the majority of work in this field, and have questioned the role of ADT in thymic regeneration [282-284].

The thymus gland is important in managing the production and maintenance of the peripheral T cell pool. However with advancing age the gland undergoes atrophy and simultaneously its function declines. The time at which the gland undergoes atrophy coincides with the onset of puberty. The specific mechanism is not known but it is postulated to be associated the increased levels of sex steroids in circulation at this period in time [285, 286]. In rat models, the administration of sex steroids has shown to inhibit thymic regeneration [287, 288].

Thymic atrophy results in the reduced production of naïve T cells and a homeostatic compensatory increase in memory T cells. Also T cell dependent antibody formation and T cell responses to antigen stimulation are all reduced with age [289, 290].

This phenomenon led immunologists to question the role of ADT in the setting of thymic atrophy and whether application of this can result in thymic regeneration and ultimately improve the immune function. Studies were initially commenced on mouse models. In postpubertal mice which either underwent surgical or chemical castration showed complete reversal of thymic atrophy and the restoration of thymic architecture [283].

In the adult mouse, the thymus gland has less than five percent cellularity in comparison to prepubertal thymus gland. The thymus gland undergoes synchronous expansion, where there is hyperplasia of thymocytes with maintenance of the proportion of thymocyte subsets. The proportion of proliferating thymocytes remained the same but the rejuvenation of thymus occurred rapidly. The thymus of these mice that were treated with ADT returned to similar cellularity to that of young adults after four weeks [283].

The increased thymopoiesis that results from ADT leads to increased thymic production and restoration of peripheral T cell population. In the mouse model there is increased production of T cells from the thymus by two weeks after ADT and the T cell levels in the periphery resemble young adult mouse by four weeks [283]. The study by Sutherland et al showed that proportion of naïve CD4+ and CD8+ T cells in post castration mice increased in comparison to aged mice. An increase of 16% of CD4+ T cells and a 24% increase in CD8+ T cells were seen in the post castration population [283]. While the naïve T cell population was increased, the memory T cell population had a corresponding decrease in number.

In the aged mouse the CD4:CD8 T cell population ratio is about 1:1 while in the young adult it is 1.5:1. This is due to a reduction of CD4+ T cells and increase in CD8+ T cells in the periphery of aged mice. However six weeks after castration, the CD4:CD8 T cell ratio was restored to the young adult level of about 2:1 [283]. The responsiveness of these T cells after the activation of their respective TCR was increased in the post castration mice to levels similar to and greater than the young adult mice [283].

In early human studies, the measure of peripheral T cell population after initiating ADT for PCa management showed a similar effect. The restoration of

peripheral lymphocytes, in particular naïve CD4+ T cells, memory and naïve CD8+ T cells [291-293]. This effect is thought to be due thymic-dependent T cell pathway. The immunomodulatory role of ADT on the immune system can have additional benefits in combination with other therapeutic modalities.

1.9 Rationale for project

The link between cancers and the immune system is well established. The failure of the immunosurveillance mechanism in eradicating the cancerous cells to the manipulation of the immune system by the cancerous cells to progress are all interesting but are poorly understood. In the case of PCa the failure of the protective mechanisms and immune manipulation of the cancer cells allow for this cancer to grow and spread. By understanding the mechanisms of tumourigenesis the immune system can be manipulated to develop treatment modalities such as immunotherapy.

The aim of this project was to understand the local immune environment in the conditions of BPH, CAP and especially PCa. This requires characterisation of the presence of infiltrating lymphocytes and the subset of T cells in each of the sample population. Particular attention will be paid to the Treg cell population and to asses if their numbers affect the particular conditions. This information will be compared to the current pathological prognostic factors to determine any correlation.

The maturation of T cell population is assessed to determine local PCa environment. The proportion of naïve, central memory, effector memory and terminal effectors cells are assessed to determine local tumour awareness. Once this subpopulation is determined, their activation status of CTLs can be assessed to see if their function is upregulated or inhibited. This is achieved by utilizing laboratory techniques of flow cytometry and immunohistochemistry which will be described in the methods chapter.

By undertaking this study, a better understanding of the local immune system in PCa is aimed to be achieved. This information could be used to assist future research in immunomodulation in the preclinical setting and in human trials.

2.0 Methods

2.1 Recruitment

The Austin Health Human Research Ethics Committee approved the studies and recruitment methods. All participants provided written informed consent.

Patients were recruited from Austin Health through the outpatient clinic department as well as pre-admission clinic.

2.2 Prostate cancer trial

Eligibility criteria –

- Competent and Consenting male above the age of 18
- Proven PCa, diagnosed on TRUS biopsy
- Having radical prostatectomy for localized PCa that is androgen sensitive
- Not on concurrent treatment or prior treatment such as androgen deprivation therapy, radiotherapy or chemotherapy
- Sign Victorian Biobank consent form for tissue donation

The control group of men who had BPH had the following criteria:

- Competent and consenting adult above the age of 18
- Diagnosis of BPH on histopathology
- Normal PSA
- Having a TURP or open prostatectomy

The patients were seen in pre-admission clinic and consented to participate in the study. Patients were seen again on the day of the procedure to confirm that they understood the study and would like to donate tissue in the study.

All patients who were undergoing retropubic radical prostatectomy whether it was an open or laparoscopic procedure had an arterial line for blood pressure monitoring. With this access 50mL of blood was drawn using aseptic technique. However those patients having a TURP for BPH did not require such invasive blood pressure monitoring and as a result a venesection was performed on the day of the operation.

The blood was taken to the laboratory for immediate Ficoll gradient centrifugation.

2.2.1 Ficoll separation protocol

50mL of blood is diluted with 50mL of phosphate-buffered saline (PBS). Four 50mL Falcon tubes are filled individually with 20mL of Ficoll solution. 25mL of the diluted blood is slowly pipetted into of each of four tubes containing Ficoll. The blood is overlaid on top of the Ficoll solution.

These tubes are then placed in the centrifuge for 25minutes at 2300rpm. The tubes are spun with slow acceleration and deceleration without brake. The buffy layer is carefully extracted using a 3mL transfer pipette before being placed in a separate 50mL Falcon tube and diluted with PBS.

The tube is then placed in the centrifuge at 1800rpm for 5minutes. The pellet is then suspended in 50mL of PBS before being placed in the centrifuge and washed a further two times. The final pellet contains peripheral blood monocyte cells (PBMC) which is then cryopreserved in 1ml volume placed I liquid nitrogen for future use.

When the prostate is dissected out of the patient it is sent fresh in a sterile container to the pathology department. Here the pathologist takes core biopsies from the cancerous regions of the prostate and distributes the samples to the Victorian Biobank and for this study.

2.2.2 Method of obtaining samples from the prostate

The prostate is first inked with black ink so that when microscopic examination is conducted, the tumour extension to the margins can be examined. Following this the prostate is bivalved (refer to figure 2.1 and 2.2).

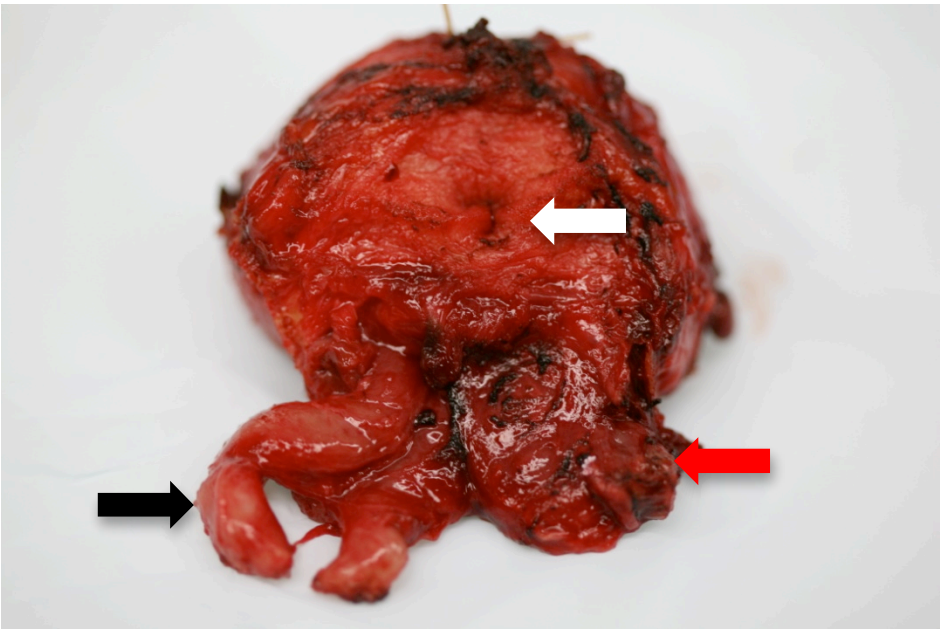


Figure 2.1 Fresh sample obtained after radical prostatectomy. White arrow shows the prostatic urethra, black arrow the vas deferens and the red arrow the seminal vesicles.

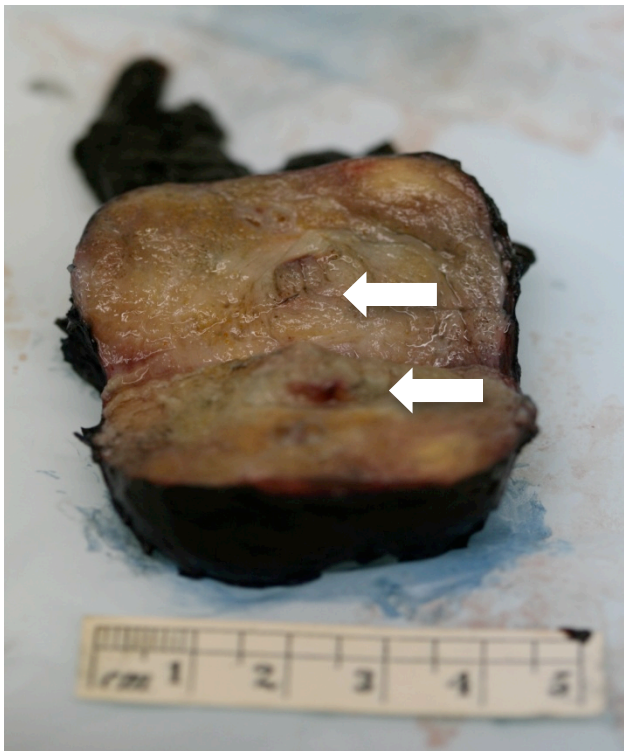


Figure 2.2 Bivalved prostate. Capsule of prostate stained with black ink. The white arrow indicates the location of the prostatic urethra.

Macroscopically it is difficult to clearly distinguish regions of cancer and normal tissue. Therefore it is important to have the TRUS biopsy result so that the regions of positive cancer can be sampled. With a 8mm core biopsy pen, a punch biopsy is taken. The biopsy sites are marked with green ink so that microscopic confirmation of positive tissue sampling can be made (refer to figure 2.3). sample slides were reviewed by a pathologist.

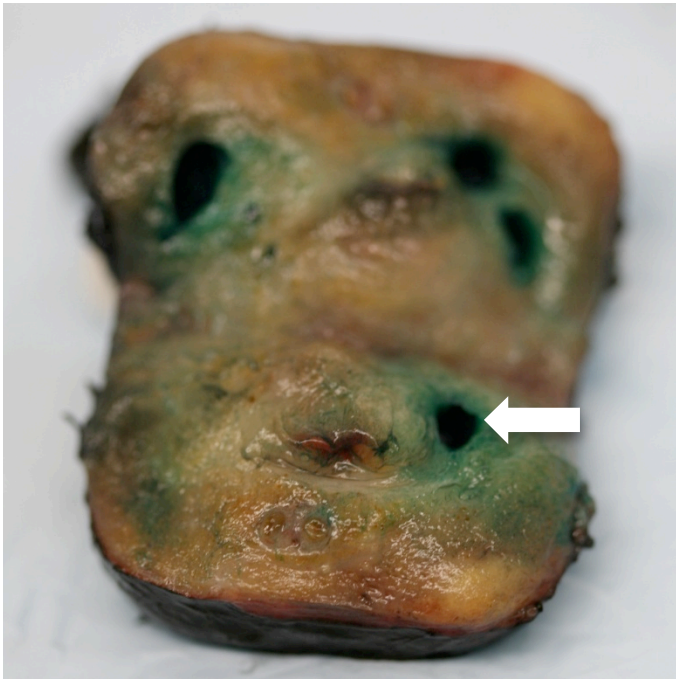


Figure 2.3 Tumour sampling. After the samples have been taken, the sites are stained green for microscopic verification (white arrow).

The core biopsies are kept in RF10 solution before the tissue is digested (refer to figure 2.4). The prostate is then restored roughly to its original shape with staples, before placing a section of a catheter to realign the prostatic urethra (refer to figure 2.5). The prostate is finally placed in formalin for further pathological processing and assessment.

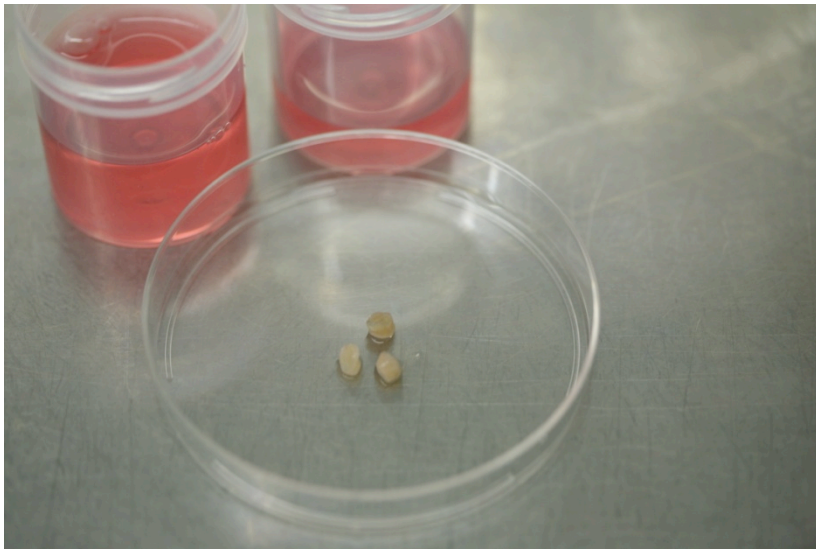


Figure 2.4 Prostate cancer samples. Cores of prostate tissue obtained for study

Figure 2.5 Reconstructing prostate shape. The shape of the prostate is restored with the staples and the portion of a urethral catheter is placed to delineate the prostatic urethra.

For those patients who underwent a TURP for BPH the method of collecting samples was different. From the entire prostate chips that were removed from the prostate, on average 200mcg was taken for the study.

2.2.3 RF10 solution

Was constituted using the following components and was stored at 4^{o}C.

- 500mL of Roswell park memorial institute medium (RPMI-1640)
- 50mL Foetal calf serum (FCS) heat inactivated
- 5mL Penicillin/Streptomycin
- 5mL Glutamine
- 250μl 2Me
- 12.5mL of HEPES

2.2.4 Tissue digestion

Throughout this study the digestion technique used has been modified to obtain the highest yield of cells in single cell suspension. The two main methods used are described below.

2.2.4.1 Method one

This is a method used very early in the study.

The prostate core biopsy samples were placed in a Petri dish with a 5 mL of RF10 to keep them moist. With a scalpel blade the samples were cleanly cut into $1mm^3$. In a separate test-tube the digestion solution is prepared. It compromises 10mL of RF10 with 10μL of collagenase D. The digestion solution is then poured into the Petri dish before being placed in a 37°C incubator overnight. The following day the digested solution containing the prostate tissue is strained through a 4μL strainer. With a plunger of a 5mL syringe, some of the remaining tissue in the strainer is expressed through. This results in a single cell suspension. A viable cell count is performed with Trypan blue. The cells are then frozen for future use.

2.2.4.2 Method two

This method was developed by the two other members in the laboratory, a postdoctoral fellow Dr Pavel Sluka and a research assistant Genevieve Whitty.

Prostate tissue was washed in 5mL PBS +100μg/mL gentamicin. Samples were cut with scalpel blades into 1-$2mm^3$ pieces. The tissue is transferred to a 15mL tube with 10mL of PBS + 100μg/mL gentamicin. It is allowed to settle

and the supernatant was discarded. The samples were washed with 10mL PBS + 100μg/mL gentamicin and allowed to settle before the supernatant was discarded. The cell pellet is washed with 10mL of PBS + 100μg/mL gentamicin and spun at 500g for 3 minutes before disregarding the supernatant. The pellet is resuspend in 5mL digestion medium before being incubated overnight (~16 hours) at 37^{o}C using a MACS mix rotor. The rotor is a battery operated device and is able to be placed in the incubator to gently mix the suspension by slow rotation.

2.2.6 Isolation of Epithelial Cells

Clumps of tissue are broken down with the pipette before being centrifuged at 300g for 5 minutes. The supernatant is discarded and washed in 5mL of wash medium. The tissues are centrifuged at 500g for 7minutes and the supernatant is discarded to isolate a pellet. This wash cycle is repeated another two times.

The cells are resuspended in 1mL Trypsin/EDTA and transferred to a 12 well dish. The dish was then Incubate at 37^{o}C for 30 minutes.

Once incubated, 1mL of wash medium is added and the tissue is broken down by passing the cells through a l8G and 23G needles, 10 times each. The cell suspension is spun at 500g for 7 minutes. It is then resuspended in 5mL of PBS and gentamicin before being centrifuged at 500g for 7 minutes. The cells were suspended in Dynabead buffer before a cell count cell was performed.

Epithelial cells were isolated according to cell number, bead number and method on invitrogen Dynabead sheet:

- Cells are resuspended with equal number of beads to cells. We assumed approx 20-25% will be the epithelial cells and the aim was to attach approx 4 beads per cell. Therefore 1 bead per cell was added.
- The dynabeads are washed with 1mL of buffer 1 in an Eppendorf tube. This will remove the azide and also coat the Eppendorf tube with some serum to make cell mixing more consistent.

- The cells are resuspended in 200μL to 500μL of buffer 1 and incubated at 4°C for 30min using a MACS mix rotor. 200μL is used if less than 5 x $l0^6$ cells or 500μL if more than 5 x 10^6 cells are counted.
- Placing the Eppendorf tube onto the magnet for 2minutes captures the bead/cells. The non-captured fraction carefully removed using an Eppendorf pipette. This fraction is kept in a separate 15mL tube. The non-bead fraction will contain cells including lymphocytes
- These cells are frozen for future use.

2.2.7 Reagents used

PBS+ 100pglmL Gentamicin – A total of 50ml is used for each sample

Wash medium - 50mL preparation is made and stored at 4°C

- RPMI (43mL)
- PenStrep (500μl)
- 100 μg/mL gentamicin (l00μl)
- 25mM HEPES (1.25mL)
- 10% FCS (5mL)

Digestion cocktail - Use 5mL for up to 200mg of tissue (Amounts for 6mL)

- Wash medium
- 225U/mL Collagenase (4.89mg of 276 U/mg solid stock)
- 125U/mL Hyaluronidase (I.04mg of 719U/mg solid stock)

Buffer 1 PBS/0.1% BSA (or FCS)
Buffer 2 PBS/0.1% BSA with 2mM EDTA (or 0.6% sodium citrate)
Buffer 3 RPMI/l% FCS and 5mM MgCl2

Once the lymphocytes and single cell suspension have been obtained they are stored for future experiments. Cell counts are performed and 5 x 10^6 cells and are suspended in 1mL of 10% Dimethyl sulfoxide (DMSO) solution before

being placed in an -80^{o}C freezer. After 24 hours these tubes are stored in liquid nitrogen.

10% DMSO solution

Consists of:

> In a 50mL falcon tube - 45mL of FCS is mixed with 5mL of DMSO (minimum 99.5%). The tube is then wrapped in aluminium foil and kept at 4^{o}C.

The single cell suspension and the PBMC were used in the studies examining Tregs, functional and activation status.

2.3 Prostatitis study

The Austin Health Human Research Ethics Committee approved this study and methods used. All participants provided written informed consent.

Men who were included in this study had the following criteria:

- Competent and consenting males above the age of 18
- Clinical diagnosis of chronic abacterial prostatitis
- Lower urinary tract symptoms such as dysuria, frequency and urgency
- Long-term treatment for prostatitis and have failed therapy
- Negative urine culture

These participants were seen in the outpatient department clinic and consented to participate in the study. They were seen again on the day of the procedure to confirm that they understood the study and would like to donate tissue in the study.

A TRUS biopsy of the prostate was performed to obtain a total of 12 samples from the apex, mid and base regions of the prostate (i.e. two from each side) (refer to figure 2.6).

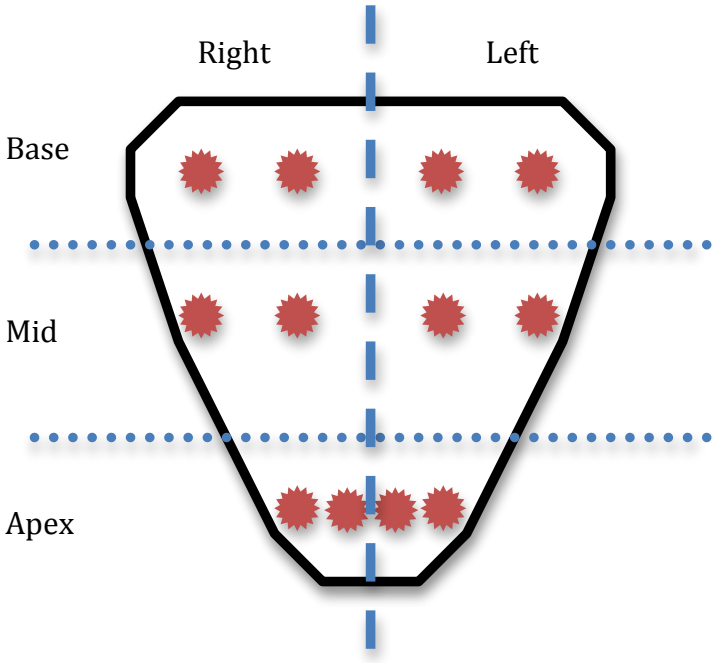


Figure 2.6 Schematic representation of twelve-core or double sextant prostate biopsy. Demonstrates the regions of the prostate where biopsies are taken (marked in red).

A venesection is taken from the participant on the day of the procedure where 50mL of blood is used for Perm buffy isolation. Six of the 12 biopsies were used for single cell suspension while the other samples are sent to pathology for immunohistochemistry analysis. The single cell suspension and PBMC are stained for CD4 and FoxP3 before flow cytometry is performed.

2.4 Androgen deprivation study

The existing Ficoll separation protocol (2.2.1) used for the prostate cancer study, was also used for this study.

The inclusion criteria for this study were:

- Competent and consenting male above the age of 18
- Men who have been diagnosed with PCa
- Hormone naïve i.e. men who have not had ADT before
- Included even if patient has had radical prostatectomy or radiation therapy
- Initiation of ADT
- Hormone therapy should be the sole treatment for three months continuous.

Participants were excluded if they have concurrent treatment such as chemotherapy or radiotherapy.

These participants were seen in the outpatient department where they were recruited. On the initial visit informed consent is obtained from the participant and also a venesection was performed to take 50mL of blood. The purpose of this is to obtain a sample of PBMC buffy before ADT was initiated. Also on the first visit, the PSA is measured to assess the pre-hormone therapy level.

At approximately three months after commencing ADT, the second and final visit is made. On this visit another 50mL of blood is taken to assess the efficacy of the hormone therapy on PSA suppression and PBMC buffy.

The PBMC collected from the two visits were stored in liquid nitrogen. The cells were not assayed until the post-treatment samples were available from all the participants. All samples were stained and analysed in the one experiment to eliminate any variability on conducting experiments at different times. The matched samples were assessed for CD4, CD8, Tregs, functional and activation status.

2.5 Surface marker staining

A 96 well U shaped bottom plate was used. A grid is drawn on this plate, starting in the top left hand corner (A1). Each of the samples have either 2 x 10^5 of PBMC or prostate tissue cells.

The compensation controls are placed as a row below the testing samples (refer to figure 2.7). In the compensation control wells, 2 x 10^5 of PBMC from an individual patient is used. The first well of the compensation control was always unstained. The subsequent wells were stained with the 1μL of each of the antibodies used in the study.

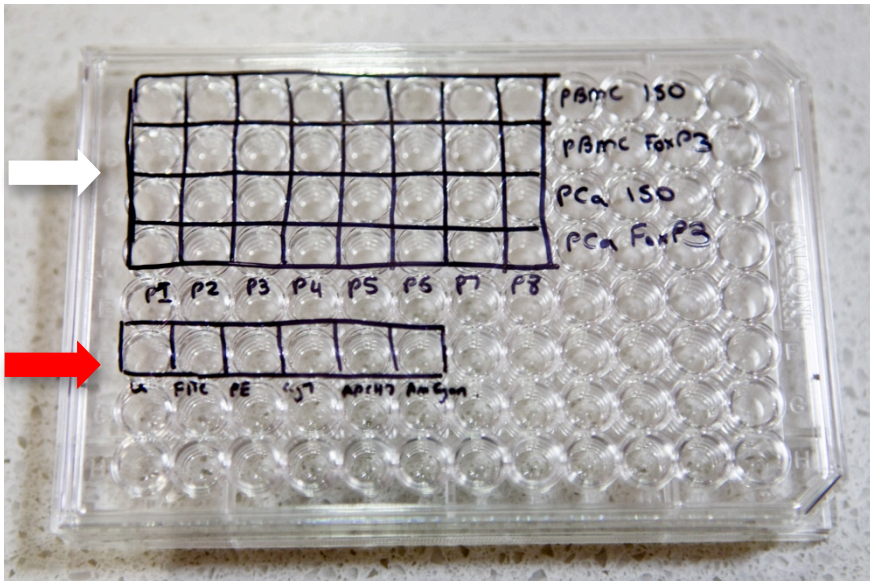


Figure 2.7 Plate set up for FoxP3 analysis using flow cytometry. Here eight patients are being studied which corresponds with the eight columns seen in the grid (white arrow) drawn starting from the top left hand side corner (A1). There are four rows for each patient. This corresponds to stained PBMC cells, PBMC isotype control, stained PCa cells and PCa isotype control. The red arrow indicates the compensation control wells.

The mixture of antibodies is created in an Eppendorf tube with PBS. The cocktail is made to a volume so that each of the wells that are to be stained has 25μL of the staining solution. When samples are examined, matching PBMC and tissue from the same patient are used to reduce variability in techniques.

2.5.1 FoxP3 staining

Stain for surface markers were undertaken in a 96 well U-bottom plate. The antibodies and the concentration used are listed below in table 2.1.

Antibody	Concentration
FITC CD4	1/20
PE CD25	1/20
Cy7 CD3	1/100
APC-H7 CD45	1/100
AmCyan CD8	1/50

Table 2.1 Antibodies used for FoxP3 staining.

The antibodies are left to stain for 30 minutes before being washed twice with PBS. 200μL of fixative is added per well and re-suspended by pipetting. It is then incubated in the fridge (4°C) strictly for 45 minutes.

The plate is centrifuged and the supernatant is removed. It is centrifuged at a stronger cycle at 960g. It is washed with 200μL per well of PBS. 200μL of permeabilisation buffer is used wash the cells. The supernatant is removed and the pellet is re-suspended in 15μL of blocking buffer.

The plate is then incubated in the fridge for 15 minutes. Following this the appropriate volume of anti-FoxP3 and isotype matched control are added. The antibodies are listed below in table 2.2.

Clone & Fluorochrome	Isotype	Stock concentration	Optimal dilution	Volume per well
236A/E7-APC	muIgG1	6.25μg/mL	1/5	8μL
Control mouse IgG1-APC	muIgG1	12.5μg/mL	1/10	3μL

Table 2.2 FoxP3 and isotype control antibodies.

The plate is incubated in the fridge for 30 minutes. Following this the plate is washed twice with 1x Permeabilisation buffer and the cells are re-suspended in 200μL of PBS per well. Soon after the plate is run on flow cytometer

Solutions used:

- Fixative – One part of Solution A (4x Fix/Perm concentrate) with three-part Solution B (Fix/Perm diluent)
- 1x Permeabilisation buffer – Solution C (10x Permeabilisation buffer) is diluted to 1/10 with Milli-Q(MQ) water. MQ water is ultrapure water that has been filtered and purified by reverse osmosis using a Millipore processor.
- Blocking buffer – Normal mouse serum is diluted to 4% in 1x Permeabilisation buffer

2.5.2 Functional analysis

A 96 well U bottom plate was used. The antibodies used for this study are listed below in table 2.3.

Antibody	Concentration
FITC CD4	1/20
Cy5 CD45RA	1/50
Cy7 CD3	1/100
APC CD45RO	1/50
APC750 CCR7	1/50
AmCyan CD8	1/50

Table 2.3 Antibodies used for functional analysis.

The antibodies are left to stain for 30 minutes before being washed twice with PBS. The samples are then ready to be examined using the flow cytometer. By conducting this study we are able to obtain populations of naïve, central memory, effecter memory and terminal effector cells. Please refer to table 2.4 for gating strategies to identify subset of T lymphocytes.

Naïve	CD45RA+	CD45RO-	CCR7+
Central memory	CD45RA-	CD45RO+	CCR7+
Effector memory	CD45RA-	CD45RO+	CCR7-
Terminal effector	CD45RA+	CD45RO-	CCR7-

Table 2.4 Gating method used to identify T cell subsets.

2.5.3 Activation analysis

Again a 96 well U bottom plate is used. The list of antibodies used for this study is shown below in table 2.5.

Antibody	Concentration
FITC CD4	1/20
PE CD69	1/20
APC-H7 CD45	1/100
AmCyan CD8	1/50

Table 2.5 Antibodies used for activation analysis.

The antibodies are left to stain for 30 minutes before being washed twice with PBS. The samples are then examined on the flow cytometer.

2.6 Flow cytometry analysis

Calibration of the flow cytometer is achieved by using a mixture containing blank as well as fluorescent microbeads with predefined levels of intensity. This analysis is plotted against the predefined fluorescence value and ranges of performance parameters.

Following calibration, the final setting is determined and the patient samples are run. The collected data is exported to the laboratory's main drive allowing for it to be analysed.

2.7 Data analysis

The Macintosh based program FlowJo version 8.8.6 was used.

2.7.1 Gating strategies for Treg analysis

The first gate used is the side scatter (SSC) on the y-axis and APC-H7 CD45 on the x-axis. This helps distinguish the lymphocyte population (refer to figure 2.8). A circular gate is drawn over this lymphocyte population and these lymphocytes are transposed onto the second.

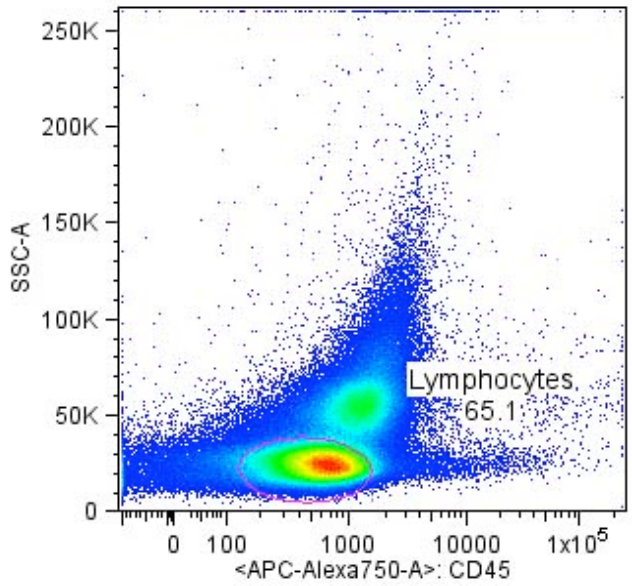


Figure 2.8 Isolation of lymphocyte population.

The next was to obtain the CD4+ T lymphocytes. This was achieved by having FITC CD4 on the x-axis and an unstained marker, PE CY5 on the y-axis (refer to figure 2.9). The reason for the use of this unstained channel is to eliminate any auto fluorescent cells that may give false positive results. A gate is drawn over the CD4+ cells.

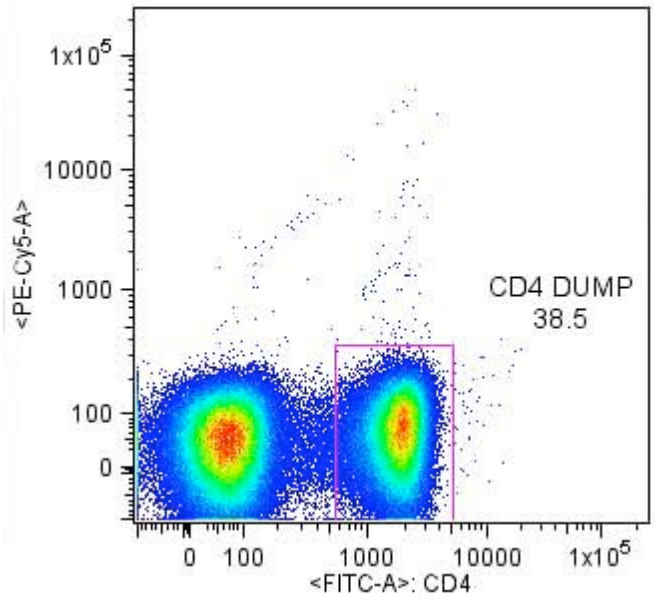


Figure 2.9 Isolating CD4 population. A dump channel is used.

The final step is to isolate the Tregs. This is performed by initially examining the isotype control. The y-axis is the PE CD25 and x-axis is APC isotype (refer to figure 2.10). The cells should not be double positive and as a result a gate is drawn over this area. The gate is then transposed over the population that has been stained with APC FoxP3 to obtain the Tregs (refer to figure 2.11).

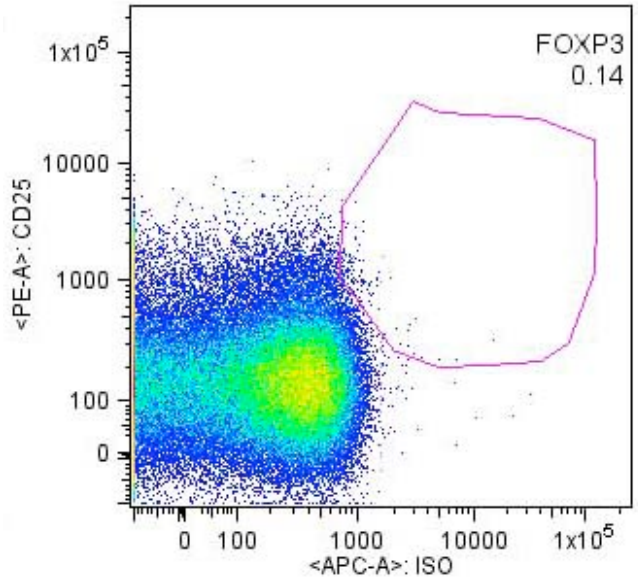


Figure 2.10 Isotype control.

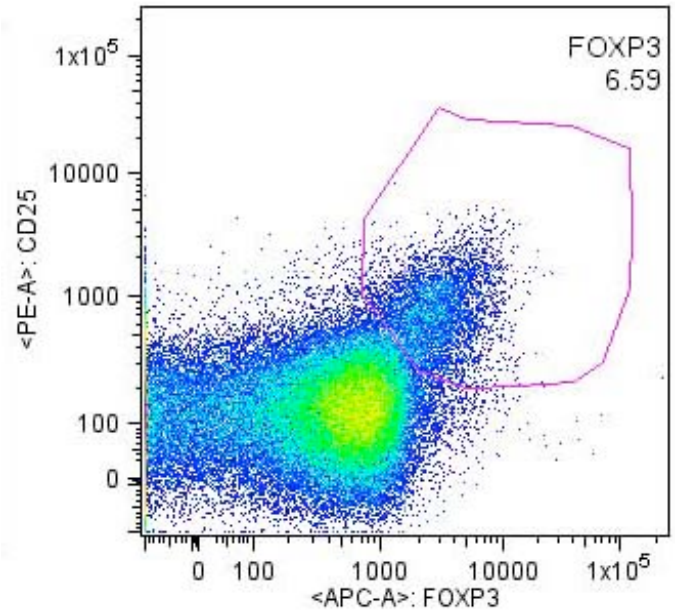


Figure 2.11 Gating for Treg cell population.

2.7.2 Gating strategy for functional analysis

Having SSC on the y-axis and forward scatter (FSC) on the x-axis isolates the lymphocytes (refer to figure 2.12). A gate isolates the population of lymphocytes.

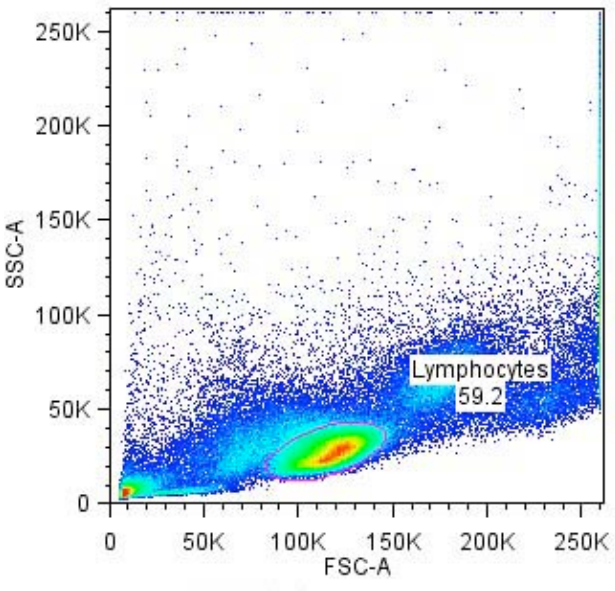


Figure 2.12 Isolating lymphocytes on forward and side scatter basis.

Having a PE dump channel on the y-axis improved detection of the CD8+ T lymphocytes and also excludes cells binding antibodies non-specifically. The x-axis was labelled as AmCyan CD8 and a gate was drawn around this positive population (refer to figure 2.13).

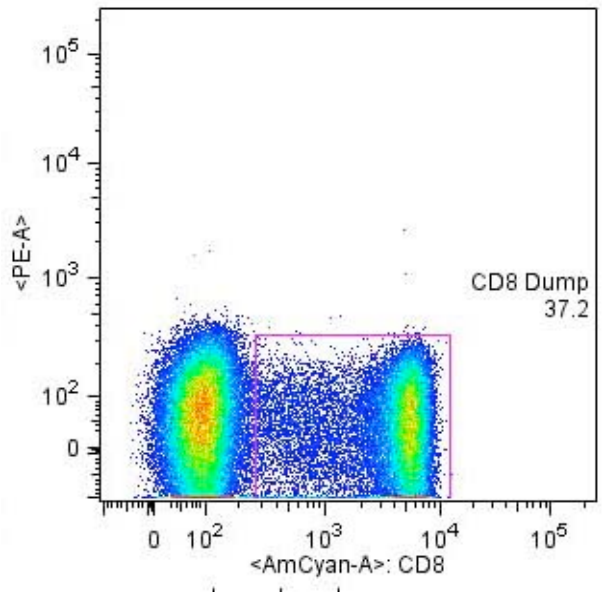


Figure 2.13 Isolating CD8 cell population.

The CD8+ T lymphocytes are then examined with APC CD45RO on the y-axis and CY5 CD45 RA on the x-axis (refer to figure 2.14). The gate is drawn by gating the true negative population of the unstained cells that were used for compensation control with the above axis. This gate is then transposed over the stained CD8+ cells

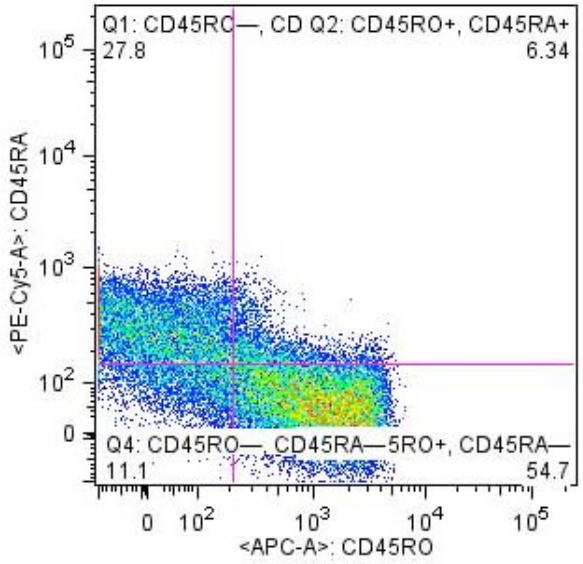


Figure 2.14 Examining the CD8 cells for CD45RA and CD45RO.

The cells that are CD45RA positive and CD45RO negative from the above plot are analysed. Here the y-axis was APC750 CCR7 and the x-axis is CY5 CD45RA (refer to figure 2.15). Again the gates are drawn after modelling the axis on the unstained population. This provides the naïve population located in quadrant two and terminal effector population quadrant four.

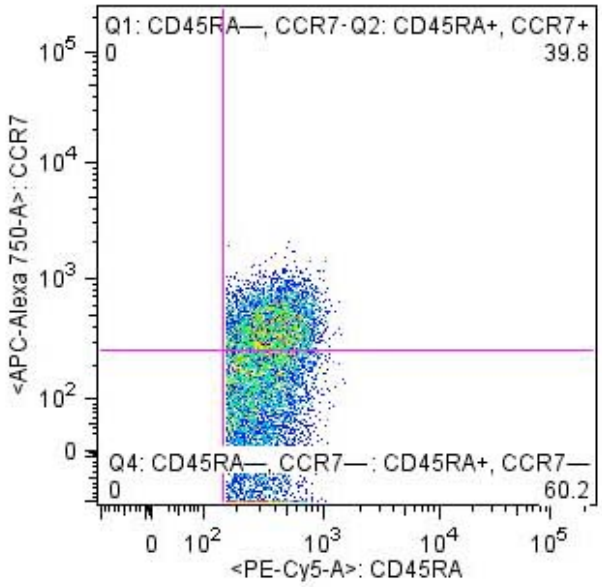


Figure 2.15 Isolating naïve and terminal effector cells.

The cells that were CD45RA negative and CD45RO positive are then examined. The y-axis is APC750 CCR7 and the x-axis is CD45RO (refer to figure 2.16). The same gating method is used where the central memory in quadrant two and effector memory quadrant four.

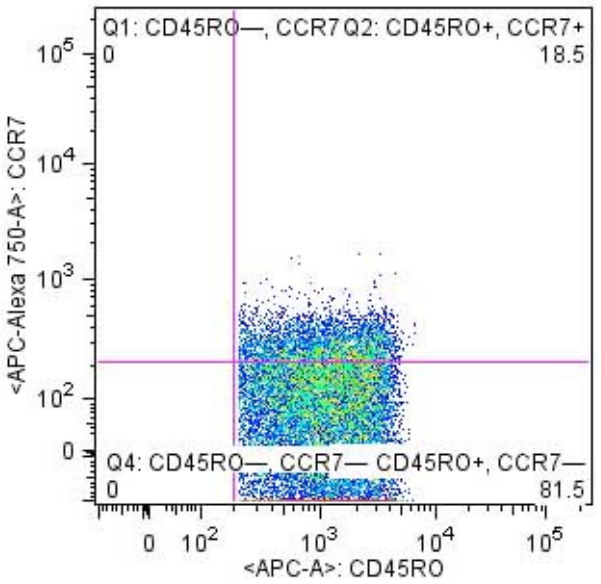


Figure 2.16 Gating for central memory and effector memory.

2.7.3 Gating strategies for activation analysis

Like the Treg analysis, gating SSC on the y-axis and APC-H7 CD45 on the x-axis isolates the lymphocytes. The CD8+ cells in the lymphocyte population are obtained by having a PE-CY5 dump channel on the y-axis and AmCyan CD8 on the x-axis (refer to figure 2.17).

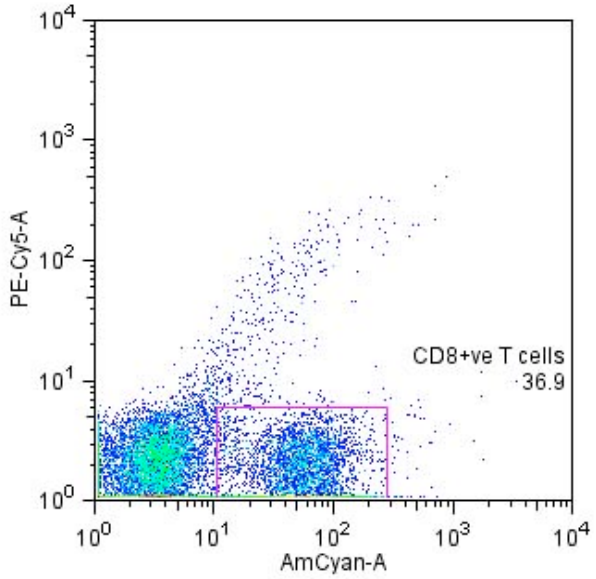


Figure 2.17 Clean CD8 samples are isolated.

The activated CD8+ T lymphocytes are obtained by assessing the marker for cell activation, PE CD69 on the y-axis and AmCyan CD8 on the x-axis (refer to figure 2.18). The gate is drawn is first drawn on the unstained cells on the calibration control and is then transposed onto the stained cells.

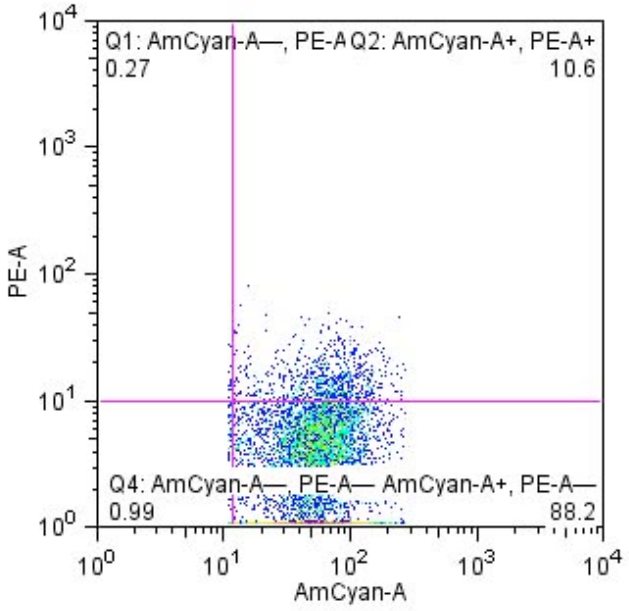


Figure 2.18 CD8 activation. Population of activated CD8 cells in quadrant three.

2.8 Wax block and slide preparation

After the core biopsy samples have been acquired, the fresh prostate is placed in formalin for 12 to 18 hours. Tissue sections are then cut into 3mm intervals for processing. These tissue samples are blocked into cassettes and are processed using automated tissue processors (PELORIS). It is where multiple dehydration steps take over 13.5 hours.

The processed tissue is then embedded in paraffin wax and tissue sections are cut at 4μm intervals and placed onto polarized slides. These slides are then ready to be used in IHC staining.

2.9 Immunohistochemistry analysis

The antibodies that were used were CD4, CD8, FoxP3, IFNγ, TGFβ and IL-10.

2.9.1 Preparation of paraffin sections

The slides are dewaxed by incubating them in an oven at 65^{o} C for 30 minutes. The slides are then washed and agitated in Xylene twice for 5 minutes each time. Then they are washed and agitated in ETOH twice for 5 minutes each time. The slides are washed in water. H_2O_2 (0.3% - i.e. 100ul of 30% in 10mL PBS) is used in the incubation of the slides for 10 minutes.

The slides are washed with tap water for 5 minutes. The slides are placed in EDTA/CITRATE retrieval buffer (DAKO). The buffer and the slides are placed in pre-heated 100^{o}C water bath for 30 minutes. It is allowed to cool on the bench for 20 minutes before washing with tap water for 5 minutes. The slides are ready for staining

2.9.2 Staining protocol

The slides are incubated in TBS for 5 minutes. Primary antibody at a volume of 200μl at the appropriate concentration is incubated for 60 minutes. The slides are washed with TBS or Wash buffer (DAKO) for 5 minutes. Envision+ polymer (anti-rabbit or anti-mouse depending on primary) is added at 3 drops per slide and incubated for 30 to 60 minutes. Again TBS wash or Wash buffer (DKAO) is used for 5minutes. Chromogenic solutions are then added:

- 50mL of PBS (warmed for 10secs in glass beaker in microwave)
- Three DAB tablets (Sigma) are dissolved in the warmed PBS
- 30μl of H_2O_2 is added to the DAB and PBS mix
- The solution is filtered into a Coplin jar using No. 4 Wattman filter paper
- The slides are placed in the Coplin jar with the solution and incubated for 10 minutes at room temp

The slides are washed in distilled water before undergoing staining. Haematoxylin wash is performed for 20 to 30 seconds before washing in tap water. Scott's Tap water wash for 30 seconds before another tap water wash. Finally the slides are dehydrated and applied a coverslip in DePeX.

3.0 Immunomodulation

3.1 Introduction

The presence of lymphocyte infiltration into the local prostate tissue environment is important in the understanding of the immune function in particular conditions. There have been studies describing both advantages and disadvantages of having an influx of lymphocytes in the local environment. The predominance of one subset of lymphocytes can swing the balance of immunomodulation. For instance, autoimmune diseases such as Type 1 diabetes mellitus, arthritis, colitis, thyroiditis, gastritis, lupus and multiple sclerosis have shown an influx of cytotoxic cells and diminished numbers of suppressive cells [294-296]. Here the immune system attacks normal tissue resulting in an autoimmune disease [297, 298]. In malignancies there is an immunosuppressive environment fostering the proliferation of cancerous cells [234, 237, 299-302].

This chapter covers work assessing the immunomodulation that takes place within the prostate and correlates it with the peripheral environment. The subsets of T cells will be quantified and their nature and properties will be taken into consideration when formulating a picture of the local environment. Particular attention will be paid to Treg cell numbers present in the three study groups of BPH, CAP and PCa. Immunomodulation was assessed using flow cytometry and IHC.

3.2 Flow cytometry data

As described in the methods chapter, the matched blood and prostate tissue of individual patients with the different pathological conditions were examined using flow cytometry. Single cell suspensions of prostate tissue and PBMC

were stained for the relevant markers and passed through the flow cytometer. The plots obtained were gated for the appropriate markers to obtain information on the percentage of CD4+, CD8+ T lymphocytes and Treg cells. See below for the gating scheme used (refer to figure 3.1).

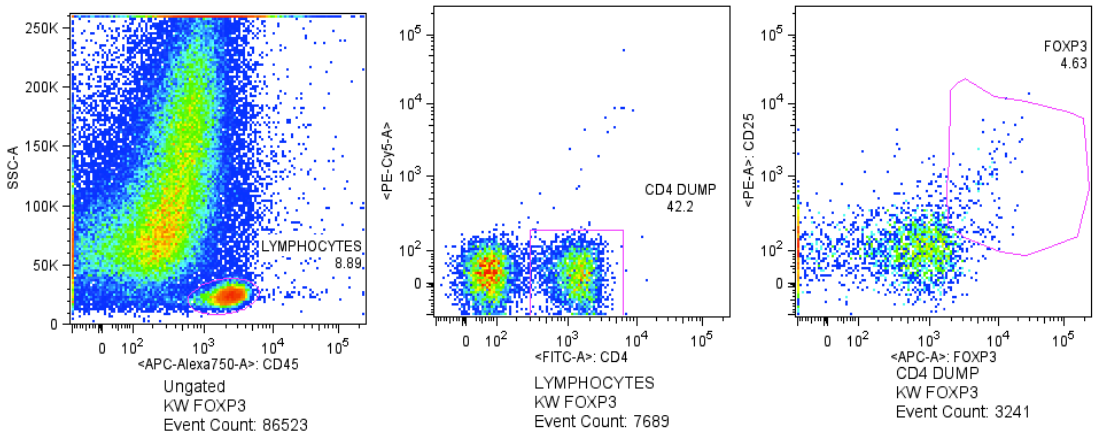


Figure 3.1 Flow cytometry plot of PCa tissue. The first plot identifies the lymphocyte population in tissue. The middle plot isolates the CD4+ lymphocyte population. The final plot shows the CD4+ CD25+ FoxP3+ Treg cells.

The percentages obtained in the gates shown above and the event counts were recorded as results for CD4+, CD8+ lymphocyte and Treg cells. The final percentage of Treg numbers was obtained by subtracting the percentage of isotype control from the FoxP3 plot.

Flow cytometry assessment, was initially performed on 10 patients with BPH, 7 with CAP and 49 with PCa. However upon analysis of the data, 3 patients with BPH, 1 with CAP and 13 with PCa were excluded on the basis on inadequate data. For example, figure 3.2 shows a patient that was excluded because the number of events in the Treg assessment was very low. Also another 6 PCa patients were excluded, as the samples did not contain tumour. This was discovered when analysing the biopsy sites marked with green ink.

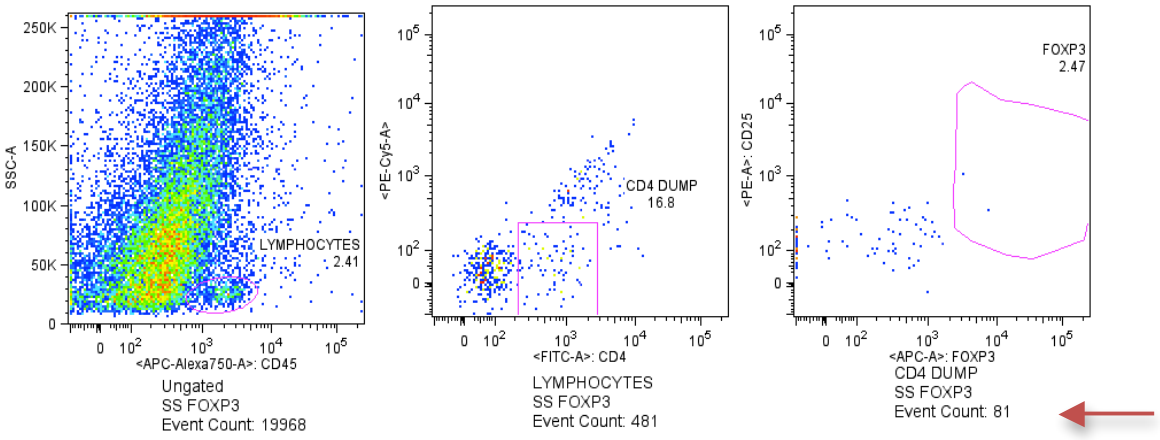


Figure 3.2 An example of a patient that was excluded due to low number of events (arrow pointing at low event count of 81). The 81 events are far too few and only two events are in the FOXP3 gate. With such small events, the outcome and can lead to falsely high or low results.

The average age of the men in the BPH group was 73.86 (95% CI 68.25 to 79.47). The mean age of the men with CAP was 56.86 (95% CI 45.96 to 67.75). In the PCa group the mean age of the participants was 61.10 (95% CI 58.98 to 63.22).

3.2.1 BPH group

In this group matched peripheral blood and prostate tissue (at time of TURP or open prostatectomy) were obtained. The tissue was processed and examined as outlined in the methods chapter. The formal histopathology reports of all the patients included in the BPH group confirmed that none contained any focus of PCa.

The initial experiment was the use of flow cytometry to assess the presence of CD4+ lymphocytes in the blood and prostate samples. The assessment of total lymphocytes was not performed, as the numbers obtained from Ficoll separation were mainly monocytes and lymphocytes and the percentage of lymphocytes will be high. This was not comparable to the low percentage of lymphocytes found in prostate stroma.

The percentage of CD4+ T lymphocytes was obtained by gating lymphocytes expressing CD4 with a dump channel. The patient data are shown in table 3.1.

Patient No.	CD4+ PBMC (%)	Number of events	CD4+ BPH (%)	Number of events	PBMC:BPH ratio
1	35.9	*153048*	13.8	*2108*	1:0.38
2	46.1	*189882*	39.2	*650*	1:0.85
3	29.2	*158763*	31.3	*3948*	1:1.07
4	48.5	*175397*	10.5	*4689*	1:0.22
5	61.5	*157303*	23.3	*1552*	1:0.38
6	41.3	*186183*	29.5	*1609*	1:0.71
7	62.4	*174565*	24.5	*5772*	1:0.39

Table 3.1 CD4+ T lymphocytes percentage and event numbers in PBMC and BPH tissue. Column 1 indicates patient number. Column 2 and 4 represent percentage of CD4 positive lymphocytes in PBMC and BPH tissue respectively. Columns 3 and 5 indicate event numbers generated on flow cytometry plot. The final column shows the PBMC and BPH ratio of individual patients.

The mean percentage of CD4+ T lymphocytes in PBMC was 46.41% (95% CI 34.96 to 55.51), while the mean CD4+ T lymphocytes in BPH was 24.59% (95% CI 15.34 to 33.83). This difference of 21.83% (95% CI 8.72 to 34.94) was statistically significant (p 0.0035). From the ratios, it could be seen that there are less CD4 cells in the tissue in comparison to the circulating population in the PBMC.

The CD8+ T lymphocyte population was assessed using CD8 positive stain with a dump channel. The percentage and event numbers are expressed in table 3.2.

Patient No.	CD8+ PBMC (%)	Number of events	CD8+ BPH (%)	Number of events	PBMC:BPH ratio
1	34.1	*159097*	29.6	*10366*	1:0.87
2	13.8	*69742*	24.2	*10818*	1:1.75
3	42.5	*194094*	27.6	*13196*	1:0.65
4	12.3	*137379*	19.7	*8135*	1:1.60
5	31.4	*65155*	11.6	*11741*	1:0.34
6	15.5	*252517*	20.1	*9079*	1:1.30
7	11.9	*106729*	22.4	*30468*	1:1.88

Table 3.2 CD8+ T lymphocytes in PBMC and BPH tissue. Column 2 and 4 shows CD8+ cells as a percentage of total lymphocytes in PBMC and BPH tissue respectively. The sixth column shows the PBMC to BPH ratios.

The mean CD8+ T lymphocyte percentage in PBMC was 23.07 (CI 11.42 to 34.73). While the percentage in BPH tissue was 22.17 (CI 16.69 to 27.66). There was very minimal difference and was statistically insignificant. Also from the ratios, four out of the seven patients had higher CD8+ cells in the tissue in comparison to the periphery.

The Treg cell population in the samples was determined by staining for FoxP3 and CD25 and they are demonstrated in table 3.3.

Patient No.	Treg PBMC (%)	Number of events	Treg BPH (%)	Number of events	PBMC:BPH ratio
1	6.9	*54927*	3.2	*291*	1:0.46
2	8.3	*87627*	7.8	*255*	1:0.94
3	5.7	*46352*	4.5	*1237*	1:0.79
4	5.0	*85092*	4.1	*492*	1:0.82
5	7.0	*76858*	8.2	*474*	1:1.17
6	5.2	*105112*	5.8	*767*	1:1.12

Table 3.3 Treg cell percentage and event numbers in PBMC and BPH tissue. Columns 2 and 4 show percentage of Treg cells in CD4+ lymphocytes obtained in PBMC and BPH tissue respectively. The final column demonstrates the ratios of PBMC and BPH.

The mean percentage of Treg cells present in PBMC was 6.26% (95% CI 4.84 to 7.68), similar to the mean Treg cell percentage in BPH of 5.58% (95% CI 3.42 to 7.74). The mean difference between the two groups was 0.75% which had no clinical or statistical significance (95% CI -1.44 to 2.94, p 0.4628). The ratios also indicate that there are predominantly more Treg cells in PBMC than the tissue.

3.2.2 CAP group

The number of CAP in the study was six as the recruitment of participants proved difficult. Participants had to undergo a TRUS biopsy for research purposes with little self-benefit except for the assessment of PCa. As a result, despite approaching numerous potential participants, informed consent was obtained from an understandably low number.

The CD4+ T lymphocytes numbers in CAP are shown in table 3.4.

Patient No.	CD4+ PBMC (%)	Number of events	CD4+ CAP (%)	Number of events	PBMC:CAP ratio
1	54.9	*222335*	35.5	*2782*	1:0.65
2	42.0	*167747*	39.8	*5488*	1:0.95
3	50.9	*171310*	35.6	*9491*	1:0.70
4	42.3	*35389*	49.1	*17575*	1:1.16
5	33.5	*196996*	14.7	*4187*	1:0.44
6	9.3	*151346*	29.5	*522*	1:3.17

Table 3.4 Percentage of CD4+ T lymphocytes in the total lymphocyte population within PBMC and CAP tissue are demonstrated in columns 2 and 4 respectively. The sixth column shows the PBMC to CAP ratios.

The mean percentage of CD4+ T lymphocytes in PBMC was 38.82% (95% CI 21.72 to 55.91). The CD4+ T lymphocytes in CAP had a mean percentage of 34.03% (95% CI 21.98 to 46.08). There were 4.78% (95% CI -13.34 to 22.91, p 0.5696) more CD4+ T lymphocytes in PBMC but this was not statistically significant. The ratios also indicate that there are more CD4+ T lymphocytes in PBMC than in the tissue.

There were not enough tissue samples obtained from TRUS biopsy to examine for CD8+ T lymphocytes. This will affect the conclusions drawn on CAP with regards to the CD8+ cell population. However the CD4+ and Treg cells analysis could be performed.

Treg cells percentage and event numbers in the CAP population is demonstrated in table 3.5.

Patient No.	Treg PBMC (%)	Number of events	Treg CAP (%)	Number of events	PBMC:CAP ratio
1	1.5	*122145*	1.0	*987*	1:0.67
2	1.5	*70427*	1.5	*2185*	1:1
3	4.9	*87206*	5.6	*3379*	1:1.14
4	3.0	*14952*	4.6	*8627*	1:1.53
5	5.1	*65909*	4.3	*616*	1:0.84
6	2.8	*14080*	2.7	*154*	1:0.96

Table 3.5 Treg cell percentage in the CD4+ T lymphocyte population in PBMC and CAP tissue is seen in columns 2 and 4. The final column demonstrates the ratios between PBMC and CAP.

The mean percentage of Treg cells in PBMC was 3.13% (95% CI 1.48 to 4.79) while in CAP tissue; it was 3.27% (95% CI 1.33 to 5.21).

There was no clinically and statistically significant difference seen between the two groups. The individual ratios were also equally matched indicating a similar Treg population in the periphery and local environment.

3.2.3 PCa Group

The CD4+ T lymphocyte population in PCa expressed as a percentage and event numbers is shown in table 3.6.

Patient No.	CD4+ PBMC (%)	Number of events	CD4+ Tissue (%)	Number of events	PBMC:PCa ratio
1	28.4	*197612*	50.9	*9371*	1:1.79
2	38.5	*197802*	46.5	*38547*	1:1.21
3	34.6	*194714*	14.7	*4187*	1:0.42
4	49.1	*198933*	10.7	*5805*	1:0.22
5	57.4	*184928*	29.4	*3529*	1:0.51
6	51.4	*149379*	20.8	*636*	1:0.40
7	37.2	*73792*	37.9	*240*	1:1.02
8	51.6	*32997*	40.4	*968*	1:0.78
9	51.2	*177927*	35.6	*9491*	1:0.70
10	44.5	*149401*	39.8	*5480*	1:0.89
11	44.8	*35495*	49.1	*17575*	1:1.10
12	18.0	*120578*	21.6	*601*	1:1.20
13	47.3	*166457*	23.0	*22016*	1:0.49
14	34.2	*188575*	38.3	*3754*	1:1.12
15	44.0	*144990*	49.0	*20527*	1:1.11
16	69.1	*148303*	38.3	*859*	1:0.55
17	49.4	*194795*	32.7	*2457*	1:0.66
18	36.7	*195783*	23.3	*1462*	1:0.63
19	48.3	*104076*	39.4	*3362*	1:0.82
20	50	*94893*	32.7	*550*	1:0.65
21	38.3	*145797*	28.1	*958*	1:0.73

22	32.5	*65534*	44.9	*13129*	1:1.38
23	42.9	*177433*	32.5	*1937*	1:0.76
24	38.4	*134521*	40.6	*5854*	1:1.06
25	9.43	*155397*	29.5	*522*	1:3.13
26	28.2	*174270*	34.4	*2608*	1:1.22
27	32.3	*127752*	26.5	*253*	1:0.82
28	41.1	*44143*	34.3	*3638*	1:0.83
29	32.5	*88371*	46.3	*33580*	1:1.42
30	56.4	*61159*	35.4	*632*	1:0.63

Table 3.6 PCa group demonstrating CD4+ T lymphocytes as a percentage of the total lymphocyte population in PBMC and tissue. The sixth column shows the ratios between PBMC and PCa.

The mean percentage of CD4+ T lymphocytes in PBMC was 41.26% (95% CI 36.78 to 45.74), while in PCa tissue it was 34.22% (95% CI 30.43 to 38.01). There were 7.04% (95% CI 1.29 to 12.78, p 0.0172) more CD4+ T lymphocytes in circulation than in the tumour environment. From the individual ratios, two thirds of patients had higher CD4+ T lymphocytes in PBMC.

Gating for CD8+ cells with a dump channel assessed for the presence for cytotoxic T cells. The results are expressed as a percentage and event numbers in table 3.7.

Patient No	CD8+ PBMC (%)	Number of events	CD8+ Tissue (%)	Number of events	PBMC:PCa ratio
1	20.9	*143100*	69.2	*504*	1:3.31
2	34.5	*55756*	37.3	*7831*	1:1.08
3	19.2	*78850*	37.6	*3036*	1:1.96
4	37.2	*86698*	27.7	*748*	1:0.74
5	20.7	*52914*	35.2	*2463*	1:1.70
6	21.4	*34641*	23.8	*19055*	1:1.11
7	21.3	*61887*	39.2	*286*	1:1.84
8	21.2	*70198*	22.7	*339*	1:1.07
9	34.9	*64427*	33.6	*1152*	1:0.96
10	16.2	*84700*	32.9	*18398*	1:2.03
11	33.4	*80591*	28.0	*1873*	1:0.84
12	37.9	*62307*	40.1	*4684*	1:1.06
13	37.1	*83994*	36.1	*5362*	1:0.97
14	39.1	*59046*	51.5	*1105*	1:0.92
15	21.7	*10497*	20.5	*3382*	1:0.94
16	36.8	*83788*	58.3	*2984*	1:2.40
17	47.8	*76081*	22.7	*4322*	1:0.47
18	34.9	*46714*	31.5	*629*	1:0.90
19	16.5	*43834*	36.9	*17200*	1:2.23
20	30.0	*57306*	34.9	*17143*	1:1.16
21	22.5	*37964*	33.6	*17277*	1:1.49

22	22.2	*84381*	36.6	*1954*	1:1.65
23	41.3	*76895*	36.5	*9925*	1:0.88
24	26.0	*83561*	24.2	*4998*	1:0.93
25	21.4	*34361*	23.8	*19055*	1:1.11
26	29.0	*38379*	35.1	*387*	1:1.21

Table 3.7 The percentage of CD8+ T lymphocytes in PBMC and PCa tissue is demonstrated in columns 2 and 4. The final column demonstrates the ratios between PBMC and PCa.

The mean percentage of CD8+ T lymphocytes in PBMC was 28.66% (95% CI 25.11 to 32.21), while in PCa tissue was higher at 34.98% (95% CI 30.51 to 39.45). There were 6.32% (95% CI -11.89 to -0.76, p 0.0267) more CD8+ T lymphocytes in local tumour environment than in the periphery. The ratios from the individual patients predominantly have higher CD8+ T lymphocytes in tissue than in PBMC.

The Treg cell population in PCa tissue and PBMC is demonstrated in table 3.8.

Patient No.	Treg PBMC (%)	Number of events	Treg Tissue (%)	Number of events	PBMC:PCa ratio
1	4.6	*67463*	4.3	*616*	1:0.93
2	7.5	*90445*	8.0	*608*	1:1.07
3	1.8	*97656*	15.1	*623*	1:8.38
4	5.9	*106137*	4.5	*1036*	1:0.76
5	15.1	*75848*	17.3	*132*	1:1.15
6	6.3	*17036*	8.4	*391*	1:1.33
7	5.6	*91094*	5.6	*3379*	1:1
8	1.2	*66461*	1.5	*2185*	1:1.25
9	2.6	*15913*	4.6	*8627*	1:1.77
10	3.2	*78704*	2.8	*5246*	1:0.88
11	3.8	*64472*	4.5	*1436*	1:1.18
12	4.5	*63789*	8.7	*10000*	1:1.93
13	5.3	*96168*	18.3	*803*	1:3.45
14	6.5	*71883*	4.6	*341*	1:0.71
15	6.7	*50264*	11.7	*1324*	1:1.75
16	7.2	*39373*	8.5	*1229*	1:1.18
17	8.9	*55892*	9.5	*269*	1:1.07
18	6.4	*21325*	7.9	*5895*	1:1.23
19	8.1	*76154*	8.8	*629*	1:1.09
20	7.8	*51603*	7.8	*2374*	1:1
21	4.6	*48118*	4.6	*483*	1:1

22	3.8	*21642*	6.2	*1062*	1:1.63
23	3.4	*12526*	7.5	*833*	1:2.21
24	7.7	*28759*	5.5	*15563*	1:0.71
25	2.5	*60890*	2.9	*1761*	1:1.16
26	5.0	*26151*	11.4	*454*	1:2.28

Table 3.8 Columns 2 and 4 show percentage of Treg cells in CD4+ T lymphocytes obtained in PBMC and PCa tissue respectively. The final column shows the PBMC and PCa ratios.

The mean percentage of Treg cells in PBMC was 5.60% (95% CI 4.46 to 6.73), while in PCa tissue was higher at 7.70% (95% CI 5.98 to 9.42). This was a difference of 2.10% (95% CI -4.11 to -0.08, p 0.0416). Majority of patients had higher Treg cells in tissue than PBMC seen on the ratios.

3.2.4 Comparison

The percentage of the lymphocyte subsets in PBMC and tissue in each of the pathological conditions are compared in tables 3.9 to 3.11.

3.2.4.1 CD4+ T lymphocytes

Medium	Comparison	Mean (%)	CI (%)	Difference (%)	CI of difference (%)	p value
PBMC	BPH vs.	46.41	34.96 to 55.51	7.60	-9.90 to 25.10	0.3599
	CAP	38.82	21.72 to 55.91			
Tissue	BPH vs.	24.59	15.34 to 33.83	-9.45	-22.55 to 3.65	0.1407
	CAP	34.03	21.98 to 46.08			
PBMC	BPH vs.	46.41	34.96 to 55.51	5.16	-5.13 to 15.44	0.3157
	PCa	41.26	36.78 to 45.74			
Tissue	BPH vs.	24.59	15.34 to 33.83	-9.63	-18.26 to -1.01	0.0296
	PCa	34.22	30.43 to 38.01			
PBMC	CAP vs.	38.82	21.72 to 55.91	-2.44	-14.00 to 9.12	0.6706
	PCa	41.26	36.78 to 45.74			
Tissue	CAP vs.	34.03	21.98 to 46.08	-0.19	-9.60 to 9.22	0.9681
	PCa	34.22	30.43 to 38.01			

Table 3.9 Comparison of CD4+ T lymphocytes in the different prostate pathology. Column one indicates the medium in which the lymphocytes are been analysed. The second column the prostatic conditions which are being compared. The third and fourth columns show the mean and 95% CI of the individual condition. The final three columns show the mean difference, 95% CI and p value between the two conditions.

From the comparison above, it is seen there are 9.63% (95% CI -18.26 to -1.01) more CD4+ T lymphocytes in the tissue of PCa than in BPH. This finding is statistically significant with a p value of 0.0296. The other comparisons did not show a statistical difference.

3.2.4.2 CD8+ T lymphocytes

Medium	Comparison	Mean (%)	CI (%)	Difference (%)	CI of difference (%)	p value
PBMC	BPH vs.	23.07	11.42 to 34.73	-5.59	-13.97 to 2.79	0.1838
	PCa	28.66	25.11 to 32.21			
Tissue	BPH vs.	22.17	16.69 to 27.66	-12.81	-21.72 to -3.90	0.0063
	PCa	34.98	30.51 to 39.45			

Table 3.10 Comparison of CD8+ T lymphocytes in BPH and PCa. See table 3.9 for the description of the columns.

There were 12.81% (95% CI -21.72 to -3.90) greater CD8+ T lymphocytes seen in PCa than in BPH tissue. This is statistically significant (p 0.0063) and indicates that there is an increased influx or reduced efflux of cytotoxic cells into the tumour environment.

3.2.4.3 Treg cells

Medium	Comparison	Mean (%)	CI (%)	Difference (%)	CI of difference (%)	p value
PBMC	BPH vs.	6.26	4.84 to 7.68	3.13	1.24 to 5.02	0.0042
	CAP	3.13	1.48 to 4.79			
Tissue	BPH vs.	5.58	3.42 to 7.74	2.32	-0.20 to 4.83	0.0675
	CAP	3.27	1.33 to 5.21			
PBMC	BPH vs.	6.26	4.84 to 7.68	0.66	-1.77 to 3.09	0.5833
	PCa	5.60	4.46 to 6.73			
Tissue	BPH vs.	5.58	3.42 to 7.74	-2.12	-5.80 to 1.56	0.2480
	PCa	7.70	5.98 to 9.42			
PBMC	CAP vs.	3.13	1.48 to 4.79	-2.47	-4.92 to -0.02	0.0485
	PCa	5.60	4.46 to 6.73			
Tissue	CAP vs.	3.27	1.33 to 5.21	-4.44	-8.09 to -0.78	0.0192
	PCa	7.70	5.98 to 9.42			

Table 3.11 Comparison of CD4+ T lymphocytes in the different prostate pathology. Refer to table 3.9 for table content description.

The Treg population in the BPH in both the periphery and the tissue were higher than CAP. This could represent diminished suppression in the prostatitis environment. There were also significant difference in the PBMC and tissue when comparing PCa and CAP. PCa had 2.47% (95% CI -4.92 to -0.02, p 0.0485) and 4.44% (95% CI -8.09 to -0.78) higher Treg cells in PBMC and tissue respectively. Also there were more Tregs in PCa tissue than in BPH but this was not statistically significant. This could result in a suppressive local tumour environment.

3.2.5 Ratios

It was important to assess both the raw numbers of the different populations but also to evaluate the proportions of the different lymphocytes subpopulation. The lymphocyte ratios of these suppressive and cytotoxic T cells can assist with predicting the net effect. Refer to table 3.12 for ratios between CD4+, CD8+ and Treg cells.

Tissue	Medium	CD4:CD8 ratio	CD4:Treg ratio	CD8:Treg ratio
BPH	PBMC	2.01:1	7.41:1	3.69:1
	Tissue	1.11:1	4.41:1	3.97:1
CAP	PBMC	-	12.40:1	-
	Tissue	-	10.41:1	-
PCa	PBMC	1.44:1	7.37:1	5.12:1
	Tissue	0.99:1	4.44:1	4.54:1

Table 3.12 The ratios of the CD4+, CD8+ and Treg cells in the patients with BPH, CAP and PCa. The first column demonstrates the prostate pathology of the patients where the samples were obtained. The second column shows whether the samples are PBMC or tissue. The final three columns show the ratios between the different lymphocytes.

The CD4:CD8 ratios in BPH and PCa were similar as they ranged between 1:1 and 2:1. The CD4:Treg ratios between BPH and PCa were also similar where PBMC had approximately 7:1 while the tissue had 4:1. In CAP there was a higher proportion of CD4+ lymphocytes in comparison to Treg cells ranging from 12.40:1 in PBMC and 10.41:1 in the tissue. This twelve and tenfold increase in the CD4+ T lymphocytes shows that there are very few Treg cells present to inhibit the actions of the immune system and thus allowing for inflammation to occur.

The CD8:Treg ratios in BPH and PCa provided similar results albeit slightly higher in PCa. There seems to be a proportionate rise in Treg cells to counter the effects of the CD8+ T lymphocytes to bring the numbers down to that seen in BPH.

3.3 Immunohistochemistry

The result that was obtained with flow cytometry needed to be verified. Immunohistochemistry was used to assess the expression T cell antigens. This provided information on the numbers and distribution of these T cells in the local tumour and surrounding environment.

Only patients with BPH and PCa were examined because only tissue samples obtained from TRUS biopsy for patients with CAP were insufficient to perform IHC staining. As a result 6 patients with BPH and 30 patients with PCa were studied.

3.3.1 BPH group

The tissue of the six participants with BPH was obtained from open prostatectomy and TURP. These samples were stained for H&E, CD4+, CD8+ and FoxP3.

The number of cells per 10hpf, also referred to as cells/mm^2, is documented in table 3.13.

Patient No.	CD4+ cells/10 hpf	CD8+ cells/10 hpf	Treg cells/10 hpf	CD4:CD8 ratio	CD4:Treg ratio	CD8:Treg ratio
1	159	244	7	1:1.53	1:0.04	1:0.03
2	87	171	0	1:1.97	-	-
3	110	132	1	1:1.20	1:0.01	1:0.01
4	56	92	3	1:1.64	1:0.05	1:0.03
5	163	158	0	1:0.97	-	-
6	192	263	18	1:1.37	1:0.09	1:0.07
Mean	127.83	176.67	4.83			
CI	73.36 to 182.30	107.80 to 245.53	-2.48 to 12.15			

Table 3.13 IHC stain for CD4+, CD8+ and Treg cells in BPH tissue. Columns two to four show the number of lymphocytes seen on IHC. Columns five to seven demonstrate the ratios of the lymphocytes in the individual patients. Row eight and nine show the mean and 95% CI of the lymphocyte population.

From the analysis, there is a higher proportion of CD8+ T cells in the tumour than the CD4+ T lymphocytes (mean 176.67 vs. 127.83). This was further demonstrated by the individual patient ratios where majority of patients had 1.2 to 2.0 times as much CD8+ T lymphocytes.

In the Treg analysis, both CD4+ and CD8+ T lymphocytes are ten times higher than the Treg cells. This represents the environment of lymphocyte homeostasis.

3.3.2 PCa group

The number of cells per 10hpf was counted within the tumour environment only. This is to examine the tumour infiltrating lymphocytes. These results are documented in table 3.14. Later in the study we will assess peripheral lymphoid tissue.

Patient	CD4+ cells/10hpf	CD8+ cells/10hpf	Treg cells/10hpf	CD4:CD8 ratio	CD4:Treg ratio	CD8:Treg ratio
1	199	110	38	1:0.55	1:0.19	1:0.34
2	35	33	2	1:0.94	1:0.06	1:0.06
3	77	80	21	1:1.04	1:0.27	1:0.26
4	57	53	17	1:0.92	1:0.30	1:0.32
5	138	75	31	1:0.54	1:0.22	1:0.41
6	142	225	37	1:1.58	1:0.26	1:0.16
7	71	82	24	1:1.15	1:0.34	1:0.29
8	284	225	6	1:0.79	1:0.02	1:0.03
9	158	89	68	1:0.56	1:0.43	1:0.76
10	37	59	17	1:1.59	1:0.46	1:0.29
11	65	112	8	1:1.72	1:0.12	1:0.07
12	192	75	38	1:0.39	1:0.20	1:0.51
13	200	215	45	1:1.08	1:0.23	1:0.21
14	53	30	17	1:0.57	1:0.32	1:0.57
15	83	53	20	1:0.64	1:0.24	1:0.38
16	68	52	10	1:0.76	1:0.15	1:0.19
17	148	191	39	1:1.29	1:0.26	1:0.20

18	88	68	32	1:0.77	1:0.36	1:0.47
19	81	100	10	1:1.23	1:0.12	1:0.10
20	21	26	4	1:1.24	1:0.19	1:0.15
21	260	282	63	1:1.08	1:0.24	1:0.22
22	45	35	11	1:0.78	1:0.24	1:0.31
23	169	185	85	1:1.09	1:0.50	1:0.46
24	30	40	5	1:1.33	1:0.17	1:0.13
25	167	117	48	1:0.70	1:0.29	1:0.41
26	12	20	1	1:1.67	1:0.08	1:0.05
27	46	118	3	1:2.57	1:0.07	1:0.03
28	64	150	12	1:2.34	1:0.19	1:0.08
29	96	115	20	1:1.20	1:0.21	1:0.17
30	52	74	10	1:1.42	1:0.19	1:0.14
Mean	104.60	102.97	24.73			
CI	77.72 to 131.48	77.24 to 128.70	16.84 to 32.63			

Table 3.14 IHC stain for CD4+, CD8+ and Treg cells in PCa tissue. Columns two to four demonstrate the lymphocyte numbers counted in the IHC stains. Columns five to seven show the ratios of the lymphocytes in the individual patients. The final two rows show the mean and 95% CI of the lymphocyte population.

In PCa there were comparable numbers of CD4+ and CD8+ T lymphocytes with means of 104.60 (95% CI 77.72 to 131.48) and 102.97 (95% CI 77.24 to 128.70) respectively. This similarity was also seen in the CD4:CD8 ratios where apart from a few outliers, the numbers were roughly proportionate.

The proportion of Treg cells was higher in PCa. This resulted in a ratio of 5:1 when compared to CD4+ and CD8+ T lymphocytes.

3.3.3 Comparison

3.3.3.1 BPH vs. PCa

The mean lymphocyte population in BPH and PCa obtained from IHC is compared in table 3.15.

Lymphocytes	Difference cells/10hpf	CI of difference	p value
CD4+	23.23	-39.85 to 86.31	0.4593
CD8+	73.70	11.50 to 135.90	0.0216
Treg	-19.90	-37.81 to -1.99	0.0305

Table 3.15 Comparison IHC findings between BPH and PCa. The first column shows the subset of lymphocyte being analysed. The second column is the difference between BPH and PCa. The final two columns depict the 95% CI and the p value generated by the t paired test.

From the findings, there was a reduction in the number of CD4+ T lymphocytes however this was not statistically significant. There were 73.70 cells/10hpf more CD8+ T lymphocytes in BPH in comparison to PCa.

There were higher numbers of Treg cells in the local tumour environment of PCa despite the total reduction of CD4+ T lymphocytes. This further supports an immunosuppressive environment.

3.3.4 Ratios

The ratios of CD4:CD8, CD4:Treg and CD8:Treg are demonstrated in table 3.16.

Tissue	CD4:CD8	CD4:Treg	CD8:Treg
BPH	0.72:1	26.47:1	36.58:1
PCa	1.02:1	4.23:1	4.16:1

Table 3.16 Lymphocyte ratios in BPH and PCa.

From the IHC data, the CD4:CD8 ratios were similar to the 1:1 ratio seen in the flow cytometry analysis. However with regards to CD4:Treg and CD8:Treg ratios the figures differed considerably. The common factor that resulted in the large differences in the ratios in BPH is the scant number of Treg cells seen.

The ratios seen for PCa in IHC were similar to the findings seen with flow cytometry.

3.3.5 Comparison with pathological markers

The current predictive markers that are available for PCa are histopathological markers. Gleason score, extraprostatic spread, lymph node involvement, lymphovascular invasion, margin involvement, perineural invasion and seminal vesicle spread are very limited in predicting the course of the disease but unfortunately they are what is available to guide clinical decision-making.

The presence of lymphocytes in cancers has been reported to show increased survival benefit [226, 237, 303]. As a result the three classes of lymphocytes, CD4+, CD8+ and Treg cells have been compared to current pathological markers to determine if a correlation is present with future outcome.

3.3.5.1 Gleason score

This is the main pathological indicator for PCa severity. The mean lymphocyte subpopulation for each Gleason score obtained from flow cytometry and IHC and is depicted in the tables 3.17 to 3.18 respectively.

	CD4+	CD8+	Treg
3+3=6 vs. 3+4=7	**PBMC:** 5.36 CI: -7.12 to 17.83 p: 0.3803 **Tissue:** -0.96 CI: -10.27 to 8.35 p: 0.8319	Only one patient Unable to calculate	**PBMC:** -0.56 CI: -3.22 to 2.09 p: 0.6550 **Tissue:** -0.37 CI: -3.23 to 2.50 p: 0.7884
3+3=6 vs. 4+3=7	**PBMC:** -2.46 CI: -16.18 to 11.26 p: 0.7050 **Tissue:** -1.15 CI: -13.51 to 11.21 p: 0.8438	Only one patient Unable to calculate	**PBMC:** -0.36 CI: -4.85 to 4.16 p: 0.8648 **Tissue:** -4.25 CI: -10.72 to 2.21 p: 0.1771
3+4=7 vs. 4+3=7	**PBMC:** -7.81 CI: -17.25 to 1.62 p: 0.0995 **Tissue:** -0.19 CI: -10.76 to 10.37 p: 0.9700	**PBMC:** 6.78 CI: -0.79 to 14.34 p: 0.0764 **Tissue:** -3.37 CI: -13.94 to 7.21 p: 0.5152	**PBMC:** 0.21 CI: -2.51 to 2.92 p: 0.8761 **Tissue:** -3.89 CI: -7.53 to -0.25 p: 0.0376

Table 3.17 Gleason score comparison with respect to lymphocyte subtypes using flow cytometry. Column one demonstrates the Gleason scores that are compared. Columns two to four show the mean difference in the PBMC and tissue in each of the lymphocyte subpopulation.

	CD4+	CD8+	Treg
3+3=6 vs. 3+4=7	Tissue: -17.08 CI: -95.22 to 61.05 p: 0.6479	Tissue: -22.28 CI: -103.88 to 59.31 p: 0.5691	Tissue: -3.70 CI: -22.98 to 15.58 p: 0.6882
3+3=6 vs. 4+3=7	Tissue: -32.18 CI: -121.83 to 57.46 p: 0.4541	Tissue: -10.29 CI: -85.49 to 64.91 p: 0.7734	Tissue: -8.11 CI: -37.54 to 21.32 p: 0.5640
3+4=7 vs. 4+3=7	Tissue: -15.10 CI: -83.56 to 53.36 p: 0.6512	Tissue: 11.99 CI: -47.80 to 71.79 p: 0.6808	Tissue: -4.41 CI: -24.82 to 16.00 p: 0.6579

Table 3.18 The mean differences between Gleason scores in relation to lymphocyte population obtained using IHC. Column one shows the Gleason score comparison. Columns two to four show the mean difference of lymphocytes in the tissue.

For the majority of the comparisons, there was no pattern seen, except for patients with Gleason score of 4+3=7 had 3.89% higher Treg cells in the tumour than patients with Gleason score 3+4=7. The presence of more immunosuppressive cells in the higher grade of PCa corresponds with poorer prognosis.

3.3.5.2 Extraprostatic spread

The spread of cancer through the capsule of the prostate is a poor prognostic indicator [304]. Flow cytometry and IHC data of CD4+, CD8+ and Tregs cells were analysed in patients with and without extraprostatic spread. The results are shown in tables 3.19 and 3.20.

Lymphocytes	Medium	Present (%)	Absent (%)	Difference (%)	CI of difference (%)	p value
CD4+	PBMC	41.43	40.17	1.26	-7.98 to 10.51	0.7811
	Tissue	35.73	32.92	2.81	-5.16 to 10.79	0.4757
CD8+	PBMC	25.04	32.04	-7.00	-13.36 to -0.65	0.0320
	Tissue	36.57	33.51	3.06	-5.62 to 11.73	0.4751
Treg+	PBMC	6.09	5.29	0.49	-1.57 to 3.16	0.4932
	Tissue	9.87	6.35	3.52	0.23 to 6.82	0.0371

Table 3.19 Lymphocyte proportion examined in patients with extraprostatic spread using flow cytometry. The first column depicts the subtype of lymphocyte being assessed. Column two shows the whether the cells are in PBMC or tissue. The third and fourth columns demonstrate the mean lymphocyte percentage of patients with and without extraprostatic spread respectively. The final three columns show the difference in the mean, 95% CI and the p value of the medium being examined.

Lymphocytes	Present (cells/10hpf)	Absent (cells/10hpf)	Difference (cells/10hpf)	CI of difference (cells/10hpf)	p value
CD4+	121.00	75.67	45.33	-14.40 to 105.07	0.1301
CD8+	122.00	93.00	29.00	-32.21 to 90.21	0.3372
Treg	23.46	20.50	2.96	-14.35 to 20.28	0.7267

Table 3.20 Lymphocyte proportion examined in patients with extraprostatic spread using IHC. Column one shows lymphocyte subtypes being examined. The second and third columns demonstrate the mean lymphocyte numbers in patients with and without extraprostatic spread respectively. The final three columns show the difference in the mean, 95% CI and the p value of the medium being examined.

From the results above, those patients with extraprostatic spread had 7% (95% CI 13.36 to 0.65, p 0.0320) lower CD8+ lymphocytes in PBMC and 3.52% (95% CI 0.23 to 6.82, p 0.0371) higher Treg cells in the local tumour environment.

This finding is consistent with an immunosuppressive environment of reduced cytotoxic T cells and increased population of Treg cells.

3.3.5.3 Lymph node involvement

The spread of PCa to local obturator lymph nodes is a poor prognostic factor. However in the process of eliminating data that did not have adequate event counts, only one patient was left with lymph node involvement. As a result, statistical calculations could not be performed.

3.3.5.4 Lymphovascular invasion

This is the identification of PCa spread into lymphatic vessels. The data are represented in table 3.21 and 3.22.

Lymphocytes	Medium	Present (%)	Absent (%)	Difference (%)	CI of difference (%)	p value
CD4+	PBMC	39.70	40.97	-1.27	-19.70 to 17.17	0.8887
	Tissue	35.00	33.50	1.50	-14.20 to 17.19	0.8460
CD8+	PBMC	34.60	28.24	6.36	-5.01 to 17.72	0.2585
	Tissue	27.27	35.95	-8.69	-23.25 to 5.88	0.2292
Treg	PBMC	5.36	5.64	-0.28	-4.02 to 3.45	0.8766
	Tissue	7.23	7.29	-0.06	-4.93 to 4.81	0.9811

Table 3.21 Lymphocyte proportion examined in patients with lymphovascular invasion using flow cytometry. Columns three and four show the mean lymphocyte percentage seen in patients with and without lymphovascular invasion.

Lymphocytes	Present (cells/10hpf)	Absent (cells/10hpf)	Difference (cells/10hpf)	CI of difference (cells/10hpf)	p value
CD4+	148.00	89.95	58.05	-23.86 to 139.96	0.1562
CD8+	136.00	102.76	33.24	-50.68 to 117.16	0.4210
Treg	31.25	20.29	10.96	-12.22 to 34.15	0.3381

Table 3.22 Lymphocyte proportion examined in patients with lymphovascular invasion using IHC. The second and third columns show the mean lymphocyte numbers in patients with and without lymphovascular invasion.

From the data above, there were no results that were statistically significant.

3.3.5.5 Margin involvement

The presence of PCa at the surgical margins is considered as a positive margin. This implies that there is a possibility of PCa still remaining in the patient and a poor prognostic indicator. The results from the analysis are demonstrated in tables 3.23 and 3.24 below.

Lymphocytes	Medium	Involved (%)	Clear (%)	Difference (%)	CI of difference (%)	p value
CD4+	PBMC	39.54	41.85	-2.31	-11.48 to 6.85	0.6084
	Tissue	35.62	32.83	2.79	-5.15 to 10.73	0.4774
CD8+	PBMC	26.02	30.92	-4.91	-11.89 to 2.09	0.1605
	Tissue	31.68	37.81	-6.12	-14.91 to 2.66	0.1633
Treg	PBMC	6.46	5.22	1.25	-1.22 to 3.71	0.3075
	Tissue	8.68	7.27	1.41	-2.35 to 5.17	0.4463

Table 3.23 Lymphocyte proportion examined in patients with margin involvement using flow cytometry. Columns three and four show the mean lymphocyte percentage seen in patients with positive and negative margins respectively.

Lymphocytes	Involved (cells/10hpf)	Clear (cells/10hpf)	Difference (cells/10hpf)	CI of difference (cells/10hpf)	p value
CD4+	119.56	87.81	31.74	-32.23 to 95.71	0.3153
CD8+	126.56	97.69	28.87	-34.95 to 92.69	0.3591
Treg	27.11	19.19	7.92	-9.82 to 25.67	0.3652

Table 3.24 Lymphocyte proportion examined in patients with margin involvement using IHC. The second and third columns show the mean lymphocyte numbers in patients with positive and negative margins.

There was not enough sample population to obtain statistically significant results.

3.3.5.6 Perineural invasion

The presence of PCa in the area surrounding nerves is indicative of perineural invasion and it is pathological marker of poor prognosis. The results from the analysis are demonstrated in tables 3.25 and 3.26.

Lymphocytes	Medium	Present (%)	Absent (%)	Difference (%)	CI of difference (%)	p value
CD4+	PBMC	41.09	38.55	2.54	-10.77 to 15.84	0.6990
	Tissue	33.88	36.03	-2.14	-13.73 to 9.44	0.7075
CD8+	Only one patient					
Treg	PBMC	5.72	4.65	1.07	-2.54 to 4.69	0.5465
	Tissue	8.03	5.18	-2.85	-2.52 to 8.22	0.2839

Table 3.25 Lymphocyte proportion examined in patients with perineural invasion using flow cytometry. The third and fourth columns demonstrate the mean lymphocyte percentage seen in patients with and without perineural invasion respectively.

Lymphocytes	Present (cells/10hpf)	Absent (cells/10hpf)	Difference (cells/10hpf)	CI of difference (cells/10hpf)	p value
CD4+	107.76	54.50	53.26	-29.25 to 135.78	0.1948
CD8+	114.00	77.00	37.00	-46.63 to 120.63	0.3695
Treg	24.00	11.75	12.25	-10.81 to 35.31	0.2832

Table 3.26 Lymphocyte proportion examined in patients with perineural invasion using IHC. Columns two and three show the mean lymphocyte numbers in patients with and without perineural invasion.

A difference could not be demonstrated with the limited number of samples.

3.3.5.7 Seminal vesicle involvement

The spread of PCa to the seminal vesicles is considered as localized extension of the tumour which is a poor prognostic indicator. The data is represented in the tables 3.27 and 3.28 below.

Lymphocytes	Medium	Involved (%)	Clear (%)	Difference (%)	CI of difference (%)	p value
CD4+	PBMC	38.77	40.96	0.77	-17.28 to 12.89	0.7675
	Tissue	43.37	33.12	10.25	-2.27 to 22.76	0.1044
CD8+	PBMC	19.63	30.36	-10.74	-20.00 to -1.48	0.0249
	Tissue	30.53	35.82	-5.30	-18.10 to 7.50	0.4007
Treg	PBMC	5.78	5.58	0.21	-3.43 to 3.85	0.9074
	Tissue	12.72	7.05	5.68	0.72 to 10.63	0.0265

Table 3.27 Lymphocyte proportion examined in patients with seminal vesicle involvement using flow cytometry. Columns three and four show the mean lymphocyte percentage in patients with and without seminal vesicle involvement respectively.

Lymphocytes	Involved (cells/10hpf)	Clear (cells/10hpf)	Difference (cells/10hpf)	CI of difference (cells/10hpf)	p value
CD4+	101.80	98.60	3.20	-75.29 to 81.69	0.9335
CD8+	108.40	108.00	0.40	-77.63 to 78.43	0.9916
Treg	22.80	21.85	0.95	-20.73 to 22.63	0.9286

Table 3.28 Lymphocyte proportion examined in patients with seminal vesicle involvement using IHC. Columns two and three show the mean lymphocyte numbers in patients with and without seminal vesicle involvement respectively.

Patients with seminal vesicle involvement had 10.74 % (95% CI 20.00 to 1.48, p 0.0249) lower CD8+ lymphocytes in PBMC and 5.68% (95% CI 0.72 to 10.63, p 0.0265) more Treg cells in the local tumour environment. This again supports the theory of evasion of immune system. Reduced CD8 and increased Treg cells demonstrate this.

3.3.5.8 PSA relapse

The European Association of Urology guidelines show that a patient is considered to have cancer recurrence after radical prostatectomy when there is two consecutive rises in PSA by greater than 0.2ng/mL [305-308]. The patients with rising PSA and stable PSA are examined in the tables 3.29 and 3.30.

Lymphocytes	Medium	Rising PSA (%)	Stable PSA (%)	Difference (%)	CI of difference (%)	p value
CD4+	PBMC	42.50	39.96	5.54	-5.69 to 16.78	0.3184
	Tissue	35.62	32.94	2.68	-6.17 to 11.52	0.5381
CD8+	PBMC	23.05	30.94	-7.89	-16.20 to 0.43	0.0618
	Tissue	32.58	33.87	-1.29	-10.20 to 7.62	0.7669
Treg	PBMC	5.90	5.01	0.89	-1.25 to 3.03	0.3967
	Tissue	10.64	6.25	4.39	0.90 to 7.88	0.0162

Table 3.29 Lymphocyte proportion examined in patients with and without PSA relapse using flow cytometry. The third and fourth columns depict the mean lymphocyte percentage in patients with and without PSA relapse respectively.

Lymphocytes	Rising PSA (cells/10hpf)	Stable PSA (cells/10hpf)	Difference (cells/10hpf)	CI of difference (cells/10hpf)	p value
CD4+	131.00	90.38	40.63	-30.87 to 112.12	0.2505
CD8+	123.29	106.06	17.22	-54.93 to 89.38	0.6248
Treg	20.00	24.13	-4.13	-24.47 to 16.22	0.6776

Table 3.30 Lymphocyte proportion examined in patients with and without PSA relapse using IHC. Columns two and three show the mean lymphocyte numbers in patients with and without PSA relapse.

Patients who had PSA relapses after radical prostatectomy had 4.39% (95% CI 0.90 to 7.88, p 0.0162) higher Treg cells in the tumour environment. This increase in immunosuppressive cells corresponds with this poor prognostic marker.

3.3.5.9 Pre-operative PSA

The level of PSA prior to surgery is an indicator of tumour burden and an elevated figure has been associated with poor prognosis [309]. Patient's pre-operative PSA were plotted in graphs with CD4+, CD8+ and Treg levels (figures 3.3 to 3.8).

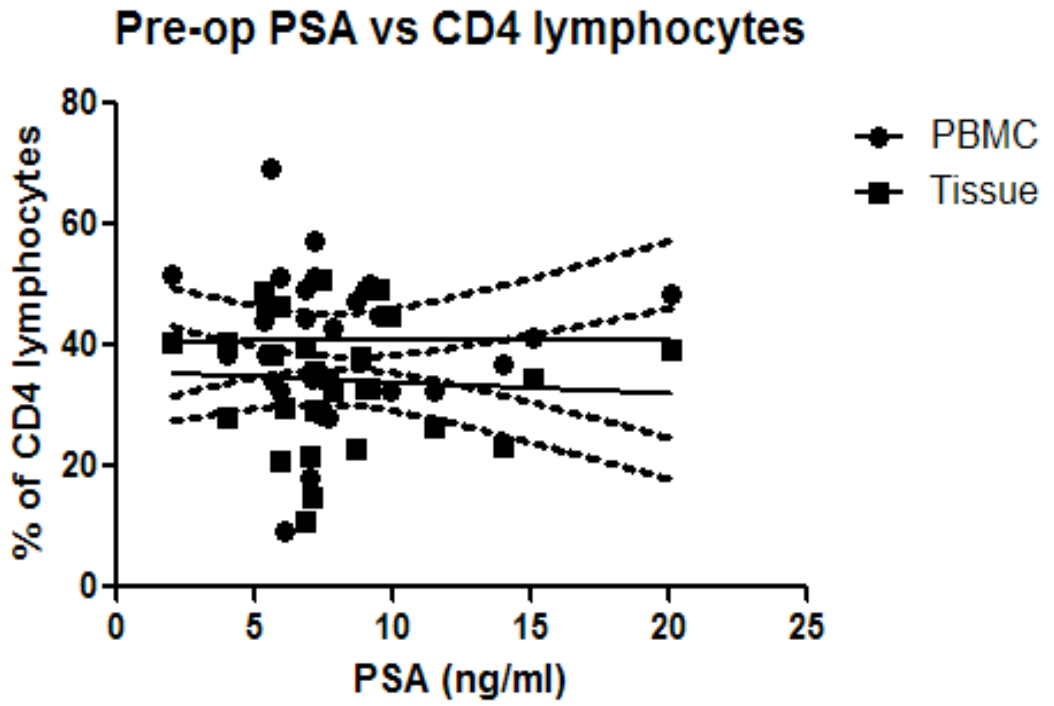


Figure 3.3 The CD4+ T lymphocytes percentage with respect to pre-op PSA using flow cytometry. The r^2 of PBMC was 0.0001 (95% CI -1.28 to 1.31, p 0.9749). The r^2 of tissue was -0.0041 (95% CI -1.31 to 0.94, p 0.7390).

Both gradients of PBMC and tissue show that there is no difference in CD4+ T lymphocytes amount at different PSA levels.

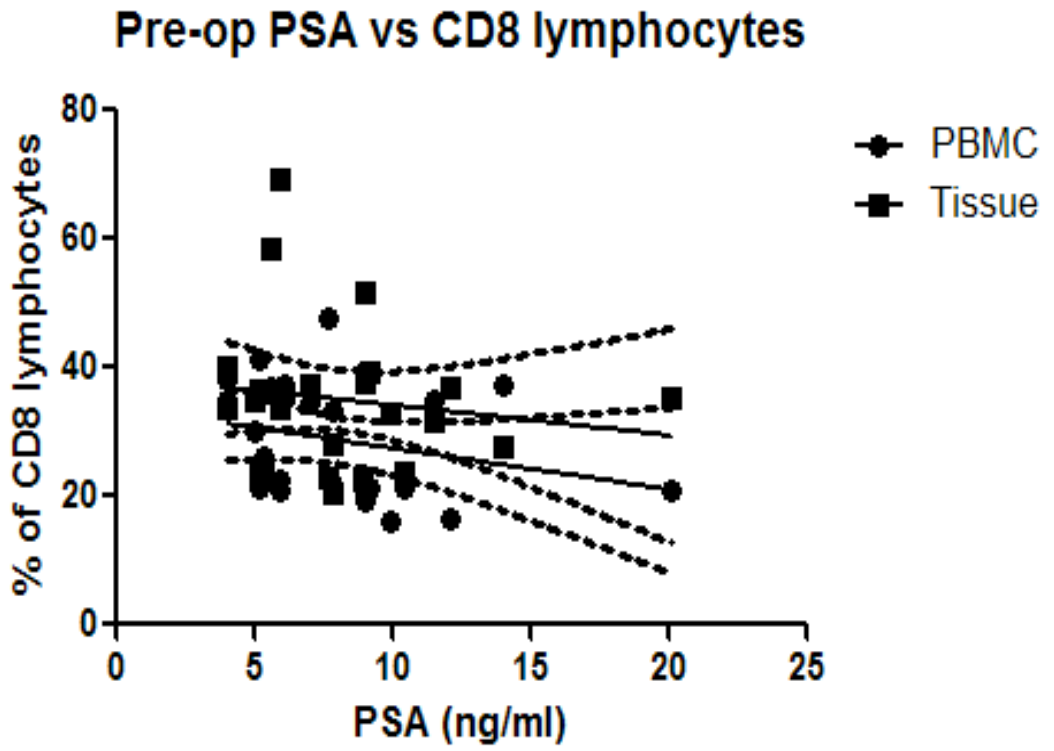


Figure 3.4 The relationship between CD8+ T lymphocytes percentage and pre-op PSA using flow cytometry. The r^2 for PBMC was -0.0673 (95% CI -1.67 to 0.39, p 0.2104). The r^2 for tissue was -0.0224 (95% CI -1.79 to 0.86, p 0.4748).

There is a slight downward trend as PSA increases however this is very marginal gradient. The 95% CI interval is wide and the results are not significant.

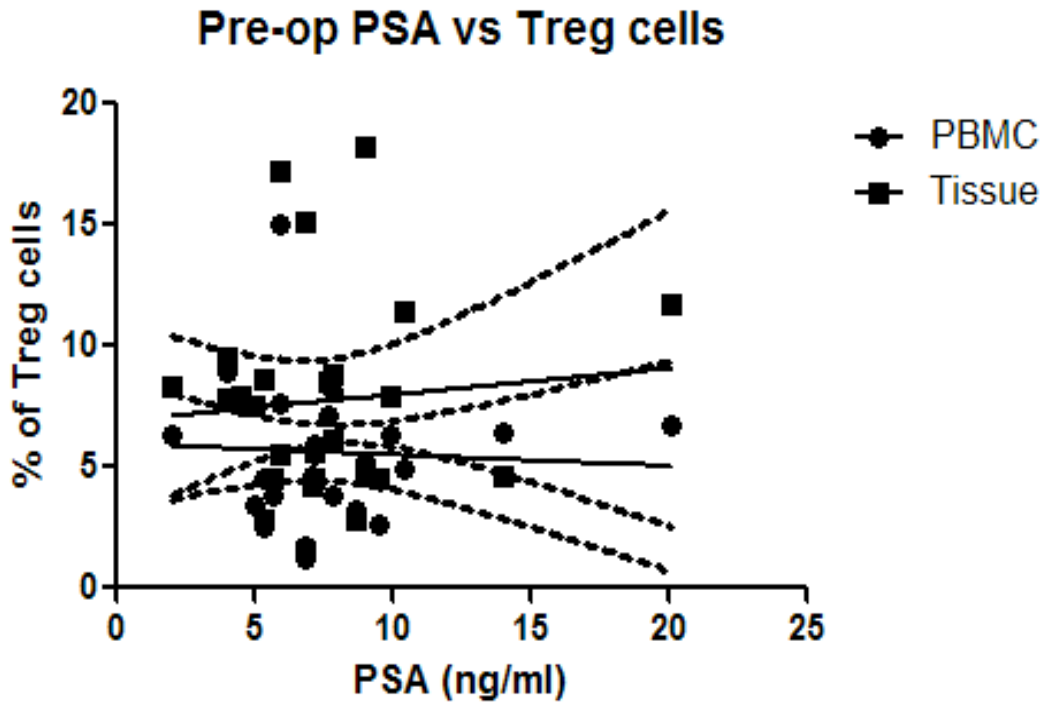


Figure 3.5 The Treg cell percentage in PBMC and tissue with respect to pre-op PSA obtained using flow cytometry. The r^2 in PBMC was -0.0033 (95% CI -0.38 to 0.29, p 0.7795). The r^2 in tissue was 0.0083 (95% CI -0.39 to 0.61, p 0.6581).

The Treg cells remained stable as PSA increased. This was seen, as the gradients remained predominantly horizontal.

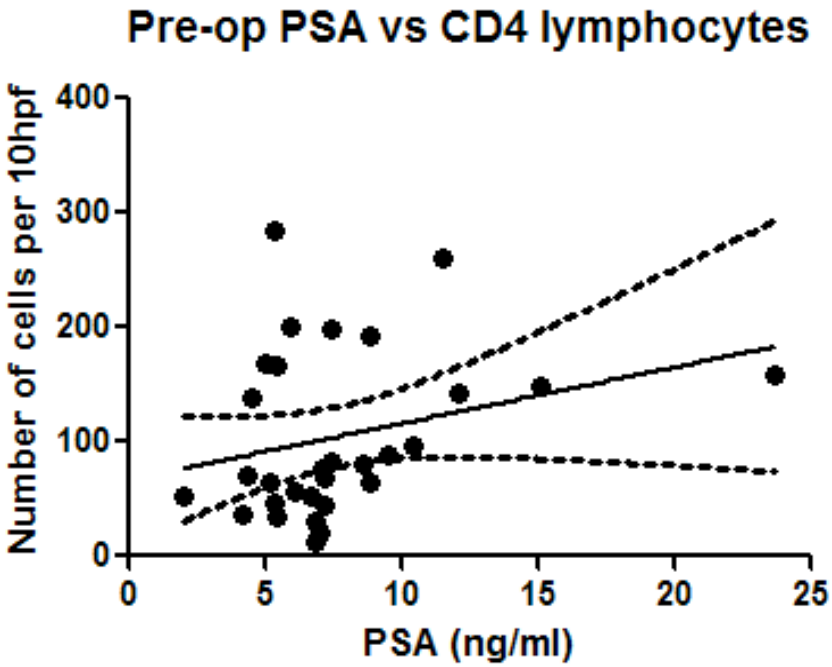


Figure 3.6 The CD4+ T lymphocyte numbers seen in relation to pre-op PSA demonstrated using IHC. The r^2 was 0.0755 (95% CI -1.74 to 11.56. p 0.1416).

There is a mild upward trend in CD4+ T lymphocytes as PSA increased. However this gradient is very minute and the results were no statistically significant.

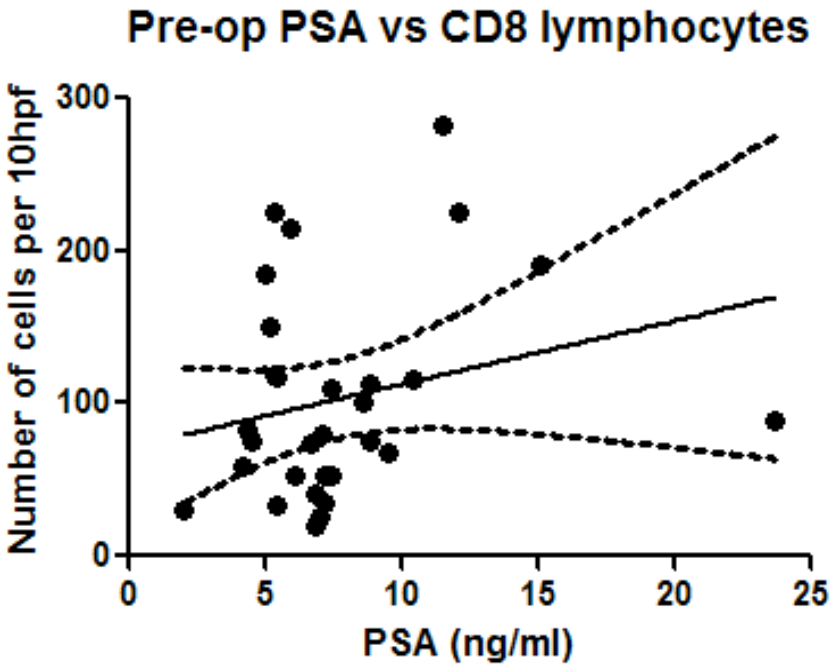


Figure 3.7 The CD8+ T lymphocyte numbers present in relation to pre-op PSA demonstrated using IHC. The r^2 was 0.0593 (95% CI -2.26 to 10.59, p 0.1949).

The CD8+ T lymphocytes had a mild rise as PSA levels increased. This again was a minor elevation in the gradient that was not statistically significant.

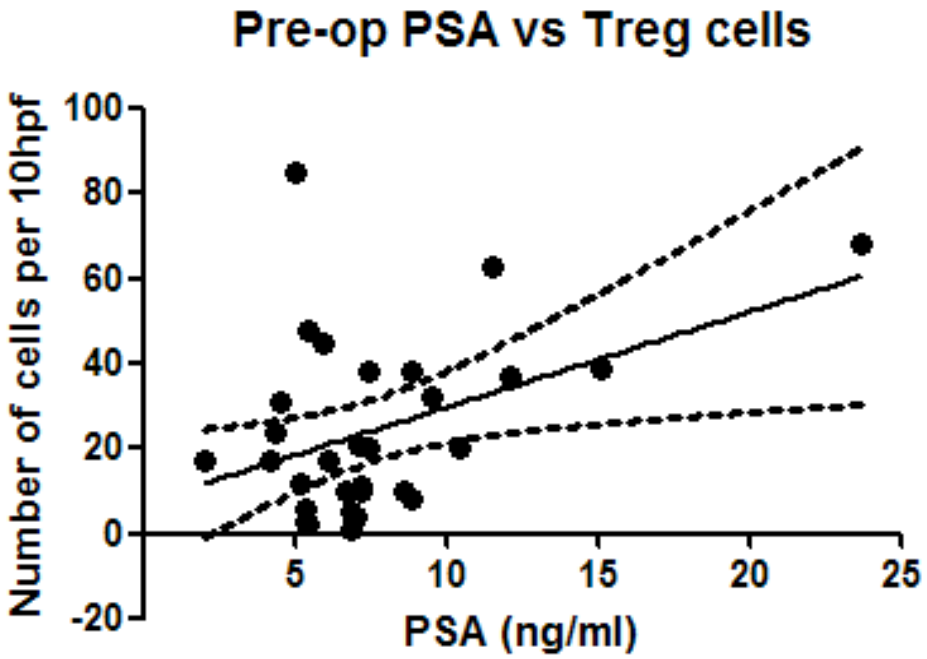


Figure 3.8 The Treg cells seen using IHC in relation to Pre-op PSA. The r^2 was 0.1834 (95% CI 0.41 to 4.08, p 0.0182).

This shows a statistical significant result where Treg cells increased as the PSA increased. The gradient is small and the clinical significance needs to be questioned. The trend seen in the flow cytometry data also had an upward trend in Treg cells. However this will require further evaluation with a large sample population to validate the results.

3.3.6 Peripheral lymphocyte aggregates

When analysing the IHC samples, the presence of lymphoid aggregates in the periphery of tumours was noted (refer to figure 3.9). These are tertiary lymphoid organs have been described in chronic inflammatory conditions such as gastritis, hepatitis and graft vs. host disease in organ transplantation [310-313].

The function of these aggregates is still disputed. Majority of the research is from chronic inflammatory conditions rather than in oncology. It has been associated with eradicating foreign pathogen and prevention of its penetration to regions of the body and support the induction of humoral immune response and T cells [314].

There have not been many studies assessing peripheral lymphoid aggregates in tumours. A study by Dieu-Nosjean et al, described the presence of lymphoid aggregates in non-small cell lung cancer and their association with increased survival [315].

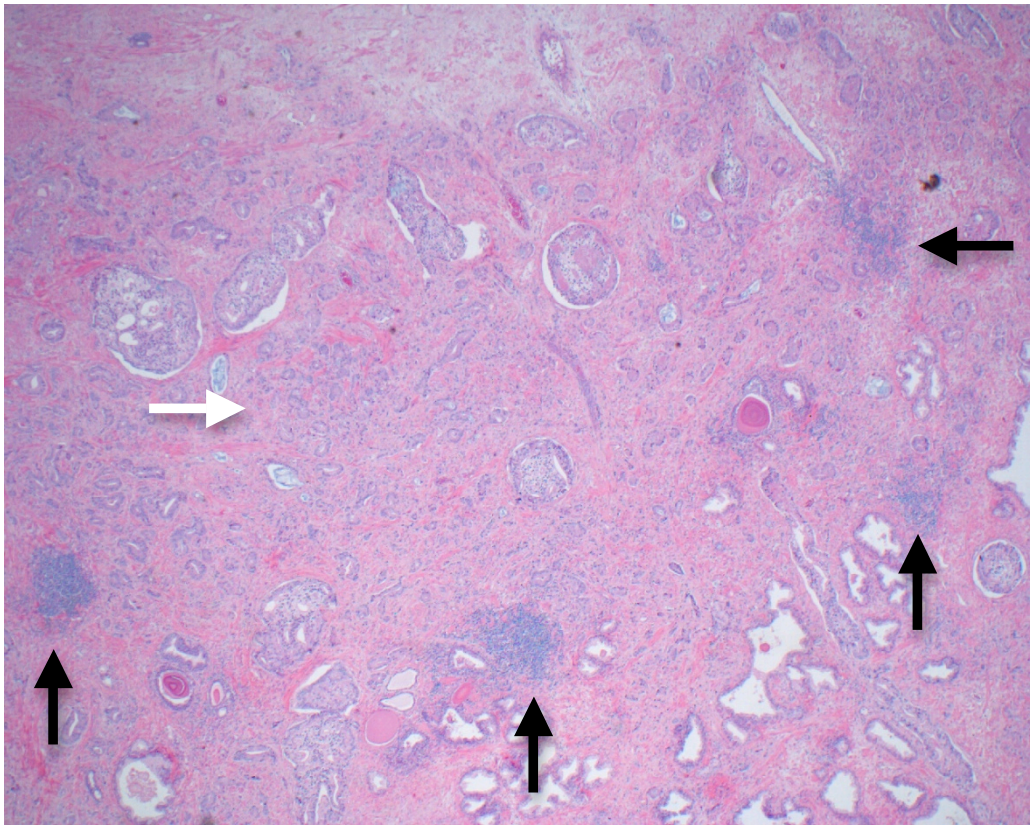


Figure 3.9 H&E staining of prostate cancer. Prostate cancer (white arrow) surrounded by aggregates of lymphocytes (black arrows) at X2 magnification.

Previously the counts were of lymphocytes within the tumour itself. Now the aggregates of lymphocytes are analysed to see their main composition. Staining for CD4+, CD8+ and Treg cells was analysed and is seen in figure

3.10. The aggregates contain an abundant number of CD4+, CD8+ and Treg cells.

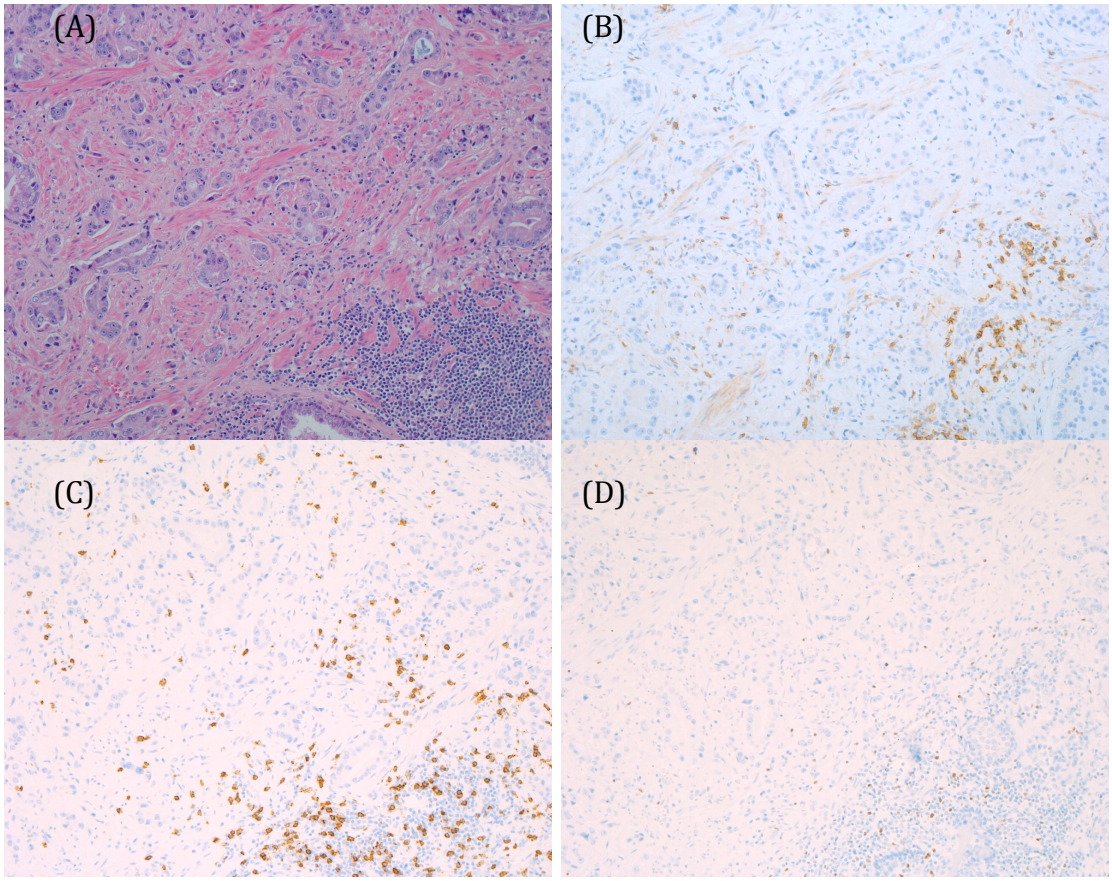


Figure 3.10 IHC staining of prostate cancer. The above images are taken of the same prostatic region with different stains at x10 magnification. The tissue was stained as the following: (A) H&E, (B) CD4+, (C) CD8+ and (D) FoxP3.

The data from previous findings were reanalysed by differentiating patients with and without the presence of these lymphoid aggregates. They are depicted in the tables 3.31 to 3.32 below.

Lymphocytes	Medium	Present (%)	Absent (%)	Difference (%)	CI of difference (%)	p value
CD4+	PBMC	42.34	38.75	3.59	-10.00 to 17.19	0.5726
	Tissue	41.97	27.23	14.74	1.51 to 27.97	0.0322
CD8+	PBMC	23.28	32.67	-9.39	-21.81 to 3.04	0.1140
	Tissue	30.68	36.80	-6.12	-15.20 to 2.96	0.1500
Treg	PBMC	4.95	3.60	1.35	-1.64 to 4.33	0.3376
	Tissue	6.41	6.96	-0.55	-5.94 to 4.84	0.8236

Table 3.31 Lymphocyte distribution analyses in patients with and without lymphoid aggregates using flow cytometry. The first column shows the subtype of lymphocytes examined. The second column demonstrates the medium in which these lymphocytes are being assessed. Columns three and four depict the mean percentage of the lymphocytes with and without lymphocyte aggregates. The final three columns show the difference in the mean, 95% CI and the p value.

Lymphocytes	Present (cells/10hpf)	Absent (cells/10hpf)	Difference (cells/10hpf)	CI of difference (cells/10hpf)	p value
CD4+	124.29	58.67	65.62	11.49 to 119.75	0.0193
CD8+	112.67	80.33	32.33	-23.51 to 88.18	0.2456
Treg	30.14	12.11	18.03	1.92 to 34.14	0.0296

Table 3.32 Lymphocyte distribution analyses in patients with and without lymphoid aggregates using IHC. The first column demonstrates the lymphocyte subtype being assessed. The second and third columns show the mean lymphocyte numbers in patients with and without lymphocyte aggregates. The final three columns show the mean difference, 95% CI and p value.

In the flow cytometry analysis, patients with peripheral lymphoid aggregates had 14.74% (95% CI 1.51 to 27.97, p 0.0322) more tumour infiltrating CD4+ lymphocytes. This is expected as these patients had lymphocyte pools resulting in an increased number. In the IHC examination, again higher number of CD4+ lymphocytes of the magnitude of 65.62 cells per 10hpf (95% CI 11.49

to 119.75, p 0.0193) was seen in patients with peripheral lymphoid aggregates. Also these patients had 18.03 more Treg cells per 10hpf (95% CI 1.92 to 34.14, p 0.0296).

3.3.6.1 Ratio

The above results were analysed in relation to each other to examine whether there is a shift in the balance of the subset of lymphocytes (table 3.33 and 3.34).

Medium	Lymphoid aggregates	CD4:CD8 ratio	CD4:Treg ratio	CD8:Treg ratio
PBMC	Present	1.82:1	8.55:1	4.70:1
	Absent	1.19:1	10.76:1	9.08:1
Tissue	Present	1.37:1	6.55:1	4.79:1
	Absent	0.74:1	3.91:1	5.29:1

Table 3.33 Lymphocyte ratios in patients with and without lymphoid aggregates using flow cytometry. Column one shows the medium being examined. The second column demonstrates whether the lymphoid aggregates are present or absent. The final three columns show the CD4:CD8, CD4:Treg and CD8:Treg ratios.

Lymphoid aggregates	CD4:CD8 ratio	CD4:Treg ratio	CD8:Treg ratio
Present	1.10:1	4.12:1	3.74:1
Absent	0.73:1	4.84:1	6.63:1

Table 3.34 Lymphocyte ratios in patients with and without lymphoid aggregates using IHC. The first column shows the medium in which the lymphocyte aggregates are being assessed. The final three columns demonstrate the ratios of the lymphocyte subsets.

The ratio of CD4:CD8 in patients with and without lymphoid aggregates was approximately 1:1. The CD4:Treg ratio was high in the PBMC group, while in the IHC analysis, they remained similar to previous results of 4:1. Finally in the CD8:Treg ratio, both modalities showed that the absent group had a higher ratio up to 9:1.

3.4 Discussion

The proportion of CD4+, CD8+ and Treg cells in the tumour environment has been shown to have both positive and negative effects on cancer cell proliferation [316-319].

The three pathological conditions, BPH, CAP and PCa are all different in their pathological processes. The BPH group was set as the benchmark to compare the other conditions. In many studies it has been used for this purpose where it was thought that the lymphocytes are in homeostasis [320-322]. Here it is assumed that there is no shift in the immune processes. However this could be incorrect as the prostate could harbour PCa or chronic infection. This is difficult to interpret but measures were taken test for this. The formal histopathological report by the pathologist of the prostate specimen was reviewed to determine if concurrent PCa or CAP were present. If this occurred, the BPH sample was excluded.

In CAP, studies have shown that the local prostate environment is infiltrated by lymphocytes initiating an immune reaction [323-326]. Alternatively in PCa the cancer cells are able to survive the multiple defensive immune mechanisms of immunosurveillance by the process of evasion and suppression. The involvement of lymphocytes in these processes has been demonstrated in PCa cell survival [327-329].

The flow cytometry analysis showed that the CD4+ T lymphocyte percentage in PCa tissue was 9.63% higher than that of BPH (p 0.0296). Also a similar trend was seen with CD4+ T lymphocytes in CAP where there was a 9.45% rise in comparison to BPH (p 0.1407). The influx of CD4+ T lymphocytes in PCa and CAP could represent detection by the immune system of abnormal pathogens and an immune response being mounted. Another explanation for this is the presence of an infection in the prostate resulting in an influx of these cells. However this reason is less likely as there was the absence of acute inflammatory cells such as neutrophils and patients had clear urine analysis.

The presence of cytotoxic CD8+ T lymphocytes in PCa tissue was 12.81% (p 0.0063) higher than that of BPH. Again this could imply that tumour recognition by the immune system has occurred which has mobilized lymphocytes to the local environment or restricted efflux. However the other factors discussed above could also be the cause for this result. Unfortunately due to the limited tissue samples available in CAP patients, the CD8+ T lymphocyte population was not able to be determined.

It was unexpected to see similarities in the mean CD4+ T lymphocytes percentages between the PCa and CAP groups. The numbers were low to draw absolute conclusions. However differences were seen in the proportion of the Treg cells. The Treg cells in PBMC of CAP were 3.13% (p 0.0042) and 2.47% (p 0.485) lower than that of BPH and PCa respectively. There was a 2.32% reduction in Treg cells in the tissue of CAP patients when compared to BPH. Similarly there was a 4.44%, (p 0.0192) reduction when compared to PCa. This shows that in CAP both peripherally and locally that the immunosuppression effect by Treg cells is low allowing for the immunogenic process to occur.

In this study the mean percentage of Treg cells in PCa tissue was 7.70%. This was consistent with other studies in showing Treg cells to be 5% to 10% of the

CD4+ lymphocyte population in PCa and other cancers such as hepatocellular carcinoma [232, 328, 330].

The percentage of Treg cells in PBMC of PCa was similar to that seen in the periphery of BPH patients. Similar findings were seen in a study by Yokokawa et al, where there were a similar number of Treg cells in the periphery of BPH and PCa patients [331]. Also another study by Unitt el al, demonstrated a similar finding of peripheral Treg cells in patients with hepatocellular carcinoma and matched healthy individuals [232]. This indicates that Treg cells play less of a role in immunosuppression systemically in patients with cancers

In the local PCa tissue environment, the result was not statistically significant however relatively there was 2.12% (p 0.2480 CI -5.80 to 1.56) higher Treg cells in comparison to BPH. This can possibly indicate a locally immunosuppressive environment but a larger population size is needed to show a difference that is statistically significant. However whether this is clinically significant could only be examined in animal or in vitro studies to monitor the growth of these tumours.

In work by Ahmadzadeh et al, where melanoma was examined, there was a ten percent higher Treg cell population in the tumour compared to peritumoural environment [332]. This percentage difference may be seen in PCa if a larger population size is used. However our study we compared patients with PCa and BPH while the study looked at the difference between intratumoural with peritumoural areas in the same patient. This study suggested that the high intratumoural Treg cells could be due to an influx of these cells resulting in a depletion of peritumoural numbers causing a falsely elevated difference.

The overall balance of the immune system in the tissue is influenced by the proportions of the subset of lymphocytes relative to each other. The ratio

between these cells such as CD8 to Treg cells has been shown to be a prognostic factor in some cancers [316, 317, 333]. In CAP there is a high CD4:Treg ratio peripherally and locally, possibly implying an active immune process however this is difficult to conclude with low numbers. In PCa, the CD4:CD8, CD4:Treg and CD8:Treg ratios were similar to that of BPH. A similar ratio was seen in a study by Shaw et al, where the CD4 to CD8 ratio was reported to be 1.84:1.51 [334]. There is a corresponding raise in Treg cells to counter the effects of CD4+ and CD8+ T lymphocytes.

The findings from the flow cytometry examination show that in CAP there is an influx of CD4+ T lymphocytes within the inflamed prostate. Concurrently there is a reduced number of suppressive Treg cells, which could allow local inflammation to occur. There is a systemic reduction in Treg cells that also supports the theory of an autoimmune process that has been reported to occur in CAP. A study by Bai et al, where blood was sampled in patients with CAP and compared with age-matched controls, found similar findings. Their results indicated FoxP3 gene messenger ribonucleic acid (mRNA) to be significantly lower than the controls [330].

There have been no studies looking at the subset of lymphocyte in prostate tissue specifically from patients with CAP. The literature shows CAP has been studied in prostate tissues from patients with BPH and PCa. Therefore, the majority of studies in CAP have only looked at urine and seminal fluid and the effect on lymphocytes. These in vitro studies show CD4+ T lymphocytes proliferate more rapidly in seminal plasma of men with CAP indicating a local environment enriched with factors to amplify an immune response [325, 335].

In CAP an autoimmune process has been implicated in the local and peripheral environment [335-337]. The actual mechanism of this action still remains elusive.

In PCa, the picture of local immunosuppression is demonstrated by the influx of tissue Treg cells. Despite the influx of CD4+ and CD8+ T lymphocytes, there is a proportional increase in Treg cells. This provides an environment for the PCa cells to evade and withstand the immune system's defences. The activity and functionality of the cytotoxic T cells will be assessed in a later chapter.

To verify the results obtained from the flow cytometry analysis, IHC was performed to examine the local environment in BPH and PCa. The number of CD4+ T lymphocytes was marginally higher in BPH but this result was not clinically significant. Also the CD8+ T lymphocyte numbers in BPH was higher than that of PCa by 73.70 cells per 10hpf (p 0.0216). The mean difference in the number of Treg cells in PCa was 19.90 cells per 10hpf higher than BPH (p 0.0305).

These quantities were then reviewed as ratios to assess the proportions in relation to the other lymphocytes. The CD4:CD8 ratios in both BPH and PCa were similar to the 1:1 figure seen in the flow cytometry data. The CD4:Treg and CD8:Treg ratios in PCa was identical to that of the result of flow cytometry where there was a 4:1 ratio in both. In the BPH analysis however the results differed considerably where in the IHC the CD4:Treg and CD8:Treg ratios were up to nine times higher. This high result is due to the high CD4+ and CD8+ T lymphocyte count and the very low Treg cell count. In BPH there are no factors such as tumour specific antigens to prime and recruit Treg cells to the local environment [328, 338-340].

The abundance of Treg cells in the tumour environment can be due to several reasons. For example, it could be due to trafficking of Treg cells by enhancing inward passage or inhibiting of outward migration from to the local tumour environment. It has been shown in ovarian cancer and associated ascitic fluid;

the trafficking of Treg cells is influenced by CCL22 [228]. The ovarian tumour cells and microenvironmental macrophages produce this chemokine allowing for the Treg cells passage to the tumour. CCL2 is not the only chemokine that recruits Treg cells to the tumour. For instance Treg cells express receptors for other chemokine such as CXCL12 and CXCR4 which have been shown to be important in mobilizing Treg cells from the bone marrow to the tumour [341]. Other chemokine receptors such as CCR4 and CCR8 on Treg cells have been shown in vitro studies to respond to chemokines such as macrophage-derived chemokine and thymus and activation-regulated chemokine [342].

In a transgenic adenocarcinoma of the mouse prostate (TRAMP) model, the recruitment of Treg cells into the local tumour and tumour draining lymph nodes was demonstrated [324]. The accumulation of Treg cells was postulated to be due to local activation of the immune system resulting in the release of chemokines.

Another factor that could result in the raised presence of Treg cells is local environment is the transformation of T cells to Treg cells and local expansion. The CD4+ T lymphocytes are transformed to Treg cell by the stimulation of their TCR without co-stimulation [343]. This is achieved by stimulation by immature DC [342]. The presence of TGF-β and IL-10 in the tumour environment produced by other Treg cells can also convert T cells into Treg cells [343].

Increased numbers of Treg cells can also be due the their ability to survive in the tumour environment. A study by Banz et al demonstrated that Treg cells were more resistant to Fas-induced apoptosis than CD25- CD4+ T cells [344]. As a result these cells are more robust and survive for a lengthier time period.

There have been studies looking into the presence of lymphocytes in cancers and the correlation with survival. Patients with a high number of T lymphocytes

in malignancies have better disease free survival [226, 237, 303]. While the presence of abundant Treg cells in cancers such as ovarian, hepatocellular, breast, pancreatic and non-small cell lung cancers have been demonstrated to have poor prognosis [228, 238, 345-347]. The subset of lymphocyte population in PCa was compared to the current pathological standards used in current clinical practice.

The most commonly used histopathological marker of cancer was aggressiveness is the Gleason sums. Here lymphocyte population was examined in patients with Gleason sums of 3+3=6, 3+4=7 and 4+3=7. The statistically significant difference of a 3.89% (p 0.0376) increase was seen in Treg cells in Gleason sum 4+3=7 in comparison to the 3+4=7 group. This result could be due to a statistical phenomenon rather than a true difference. A larger sample population is required to see establish if a difference is present in all the Gleason score range including high-grade disease. Although the remainder did not have enough power to show statistical significance, some general trends were seen. In the flow cytometry analysis the CD8+ T lymphocytes and Treg cells in the local environment showed a gradual increase as the Gleason sum rose. Also the CD4+ T lymphocytes in IHC increased with higher Gleason sum. However larger population is needed to clarify these trends.

Patients with extraprostatic spread had 7.00% (p 0.0320) lower CD8+ T lymphocytes in PBMC and 3.52% (p 0.0371) higher Treg cells in the tissue. These findings provide an image of active immune awareness where the harmful effects of cytotoxic cells are neutralized by local immunosuppression implemented by Treg cells. A similar picture was seen with seminal vesicle involvement. Those who had seminal vesicle extension of PCa had 10.74% (p 0.0249) lower CD8+ T lymphocytes in PBMC and 5.68% (p 0.0265) higher Treg cells in the prostate tissue.

The other histopathological markers; lymphovascular invasion, lymph node involvement, margin involvement and perineural invasion did not have enough power to generate statistical significance. This should be investigated with further increasing the sample population.

The association of Treg cells as a prognostic factor was again seen in the analysis of PSA relapse. Patients were followed in clinic with subsequent PSA; majority of patients reached PSA nadir and some patients were lost to follow-up as their private urologists managed their post operative care. Patients with positive surgical margins and positive lymph node involvement were excluded.

Patients with PSA relapse had 4.39% (p 0.0162) higher Treg cells in the tissue than those patients who had stable PSA. The presence of higher Treg cells in the original sample could represent a cancer that is more aggressive and having micrometastasis at the time of prostatectomy. A high Treg cell count in the tumour is related to poor prognosis as it has a higher risk of PSA relapse. Clinically the relevance of this could be patients found to have a high proportion of Treg cells need to monitored more closely or be offered adjuvant radiotherapy.

The preoperative PSA levels did show possible trend with CD8+ T lymphocyte decrease as the PSA levels rose. However the results from this analysis were not convincing and definite conclusions cannot be made.

An inflammatory process after the TRUS biopsy may have resulted in a spurious result both locally and peripherally. It was hypothesized a local inflammatory process would have occurred after biopsy and if there was a short interval between biopsy and radical prostatectomy a disproportionately high influx of inflammatory lymphocytes would have been seen. However there was no clear convincing trend seen (data not shown) to indicate if the time taken

between biopsy and surgery influenced the readings of the lymphocytes numbers in PBMC and tissue.

This could be better evaluated in the future by assessing the lymphocyte subsets in the PBMC at regular intervals in individual patients from biopsy to time of radical prostatectomy. However the more important local cancer environment cannot be regularly examined with ease.

There have been conflicting studies in PCa with regards to the prognostic value of lymphocytes. Studies by McArdle et al and Irani et al, show that the presence of abundant CD4+ T lymphocytes in PCa had early cancer recurrence and poor cancer survival [348, 349]. These studies postulated that the possibility for the poor outcome was that the T lymphocytes were inactive and downregulation of MHC I expression allowed for the PCa to evade destruction [350]. One deficiency of these studies was that the subsets of CD4+ T lymphocytes were not examined. The Treg cell population was not determined and as a result the proportion of suppression imparted by these cells would have influenced the prognostic outcome.

A study by Vesalainen et al showed the presence of dense T lymphocytes in PCa tissue in comparison to those patients without or reduced numbers of T lymphocytes had better survival outcomes [351]. This study also failed to assess the subset of lymphocytes individually in relation to prognosis.

The results from this study do show that especially high Treg cell numbers is a poor prognostic marker. Other studies such as the one by Fox et al have looked at similar correlation with Treg cells and tumour stage, capsular invasion, urethral margin, vascular invasion, Gleason score, pre-operative PSA and PSA relapse but did not find any correlation [352]. Leaders in immunology have advocated for the use of Treg cells as a biomarker [353]. However,

consensus is required for clinically meaningful levels to assist decision-making. This has potential to assist clinicians at times of TRUS biopsy and after a prostatectomy to determine which patients should be treated aggressively and those who would be better managed conservatively with indolent tumours.

The presence of peripheral lymphoid organs in PCa was noted in some patients upon examining the IHC samples. Aggregates of lymphocytes were found at the edge of the tumour where CD4+, CD8+ and Treg cells were seen penetrating the tumour environment as TILs. In BPH tissue there were no evidence of lymphoid aggregates, the lymphocytes that were seen were usually individually scattered amongst the local environment.

The PCa patients with and without these lymphoid aggregates were divided into their respective groups and were analysed. The findings showed that patients with lymphoid aggregates had 14.74% (p 0.0322) higher CD4+ T lymphocytes in the tissue on flow cytometry. This was supported by the IHC analysis where there were 65.62 (p 0.0193) more CD4+ T lymphocytes cells per 10hpf in the patients with lymphoid aggregates. Also these patients had 18.03 (p 0.0296) more Treg cells per 10hpf.

A study by Miller et al also demonstrated the presence of inflammatory infiltrates surrounding PCa tissue [327]. This study similarly showed an abundant number of Treg cells was also isolated. Two studies by Ebelt et al in 2008 and 2009, also demonstrated that PCa islets were surrounded by T lymphocytes including Treg cells [320, 321]. As a result it could be concluded that patients with peripheral lymphoid aggregates around PCa cells had a higher number of TILs.

The ratios of the two groups were compared. The CD4:CD8 ratios in flow cytometry and IHC were approximately 1:1 in patients with and without the

presence of lymphoid aggregates. In the flow cytometry data, the CD4:Treg ratios were inconsistent however in the IHC showed a 4:1 ratio in both groups. Finally the CD8:Treg in patients with lymphoid aggregates, in both flow cytometry and IHC showed a ratio of approximately 4:1. In the absent group, there was a higher ratio ranging from 9 to 5:1. This result is in contrary to previous findings where these patients have high CD8+ T lymphocytes to Treg cells indicating the cytotoxic cells are relatively less inhibited. The activity and functionality of these CD8+ T lymphocytes will be investigated in the following chapter.

In the IHC analysis of PCa tissue, multiple lymphoid aggregates were seen surrounding the tumour islets of some patients. The presence of these lymphocytes in clusters around the tumour cells of non-small cell lung cancer has been reported as a good prognostic predictor [315]. It is thought that these cells are at the cusp of the tumour and are waiting to penetrate the tumour to initiate an anti-tumour effect [354]. Other positive prognostic results were seen in tumours of patients who had high CD8+ T lymphocyte numbers and those with high CD8:Treg ratios [237, 303, 355]. It is important assess the subpopulation of these lymphocyte aggregates to determine its prognostic value. In a study looking at breast cancer patients, the presence of abundant Treg cells in the lymphoid population had poor survival outcome [356].

From the findings, there is generally a proportionate distribution of lymphocytes in patients with and without the lymphoid aggregates. In patients with high TILs, Treg cells counter the numbers of abundant CD4+ and CD8+ T lymphocytes. This balance between cytotoxic and suppressive cells allows for the PCa to survive.

Despite the high number of CD8+ T lymphocytes present, there are proportionately high inhibitory Treg cells. This led to the question of assessing

whether these cytotoxic T cells were activated and what was their functional status. This will be examined in the following chapter.

From this study, the distribution of the subset of T cell population in the conditions afflicting the prostate gland was demonstrated. In CAP the up regulation of the immune system's destructive mechanism was seen both locally and systemically. In PCa, patients with lymphoid aggregates surrounding the PCa islets had higher number of TILs. These patients had a proportionate increase in Treg cells to counter the influx of CD4+ and CD8+ T lymphocytes. This demonstrates that the local environment has mechanisms to appropriately recruit these suppressor cells to allow the PCa cells to evade the immunosurveillance processes.

From this study, questions were raised with regards to the characteristics of the CTL that present within the tumour environment. Despite the presence of these T cells, questions regards their effectiveness in attacking the tumour cells needs to be assessed. The functional characteristics and their activation status in mounting a response will be studied in the following chapter.

4.0 T cell maturation and activation in prostate cancer

4.1 Introduction

An influx of TILs was seen in the study population with PCa. CD8+ T lymphocytes were found to be in abundant numbers in the PCa local environment. The presence of CD8+ T lymphocytes has been shown to have positive survival outcome in patients with cancer [237, 303, 355]. However despite their presence, the PCa cells continue to evade immunosurveillance and multiply and propagate. The question is raised about their functionality and activation status.

An overall high CD8+ T lymphocyte population can be misleading. Further analysis is essential to increase our understanding. They should be subdivided into smaller subsets based on their functional activities. CD8+ T lymphocyte subsets are categorized into naïve, terminal effector, central memory and effector memory cells. The distribution and relative proportion with respect to each other is important in understanding the dynamics of their overall influence in an inflammatory process. If there is skew towards a certain cell population, the picture could differ from being an effective and active process to a dormant holding state.

Despite the presence of these cells, whether these CD8+ T lymphocytes are activated to produce their cytotoxic effect needs to be investigated. The activation status of CD8+ T lymphocytes was assessed by surface CD69 expression. CD69 also known as induced activator molecule is one of the earliest activation marker that is expressed on the cell surface of T

lymphocytes, B cells and macrophages [357-360]. It is expressed within two to four hours after the stimulation of TCR [361].

The activation of the cytotoxic T cells would correlate with increased killing of the tumour cells. One of the major cytokines produced by the cytotoxic T cells in the pro-inflammatory process is IFNγ. It is involved in a variety of immunological processes resulting in the inhibition of growth and apoptosis of tumour cells [362, 363].

The local tumour environment interplays with many cytokines which are pro-inflammatory and inhibitory. There are proteins called perforin and serine proteases such as granzymes associated with cytolysis. On the other hand tumour and bystander cells produce and promote inhibitory cytokines such as TGFβ and IL-10 to counter the effects of pro-inflammatory effects. The distribution and ratios of these effects with reference to each other will provide the direction of the immune system.

This chapter will assess the functional and activation status of CD8+ T lymphocytes in the PCa local and systemic environment. Measurement of the subset of CD8+ T lymphocytes in the functional analysis and the assessment of activation with regards to the expression of CD69 was carried out using flow cytometry. The cytokine profile in the local prostate environment was determined using IHC.

4.1.1 Flow cytometry

As described in the methods chapter (section 2.7.2), matched blood and tissue was obtained from patients with PCa and BPH. The functional analysis was conducted using cells stained for CD8, CD45RA, CD45RO and CCR7. These are cell surface markers that are used to differentiate the subpopulation of

CD8+ T lymphocytes. They allow for the identification of naïve, central memory, effector memory and terminal effector cells. The patterns of surface antigen expression by these cells are demonstrated in table 4.1.

	CD45RA	CD45RO	CCR7
Naïve	+	-	+
Central memory	-	+	+
Effector memory	-	+	-
Terminal effector	+	-	-

Table 4.1 The expression of CD45RA, CD45RO and CCR7 show the functional subset of CD8+ T lymphocytes.

With the understanding of different antibody expression of the subset of CD8+ T lymphocytes, the PBMC and tissue samples were accordingly stained for flow cytometry. The functional subsets are expressed as a percentage of the CD8+ T lymphocyte population. These percentages and the number of events are tabulated.

The activation status was examined in a separate experiment where the cells in PBMC and tissue samples of patients with BPH and PCa are stained for CD69 expression. The gates are positioned after examining cells which were not stained for CD69 as a negative control. The gates are placed around the negative cell population so that the cells that express CD69 above this reference gate, are considered to be positive.

The results are expressed as a percentage of CD8+ T lymphocytes and the number of events is noted to verify the validity of the data.

4.1.2 Immunohistochemistry

The cytokine profile in the local PCa tissue environment was examined using IHC. The purpose of this was to gain some insight into the cytolytic and suppressive cytokines that are present in the tumour environment.

Tissue samples of patients with BPH and PCa were used in the IHC study. The stains that were used were H&E, IFNγ, TGFβ and IL10. The antibody concentration for staining was titrated on control tissue prior to application on the prostate tissues. This reduced the possibility of weak staining and background staining. The samples were examined at 10hpf. Ten intratumoural regions were examined and an average was documented.

4.2 Functional analysis

4.2.1 BPH group

Results from 11 patients in the BPH group were analysed as there were sufficient event numbers on the flow cytometry analysis. The CD8+ T lymphocyte population was first analysed.

4.2.1.1 CD8+ T lymphocytes

The CD8+ T lymphocyte population in both PBMC and tissue in patients with BPH are demonstrated in table 4.2.

Patient No.	CD8+ PBMC (%)	Number of events	CD8+ BPH (%)	Number of events
1	36.2	43085	2.16	14745
2	39.7	79836	23.5	9473
3	17.9	128259	11.1	12149
4	15.2	84863	25.9	10937
5	48.1	112036	41.6	15675
6	19.5	56285	59	13625
7	15.2	68975	18	15274
8	37.5	36234	18.1	11869
9	17.7	44653	19.3	12746
10	14.2	81974	35.8	13120
11	23.9	77436	19.2	10292

Table 4.2 CD8+ T lymphocytes in the BPH group.

The mean percentage of CD8+ T lymphocytes in patients with BPH was 25.92% (CI 17.78 to 34.05) in PBMC which was a fraction higher than the 24.88% (CI 14.41 to 35.35) in tissue.

4.2.1.2 Naïve T cells

The percentage of naïve T cells (CD45RA+, CD45R0- and CCR7+) in BPH is demonstrated in table 4.3.

Patient No.	Naïve PBMC (%)	Number of events	Naïve BPH (%)	Number of events
1	38.8	4721	50	652
2	44.1	6459	35.9	537
3	19.3	8812	31.2	284
4	18.6	2767	24	499
5	35.7	7408	33.2	1036
6	27.7	4713	20.8	753
7	39.4	2867	23.9	379
8	38.5	6294	15.9	437
9	27	5354	23.2	294
10	25.6	5171	8.1	617
11	16.9	7520	15	373

Table 4.3 Naïve T cells in the BPH group.

The mean percentage of naïve cells in BPH PBMC was 30.15% (CI 23.71 to 36.59) which was higher than the 25.56% (CI 17.79 to 33.33) found in tissue.

4.2.1.3 Terminal effector T cells

The lymphocytes in BPH that were CD45RA+, CD45RO- and CCR7- were classified as terminal effectors. The percentage of these cells in PBMC and BPH are shown in table 4.4.

Patient No.	Terminal eff PBMC (%)	Number of events	Terminal eff BPH (%)	Number of events
1	61.2	4721	50	652
2	55.9	6459	65	537
3	80.7	8812	57.9	284
4	81.4	2767	74	499
5	64.3	7408	66.2	1036
6	72.3	4713	74.2	753
7	60.6	2867	67.4	379
8	61.5	6294	84.1	437
9	73	5354	75.1	294
10	74.3	5171	91.9	617
11	83.1	7520	85	373

Table 4.4 Terminal effector T cells in the BPH group.

The mean percentage of terminal effectors in PBMC was 69.55% (CI 63.41 to 76.28) while in the BPH tissue it was 71.89% (CI 63.62 to 80.16).

4.2.1.4 Central memory T cells

The lymphocytes in BPH that were CD45RA-, CD45RO- and CCR7+ were classified as central memory T cells. The percentage of these cells in PBMC and BPH are shown in table 4.5.

Patient No.	Central mem PBMC (%)	Number of events	Central mem BPH (%)	Number of events
1	21.7	4792	29.2	3904
2	27.5	2936	30.4	2794
3	20	8926	38.2	1037
4	34.3	7260	34.1	4026
5	22.1	2833	37.2	1639
6	28.9	8027	31	2937
7	33.8	6268	33	2534
8	21.8	2946	28.5	4926
9	23.1	1927	32.1	1527
10	21	3786	10.4	3501
11	9.16	7488	13.8	1284

Table 4.5 Central memory T cells in the BPH group.

The mean percentage of central memory T cells in PBMC was 23.94% (CI 19.20 to 28.63) and in the BPH tissue it was 28.90% (CI 22.94 to 34.86).

4.2.1.5 Effector memory T cells

The lymphocytes in BPH that were CD45RA-, CD45RO+ and CCR7- were classified as effector memory T cells. The percentage of these cells in PBMC and BPH are shown in table 4.6.

Patient No.	Effector mem PBMC (%)	Number of events	Effector mem BPH (%)	Number of events
1	78.3	4792	70.3	3904
2	72.5	2936	69.6	2794
3	80	8926	61.8	1037
4	65.7	7260	65.8	4026
5	77.9	2833	62.9	1639
6	28.9	8027	31	2937
7	33.8	6268	33	2534
8	21.8	2946	28.5	4926
9	23.1	1927	32.1	1527
10	21	3786	10.4	3501
11	9.16	7488	13.8	1284

Table 4.6 Effector memory T cells in the BPH group.

The mean percentage of effector memory T cells in PBMC was 46.56% (CI 27.75 to 65.37) while in BPH tissue it was 43.56% (CI 28.23 to 58.89).

4.2.2 PCa group

From the initial 26 patients that were sampled, only 19 were taken into consideration. This was because only these patients had adequate event numbers and as a result the percentages obtained were considered to be reliable. It should therefore be noted that 7/26 patients have very few infiltrating lymphocytes and the mechanism of immune dysregulation in these patients, if it exists, is probably mediated by some means other than Treg and cytokine

expression by lymphocytes. For example, these tumours could express factors that actively prevent lymphocyte ingress.

4.2.2.1 CD8+ lymphocytes

Data relating to CD8+ T lymphocytes in PCa participants are demonstrated in table 4.7.

Patient No.	CD8+ PBMC (%)	Number of events	CD8+ BPH (%)	Number of events
1	41.3	76895	36.5	9925
2	26	83561	24.2	4998
3	21.4	34641	23.8	19055
4	41.1	54656	17.1	16212
5	22.5	37964	33.6	17277
6	39.1	59046	51.5	1105
7	21.7	10497	20.5	3382
8	37.1	83994	36.1	5362
9	34.9	64427	33.6	1152
10	33.4	80591	28	1873
11	37.9	62307	40.1	4684
12	19.2	78850	37.6	3036
13	20.7	52914	35.2	2463
14	34.5	55756	37.3	7831
15	39.3	41889	22.2	14467
16	22.2	84381	36.6	1954
17	31.4	81793	34.2	14799
18	16.2	84700	32.9	18396
19	47.8	76081	22.7	4322

Table 4.7 CD8+ T lymphocytes in the PCa group.

The mean percentage of CD8+ T lymphocytes in patients with PCa was 31.77% (CI 27.73 to 35.81) in PBMC which was marginally higher than the 30.93% (CI 26.44 to 35.42) found in tissue.

4.2.2.2 Naïve T cells

The data related to the naïve T cell population in PCa tissue and PBMC are demonstrated in table 4.8.

Patient No.	Naïve PBMC (%)	Number of events	Naïve BPH (%)	Number of events
1	26.7	10937	15.8	284
2	36.5	8647	17.4	109
3	35.8	4469	15.3	459
4	38.4	7678	20.7	82
5	15.4	5120	24.1	174
6	37	12750	14.3	28
7	38.4	904	20.9	43
8	20.2	14970	16.8	101
9	25.4	8633	10.4	48
10	19.7	15634	10.5	76
11	18.2	11209	16.7	90
12	16.2	6832	17.9	123
13	26.3	7633	34.4	90
14	19.5	9800	7.59	79
15	10.6	7449	8.57	70
16	26.8	11036	2.08	48
17	14.1	10565	11.3	141
18	12.1	7351	20	120
19	9.23	24895	13.6	308

Table 4.8 Naïve T cells in the PCa group.

The mean percentage of naïve T cells in PCa PBMC was 23.50% (CI 18.74 to 28.26) and tissue was 15.70% (CI 12.32 to 19.08). There were numerically significantly more naïve cells in PBMC than in tissue with the p value of 0.0080.

4.2.2.3 Terminal effector T cells

The percentage of terminal effector T cells in participants with PCa is shown in table 4.9.

Patient No.	Terminal eff PBMC (%)	Number of events	Terminal eff BPH (%)	Number of events
1	73.3	10937	84.2	284
2	63.5	8647	82.6	109
3	64.2	4469	84.7	459
4	61.6	7678	79.3	82
5	84.5	5120	75.9	174
6	63	12750	85.7	28
7	61.7	904	79.1	43
8	79.8	14970	83.2	101
9	74.6	8633	89.6	48
10	80.3	15634	89.5	76
11	81.8	11209	83.3	90
12	83.8	6832	82.1	123
13	73.6	7633	65.6	90
14	80.5	9800	92.4	79
15	89.4	7439	91.4	70
16	73.2	11036	97.9	48
17	85.9	10565	88.7	141
18	87.9	7351	80	120
19	58.8	24895	79.9	308

Table 4.9 Terminal effector T cells in the PCa group.

The mean percentage of terminal effectors T cells in PBMC was 74.81% (CI 69.98 to 79.64) while in the PCa tissue it was 83.95% (CI 80.55 to 87.35). There was a significant difference between the two groups (p 0.0025), where there were 9.14% more terminal effectors in the local tissue environment than in the periphery.

4.2.2.4 Central memory T cells

The central memory T cells in PCa tissue and PBMC are shown as a percentage in table 4.10.

Patient No.	Central mem PBMC (%)	Number of events	Central mem BPH (%)	Number of events
1	24.3	13860	21.1	3000
2	44.1	8385	25	963
3	27.4	1827	21.3	3407
4	31.6	11265	25.4	2492
5	34.5	1985	32.8	5246
6	31	5701	28	508
7	20.7	937	24.7	534
8	25.1	8495	33.6	1606
9	27.4	10325	24.3	259
10	28.5	6871	28	386
11	27.2	9554	23.9	1538
12	18.7	5286	21.8	861
13	26.4	1777	33.1	617
14	26	6496	12.9	2525
15	11.1	5139	6.05	2892
16	10.5	3273	3.74	561
17	13.9	9573	6.73	4446
18	10.4	3593	8.83	5592
19	4.07	5059	4.41	431

Table 4.10 Central memory T cells in the PCa group.

The mean percentage of central memory T cells in PBMC was 23.31% (CI 18.56 to 28.06) and in the PCa tissue it was 20.30% (CI 15.46 to 25.14). There was no statistically significant difference between the two groups (p 0.3572).

4.2.2.5 Effector memory T cells

The percentage of effector memory T cells in PCa patients are demonstrated in table 4.11.

Patient No.	Effector mem PBMC (%)	No of events	Effector mem BPH (%)	No of events
1	75.7	13860	78.9	3000
2	56	8385	75	963
3	72.6	1827	78.7	3407
4	68.5	11265	74.7	2492
5	65.5	1985	67.2	5246
6	69	5701	72	508
7	79.2	937	75.3	534
8	74.9	8495	66.2	1606
9	72.6	10325	75.7	259
10	71.6	6871	72	386
11	72.8	9554	76	1538
12	81.2	5286	78.2	861
13	73.5	1777	66.8	617
14	74	6496	87.1	2525
15	88.9	5139	93.9	2892
16	89.5	3273	96.3	561
17	86.1	9573	93.3	4446
18	89.6	3593	91.2	559
19	94.3	5059	95.1	431

Table 4.11 Effector memory T cells in the PCa group.

The mean percentage of effector memory T cells in PBMC was 76.61% (CI 71.95 to 81.26) while in PCa tissue it was 79.66% (CI 74.83 to 84.50). There was no statistical significant difference between the two means (p 0.3449).

4.2.3 Comparison

The patients in the BPH and PCa group were compared to determine local and peripheral differences of the functional activity of the CD8+ T lymphocytes.

4.2.3.1 Naïve T cells

The comparison of the naïve CD8+ T lymphocytes in the two groups is demonstrated below in table 4.12. This table shows that there are a higher proportion of naïve T cells in BPH PBMC and tissue in comparison to PCa.

Medium	Tissue	Mean (%)	CI (%)	Difference (%)	CI of difference (%)	p value
PBMC	BPH	30.15	23.71 to 36.59	6.64	-0.94 to 14.23	0.0836
	PCa	23.50	18.74 to 28.26			
Tissue	BPH	25.56	17.79 to 33.33	9.86	2.95 to 16.77	0.0068
	PCa	15.70	12.32 to 19.08			

Table 4.12 The differences between naïve T cell percentages in BPH and PCa groups.

There were 9.86% (p 0.0068) more naïve T cells in BPH tissue than in PCa.

4.2.3.2 Terminal effector T cells

The comparison of the terminal effector T cells in the two groups is demonstrated below in table 4.13. There is a higher terminal effector population in PCa in both PBMC and tissue.

Medium	Tissue	Mean (%)	CI (%)	Difference (%)	CI of difference (%)	p value
PBMC	BPH	69.84	63.41 to 76.28	-4.97	-12.62 to 2.69	0.1948
	PCa	74.81	69.98 to 79.64			
Tissue	BPH	71.89	63.62 to 80.16	-12.06	-19.26 to -4.86	0.0019
	PCa	83.95	80.55 to 87.35			

Table 4.13 The differences between terminal effector T cell percentages in BPH and PCa groups.

There were 12.06% (p 0.0019) more terminal effector T cells in BPH than in PCa tissue.

4.2.3.3 Central memory T cells

The comparison of the central memory CD8+ T lymphocytes in the two groups is demonstrated below in table 4.14. The PCa patients had a lower number of central memory T cells in PBMC and tissue when compared with BPH patients.

Medium	Tissue	Mean (%)	CI (%)	Difference (%)	CI of difference (%)	p value
PBMC	BPH	23.94	19.20 to 28.68	0.63	-6.32 to 7.58	0.8534
	PCa	23.31	18.56 to 28.06			
Tissue	BPH	28.90	22.94 to 34.86	8.60	1.12 to 16.08	0.0258
	PCa	20.30	15.46 to 25.14			

Table 4.14 The differences between central memory T cell percentages in BPH and PCa groups.

BPH tissue had 8.60% (p 0.0258) more central memory T cells when compared to PCa tissue.

4.2.3.4 Effector memory T cells

The comparison of the effector memory T cells in the two groups is demonstrated below in table 4.15. The effector memory T cell population in PCa patients in both PBMC and tissue was higher than the BPH group.

Medium	Tissue	Mean (%)	CI (%)	Difference (%)	CI of difference (%)	p value
PBMC	BPH	76.06	71.33 to 80,80	-0.54	-7.39 to 6.30	0.8724
	PCa	76.60	71.95 to 81.26			
Tissue	BPH	71.04	65.07 to 77.00	-8.63	-16.10 to -1.15	0.0253
	PCa	79.66	74.83 to 84.50			

Table 4.15 The differences between effector memory T cell percentages in BPH and PCa groups

There were 8.63% (p 0.0253) more effector memory T cells in PCa than in BPH tissue.

4.3 Activation analysis

4.3.1 BPH group

Of the ten patients in the BPH group that were stained for CD69, one was excluded due to inadequate events on the flow cytometry analysis. The results for CD8+ T lymphocytes and CD69 staining are represented in tables 4.16 and 4.17.

Patient No.	CD8+ PBMC (%)	Number of events	CD8+ BPH (%)	Number of events
1	34.1	159097	29.6	10366
2	13.3	194746	6	8114
3	13.8	69742	24.2	10818
4	42.5	194094	27.6	13196
5	15	238015	39.8	18150
6	12.3	137379	19.7	8135
7	31.4	65155	11.6	11741
8	15.5	252517	20.1	9079
9	11.9	106729	22.4	30468

Table 4.16 The CD8+ T lymphocytes in the BPH group.

There were similar proportions of CD8+ T lymphocytes in PBMC and BPH tissue. There was 21.09% (CI 12.17 to 30.01) and 22.33% (CI 14.73 to 29.94) respectively.

Patient No.	CD69+ PBMC (%)	Number of events	CD69+ BPH (%)	Number of events
1	3.76	54279	28.8	3070
2	4.02	25978	30.6	487
3	4.9	9593	21.8	2622
4	3.03	82416	23.4	3638
5	4.01	35736	26.8	7227
6	3.74	16926	22	1600
7	4.63	20451	18.1	1363
8	3.64	39236	17.3	1825
9	1.44	12677	22.1	6833

Table 4.17 CD69 expression in the BPH group.

The percentage of CD8+ T lymphocytes that expressed CD69 in PBMC was 3.69% (CI 2.91 to 4.46) while in BPH tissue was 23.43% (CI 19.95 to 26.91). This indicated that there was 19.75% (p 0.0001) more activated CD8+ T lymphocytes in BPH tissue than in the peripheral blood.

4.3.2 PCa group

In this group a total of 22 patients had sufficient flow cytometry events to be included in the analysis. The percentage of CD8+ T lymphocytes and their expression of CD69 have been tabulated in tables 4.18 and 4.19.

Patient No.	CD8+ PBMC (%)	Number of events	CD8+ PCa (%)	Number of events
1	40.9	103739	48	6187
2	22.6	51665	30.3	2792
3	16.8	25957	24.5	9896
4	37.7	48683	26.3	15831
5	19.6	44421	34.5	19302
6	31.9	94240	34.5	2294
7	35.1	61509	45.2	6604
8	13.7	58275	11.1	5255
9	38.7	62149	35	6640
10	16.8	75063	34	1068
11	30.2	43403	27.9	2687
12	33.8	104985	2.77	3608
13	34.5	87643	37.5	13915
14	19.8	81637	32.5	4894
15	30.8	99678	6.88	5956
16	15.6	106665	19.9	15952
17	14.2	90042	29	6602
18	26.2	97201	20.6	21779
19	30.9	27746	19.8	13738
20	19.2	48837	38.6	2438
21	27.2	58647	30.8	13660

22	16.5	43834	36.9	17200

Table 4.18 The CD8+ T lymphocytes in the PCa group.

The mean CD8+ T lymphocyte percentage in PBMC was 26.03% (CI 22.12 to 29.94) and in PCa tissue 28.48% (CI 23.41 to 33.55).

Patient No.	CD69+ PBMC (%)	Number of events	CD69+ PCa (%)	Number of events
1	1.18	42388	40.3	2971
2	1.55	11698	19.9	845
3	1.9	4361	41.7	2422
4	1.06	18331	13.8	4160
5	1.75	8720	41.6	6650
6	2.06	30079	27.8	791
7	1.68	21573	38.1	2987
8	0.87	8007	33.3	583
9	2.7	24069	36.3	2323
10	1.78	12611	44.6	363
11	1.12	13090	44.7	751
12	3.67	35482	51.2	998
13	1.08	30226	54.3	5217
14	1.52	16148	16.2	1590
15	0.89	30666	36.3	410

16	1.06	16606	21.6	3170
17	2.12	12790	51.4	1914
18	1.47	25439	30.5	4482
19	14.7	8568	47	2715
20	7.05	9367	58.9	940
21	6.49	15933	49.1	4211
22	1.7	7249	10.6	6352

Table 4.19 CD69 expression in the PCa group.

The percentage of CD8+ T lymphocytes that were activated in PBMC was 2.70% (CI 1.31 to 4.09). The percentage in PCa tissue was 36.78% (CI 30.71 to 42.86). From the analysis, 34.08% more CD8+ T lymphocytes were activated in tissue in comparison to PBMC (p 0.0001).

4.3.3 Comparison

The sample populations of BPH and PCa are compared to determine local and peripheral differences. The CD69 expressing lymphocyte population is examined below.

4.3.3.1 CD69+ T lymphocytes

The comparison of the activation status of the CD8+T lymphocytes in the two sample groups is demonstrated below in table 4.20.

Medium	Tissue	Mean (%)	CI (%)	Difference (%)	CI of difference (%)	p value
PBMC	BPH	3.69	2.91 to 4.46	0.99	-1.21 to 3.19	0.3680
	PCa	2.70	1.31 to 4.09			
Tissue	BPH	23.43	19.95 to 26.91	-13.35	-22.98 to -3.72	0.0083
	PCa	36.78	30.71 to 42.86			

Table 4.20 The difference in CD69 expression in BPH and PCa groups.

The results show that in PCa tissue there were a significantly higher number of activated CD8+ T lymphocytes in comparison the BPH tissue. There is a 13.35% difference with a p value of 0.0083.

4.4 Assessment of local pro-inflammatory and inhibitory cytokines

The cytokines in the local tumour environment show the actual end result of the activated immune cells. Even though there may be an up regulation of CTL or Treg cells, their effectiveness in producing specific cytokines to induce their actions is important. Defective and altered actions of these lymphocytes in the local tumour environment would alter the production of cytokines.

For the next stage of the study, the pro-inflammatory cytokines IFNγ and inhibitory cytokines TGFβ and IL-10 were examined. Assessment of the local tumour environment for these cytokines could help with the understanding of whether these activated T cells are functioning appropriately.

The control BPH group and the PCa group prostate tissues were stained for CD8, IFNγ, TGFβ1 and IL10 and examined using IHC.

4.4.1 BPH group

There were a total of six patients whose prostate tissue was examined and the results are shown in table 4.21. Due to the extensive staining and analysis required for this study, six patients had sufficient samples for this to be achieved.

Patient No.	CD8+ (cells/10hpf)	IFNγ (cells/10hpf)	TGFβ (cells/10hpf)	IL10 (cells/10hpf)
1	244	28	0	25
2	171	8	0	25
3	132	2	0	13
4	92	7	0	16
5	158	6	0	8
6	263	4	0	26

Table 4.21 Cytokine staining results of BPH tissue.

4.4.2 PCa group

In the PCa group a total of ten patients were studied and the results are demonstrated in table 4.22. Again ten patients had enough tissue samples to perform this study.

Patient No.	CD8+ (cells/10hpf)	IFNγ (cells/10hpf)	TGFβ (cells/10hpf)	IL10 (cells/10hpf)
1	225	0	0	0
2	185	0	0	0
3	215	0	17	36
4	191	2	0	7
5	282	0	0	10
6	115	0	0	32
7	225	27	0	1
8	150	27	0	29
9	117	0	7	2
10	118	0	0	0

Table 4.22 Cytokine staining results of PCa tissue.

4.4.3 Comparison

The results of the stains in the two sample groups are compared below in table 4.23.

Stain	Tissue	Mean (%)	CI (%)	Difference (%)	CI of difference (%)	p value
CD8	BPH	176.67	107.80 to 245.53	-5.63	-71.98 to 60.72	0.8581
	PCa	182.30	141.90 to 222.70			
IFNγ	BPH	9.17	-0.78 to 19.11	3.57	-8.26 to 15.40	0.5283
	PCa	5.60	-2.48 to 13.68			
TGFβ	BPH	0.00	0.00 to 0.00	-2.40	-7.36 to 2.56	0.3166
	PCa	2.40	-1.59 to 6.39			
IL10	BPH	18.83	10.88 to 26.78	7.13	-6.85 to 21.12	0.2925
	PCa	11.70	1.18 to 22.22			

Table 4.23 Comparison of cytokine staining of BPH and PCa groups.

From the analysis, no statistically significant results were seen and as a result conclusions on local cytokine production cannot be made.

4.5 Discussion

A greater understanding of the workings of the immune system can be achieved by assessing the functional characteristics of CD8+ T lymphocytes. The local and systemic distribution of these cells in the two pathological conditions was examined.

Differences were seen in the CD8+ T lymphocyte population in the tissue of the two pathological conditions. There was a 9.86% (p 0.0068) higher naïve T cell population in the BPH tissue in comparison to PCa. The naïve T cell population after migration from the thymus to the periphery is generally maintained at a

constant level [170, 364-366]. The thymus continues to produce naïve T cells in order to balance the losses in the periphery. The naïve T cells become activated after encountering a specific antigen on APC. This results in rapid expansion to produce effector T cells.

From the low number of naïve T cells present in the tissue environment, it could be postulated that there is a depletion of these cells. The depletion could be attributed to the activation of these cells and the conversion to effector T cells.

The theory of naïve T cell depletion and the increased production of terminal effector T cell was statistically significant. In PCa tissue, there were 12.06% (p 0.0019) more terminal effector T cells in the prostate tissue when compared to BPH tissue. The expansion of these effector T cells indicates a shift in the cells in the tissue environment after recognition of tumour antigens. This leads to the possibility of immune recognition of tumour cells and a cytolytic response initiation.

There are numerous models on the development of memory T cells. The most commonly accepted model is the direct descendant from naïve T cells that have encountered antigen with co-stimulatory signals. The naïve T cells become activated and differentiate into effector T cells where they acquire effector function and the capability to migrate to the site of the pathogen. These cells undergo expansion of up to 10000 fold at a rate of four to six hours per cell cycle [162, 367, 368]. Once the pathogen has been eradicated the numbers of pathogen specific effector T cells rapidly decline by apoptosis via a contraction phase [368, 369]. This results in a substantial reduction in effector numbers of up to 95% [370]. The remaining population of effector T cells that survive matures into memory T cells [162, 371-373].

These memory T cells persist lifelong and provide protection against the same pathogen if re-encountered [164, 374]. Without the presence of antigen, the memory T cell population remains stable. These cells undergo self-renewal by homeostatic turnover and this influenced by interaction with IL-7 and IL-15 [162, 163, 167, 367].

There were no statistical significant results seen in the PBMC when the two pathological conditions were compared. However, in the PCa group, there were more naïve T cells in the general circulation than what was found in the tumour environment. There were 7.80% more naïve T cells in PBMC (p 0.0080) than in the tissue. Correspondingly there were 9.14% more terminal effector T cells in the tissue than in the periphery. This signifies a local inflammatory process underway.

The memory T cell population is divided into two main populations, central memory and effector memory T cells. These two subsets are divided on their expression of lymphoid homing receptors CD45RA, CD45RO and CCR7. Unfortunately there is no single phenotype marker to precisely identify memory T cells; another marker that is commonly used is L-selectin. Central memory T cells CD45RA+, CD45R0+ and CCR7+ are mainly located in lymphoid tissues such as lymph nodes and spleen. The effector memory T cells $CD45RA^{-}$, $CD45RO^{+}$ and $CCR7^{-}$ are located in the periphery in organs such as lungs, liver and the gastrointestinal tract [375].

The development of the subset of memory T cells is also controversial. The commonly accepted theory is that after the clearance of the pathogen, the initial antigen-specific memory T cell population mainly comprises effector memory T cells. These effector memory T cells over time convert to the central memory T cell population [376].

The effector function of the memory T cells subset responds differently when the same pathogen is re-encountered. When these cells are activated, they both equally produce considerable amount of effector cytokines IFNγ and TNF. Central memory T cells produce large quantities of IL-2 which increases proliferation in response to the antigen [377]. Effector memory T cells have pre-formed mRNA transcripts that encode for their potent lytic activity. They produce copious amount of perforin and granzyme B rapidly resulting in early and effective killing of hazardous cells [378, 379]. The potent cytolytic activity of effector memory T cells correlates with their direct origin from effector cells.

The effector memory T cells have two roles in the periphery. Firstly, they alert the immune system of the invading pathogen by producing pro-inflammatory cytokines and chemokines resulting in an inflammatory reaction. Secondly they commence destruction of the abnormal cells with their cytolytic properties.

A study by Vezys et al, showed that repeated vaccination resulted in accumulation of a large population of effector memory T cells [380]. With the presence of PCa antigen in the prostate tissue, this explains the increased numbers of effector memory T cells in the periphery. From our laboratory, vaccines have been studied for melanoma. We have shown that there is an increase in Treg cell numbers after vaccination especially in advanced disease [381-383].

The central memory T cells in PCa tissue were 8.60% (p 0.0258) less than that were seen in BPH tissue. While the percentage of effector memory T cells in PCa patients was 8.63% (p 0.0253) higher than the numbers seen in BPH tissue. These findings imply that in the PCa tissue there is an early inflammatory process taking place.

The presence of high effector cells and effector memory T cells in the prostate tissue of patients with PCa raised the question of whether these cells were activated. The presence of these cells and the persistence of cancer cells are conflicting. Therefore the activation of these cells was assessed.

The findings from this study showed that 13.35% (p 0.0083) more CD8+ T lymphocytes in PCa tissue were activated when compared to the control BPH group. This result indicates that the CD8+ T lymphocytes in the PCa are activated and therefore should be executing their cytolytic action. On IHC staining for CD69, lymphocytes within the PCa local environment were further activated when compared to surrounding normal tissue and separate patients with BPH [320]. This study did not differentiate between CD4+ and CD8+ T lymphocytes or the profile of cytokine production. However it did demonstrate the activation of the entire lymphocyte population.

The findings from this study cannot be regarded as absolute. The assessment of the activated cells should have individually studied the subset of CD8+ T lymphocytes rather than the entire number as a whole. This could have clarified the local PCa tissue environment with respect to the activation of the effector cells.

In order to study the subset CD8+ T lymphocyte activation status, the two experiments needed to have been combined. Unfortunately the study was limited due to the number of stains that could be performed in the one instance on the flow cytometer.

Another method could also have been used to verify the results. The use of another activation marker, CD107a, could have been used in a separate experiment. CD107a also known as lysosomal-associated membrane protein-1

(LAMP-1), is a marker of degranulation of both CD8+ T lymphocytes and NK cells [384].

CD107a is sensitive marker for cytolytic activity [357, 385, 386]. Its increased expression on the cell surface correlates with loss of perforin [384, 387]. It is expressed later than CD69 which is expressed during the early stages of cell activation [359, 360, 388]. However the samples that were obtained from the digestion of prostate tissue were limited and therefore there was not enough tissue to perform a second experiment.

Despite the possibility of confounding factors, the overall activation of CD8+ T lymphocytes was higher in PCa when compared to BPH patients. This raised the question of why the cancer cells survived despite the presence of these activated cells.

One of the possibilities was that the function of the activated cells was defective and as a result the cytolytic cytokines such as IFNγ was not being secreted. Another factor is that the local tumour environment is present with cytokines such as TGFβ and IL-10 countering the effects of the activated CD8+ T lymphocytes.

These cytokines were examined using IHC in both BPH and PCa tissue. However from the data obtained there was no clear picture of the local tumour environment. The results were not statistically significant. This study was limited by the study sample where there were six in BPH and ten in PCa. Only this limited number of tissue samples was available to perform this analysis. Analysis of the individual counts could show some trends. In the IFNγ groups, the BPH patients had some presence of this cytokine. In the PCa group, apart

from the three patients, the remainder did not have any IFNγ staining present. Therefore the results in the PCa group could have been falsely elevated.

There are many studies showing the immunoprotective qualities of IFNγ. One of its properties is the prevention and development of cancers [389-391]. Cells such as T lymphocytes, NK and NKT cells are the main producers of IFNγ when the immune system is activated [362, 363].

There are many ways in which IFNγ prevents and inhibits tumour growth. It can exert anti-proliferative and anti-metabolic effects on tumour cells resulting in apoptosis [392-395]. IFNγ has the ability to indirectly inhibit angiogenesis within tumours by promoting the chemokines such as IP-10, Mig and I-TAC that exert potent angiostatic effects [396-400]. The induction of the immune system via the innate and adaptive responses is influenced by IFNγ. It is a macrophage-activating factor, and as a result induces non-specific tumour killing [401]. The IFNγ produced at the local tumour environment further induces the production of more IFNγ by cells such as NK cells that have recently arrived to form part of the innate response [402].

The rich IFNγ environment enhances the adaptive immune response, where the development of CD4+ Th1 anti-tumour response leads the formation of the cytotoxic CD8+ T lymphocytes [403]. These CD8+ T lymphocytes not only kill the tumour cells directly via perforin and granzyme, they produce a substantial quantity of IFNγ resulting in the amplification of the response.

There have been studies looking into the effect of IFNγ on PCa cells. A study with a mouse model using TRAMP-C2 PCa cell line showed that IFNγ was vital in the antitumour T cell mediated memory response [404]. This resulted in successful elimination of most PCa cells. Another study by Selleck et al

showed that IFNγ increased the sensitivity of Fas-mediated cell death in PCa cells when they were pre-treated with IFNγ [405]. The effects of IFNγ on PCa cells have been studied and plans for gene therapy have been put forward.

In the TGFβ group, there was no staining seen in patients with BPH. In the PCa population, again only two patients had staining for this cytokine and could have elevated the final result artificially. It is a very important cytokine that controls and maintains immune homeostasis. It is able to control cell survival, proliferation, differentiation and as well as regulate the microenvironment [406-408].

TGFβ is capable of affecting all human cell types. It has a duel role, in the early stages is prevents developing tumours form progressing but in the later stages it promotes tumour growth. The tumour manipulates the TGFβ signalling and supports tumour proliferation, invasion, evasion of immune surveillance and cancer cell dissemination and metastasis [409, 410].

In the tumour environment, TGFβ is produced by tumour cells as well as cells in the stroma. It is capable of inhibiting the action of both the innate and adaptive immune response. Whereby the cells such as CD4+ T lymphocytes (Th1 and Th2), CTL, DC, NK cells and macrophages are inhibited [411-413]. On the other hand TGFβ enhances the production of Treg cells resulting in further immunosuppression [204, 412, 414, 415].

In the normal prostate tissue, TGFβ stimulates cell differentiation and controlling epithelial proliferation via apoptosis [416-419]. However in PCa, high levels of namely TGFβ1 are found in serum and tissue which have been related to poor prognosis [420-425].

In a PCa mouse model using TRAMP-C2 cells, CTL were rendered insensitive to TGFβ ex vivo and were transferred into the mouse with PCa. This resulted in the CTL penetrating into the tumour environment resulting in apoptosis of the PCa cells [426].

Finally in the IL-10 analysis, there were events recorded in both sample populations. However there was no clear trend seen and a result a conclusion could not be made without more power. IL-10 is an immunosuppressive cytokine produced by many cells such as DC, macrophages, B cells, NK and NKT cells and the different subset of CD4+ T lymphocytes (TH1, TH2, TH17 and Treg cells) [427, 428]. It has been also shown that another source of IL-10 is from effector cells [429]. The production of IL-10 by these effector cells occurs in the periphery to protect the surrounding normal tissue from damage. These cells are the highest generators of IL-10 at the peak of the cytolytic activity but are taken over by CD4+ T lymphocytes in the later phase.

The IL-10 producing effector cells are very potent cytolytic cells. They do not originate from a different lineage of effector cells. However their secretion of the immunosuppressive cytokine is a transient and reversible state that occurs at the height of the cytolytic phase [430]. A study by Quatan et al, performed functional cytokine assays of PBMC in patients with PCa. They found that IL-10 was higher in patients with PCa but their results were not statistically significant [431].

In order to improve accuracy of the cytokine staining, a larger sample population could have provided a greater power to obtain statistically significant data. Another drawback of this study was that only small number of cytokine staining of the cells was seen. This could be due to weak target staining but this was attempted to be reached by test titrating of the antibodies on control tissue prior to applying them on the prostate tissues. The other possibility is that there are only a small number of cytokines in the samples being tested.

This could by overcome by using a more sensitive test such as flow cytometry to detect this minute population.

A problem with the analysis of IHC stains is the human error that could arise when counting the samples. This could lead to either false positive or negative results. Measures were taken to overcome this by having a pathologist count the slides twice and an average taken. Another method that could be used is to take photographs of the slides and process it through a computer program that quantifies positive stains.

Other cytokines such as perforin and granzyme could be also evaluated in future studies. These cytokines are important in the initial immune response against offending pathogens. This could be ideally achieved by increasing the number of stains and undertaking the experiment on a flow cytometer with increased colour resolution to reduce the incidence of variability with separate experiments.

In conclusion, the detection of the immune system of tumour cells is evident with the up regulation of the number of effector T cells and effector memory T cells in PCa local environment. The increased number of activated CD8+ T lymphocytes points to a pro-inflammatory process underway. However the data obtained in this study was not sufficient to determine the effectiveness of these cells in producing cytokines to kill the tumour cells. Further studies are required to profile the cytokines in the local tumour environment to understand the inflammatory process that is taking place.

5.0 Androgen deprivation therapy effects on T cell population

5.1 Introduction

The use of ADT was a major breakthrough in the management of locally advanced and metastatic PCa. By suppressing the production of testosterone, tumour growth is impeded. This reduced PCa related complications and increasing survival [131, 432-435].

The other broader biological effects of ADT include metabolic and cardiovascular disease and less well-known effects such as thymic regeneration [436-440]. The common effects include bone loss, insulin resistance, diabetes, vasomotor symptoms and anaemia [441]. As age increases, the thymus atrophies and as a result its involvement in the production and maintenance of the peripheral T cell population diminishes. This decline in function commences at the time of puberty where there is an increased production of sex steroids.

It has been shown that over time the adaptive immune system function declines with age [442, 443]. This is due to the reduction in T cell number and function. With increasing age there is thymic involution where there is reduction in the size, weight and cellularity of the thymus. This leads to the reduction in the number of naïve T cells entering the periphery which then results in expansion of the pre-existing memory cells and decreased response to new and previously handled antigens [444-446]. Clinically, reduced efficacy of the immune system can lead to opportunistic infections, cancers and autoimmunity.

The use of ADT to counter the effects of the increased sex steroids in circulation led scientist to attempt to reverse thymic atrophy.

Experiments performed in mouse models have shown the restoration of the peripheral population of T cells and thymic regeneration. A study by Sutherland et al showed a 16% increase in the CD4+ T lymphocytes and a 24% increase in the CD8+ T lymphocyte population after castration [283]. There was an increase in the naïve cell population with a corresponding reduction in the memory T cell population.

There have been some early studies in men with PCa. These studies show that there is evidence of thymic regeneration as well as an increase in peripheral lymphocyte numbers [291-293].

The use of ADT could be very important not only in the management of PCa but also in heightening the T cell response in all cancers. This could lead to the use of ADT prior to all chemotherapy and immunotherapy so that maximum responses can be achieved.

A pilot study was conducted to assess the profile of T cells of men who are undergoing androgen deprivation therapy, including assessment of lymphocyte subsets and their functional and activational status.

5.2 Method

The participants were recruited from the uro-oncology outpatient clinics at Austin Health. The inclusion criteria were any men who were to start ADT for PCa. This resulted in patients recruited at varying stages of disease. Some were men who had locally advanced cancer while others had organ-confined

cancer that received ADT prior to radiotherapy. All men received a four-week course of anti-androgen and a three monthly acting LHRH agonist.

After obtaining informed consent from these participants, 50mL of blood was obtained via venesection for PSA and flow cytometry analysis. The lymphocytes were isolated using Ficoll separation. The samples obtained prior to the commencement of ADT were stored in liquid nitrogen. At the three-month review, the participants had the second venesection prior to their second injection of LHRH agonist. Again the samples were analysed for PSA and lymphocyte numbers. The lymphocyte samples were also stored in liquid nitrogen until all participant samples were gathered.

The PSA was examined using the pathology services at the Austin Hospital where the same analyser was used to prevent discrepancies from different machines. The pre and post ADT samples of PBMC were thawed from liquid nitrogen and were analysed using flow cytometry. All ten samples were analysed in the one experiment so that experimental variables were minimised.

PBMC were analysed for the total lymphocyte and subpopulation using stains for lymphocytes, CD4, CD8 and FoxP3. The functional differentiation of CD8 lymphocytes was obtained by staining for CD45RO, CD45RA and CCR7. Finally the effect of ADT on activation status was examined by staining for CD69.

5.3 Patient demographic and PSA response

The patients that were recruited were of varying ages and were at various stages of their PCa management. The patient demographics and PCa profile are demonstrated in table 5.1. The method of diagnosis was included because it shows the regions where tissue was sampled. In TRUS bx, the peripheral

zones are examined while in TURP samples the transitional zones and to some extent the peripheral zones are investigated.

Patient no	Age	Diagnosis method	PCa profile	Disease process
1	74	TRUS bx (12 cores)	Right side (6/12 cores) – Gleason 3+4=7	Localised PCa – ADT + radiotherapy
2	71	TRUS bx (12 cores)	Bilateral all cores (12/12 cores) - Gleason 4+4=8	Localised PCa – ADT + radiotherapy
3	79	TRUS bx (1 core of nodule)	One core taken – Gleason 4+5=9	Metastatic PCa – ADT
4	74	TRUS bx (12 cores)	All cores except right base (10/12 cores) – Gleason 3+3=6	Locally advanced PCa – ADT
5	55	TRUS bx (12 cores)	Right side (6/12 cores) – Gleason 4+5=9	Localised PCa – ADT + radiotherapy
6	71	TURP	Gleason 4+5=9 in 40% of specimen	Metastatic PCa – ADT
7	66	TRUS bx (12 cores)	Left side + right mid (8/12 cores) – Gleason 4+5=9	Post RRP, +ve margin - ADT
8	58	TURS bx (12 cores)	Left mid (2/12 cores) – Gleason 3+4=7	Localised PCa – ADT + radiotherapy
9	81	TRUS bx (14 cores)	Right apex and mid (4/14 cores) – Gleason 4+4=8	Locally advanced PCa - ADT
10	83	TURP	Gleason 4+5=9 in 5% of specimen	Metastatic PCa - ADT

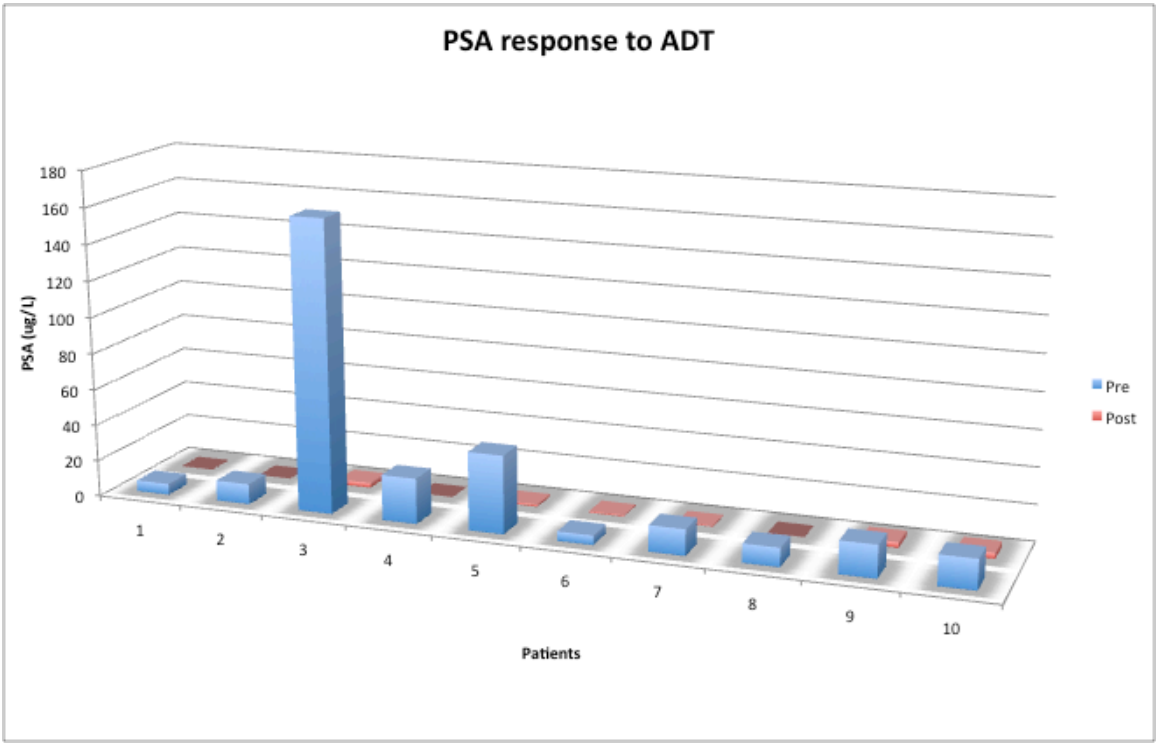
Table 5.1 Patient demographics and PCa profile.

The median age of the participants was 71. Most of these patients had high-risk disease, defined as having poorly differentiated adenocarcinoma with Gleason score greater than seven [447-451].

Some patients had organ-confined disease and were having ADT prior to curative radiotherapy. Some had positive margins after radical prostatectomy and were having salvage therapy, while others had disseminated disease and ADT was commenced for disease control. It could be seen from this demographic that patients who were involved in the study had PCa at different stages.

PSA values for patients prior to and three months after commencement of treatment are shown in figure 5.1.

Figure 5.1 The PSA pre and post androgen deprivation therapy.



All patients had a PSA response to ADT. This is expected as PSA halving time has been reported to be a median of 18 days [452].

5.4 ADT impact on immune system

5.4.1 Lymphocyte population

Enumeration of the lymphocyte populations as a percentage in the pre and post ADT groups is demonstrated in table 5.2.

Patient No	Pre ADT (%)	Number of events	Post ADT (%)	Number of events	Relative proportional change (%)
1	85.6	132843	84.5	104723	-1.3
2	70.7	81472	61.6	68493	-12.9
3	78	127698	84.1	108927	7.8
4	51.6	113984	75	98204	45.3
5	75.6	140275	66.4	102128	-12.2
6	62.1	208769	52.7	235785	-15.1
7	77.6	164908	73.5	134699	-5.3
8	75	120736	63.5	149625	-15.3
9	61.1	176352	71.2	138004	16.5
10	73.9	108378	74.2	167364	0.4

Table 5.2 Lymphocyte populations as a percentage of the PBMC sample in the pre and post ADT groups is seen in columns two and four. The event numbers generated from the flow cytometer are shown in columns three and five. The relative change in the lymphocytes between the pre and post treatment group is seen in the final column.

The mean lymphocyte percentage pre ADT was 71.12% (95% CI 63.95 to 78.29), while the post ADT percentage was 70.67% (95% CI 63.57 to 77.77).

There was a slight 0.45% decrease in the lymphocyte population after treatment (95% CI -8.92 to 9.82) with a p value of 0.9208. In the relative proportional change six out of the ten patients had a decrease in lymphocytes after ADT. This was not a convincing difference to draw a clinical conclusion.

5.4.2 CD4+ T lymphocyte

The CD4+ T lymphocytes in the pre and post ADT groups are shown in table 5.3.

Patient No	Pre ADT (%)	Number of events	Post ADT (%)	Number of events	Relative proportional change (%)
1	62.3	113714	65.8	88491	5.6
2	50	57601	46.9	39728	-6.2
3	45.8	99604	58.3	91608	27.3
4	14.7	58816	18.3	73653	24.5
5	46	106048	40	67813	15
6	37.8	129646	38.5	124259	1.9
7	33	127969	45.2	99004	37
8	50	90552	47.3	95012	-5.4
9	48.2	107751	41.1	98259	-14.7
10	45.6	80091	47	124184	3.1

Table 5.3 CD4+ T lymphocytes percentages of total lymphocytes in pre and post ADT groups are seen in columns two and four. The third and fifth columns show the event numbers. The sixth column shows the relative difference in the individual patients after ADT.

The mean CD4+ T lymphocytes percentage pre ADT was 43.34% (95% CI 34.27 to 52.41). While the post ADT percentage was 44.80% (95% CI 35.86 to 53.82).

There was a 1.50% (95% CI -13.36 to 10.36) increase in the post ADT group. However this result was not statistically significant (p 0.7934). Seven out of the ten patients had a relative increase in CD4+ T lymphocytes after ADT.

5.4.3 CD8+ T lymphocytes

The CD8+ T lymphocytes in the pre and post groups are displayed in table 5.4.

Patient No	Pre ADT (%)	Number of events	Post ADT (%)	Number of events	Relative proportional change (%)
1	7.9	115937	6.8	85278	-13.9
2	13.5	57392	12.8	43782	-5.2
3	6.6	95627	5.5	92901	-16.7
4	7.5	51028	9.6	79832	28
5	16.9	112524	18.5	61020	9.5
6	18.5	117293	17.9	116381	-3.2
7	25.7	122038	27.3	107257	6.2
8	29.3	92076	30.7	98975	4.8
9	15.6	100271	27	96781	73.1
10	16.3	85780	21.5	117178	31.9

Table 5.4 CD8+ T lymphocytes as a percentage of lymphocytes in the pre and post ADT groups are seen in second and fourth columns. In columns three and five, the event numbers generated from flow cytometry is seen. The final column shows the relative change in the individuals as a percentage.

The mean CD8+ T lymphocyte percentage in the pre ADT group was 15.79% (95% CI 10.40 to 21.17). While the post ADT percentage was 17.76% (95% CI 11.36 to 24.16).

There was a 1.97% increase in post treatment group (95% CI -9.74 to 5.80, p 0.6003. There were six patients who had an increase in the CD8+ T lymphocyte after ADT. This difference was more of a trend rather than a clinically significant result.

5.4.4 Treg cells

Table 5.5 demonstrates the differences between the Treg cells in the patients' pre and post ADT.

Patient No	Pre ADT (%)	Number of events	Post ADT (%)	Number of events	Relative proportional change (%)
1	3.1	70844	2.8	58227	-9.7
2	5.8	28801	4.2	18632	-27.6
3	2.8	45619	4.1	53407	46.4
4	3.8	8646	6.7	13478	76.3
5	4.0	48782	4	27125	No change
6	5.9	49006	5.6	47840	-5.4
7	6.9	42230	7.2	44750	4.3
8	5.4	45276	5.9	44941	9.3
9	5.4	51936	5.4	40384	No change
10	5.0	51936	3.7	58366	26

Table 5.5 Treg cells as a percentage of CD4+ T lymphocytes in the pre and post ADT groups is seen in columns two and four. The event numbers are demonstrated in columns three and five. The sixth column shows the relative change in the individual patient.

The Treg cell percentage in the pre ADT group was 4.82% (95% CI 3.86 to 5.77). While the post ADT group percentage was 4.96% (95% CI 3.94 to 5.98).

There was a slight increase in the Treg cell numbers after ADT by 0.15% (95% CI -1.44 to 1.15) with a p value of 0.8129. Five out of the ten patients had an increase in Treg cells after ADT while two had no change and three had a decrease. These numbers are low to draw conclusions.

5.4.5 Naïve T cells

The naïve T cells in the pre and post ADT groups are exhibited in table 5.6.

Patient No	Pre ADT (%)	Number of events	Post ADT (%)	Number of events	Relative proportional change (%)
1	17.7	52404	17.4	38375	-1.7
2	32.8	23301	35	19045	6.7
3	7.3	39302	10.2	42920	39.6
4	2.8	27402	4.3	39516	53.6
5	2.5	38708	2.4	24286	-4.0
6	6.2	46683	4.7	48182	-24.2
7	10.8	58700	10.4	51054	-3.7
8	24.6	53496	17.4	46914	-29.3
9	10.8	46525	5.7	43648	-47.2
10	17.6	38086	12.8	52613	-27.3

Table 5.6 Naïve T cells shown as a percentage of CD8+ T lymphocytes in the pre and post ADT groups is seen in the second and fourth columns. The event numbers are demonstrated in columns three and five. The final column shows the relative proportional change in naïve cells in the individual patient as a percentage after ADT.

The mean naïve T cell percentage in the pre ADT group was 13.31% (95% CI 6.27 to 20.35). While the post ADT group percentage was 12.03% (95% CI 5.13 to 18.94).

There was a1.28% decrease in naïve T cells after ADT (95% CI-7.88 to 10.43) and a p value of 0.7729. Although this result was not statistically significant,

there were seven out of the ten patients who had a reduction of naïve T cells after ADT. This trend could be further evaluated with a large sample population.

5.4.6 Terminal effector T cells

The terminal effector T cells pre and post ADT are shown in 5.7.

Patient No	Pre ADT (%)	Number of events	Post ADT (%)	Number of events	Relative proportional change (%)
1	82.3	52404	82.6	38375	0.4
2	67.2	23301	65	19045	-3.3
3	92.7	39302	89.8	42920	-3.1
4	97	27402	95.2	39516	-1.9
5	97.1	38708	97.4	24286	0.3
6	93.3	46683	94.8	48182	1.6
7	89.2	58700	89.6	51054	0.4
8	75.4	53496	82.6	46914	9.5
9	89.2	46525	94.3	43648	5.7
10	82.4	38086	87.2	52613	5.8

Table 5.7 Columns two and four demonstrate terminal effector T cells as a percentage of CD8+ T lymphocyte population in the pre and post ADT groups. The columns three and five show the event numbers from flow cytometry. The sixth column demonstrates the percentage change in the individual patients.

The mean terminal effector T cell percentage in the pre ADT group was 86.58% (95% CI 79.62 to 93.54). While the post ADT group percentage was 87.85% (95% CI of 81.02 to 94.68).

There was a 1.27% increase in terminal effector T cells after ADT with (95% CI-10.32 to 7.78) a p value of 0.7716. Although this was not statistically significant, seven out of the ten patients had an increase in terminal effector T cells after ADT. This trend could be further evaluated with a larger sample population.

5.4.7 Central memory T cells

The central memory T cells in the pre and post ADT groups in table 5.8.

Patient No	Pre ADT (%)	Number of events	Post ADT (%)	Number of events	Relative proportional change (%)
1	17.8	36172	17.6	33273	-1.1
2	25.7	23186	25.8	17294	0.4
3	26.4	35573	34.4	35860	30.3
4	4.8	13369	6.8	25945	43.4
5	6.2	49060	6.7	23615	7.4
6	5.6	48325	4.7	46203	-16.1
7	7.0	34049	7.2	42796	2.9
8	9.4	23571	6.9	39986	-26.6
9	9.1	42214	5.2	24354	-42.9
10	10.8	27964	8.7	50855	-19.4

Table 5.8 Central memory T cells are shown as a percentage of CD8+ T lymphocytes in the pre and post ADT groups in columns two and four. The third and fifth columns show the event numbers. The proportional change in the individual patient after treatment is seen in final column.

The mean central memory T cell percentage pre ADT was 12.28% (95% CI 6.46 to 18.10). While the post ADT group percentage was 12.39% (95% CI 5.09 to 19.69).

There was marginal increase of 0.11% after ADT therapy (95% CI -8.78 to 8.56, p 0.9796). This is clinically and statistically not significant. No trend was seen either as half the patients had an increase in the central memory numbers after ADT.

5.4.8 Effector memory T cells

Table 5.9 shows the differences in effector memory T cells in the pre and post ADT groups.

Patient No	Pre ADT (%)	Number of events	Post ADT (%)	Number of events	Relative proportional change (%)
1	81.9	36172	82.3	33273	0.5
2	74	23186	73.9	17294	-0.1
3	73.6	35573	65.3	35860	-11.3
4	95.2	13369	93.2	25945	-2.1
5	93.8	49060	93.3	23615	-0.5
6	94.4	48325	95.3	46203	1.0
7	93	34049	92.8	42796	-0.2
8	90.6	23571	93.1	39986	2.8
9	90.9	42214	94.8	24354	4.3
10	89.2	27964	91.3	50855	2.4

Table 5.9 Effector memory T cells in CD8+ T lymphocytes in the pre and post ADT are shown as a percentage in columns two and four. The third and fifth columns show the events generated with flow cytometry. The sixth column demonstrates the proportional change in the individual patient after ADT as a percentage.

The mean effector memory T cell percentage in the pre ADT group was 87.66% (95% CI 81.78 to 93.54). While the post ADT group percentage was 87.53% (95% CI 80.15 to 94.91).

There was a minimal decrease of 0.13% seen after ADT (95% CI -8.64 to 8.90, p 0.9755). This was not significant difference seen and individually only half the patients had a decrease in effector memory T cells.

5.4.9 Activation status of CD8+ T lymphocytes

Table 5.10 shows the differences in activation status of CD8+ T lymphocytes in the pre and post ADT groups.

Patient No	Pre ADT (%)	Number of events	Post ADT (%)	Number of events	Relative proportional change (%)
1	5.7	9171	12.5	5756	119.3
2	12.2	7748	8.7	5604	-28.7
3	6.9	6340	9.1	5119	31.9
4	9.6	3832	3.2	7672	-66.7
5	3.7	19017	8.2	11289	121.6
6	3.4	21699	10.1	20832	197.1
7	2.3	31364	1.8	29281	-21.7
8	1.9	26978	1.6	30385	-15.8
9	1.9	15642	1.0	26131	-47.4
10	1.6	13982	1.4	25193	-12.5

Table 5.10 Activation status of CD8+ T lymphocytes in the pre and post ADT groups is demonstrated in columns two and four. The third and fifth columns show the event numbers. The final column expresses the proportional change in the individual patients.

The mean percentage of activated CD8+ T lymphocytes in the pre ADT group was 4.93% (95% CI 2.32 to 7.53). While the post ADT group percentage was 5.75% (95% CI 2.61 to 8.88).

There was a 0.82% increase in the activation status of CD8+ T lymphocytes after ADT (95% CI -4.60 to 2.96, p 0.6547). Six out of the ten patients had a decrease in the number of activated CD8+ T cells after ADT. These numbers are not significant to draw firm conclusions on.

5.5 Discussion

In this study, all the participants started on ADT and had a good biochemical response at three months which was demonstrated with a drop in PSA. A good PSA response to ADT is associated with longer survival [453].

This pilot study aimed to evaluate the potential benefit of ADT in thymic regeneration and replenishment of peripheral T cell pool. From our analysis there was no difference seen in the lymphocyte subpopulation in their numbers, function and activational status in the post ADT population. There were some trends seen with the proportional change in individual patients. Clinically significant differences could not be confidently distinguished.

One reason why a difference was not seen could be due the limited time period of the study was conducted. A three-month interval between the pre and post samples could have been too close to see a difference. However the study by Mercader et al, gave ADT at 7, 14, 21 and 28 days before radical prostatectomy showed a T cell increase in all these time periods [454].

In order to determine the potential effects of ADT, modification of the study is needed. The participants should be followed for a longer time course and have blood analysis on a more frequent basis. Ideally, patients should be followed until they become hormone refractory and they cease ADT. The changes to the immune cell profile could be assessed to see if there is a decline in immunity.

It is also important to verify the effects of ADT. In this study, the drop in PSA is seen and as a result indirectly indicates the effects of ADT. It would be useful to also assess the testosterone level, to verify the effects of androgen ablation. In this study testosterone levels were not assessed, as there is no consensus on the level and timing at which suppression occurs. Most studies assess levels at a six-month period and performing an analysis at three months would have been too early [455, 456]. In the literature patients who are on ADT and achieved testosterone levels less than 50ng/dL were classified as being castrated [457-459]. However a significant proportion of patients, 13 to 42% have androgen levels that do not reach castrate levels [455, 456, 458, 460].

This study assessed the possible effects of ADT on lymphocyte subpopulations in a heterogeneous population of men. In order to obtain statistically significant results the power of the study needs to be increased.

The participants' ages in the study ranged from 55 to 83. As the action of the immune system declines over time, the numbers, function and activational status of immune cells would be different. Also the ability and rate at which the immune system responds varies with age. This could be rectified in future studies by grouping patients by age bands. However no obvious pattern was seen in the different age groups.

In future studies it would also be useful to have a control group. Although the pre ADT sample is used as a control group and the post ADT sample is a labelled as the treatment group. An ideal control would be having a group of men with PCa who are on active surveillance or on the waiting list for a radical prostatectomy. They could have bloods taken at three monthly intervals to assess the changes in the immune cells in the natural course of PCa.

Another alteration in future studies is to minimise the variables in the patients. For instance, the stage of disease is very important, the tumour bulk in localised PCa is considerably low in comparison to those patients with distant metastatic disease which could influence the effects on the immune system in its activation and inhibition. Subdividing patients according to Gleason score and cancer staging could rectify this in future.

Alternatively studies by Gannon et al and Mercader et al have demonstrated an influx of immune cells in the local prostate environment after ADT [454, 461]. They performed their studies by giving patients ADT up to one month prior to radical prostatectomy and then analysing the prostate tissues for immune cells and comparing the numbers with matched controls. One of the variables with this method is that after ADT, there is apoptosis of cancer cells and this can lead to an influx of immune cells resulting in a false positive.

From this pilot study, a difference in lymphocyte numbers, functional and activational status after ADT could not be seen. However lessons on establishing further studies have been learnt. This study will assist in the devising larger studies, looking at an identical demographic of PCa patients with matched controls over a longer period of time. We plan to assess the same T cell population, functional and activation status.

6.0 Conclusion and Future directions

6.1 Conclusion

Prostate cancer is a prevalent disease that affects many men around the world. The detection of PCa has increased over the last few decades due to improved screening and diagnostic techniques. However there is suboptimal understanding of the illness in terms of which prostate cancers need immediate intervention and which do not because of their indolent behaviour. One of the major potential factors is the interaction of the immune system with PCa cells.

The aim of this study was to obtain insight into profile of the tumour infiltrating lymphocytes in patients with PCa. This was evaluated by studying the interaction of the lymphocytes in both the local (intratumoural) and peripheral blood environment. Sufferers of PCa were compared with patients with other prostate pathology such as BPH and CAP to assess the activity of the immune system.

In the study, BPH was the control group where the lymphocyte population was considered most likely to be at homeostasis. The lymphocyte profile of participants with CAP was determined to support an autoimmune component to its pathogenesis. In future research it would be interesting to pursue the CAP pathway with larger a population and examine the full lymphocyte profile and activational status. It is important to have a better understanding to develop treatment options for this clinically significant condition as it causes considerable morbidity and impairs quality of life of sufferers .

In the PCa local environment, there was a proportional rise in cytotoxic cells and immunosuppressive lymphocyte subsets. This allowed for the development of a neutral environment permitting for the survival of PCa cells. This equal rise in lymphocyte numbers could be a result of defective lymphocyte function resulting in an environment that could favour or inhibit tumour cell growth.

Examination of the functional status of CD8+ T lymphocytes in the PCa local environment revealed that there were increased numbers of terminal effector T cells and effector memory T cells with a corresponding reduction in naïve and central memory T cells. This functional profile indicated that the immune system has recognised tumour antigens and has shifted the dynamics to favour a cytolytic profile. This was supported by the presence of activated CD8+ T lymphocytes. Despite the findings of these primed and activated lymphocytes, there were no overt signs of tumour cell death. This raised the question whether these activated immune cells were actually effective in secreting the cytokines and other effector.

The presence of some cytokines in the local tumour environment was evaluated using IHC. The IHC stains for the cytokines IFNγ, IL-10 and TGFβ did not show any significant difference within the PCa local environment when compared to the control group. There were only a small number of cells expressing the cytokines and as a result in future studies a more specific method of detection should be used. For instance techniques such as flow cytometry could be used to evaluate the cytokine profile in the local tumour environment. With this method more cytokines could be examined and also only a small quantity of tissue is needed to perform these studies.

The current method of determining the severity and prognostic characteristics of PCa is determined from pathological markers. The study examined the correlation of lymphocyte subsets with current pathological markers of poor

outcome. Patients with poor prognostic factors such as extraprostatic spread, seminal vesicle involvement and PSA relapse all had higher Treg cell counts in the tumour environment than those patients with favourable pathological markers. This could be a statistical anomaly but requires further evaluation with larger numbers to determine if Treg cells could be used as a prognostic marker. The implication in the management of PCa could be that patients with high Treg cells might require earlier therapy such as salvage radiotherapy rather than monitoring and waiting for PSA relapse. These clinical correlations remain to be tested.

In the literature there is evidence showing ADT for PCa could play an additional role in rejuvenating the thymus to strengthen the immune system. We conducted a pilot study to evaluate this theory. From the findings, there was no clear difference seen in the percentage, functional and activational status of the lymphocytes in the periphery of patients after ADT. Although no difference was seen, this study will allow for the development of future larger population studies. The study needs to be extrapolated to looking at fixed demographic of patients with similar cancer profiles. The assessment needs to be for longer periods from time of diagnosis to time of hormone resistance. Also tissue sampling in the form of TRUS biopsy or TURP would be helpful in assessing the local tumour environment. Also differences can be assessed in patients at different stages of disease such as pre ADT, during ADT and hormone refractory PCa. If a true rejuvenation of thymocytes and upregulation peripheral T cell population is obtained with ADT, its use as a concurrent therapy in many cancers might be significant.

6.2 Future direction

The future direction of this study will be in the further evaluation of the cytokine profile in the local tumour environment. This would be assessed with flow cytometry so that a broad spectrum of cytokines can be evaluated. This will

provide a better understanding in the of the local tumour environment to see if it is a hostile or a nurturing environment of the tumour cells.

If the study is continued and a larger sample population is recruited, some of the trends that have been seen could be verified and more statistically significant data could be attained. The patients could be followed to see the nature of their PCa and correlated with the lymphocyte profile. Attention could be given to obtaining and evaluating patients with high grade PCa, that is Gleason score greater than seven, to assess their lymphocyte profile. By increasing the sample population, this group of patients can be captured and compared to the low and intermediate PCa patients.

The other direction is the evaluation of ADT in PCa in a larger and longer-term study. Conducting the study for a longer duration patient numbers can be increased and monitoring the lymphocyte population over the course of the disease can be evaluated. Assessing the lymphocyte profile on a regular basis will provide a better understanding of immunomodulation at different stages of the disease which has not been evaluated before in literature.

This study has given an insight into aspects of the immunological processes that take place in prostate pathology, in particular PCa. It has shown the lymphocyte profile in PCa, its correlation with current prognostic markers and response to ADT. This will hopefully lead to further studies to understand the immunomodulation processes that occur so that immune based therapies can be developed to combat this disease.

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