

**Prostate stromal fibroblasts as
immune regulators and effectors**

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Abstract

Prostate cancer (PCa) is the most common cancer diagnosis in males and the second leading cause of cancer related male deaths. Local microenvironments containing stromal fibroblasts are vitally important in the normal development and homeostatic regulation of the prostate, and have key roles in supporting prostate cancer progression. Local chronic inflammation has been associated with the development of prostate cancer. The potential impact of local immune cell derived inflammatory mediators on prostate stromal and epithelial/tumour cells have been studied, however the reciprocal impact on infiltrating immune cells has not been fully explored. Advancements in immunotherapy through clinical applications in checkpoint molecule inhibition have led to significant progress in the treatment of melanoma and lung cancer in recent years. However, for unknown reasons, immunotherapies thus far have widely failed to have therapeutic efficacy in prostate cancer patients.

By utilising primary human prostate tissue samples from patients with benign prostatic hyperplasia (BPH) or PCa using both *in vitro* culture systems combined with gene expression profile analysis, imaging and flow cytometry, it has been shown that prostate stromal cells exhibit a conserved capacity to interact with local immune cells. Prostate stromal cells potently express an array of molecules known to negatively regulate immune cells, either endogenously, or in response to local immune activity through TGF- β , IDO and PD-L1. The expression of these molecules drives inhibition of local anti-tumour T cells and ultimately, tumour immune evasion. Furthermore, an experimental protocol to analyse the prostate infiltrating immune cells by flow cytometry was developed and used to demonstrate preliminary evidence for an enrichment of cytotoxic T lymphocytes in the tissue compared to peripheral blood. Importantly, these T cells have an increased surface expression of PD-1, the receptor that binds PD-L1 to induce T cell inhibition.

Prostate tissue contains large numbers of stromal fibroblasts, even in cases of high-grade cancer. This study indicates prostate stromal cells tip the balance toward immunosuppression, which in the context of prostate cancer may lead to tumour immune escape. This is an important consideration for future studies in the field of immunotherapy in prostate cancer, since prostate infiltrating immune cells reside in the stromal compartment. Therefore, the success of PCa immunotherapy likely relies on targeting tumour fibroblasts.

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Supplementary material

This consists of a digital archive of plots derived from nanoString analysis. All genes contained within the analysis have been plotted and contained as separate PDF files, with the file name saved as the corresponding gene name.

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Author's Declaration

I, Katrina Reilly, declare that this thesis is a presentation of original work and I am the sole author. This work has not previously been presented for an award at this, or any other University. All sources are acknowledged as references.

Chapter 1

Introduction

1.1. The human prostate gland

1.1.1. Gross structure and function

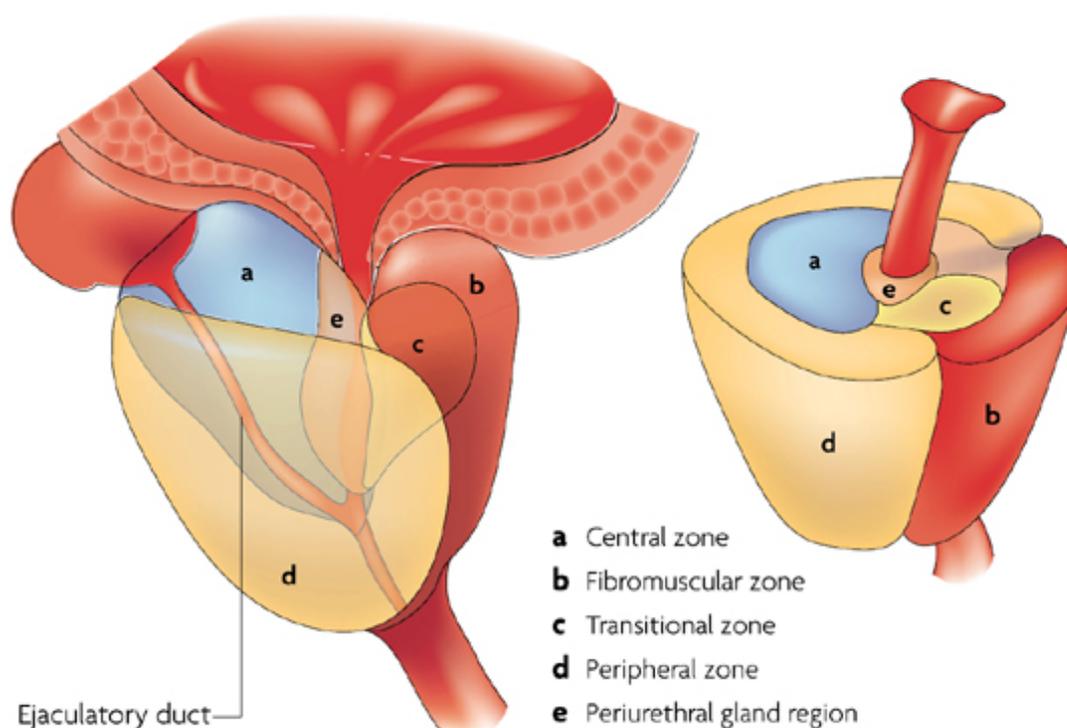
The male prostate gland is a walnut-sized exocrine gland of the reproductive system. It is located at the base of the bladder and surrounds the urethra. Pathologically, it can be divided into distinct zones: the peripheral zone (PZ), central zone (CZ), transitional zone (TZ) and the anterior fibro-muscular zone (Figure 1.1) (McNeal, 1988). The gland functions to discharge an alkaline secretion that together with the seminal vesicle secretions and sperm, makes up the semen. Given that the vaginal environment is largely acidic, the basic fluid provided by the prostate and seminal vesicles is important for both the survival and motility of spermatozoa in this milieu. Although females do not strictly have a prostate gland, there is substantial evidence that the skene's gland is the undeveloped female equivalent.

1.1.2. Embryonic development of the prostate

The prostate arises from the urogenital sinus (UGS) at around 10 weeks after gestation in humans (Lowsley, 1912). In contrast to most reproductive organs, which derive from the Wolffian ducts and are mesodermal in origin, the UGS has endodermal origins (Lowsley, 1912). Urogenital epithelial (UGE) cells bud from the UGS and migrate in succession into the surrounding mesenchyme (UGM). Once implanted in the UGM, the UGE depend on prompts from the UGM to form interconnecting branches (Prins and Putz, 2008; Timms *et al.*, 1995). Thus, the development of the prostate is highly dependent on stromal cells. Subsequently, UGE derived signalling causes differentiation of the UGM into mature smooth muscle cells and fibroblasts that form the non-haematopoietic stroma of the adult prostate (Cunha *et al.*, 1996; Hayward *et al.*, 1996). Androgen steroid signalling between stroma and epithelia is essential in both this developmental phase of the foetal prostate and the homeostasis of the adult prostate (Prins and Birch, 1995). It is mediated via stromal derived molecules collectively referred to as andromedins. Andromedins act on androgen receptor (AR) negative basal cells, triggering their differentiation into the epithelial luminal cells (Berry *et al.*, 2008). These same interactions are thought to be involved in the counter direction following castration, whereby a stromal-dependent reduction of the prostate occurs (Kurita *et al.*, 2001).

1.1.3. Microanatomy

Histologically, prostate tissue can be further divided into the epithelial and stromal compartments (Figure 1.2). Epithelial acinar structures are composed of a pseudostratified columnar epithelium, which perform the secretory function of the gland. Within the basal layer of the acini reside the committed basal and stem cells, the latter of which differentiate to provide the cells of the luminal layer: a mixture of transit amplifying and terminally differentiated luminal epithelial cells. The basal epithelial cells are surrounded by a basement membrane, providing a protective barrier between them and the stromal compartment.



	Prostate zone		
	Peripheral	Transition	Central
Focal atrophy	High prevalence	Medium-high prevalence	Low prevalence
Acute inflammation	Low prevalence	Low prevalence	None
Chronic inflammation	Medium-high prevalence	Medium-high prevalence	Low prevalence
Benign prostatic hyperplasia	None	High prevalence	Low prevalence
High-grade PIN	Medium-high prevalence	Low prevalence	Low prevalence
Carcinoma	Medium-high prevalence	Low prevalence	None

■ High prevalence ■ Low prevalence
■ Medium-high prevalence ■ None

Figure 1.1: Gross anatomical structure of the prostate gland and associated prostatic diseases

The prostate gland can be broadly separated into distinct zones the PZ, CZ, TZ and the anterior fibromuscular zone. Each zone has associated diseases, for example, BPH most often presents in the TZ, rarely in the CZ and never in the PZ. Conversely PCa occurs more often in the PZ than the TZ and never in the CZ. The occurrence of PCa correlates exactly with the prevalence of prostatic intraepithelial neoplasia (PIN), thought to be a PCa precursor. Figure taken from: (De Marzo *et al.*, 2007)

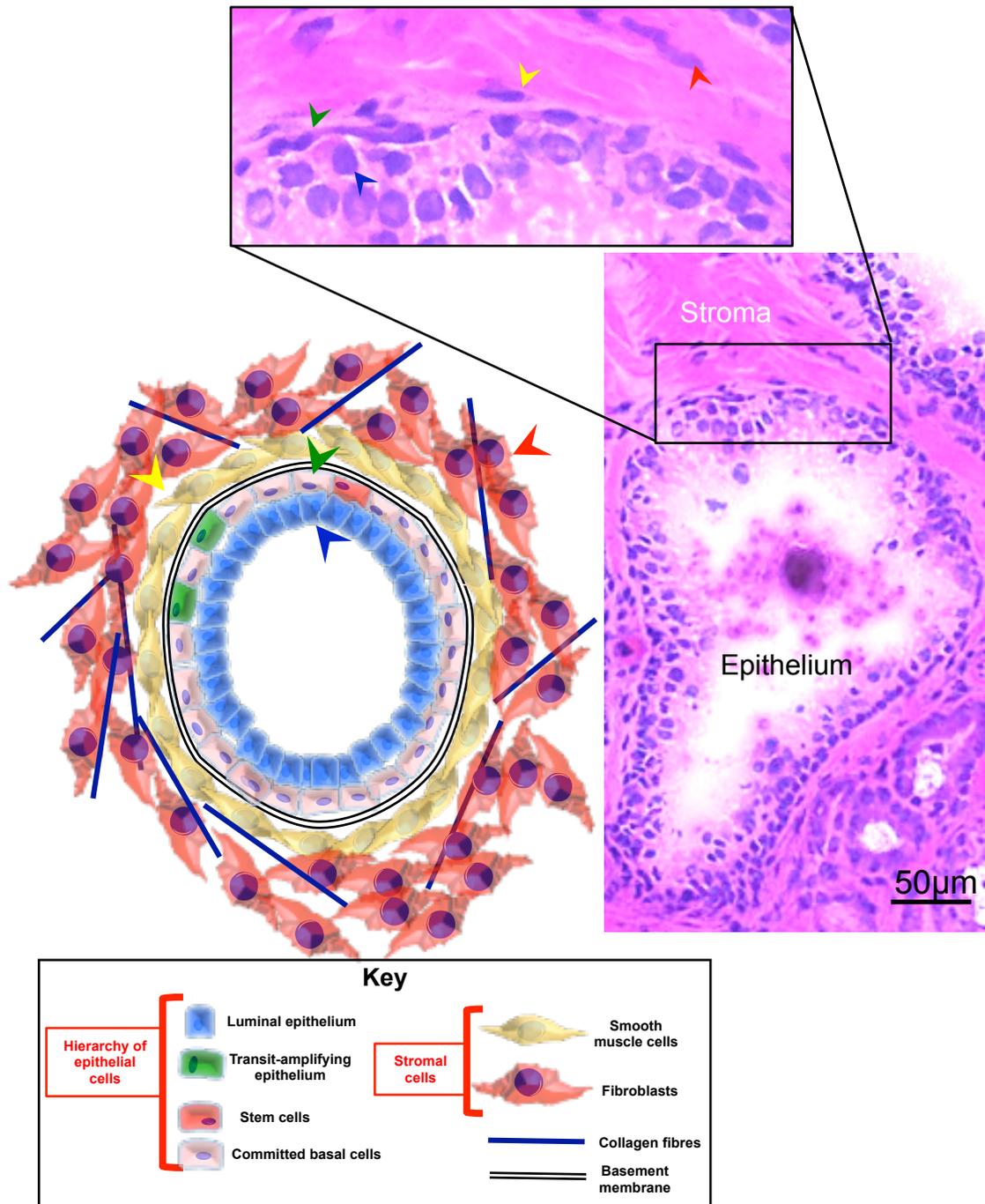


Figure 1.2: Microanatomy of the prostate

The diagram depicts a cartoon representation of the cellular composites of the human prostate architecture. To demonstrate this histologically an example of an intact acinus is shown on the right from BPH tissue. Epithelial acini are composed of a hierarchy or epithelial cells in a basal (green arrowhead; committed basal, stem and Trans-amplifying cells) and luminal layer (blue arrowhead). The acinus is surrounded by an intact basement membrane, which is lined by smooth muscle cells (yellow arrowhead). The stromal compartment contains mostly fibroblasts (red arrowhead) in an interconnecting ECM. Note the multiple layers of columnar epithelium of the luminal layer typical of luminal epithelial BPH.

1.2. **Stromal cells**

The biological term “stroma” can be ambiguous and cause confusion since it incorporates many cell types (ranging from haematopoietic cells to fibroblasts) in any organ. Essentially it includes any cell that provides a supportive role in fulfilling the primary function of the organ in which it resides. It is perhaps this “supportive” role allocated to stroma that has resulted in the under-representation of stromal biology in research until relatively recent years. It is now well appreciated that stromal cells provide a vital backdrop to many biological developmental and homeostatic processes, and stromal cell dysfunction can contribute to the development of most diseases. Although immune cells may be included in this broad stromal term, for the purpose of this research project, the immune component of stroma are considered separately, so **only the non-immune stroma shall henceforth be designated stroma.**

1.2.1. Prostate stromal cells

All stromal cells originate embryonically from the mesoderm. As discussed earlier in this chapter, the stroma of the immature prostate is essential for the normal development of an adult prostate and for healthy homeostatic regulation in the prostate. Prostate stromal cells, which are predominantly smooth muscle cells (SMCs) and fibroblasts, have gained increasingly more attention due to established roles in prostatic diseases such as benign and malignant transformation of prostate epithelia (Condon and Bosland, 1999; Hagglof and Bergh, 2012; Ishigooka *et al.*, 1996; Wang *et al.*, 2016). SMCs of the adult prostate are positioned in the stromal compartment surrounding the basement membrane. The prostate fibroblasts compose the majority of the stromal compartment. Fibroblasts lay down and orientate collagen and fibronectin fibres, which form the extracellular matrix (ECM) that SMCs are bound and upon contraction facilitate the expulsion of prostate secretions from the lumen of the acini. Though, “fibroblasts” themselves are a misleading term as their gene expression and functions vary from tissue to tissue, depending on their localised microenvironment (Chang *et al.*, 2002). For this reason Komuro suggested fibroblasts should be further defined by the main functions they exhibit, although this has not been widely implemented (Komuro, 1990).

Culturing primary human prostate stromal cells *in vitro* is a valuable technique used to elucidate mechanisms underlying stromal mediated disease progression. However, this is a practice that can lead to unreliable conclusions if the caveats are not taken into account. Not only do stromal cells become senescent if cultured for extended periods, they have also demonstrated a loss of physiologically important features after a just few passages (Hall *et al.*, 2002; Janssen *et al.*, 2000; Peehl, 2005). Characterisations of prostate stromal cultures in the past have concluded that fibroblasts and myofibroblasts predominate (Gravina *et al.*, 2013). SMCs and endothelial cells form only a small fraction and are lost early in culture. Cultured prostate stroma have been shown to exhibit features reminiscent of the stromal compartment of origin (i.e. normal and cancer),

making *in vitro* investigations of prostate stroma an important technique to utilise to improve understanding regarding stroma and disease (Hall *et al.*, 2002; Kopantzev *et al.*, 2010).

1.2.2. Multipotent mesenchymal stromal cells (MSCs)

Due to the contentious issues surrounding the nomenclature of traditionally named mesenchymal *stem* cells (MSCs), the international society of cell therapy (ISCT) published a position statement to address inconsistencies between the classification of MSCs and the biological properties they exhibit (Dominici *et al.*, 2006). Hence, it was declared that, unless cells meet the true stem cell criteria, heterogenous adherent cells isolated from tissues would be termed mesenchymal *stromal* cells. MSCs may be isolated from many types of tissues, including but not limited to bone marrow (BM), adipose tissue, dental pulp and umbilical cord (da Silva Meirelles *et al.*, 2006). BM-derived MSCs (BMSCs) are most well studied. To warrant the MSC classification, cells must meet a number of criteria, according to the ISCT. First, cells must be plastic-adherent. Secondly, they must express a number of cell surface markers (e.g. CD105 and CD90) in the absence of haematopoietic markers such as CD45 and CD14. Lastly, they must exhibit multipotency. That is; the ability to give rise to a number of different mesenchymal progeny. The last is potentially a remaining sticking point for those working in the MSC specialty. Since MSCs are heterogenous in nature, not all are able to differentiate into all three (osteogenic, adipogenic and chondrogenic) lineages (James *et al.*, 2015). Rather, there are variabilities in the potency of differentiation among the MSC cultures, from tripotent (the ability to give rise to all 3 lineages) to nullipotent (unable to differentiate). Only the first (together with the other criteria) merits the stem cell terminology (Muraglia *et al.*, 2000; Okamoto *et al.*, 2002).

MSC research has focused substantially on a role in immunoregulation, potentially due to the haematopoietic niche that BMSCs inhabit (Bernardo and Fibbe, 2013; Nauta and Fibbe, 2007; Puissant *et al.*, 2005). However it is not only BMSCs that have the capacity to modulate immune cell function, even those isolated from non-haematopoietic tissues share this ability (Bartholomew *et al.*, 2002). They are able to facilitate inhibition of innate and adaptive immunity, depending on the immunological context due to plasticity (usually with on-going inflammation), but may also provide stimulatory signals (Wang *et al.*, 2014; Weinstock *et al.*, 2015). Through expression of transforming growth factor- β 1 (TGF- β 1), indoleamine 2,3-deoxygenase (IDO) and immune checkpoint molecules such as programmed death ligand 1 (PD-L1), MSCs are able to prevent immune cell proliferation and activity, improving self-tolerance and preventing autoimmunity (Abumaree *et al.*, 2013; DelaRosa *et al.*, 2009; Nemeth *et al.*, 2010; Spaggiari *et al.*, 2008). Alternatively, given differing immune signals (e.g. TLR4 agonists) MSCs are able to fulfil a pro-inflammatory role, in order to improve local immune cell activity through either cell-cell contact or secretion (Tomchuck *et al.*, 2008; Waterman *et al.*, 2010).

Populations of progenitor MSCs reside in all adult organs, including non-haematopoietic organs such as the prostate where they are believed to provide a source of mature stromal cells to

facilitate regeneration (Crisan *et al.*, 2008; da Silva Meirelles *et al.*, 2006). There is also evidence to suggest that in response to inflammation, BMSCs are liberated from the BM to colonise elsewhere (Kassis *et al.*, 2006).

1.2.3. Stromal cells in lymphoid organogenesis and adult lymphoid tissues

Non-haematopoietic stromal cells of lymphoid organs such as the lymph node (LN) are key in maintaining an environment that permits the development and sustenance of lymphoid reactions. The development of secondary lymphoid organs (SLO) is regulated by cross talk between stroma and lymphocytes (Mueller and Germain, 2009). Early haematopoietic lymphoid tissue inducer (LTi) and lymphoid tissue initiator (LTin) cells provide resident stroma (LT organisers; LTo) with signals that encourage stromal mediated retention of haematopoietic cells. These signals are predominantly lymphotoxin (LT) dependent. LTi cells release $LT\alpha 1\beta 2$, which upon binding to $LT\beta R$ on LTo cells, triggers upregulation of (1) chemokines that attract further LTi cells and (2) adhesion molecules (AM) such as intercellular adhesion molecule 1 (ICAM1) and vascular cell adhesion protein 1 (VCAM1), which are vital for LTi retention (Adachi *et al.*, 1997; Honda *et al.*, 2001). Initiation of a positive feedback occurs when LTo cells release interleukin-7 (IL-7) and TNF related activation-induced cytokine (TRANCE, also known as RANKL), acting to upregulate release of $LT\alpha 1\beta 2$ by LTi and potentiate the development (Meier *et al.*, 2007). IL-7R mediated signalling is only partially required in lymph node (LN) development though, as this occurs in the absence of IL-7 signalling, whereas in Peyer's patch formation it is a complete requirement (Adachi *et al.*, 1997; Luther *et al.*, 2003). Interestingly, it is the stroma that dictates the initiation of SLO development, as LTo cells are primed before LTi infiltration, however the signals that trigger the stromal maturation are yet to be elucidated (Benezech *et al.*, 2010; Brendolan and Caamano, 2012).

In fully developed LN, $LT\beta R$ signalling is important for the upkeep of stromal organisation and function and can contribute to the development of disease (Gommerman *et al.*, 2002; Mackay *et al.*, 1998). There are 3 *principal* stromal populations recognised in the human LN that promote lymphocyte homeostasis and activation through the generation of distinct anatomical niches: marginal reticular cells (MRCs) which reside in the subcapsular sinus, follicular dendritic cells (FDCs) and follicular reticular cells (FRCs), which reside in the B cell follicles and T cell zones respectively. Reciprocal interactions between LN stromal cells and the corresponding lymphocyte maintain respective stromal phenotypes and structural integrity of lymphocyte segregation (Boulianne *et al.*, 2012; Endres *et al.*, 1999). During immune responses, the LN undergoes dramatic remodelling through reorganisation and expansion of the stromal cell network. This permits LN hypertrophy (influx of lymphocytes) and formation of the germinal centre, both of which are essential for generating a successful immune response (Allen and Cyster, 2008; Vu *et al.*, 2008). In certain immunological scenarios it can be recognised that loss in the structural integrity of lymphocyte segregation (and respectively the stroma) causes detrimental effects on

generation of an appropriate immune response (Mackay and Browning, 1998). An example of this is the loss of FRCs in response to Lymphocytic Choriomeningitis Virus (LCMV), which prevented immunological response to secondary infections (Scandella *et al.*, 2008). This highlights the importance of immune-stromal cell interactions under both homeostatic and immune responses.

1.2.4. Stromal-immune interactions in disease

Stromal cells from non-haematopoietic organs probably share the ability to regulate immune infiltrates upon activation (Barone *et al.*, 2012). Given the potential destruction that can occur as a result of either overactive or under active immune responses, it is logical that mechanisms exist in peripheral tissues to regulate immune cells. This is particularly important considering populations of regulatory immune cells are scarce and lymphoid stroma are absent. Resident stroma has become a focus for clinical research particularly in immunological related conditions. For example, the immune suppressive abilities of MSCs have gained them attention as a potential treatment to reduce overactive immune activity in autoimmune diseases such as systemic lupus erythematosus (SLE) and organ transplantation (Reinders *et al.* 2013; Wang *et al.* 2014a). Although reactive prostate stroma has not been shown to directly modulate immune cells, they do express chemokines and cytokines, known inflammatory mediators (De Marzo *et al.*, 2007; Niu and Xia, 2009).

Formation of tertiary lymphoid structures (TLS) occurs during chronic inflammation and often close to tumours. It is likely that similar mechanisms involved in the formation of SLO are conserved in the formation of TLS, and that reciprocal signalling between stroma and immune cells promotes this. Its presence close to tumours is most often found to be a positive prognostic indicator for patients, though the molecular mechanisms and whether they can support generation of anti-tumour immunity are yet to be clarified (Dieu-Nosjean *et al.*, 2008; Germain *et al.*, 2014; Goc *et al.*, 2014; Ladanyi *et al.*, 2007).

1.3. Prostatic disease

1.3.1. Prostatitis

Prostatitis is an inflammatory condition of the prostate. It is sometimes associated with acute or chronic bacterial infections (acute or chronic bacterial prostatitis), but usually the aetiology cannot be identified (chronic prostatitis/chronic pelvic pain syndrome; CP/CPPS or asymptomatic prostatitis). Its prevalence overall is reportedly between 2-10% and most are CP/CPPS diagnosed (Krieger *et al.*, 2002). Over the years there have been many attempts to understand the association between prostatitis and prostate cancer (PCa), with inconsistent results. In one study, CP was directly associated with benign prostatic hyperplasia (BPH) but was found to occur at similar rates close to both normal and cancerous glands (DeLongchamps *et al.*, 2008). Another found a weak positive correlation between CP and PCa (Davidsson *et al.*, 2011). CP was directly associated with

the development of proliferative inflammatory atrophy (PIA), but this was *not* found to correlate with the development of prostatic intraepithelial neoplasia (PIN; a condition considered by many as a precursor to PCa), or PCa (Vral *et al.*, 2012).

1.3.2. Benign Prostatic Hyperplasia (BPH)

BPH is a non-malignant hyperplastic disease of the prostate that is increasingly prevalent with age (Berry *et al.*, 1984). Since both prostate epithelial and stromal cells undergo hyperplasia in BPH, it is an indication that it is not a clonal disease (Tang and Yang, 2009). BPH is not accompanied by disruption of the basement membrane and so does not result in invasion of epithelium. As microanatomical expansion occurs, the prostate gland becomes significantly enlarged. The anatomical position means this enlargement causes compression of the upper urethra. Due to the resulting lower urinary tract symptoms (LUTS), BPH causes a great deal of financial stress on the NHS (Speakman *et al.*, 2015). Initially, patients are treated with alpha-blockers (e.g. Flomaxtra/Tamsulosin), which through relaxation of the prostate, neck of the bladder and thickened urethra wall, permit easier passage of urine through the obstructed urethra (Kenny *et al.*, 1996; Lepor, 2007). Avodart, a 5 α -reductase inhibitor, may alternatively be used, although the drug has reduced efficacy for directly relieving urine flow complications (Tarter and Vaughan, 2006). In patients that respond less well on Flomaxtra, Avodart may prevent the need for surgical intervention through overall reduction in the size of the prostate (Emberton *et al.*, 2007). In patients with advanced BPH, where urethral blockage is extensive, a transurethral resection of the prostate (TURP) is performed. A resectoscope (a tube containing a resection loop, camera and light) is passed through the urethra to the point of obstruction. The resection loop heats when a current is passed through it and facilitates the removal of tissue blocking the urethra. The tissue is removed in sections (or chips), which can be examined histologically.

The exact aetiology of BPH is unknown, but it has been closely associated with chronic infections, inflammation of the prostate, and suspected interference of paracrine signalling within the microenvironment that control homeostatic regulation. Activated infiltrating immune cells are common in BPH tissue, however it is unclear whether this is a causative or aggravating effect (Kramer *et al.*, 2007). Cytokines produced by activated immune cells may either induce or inhibit growth of prostate epithelial and stromal cells. For example, IL-4 (mainly derived from T helper 2; TH2 cells) inhibits SMC growth while inducing clonal expansion of fibroblasts (Kramer *et al.*, 2002; Steiner *et al.*, 2003). IL-17 stimulates cytokine production (IL-6 and IL-8) by stromal cells and is expressed mainly by T cells derived from BPH, and to a lesser extent in the corresponding prostate epithelium (Steiner *et al.*, 2003). Activated TH1 and cytotoxic T lymphocytes (CTLs) are potent interferon- γ (IFN γ) expressers, which was found to induce growth of BPH stroma and epithelial separately (Deshpande *et al.*, 1989; Kramer *et al.*, 2002; Steiner *et al.*, 2003). And TGF- β is understood to induce transdifferentiation of prostate fibroblasts to myofibroblasts (Huang and Lee, 2003; Untergasser *et al.*, 2005). It is unlikely however that the activated immune infiltration occurs

spontaneously. As such, BPH is associated with recurring urinary tract infections (UTI), which are postulated to induce chronic inflammation and consequently hyperplasia of the prostatic tissue. McNeal performed key studies that introduced the embryonic reawakening theory (McNeal, 1978). He suggested that prostate stromal cells regain the embryonic functions that stimulated the initial prostate morphogenesis. Many believe the aforementioned inflammatory effects on stroma propagate stromal mediated epithelial hyperplasia and therefore contribute to the embryonic reawakening theory, but the initiating event remains undetermined.

Another potential mechanism of BPH development is persistent androgen signalling, which normally stimulates prostatic growth through induction of growth hormones. Prostate stromal cells convert testicular testosterone to dihydrotestosterone (DHT), which has higher affinity for the AR. This is facilitated in the prostate by type II 5 α -reductase (hence the use of Avodart to reduce prostate enlargement) (Makela *et al.*, 1990). With age, androgens decrease in the peripheral blood however they continue to be present at high levels in the prostate (Marberger *et al.*, 2006). It is this persistent presence of *local* androgens that could contribute to BPH development.

1.3.3. Prostate Cancer (PCa)

PCa has replaced lung cancer as the most commonly diagnosed male cancer in the UK and is expected to remain so up to 2035 (Smittenaar *et al.*, 2016). It is the second leading cause of cancer related death in men, with over 11,000 documented annually (Cancer Research UK). Although 5-year survival is high (98.6%) for early stage localised PCa, 55-65% of these patients are estimated to develop incurable metastatic disease, for which the 5-year survival is markedly lower (32.6%) (McPhail, 2008; NICE, 2013). The ability to discriminate patients whose disease is likely to advance from those with indolent disease is not currently possible, despite many years' research focus on this objective.

PCa treatment and prognosis is assessed during diagnosis according to the Gleason Pattern Scoring System. First developed in the 1960's by pathologist Dr Donald Gleason (Gleason, 1966), the system is still widely implemented in medicine today. Pathological examination of haematoxylin and eosin (H&E) stained biopsy tissue is evaluated on the basis of architectural features including the degree of similarity of the sample to normal prostate tissue, acinus formation and invasion to surrounding tissues (McNeal and Gleason, 1991). An overall Gleason score (between 2 and 10) is calculated according to the combined major and minor patterns (1 to 5) observed in the sample, and therefore takes into account a degree of the heterogeneity that exists in PCa. Higher Gleason scores are indicative of aggressive/advanced and poorly differentiated disease, increased risk of metastasis and a worse prognosis. For example, tissue where the majority (major pattern) exhibits characteristics fitting with Gleason pattern of 5 and minor pattern of 4, the diagnosis would be Gleason score of 5+4=9. The loss of cellular architecture during in PCa is depicted in Figure 1.3 and Gleason scoring system is demonstrated in Figure 1.4 (Epstein *et al.*,

2016). The stage of disease positively correlates with substantial changes in the local stromal compartment, originally described as a co-evolutionary process (Tuxhorn *et al.*, 2002). This “co-evolution” term and the nature of the Gleason scoring system suggests a transitional progression of PCa, implying that lower Gleason scoring tumours become “more malignant” over time. This is a difficult concept to prove, as repeated biopsies would have a number of limitations, including inaccuracy and a resulting local inflammatory response and cytokine release that would influence tumour growth. An additional theory for PCa progression has been the existence of separate cancer stem cells (CSCs) giving rise to the distinct tumour grades observed in multifocal tumour tissue (Packer and Maitland, 2016). PCa cells are phenotypically luminal, though the CSC theory conveys that populations of basal-like tumour initiating cells (CSCs/TICs) residing in the niche uncontrollably give rise to progeny of luminal epithelial cancer cells (Maitland and Collins, 2005). This hierarchical model is well defined in haematological malignancies, which have been better studied due to the accessibility of peripheral blood (Bonnet and Dick, 1997). It proposes that only a fraction of the tumour cells are able to initiate tumours and are therefore responsible for recurrence post-treatment (Boman and Wicha, 2008; Maitland and Collins, 2008).

As with embryonic development, normal function and BPH, AR signalling is important in the early stages of PCa. Activation of the AR upon binding of DHT or testosterone results in translocation to the nucleus, where it mediates transcription of AR response genes such as prostate specific antigen (PSA) and promotes survival and proliferation of luminal cells. In patients whose disease progresses beyond locally confined disease after radical prostatectomy (RP) or radiotherapy, androgen deprivation therapy (ADT; e.g. Enzalutamide) is used to prevent AR mediated tumour cell survival. Consistent with this, ADT is effective in dramatically reducing the size of prostate tumours. However, this is a transient effect, and ultimately almost every patient will become refractory to ADT and develop what is termed castrate resistant prostate cancer (CRPC). Response to ADT is monitored by measuring serum concentration of PSA, where increases in PSA are indicative of revival of AR signalling and resistance to ADT. PSA had been considered a useful PCa biomarker, instigating a PCa screening program, although this has had considerable controversies associated due to inaccuracies and has since been advised against (Moyer, 2012). CRPC and metastatic-CRPC (mCRPC) is incurable and treatments are mainly palliative, e.g. chemotherapy (docetaxel and abiraterone) or bone directed radiotherapy and bisphosphonates to ease bone pain (due to the high propensity of PCa to metastasis to the bone).

Like with BPH, cytokine release by infiltrated immune cells is associated with progression of cancer due to many of the same signals previously described. Packer and colleagues describe CSC cytokine addiction as an initiator of positive feedback loop that contributes to the development of PCa (Packer and Maitland, 2016). PCa cells gain the ability to produce cytokines (such as IL-6 and IL-8) and in doing so establish an autocrine loop that facilitates their expansion. It is observations such as these that have supported the inflammatory cancer theory, which is the

role of chronic inflammation in promoting development of cancer. This is a concept explored throughout this thesis.

1.4. **Reactive Stroma**

PCa, like many tumours, has cancer-associated localised changes to stroma. Whether these changes are due to either epigenetic or genetic have been investigated, with variable results. Using laser capture microdissection (LCM) and downstream genetic/epigenetic analysis, a number of studies found have found tumour stroma to have some genetic alterations, although the use of LCM could produce results difficult to interpret (Hanson *et al.*, 2006; Hu *et al.*, 2005). It is unclear exactly what signals may cause the stromal compartment to change local to the tumour or indeed the source of the transformed stromal cells (i.e. whether they arise due to differentiation of resident mature stroma, local MSC progenitors or BMSC) (Ishii *et al.* 2015). Nevertheless, the presence of this tumour microenvironment is absolutely essential for tumour survival, progression and migration (Olumi *et al.*, 1999; Tuxhorn *et al.*, 2002; Yang *et al.*, 2005). This knowledge has sustained the concept that inhibition of tumour recurrence after treatment could be achieved by undoing the cancer-promoting changes in the tumour stroma (Hiscox *et al.*, 2011).

The altered stromal compartment local to PCa is referred to as reactive stroma. This can be characterised by increased myofibroblast frequency (also referred to as cancer associated fibroblasts (CAFS)), increased expression of TGF- β , loss of SMCs and ECM remodelling (Barron and Rowley, 2012; Tuxhorn *et al.*, 2002). Development of a reactive stromal compartment though, is not necessarily tumour specific and occurs in many conditions with an associated inflammatory component. An increased proportion of myofibroblasts/CAFs is found in a variety of physiological conditions, including wound healing (granulation tissue), PIN and BPH (Darby *et al.*, 2014; Schauer and Rowley, 2011; Tuxhorn *et al.*, 2001; Xue *et al.*, 1998). Most strikingly, reactive stroma is similar in phenotype to tertiary lymphoid tissue (TLT) stroma, suggesting the associated inflammatory signals have a significant impact on stromal phenotype (Peduto *et al.*, 2009). TGF- β 1 has been found to induce conversion of fibroblasts to myofibroblasts *in vitro* and *in vivo*. Potentially then, the development of reactive stroma may be an indirect consequence of cancer and could be the result of increased inflammation.

In comparison to normal and BPH associated stroma, PCa stromal cells have been shown to provide functionally distinct roles to the corresponding epithelial cells (and vice versa). Hall and colleagues characterised 3-dimensional (3D) co-cultures of prostate stroma and epithelial cells from BPH and PCa diagnosed patients in collagen gels, which revealed intrinsically different features (Hall *et al.*, 2002). PCa stroma demonstrated a reduced capacity to contract collagen gels when co-cultured with BPH epithelium. This effect was reversed when PCa epithelium populated the surface. Correspondingly, migration of prostate epithelium into collagen gels was governed by the stroma within the gel. BPH stroma but **not** tumour stroma permitted single cell tumour epithelial invasion. Contrastingly, BPH epithelium migrated and formed acini-like structures in

BPH stromal gels but not tumour stroma gels. Altogether this study conveyed that tumour stroma de-regulated BPH epithelial organisation but was also able to **prevent** tumour cell invasion, whereas BPH stroma was not. It is clearly evident from this study that epithelium and stromal cells are less able fulfil normal function when in close range to counterparts from different diseases, indicating differential signalling. Studies *in vitro* and *in vivo* have demonstrated more drastic changes imparted on epithelium by tumour stroma. Hayward's and Cunha's labs have contributed considerably to prostate stroma studies. They demonstrated that only when human prostate CAFs are grafted with human immortalised BPH epithelial cells (BPH-1) into the renal capsule of mice, are tumours able to arise and neither could form a tumour in isolation (Cunha *et al.*, 2002; Hayward *et al.*, 1998). Similar to the described study by Hall *et al.*, this was unique to tumour stroma and did not occur when BPH stroma and BPH-1 epithelium were mixed. Moreover, they demonstrated that tumour stroma induced neoplastic growth of non-tumourogenic (BPH-1) prostate epithelium (Hayward *et al.*, 2001). Reactive stroma is not present in immunodeficient mice, indicating a requirement for immune cells for its initiation.

A number of mechanisms may account for these described functional differences between normal, BPH and PCa stromal cells. Metallomatrix proteases (MMPs) are commonly found to be upregulated in CAFs compared to the normal equivalents, providing a mechanism for invasion by degradation of the basement membrane and ECM fibres (Stearns and Stearns, 1996). Reactive stromal cells are known expressers of chemokines and cytokines, which may invoke autocrine and paracrine signals between stroma and epithelium promoting tumour proliferation and migration (Jung *et al.*, 2010; Orimo *et al.*, 2005). An emerging field of exosome-mediated communications has led to increased investigation of tumour stroma derived exosomes. Some early investigations find that prostate stroma-derived exosomes containing microRNA may modulate prostate tumour cell resistance to therapy (Fletcher *et al.* unpublished). They can be potent expressers of growth factors such as fibroblast growth factor (FGF) and hepatocyte growth factor (HGF), which can reportedly directly induce invasion of tumour cells and select for a CSC-like phenotype respectively (Henriksson *et al.*, 2011; Vermeulen *et al.*, 2010). Expression of AM may also influence migratory capacity of tumour stroma and consequently tumour cells. Lakins *et al.* demonstrate that increased expression of podoplanin, ICAM1 and VCAM1 (similar to TLT stroma) corresponds to increased migration by tumour stroma (Lakins, 2012). This phenotype was mimicked in BPH and high passage stroma when treated with IL-4, LT β & TNF α , although the migratory capacity of high adhesion molecule (i.e. inflammatory cytokine treated) BPH stroma was not investigated. The authors suggest that migration of tumour stroma formed a path of least resistance, allowing tumour cells to follow sequentially. *Stromal cell phenotype in BPH and PCa will be explored further in Chapter 5.*

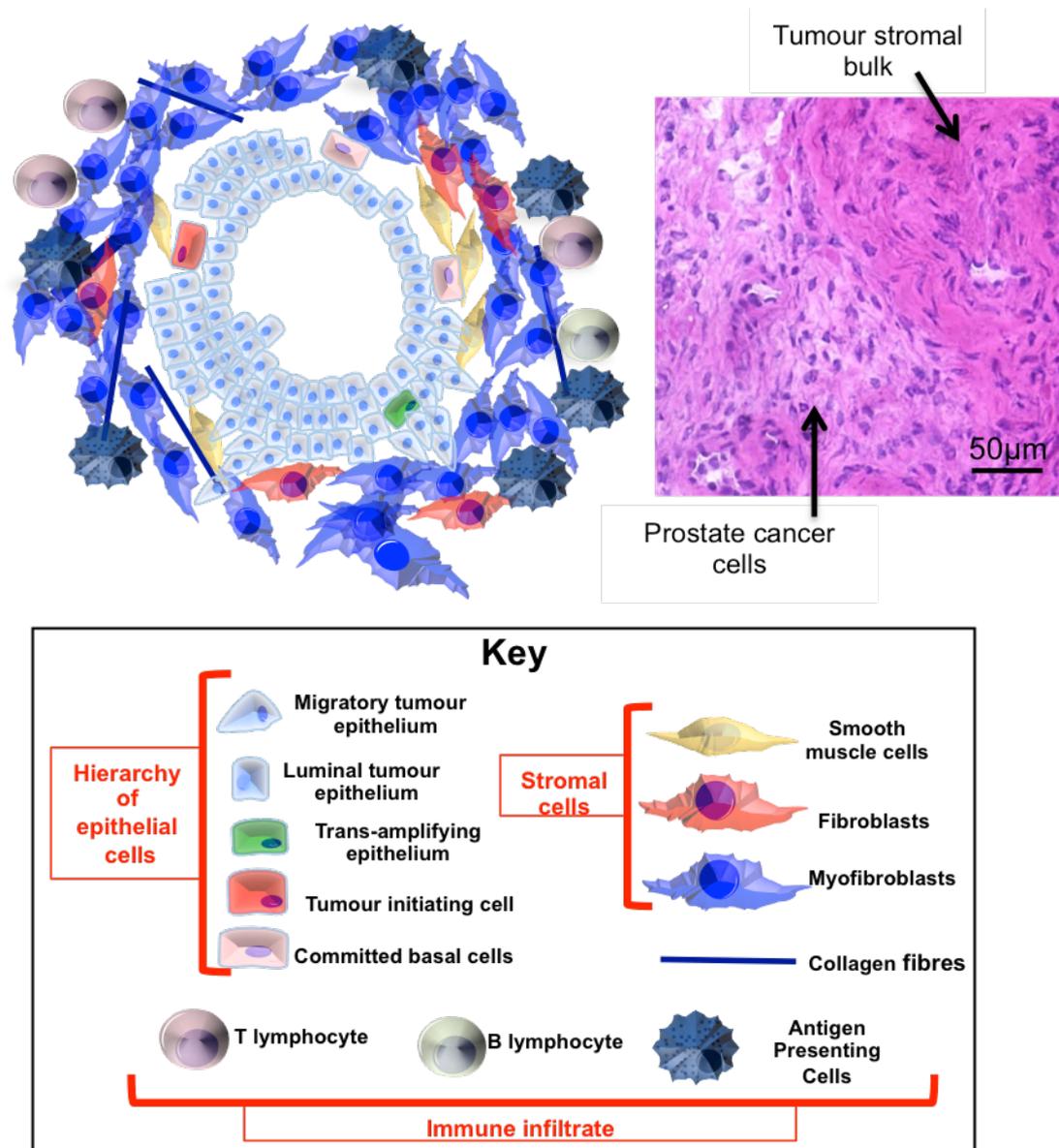


Figure 1.3: Prostate cancer results in a loss of normal architecture

Prostate cancer involves over expansion of the epithelium and loss of the basement membrane. Although the cancer cells adhere to the luminal phenotype they expand due to over-proliferation of tumour initiating cells giving rise to luminal progeny at an increased, uncontrolled rate. Loss of the basement membrane means malignant cells can invade the stromal compartment. Notable changes occur in the stromal compartment including infiltration of immune cells, accretion of myofibroblasts and loss of SMCs. An example of histology of high Gleason grade PCa is shown on the top right. High grade PCa is characterised by the complete loss of structure. Epithelial tumour cells grow in sheets, becoming mixed with activated stromal and immune infiltrating cells. Although the cells are mixed in the image, the bottom left corner contains mostly tumour cells and the top right corner mostly stromal cell, recognisable by the high amount of collagen fibres (dark pink).

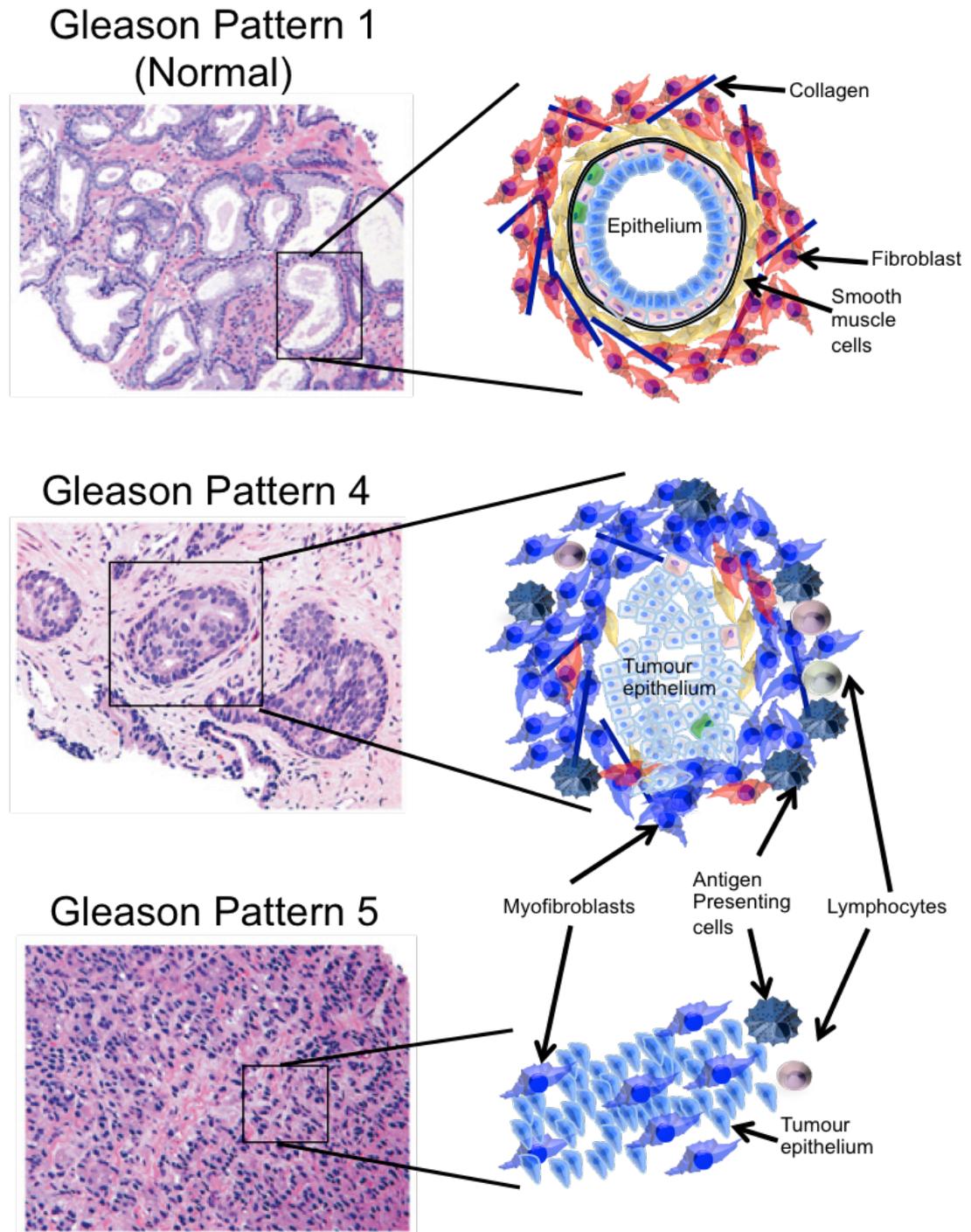


Figure 1.4: Gleason Pattern Grading System

Gleason Pattern 1: Neoplastic tissue is well differentiated and most similar to normal prostate tissue. Glands are well packed and formed.

Gleason Pattern 2: Glands are large and well-formed but have more stromal tissue between.

Gleason Pattern 3: Glands stain darker and show signs of randomised structure. They seem to be invading surrounding tissue.

Gleason Pattern 4: Glands may be poorly formed and cribriform glands may be present with a few recognizable glands.

Gleason Pattern 5: There are no recognizable glands mostly cribriform glands are present. Cells with distinct nuclei appear in sheets.

H&E images taken from: (Epstein *et al.*, 2016)

1.5. **Tumour Immune evasion**

The ability of cancer cells to evade immune mediated destruction became a new addition to the original hallmarks of cancer more than a decade after first introduced (Hanahan and Weinberg, 2011). Tumour immune escape occurs when any step in the cancer-immunity cycle fails, as a result, there is an absence in mounting an adequate immune response and so cancer presents clinically (Chen and Mellman, 2013; 2017). Failures in the immunity cycle can equally result in loss of self-tolerance, when an unwarranted immune response is triggered against self-peptide resulting in autoimmunity. Hence, a subtle equilibrium exists, which if disturbed either way is detrimental to the host. It is the necessity of this balance that makes cancer (as aberrant self-cells) a challenge for the immune system. For an effective anti-cancer T cell response to be initiated, a number of steps must occur, each reliant on various factors. Cancer cells must express recognisable antigens, which can be processed and presented by antigen presenting cells (APCs) tasked with immune surveillance (Galon *et al.*, 2013; Zitvogel *et al.*, 2013). Upon priming and activation in SLO (tumour draining LN), CTLs migrate and infiltrate tumour tissue. Cancer cells expressing cognate antigen on Major Histocompatibility Complex (MHC) I molecules are specifically recognised and targeted for immune mediated death, and so releasing further cancer antigens, propagating the cycle (Figure 1.5).

Needless to say, microevolution of the tumour can result in evasion of immune mediated cell death at any point in the cycle, particularly accentuated in the presence of moderate immune pressure, allowing for “immune editing”. Alternatively, immune cells can be actively restrained (rather than evaded) so that effector T cell function is prevented, consequently resulting in reduced tumour cell killing (Motz and Coukos, 2013). This may be accomplished by a number of means including: over representation of T regulatory cells (Treg); reduced expression of pro-inflammatory cytokines; over expression of inhibitory and checkpoint molecules; loss of MHCI expression on cancer cells; altogether resulting in a tolerogenic, rather than immunogenic, response. Tumour immunogenicity can be defined by the propensity of tumour cells to be recognised by immune cells (Blankenstein *et al.*, 2012). It can be ranked according to the amount of distinctive cancer-antigens, as well as the degree of similarity to self-antigens and thereby controls the power of the immune response. Melanoma is one of the most immunogenic tumours in humans, characterised by a high degree of mutations and strong immune responses. In contrast, prostate tumours have one of the least detectable mutagenic burdens, slow growth and low immunogenicity. This therefore makes PCa one of the more difficult cancer types for immune cells to “see” and intercept.

Cancer immunotherapy aims to devise a way to harness the killing ability immune cells possess to target cancer (Mellman *et al.*, 2011). One such way has been neutralisation of immune checkpoint inhibitors, which has had variable success in clinical trials, partly due to the accompanying adverse effects. Inhibition of the cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and programmed death-1 (PD-1) pathways have shown particular promise in clinical trials for a number of malignancies, the largest achievement perhaps being melanoma (Krummel

and Allison, 1995; Parish, 2003; Prieto *et al.*, 2012). In prostate cancer specifically there has been little success with immune checkpoint inhibition, with a recent phase III trial showing no difference in placebo and Ipilimumab (anti-CTLA-4) treated patients (Kwon *et al.*, 2014a). The use of immunotherapy in PCa is explored in section 4.2. Adoptive T cell transfer has also proven successful for melanoma (Kalos and June, 2013). This is when immune cells are extracted from the patients' tumour or tumour draining LN with the appreciation that a proportion of lymphocytes homing to these sites have specificity for tumour-specific antigens (TSAs) or tumour-associated antigens (TAAs). They are expanded *ex vivo* and reintroduced back to the original patient; in a fraction of patients either partial or complete responses (PR; CR) have been achieved. Cancer vaccines could be utilised and particularly useful in patients who have failed to mount an adequate immune response, compared to those exhibiting immunosuppressive tumour microenvironment (Palucka and Banchereau, 2013; van den Boorn and Hartmann, 2013).

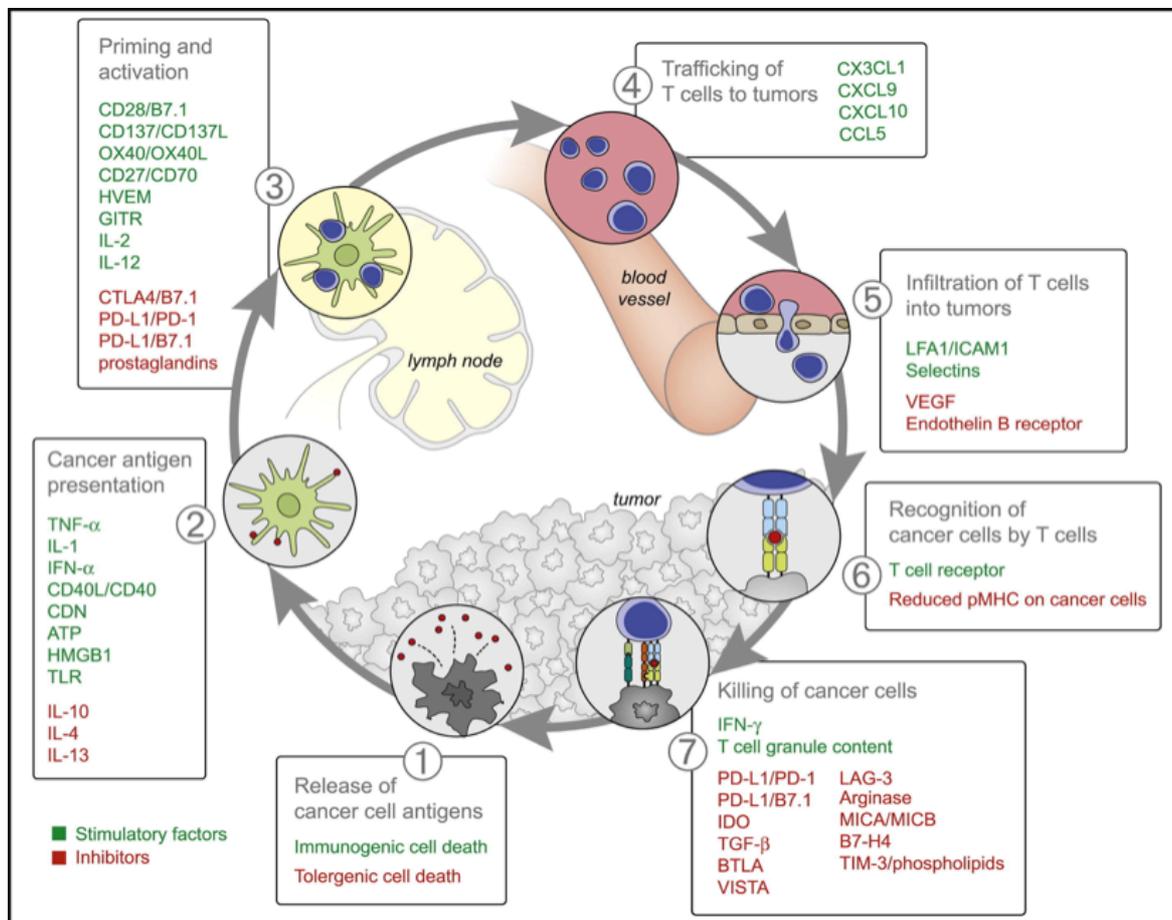


Figure 1.5: Cancer immunity cycle and potential mediators

For activation of anti-tumour immunity tumour cells must express unique cancer antigens (TSA or TAA), which are presented on APCs at the tumour draining lymph node. Here they interact with cancer specific lymphocytes in order to induce adaptive immunity. T lymphocytes migrate to the tumour where they recognise cancer antigens on tumour cells inducing T cell mediated killing. This process may be inhibited at any point and result in failure of effective adaptive immunity. Figure taken from: (Chen and Mellman, 2013)

1.6. **Interferon- γ (IFN γ)**

Interferons were so named due to the ability to interfere with viral infections. IFN γ is the only member of the type II IFNs and differs from the Type I family in chromosomal location and lacks any sequence homology (Pestka *et al.*, 2004). Upon dimerization it binds and signals through a heterodimeric receptor formed of IFN γ R1 (which confers ligand binding capacity) and IFN γ R2 (the signal transducing component), and transmits signal predominantly through the JAK-STAT1 (Janus Kinase- Signal transducer and activator of transcription) classical pathway (Figure 3.2) (Bach *et al.*, 1997; Pestka *et al.*, 1997). IFN γ dependent transcription is reliant on IFN γ activated site (GAS) promoter elements in genes, which are bound by phosphorylated STAT1 (pSTAT1) homodimers (Darnell, 1997; Darnell *et al.*, 1994). This canonical model of IFN γ (and many other cytokines) signal transduction has been enduring until recently when a non-canonical model was introduced (Johnson *et al.*, 2013). The authors suggest IFN γ R1-IFN γ -JAK/STAT1 complexes are endocytosed and translocate to the nucleus to permit specific transcription of IFN γ response genes. The non-canonical pathway addresses issues surrounding how enumerable cytokines that transmit through the same signalling molecules go on to induce different results. IFN γ can also result in signalling via other pathways, such as PKC δ (via PI3K). PKC δ facilitates phosphorylation of the STAT1 homodimer at the Serine residue position 727 and this is required for transcriptional regulation of IFN stimulated genes (ISGs) (Huang and Lee, 2003). When this phosphorylation is inhibited, fibrosarcoma cells have an increased susceptibility to apoptosis by the chemotherapeutic drug Etoposide (DeVries *et al.*, 2004). Depending on the signalling pathways that are co-stimulated, certain transcription factors will be recruited to STAT1 in the nucleus. For example, recruitment of C/EBP β by MEK-ERK signalling allows transcription of IFN γ activated transcriptional elements (GATEs), which have been implicated in promoting IFN γ mediated cell death (Gade *et al.*, 2008; Roy *et al.*, 2000). A less appreciated feature of IFN γ is its strong binding to the glycosaminoglycan (GAG) heparin sulfate (HS), which comprise part of the ECM (Saesen *et al.*, 2013). By binding to HS, IFN γ forms repositories so that concentrations vary immeasurably within tissues. In this form IFN γ is protected from proteolytic degradation so increasing IFN γ functionality.

In the context of PCa, IFN γ has been shown to negatively impact tumour cell invasive capacity by repressing Annexin2, an adhesion molecule that facilitates cell-ECM interactions (Hastie *et al.*, 2008). Fang *et al.* demonstrated in a STAT1-mTOR dependent manner, IGFBP-3 sensitised prostate tumour cells to IFN γ induced cell death (Fang *et al.*, 2008). Interestingly, there may be a link between presence of cytokine in the microenvironment and progression of PCa to androgen independence prostate cancer (AIPC); when AIPC cell lines were treated *in vitro* with nerve growth factor (NGF) and IFN γ in combination, there was a loss of proliferation, increased apoptosis and reduction in AI associated with downregulation of fibroblast growth factor receptor 2 (Chen *et al.*, 2012). In a small clinical study of 10 CRPC patients treated with immunotherapy, clinical benefit (assessed by reduction in PSA levels) was positively correlated with serum

concentrations of IFN γ , indicating the therapeutic benefit was due to increased immunity (Yuan *et al.*, 2009). Importantly though, out of the 8 patients that did respond, all suffered adverse effects associated with autoimmunity.

While there is evidence IFN γ can directly induce tumour cell death, it mainly contributes to anti-tumour immunity by indirect mechanisms. Classic IFN γ induced genes include the chemokines CXCL9, CXCL10 and CXCL11, which propagate inflammation most probably not redundantly but rather synergistically and temporally (Groom and Luster, 2011; Singh *et al.*, 2003). These chemokines induce chemotaxis of further inflammatory CXCR3 positive T cells. IFN γ further stimulates (1) the proliferation of CTL, NK and TH1 cells and (2) preferential differentiation of TH1 cells from naive T cells. MHC I molecules are also inducible through IFN γ , which may paradoxically increase immune recognition of tumour cells through presentation of tumour antigens and facilitate tumour cells to inhibit NK cells directly (Fruh and Yang, 1999; Zhou, 2009). Of note, IFN γ has been associated with the upregulation of a number of immune inhibitory molecules including checkpoint ligands and IDO, an enzyme that indirectly inhibits T cells by depleting local amino acid availability (Zaidi and Merlino, 2011).

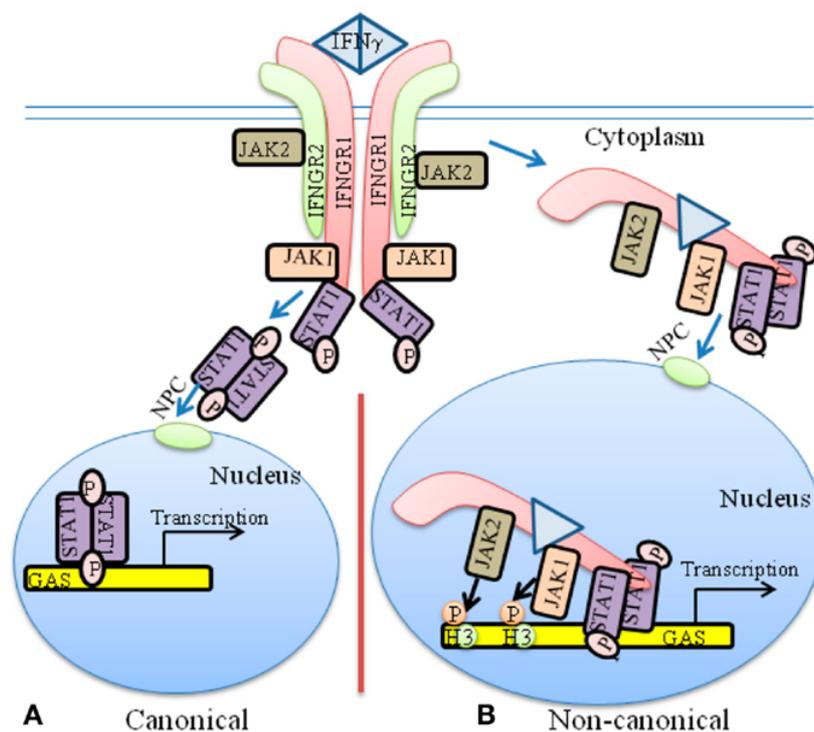


Figure 1.6: The canonical and non-canonical pathways of IFN γ signaling.

Homodimeric IFN γ binds to the heterodimeric receptor consisting of IFNGR1 and IFNGR2 and initiates either canonical (A) or non-canonical (B) signaling. In the canonical signaling model, receptor ligation results in phosphorylation of JAK1/JAK2 and recruitment of STAT1. STAT1 forms a phosphorylated homodimer, which translocates to the nucleus and modulates transcription of IFN γ response genes. In the non-canonical model the IFNGR1 receptor is internalised upon ligation and forms a complex of IFN γ /IFNGR1/JAK1/JAK2/pSTAT1, which is actively transported to the nucleus. Here, it mediates transcription of IFN γ response genes. Figure taken from: (Johnson *et al.*, 2013).

1.7. **Interleukin-4 (IL-4)**

TH2 cells produce IL-4, which has pleiotropic effects, but its role in suppressing effector TH1 functions gained it an anti-inflammatory reputation (Cohn *et al.*, 2001; Sadick *et al.*, 1990). IL-4 binds to the heterodimeric IL-4 receptor composed of an IL-4-R α subunit and either the common γ chain (γ c) (Type-I) or the IL-13R α 1 subunit (Type-II), allowing activation of variable downstream signalling pathways (Figure 3.3) (He and Malek, 1995; Johnston *et al.*, 1994; LaPorte *et al.*, 2008; Obiri *et al.*, 1995). Expression of the γ c subunit is normally restricted to haematopoietic cells, whereas the IL-4R α and IL-13R α 1 subunits have broader expression profiles (Orchansky *et al.*, 1999; Ul-Haq *et al.*, 2016; Witthuhn *et al.*, 1994). IL-4 receptor ligation in either form induces responses through STAT6 homodimer mediated transcription (Malabarba *et al.*, 1996; Rolling *et al.*, 1996). In Type-I receptor signalling JAK1/JAK3 precedes this and so can activate both STAT6 and insulin receptor substrate-2 (IRS-2), whereas in Type-II signalling JAK1/JAK2 and Tyk2 transduce the signal to STAT6 (Malabarba *et al.*, 1996; Murata *et al.*, 1998; Rolling *et al.*, 1996; Schnyder *et al.*, 1996). In T cells, STAT6 activates Gata3, the master regulator of TH2 differentiation (Ranganath *et al.*, 1998). To induce survival and proliferation, STAT6 lessens cyclin-dependent kinase inhibitor 1B (CDKN1B) mediated inhibition of cell cycle (Liu *et al.*, 2000). IL-4 mediated activation of STAT6 in B cells induces Immunoglobulin class switching, promoting IgE and IgG1 antibodies (Gascan *et al.*, 1991).

While IL-4 potently represses IFN γ expression and TH1 effector functions, it can also be considered pro-inflammatory due to strong associations with pathological allergic responses (Grunewald *et al.*, 1998). Mechanistically this is due to the requirement of TH2 cells for B cell class switching (Foote *et al.*, 2004; Morris *et al.*, 2000). Therefore, imbalance in IL-4 results in overrepresentation of TH2 cells, increased B cell activation and antibody production, leading to pathological disease mediated through humoral immunity. IL-4/STAT6 mediated transcription induces upregulation of AM such as VCAM1, particularly synergistically with TNF α (Iademarco *et al.*, 1995; Thornhill *et al.*, 1991). This effect initially directly associated IL-4 signalling with extravasation of leukocytes to inflamed tissues and in cancer has been associated with increased tumour cell migration and invasion to the vasculature (DeNardo *et al.*, 2009; Li *et al.*, 2008). Moreover, it has been shown to increase survival in tumour cells, and there is evidence for increased clonogenic potential in PCa CSCs as a result of IL-4 (Nappo, 2016; Prokopchuk *et al.*, 2005; Roca *et al.*, 2012). This is of particular clinical significance as IL-4 and IL-4R α is increased in PCa patients (Wise *et al.*, 2000). Further, immunosuppressive cytokines, including IL-10 and TGF- β are inducible by IL-4 and strongly inhibit effector immune cell proliferation, activity and increases regulatory immune cell activity (Fiorentino *et al.*, 1989).

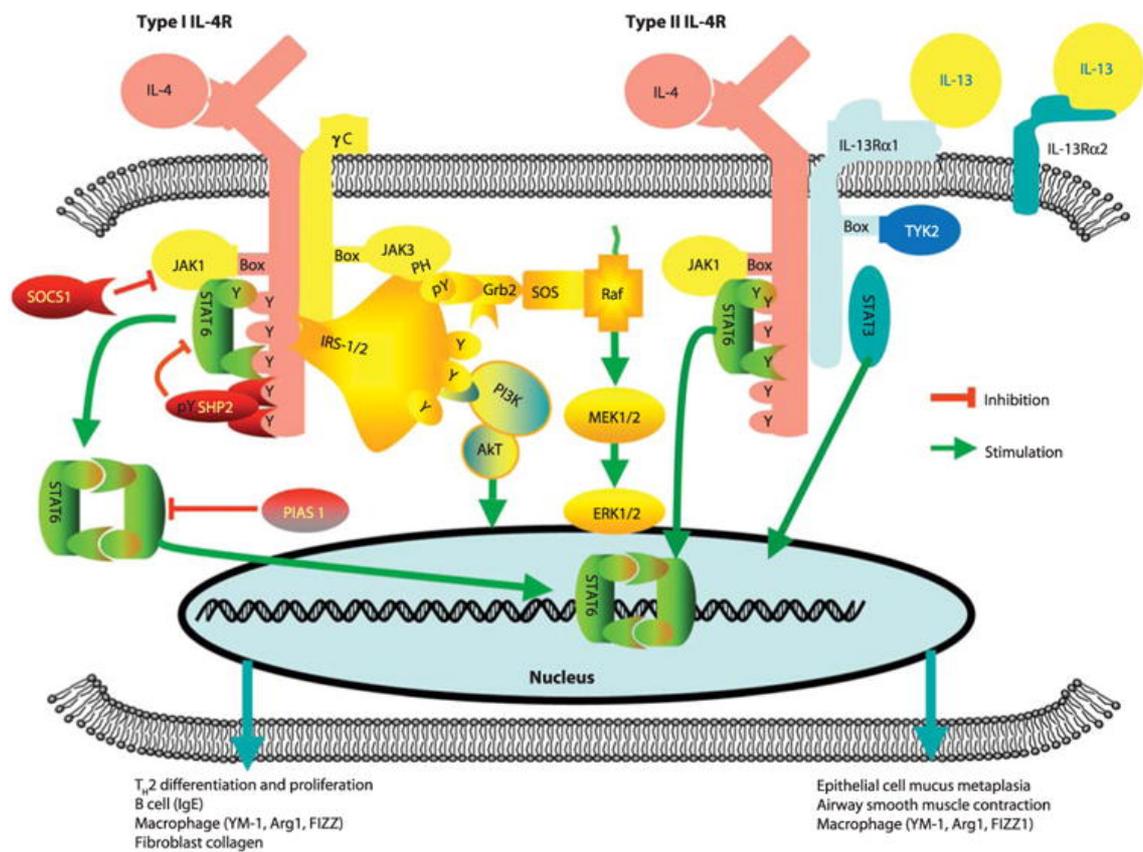


Figure 1.7: The potential mechanisms of IL-4 signalling

IL-4 signalling can occur through either Type I or Type II receptors. The type I receptor is a heterodimer of γ_c and IL-4R α . Both IL-4 and IL-13 can initiate signalling via the type II receptor constructed of IL-4R and IL-13R α 1. IL-13R α 2 has specificity for IL-13 only and is largely thought to act as a “decoy”. IL-4 mediated activation of the type I receptor stimulates JAK1/JAK3 signalling and formation of pSTAT6 homodimer. Binding of IL-4/IL-13 to the type II receptor results in activation of JAK/Tyk2 and pSTAT6. pSTAT6 modulates transcription of IL-4/IL-13 response genes. Figure taken from: (Wills-Karp and Finkelman, 2008)

1.8. **Tumour necrosis factor- α (TNF α)**

TNF α belongs to the TNF superfamily and is so named due to evidence of direct apoptotic/necrotic effects on tumour cells (Beutler and Cerami, 1988; Oettgen *et al.*, 1980). It can be produced by TH1, CTL and APCs to name a few, indicating the probability of its magnitude in many inflammatory scenarios including tumours (Mukhopadhyay *et al.*, 2006). However, it has been implicated in divergent biological processes, exhibiting remarkable dual functionality (Bertazza and Mocellin, 2010). The reason for this may depend on the contextual environment, as it has been shown to complement other cytokines synergistically (Badalyan *et al.*, 2014; Dong *et al.*, 2013; Enderlin *et al.*, 2009; Ray *et al.*, 2009).

TNF α has dual specificity for receptors TNFR1 and TNFR2, which may alternately/additionally account for these contradictory roles (Wajant *et al.*, 2003). TNFR1 and TNFR2 have differential expression and specificity; TNFR1 binds only TNF α and is expressed in most cell types, while TNFR2 can bind both TNF α and TNF β , and its expression is more restricted (Figure 3.4) (Grell *et al.*, 1995; Li *et al.*, 2002; Ware *et al.*, 1991). Transmitting signals through both its cytoplasmic death domain (DD) and indirect activation of the TNFR-associated factor (TRAF) signalling molecules means that TNFR1 can induce both apoptosis and survival. The DD of TNFR1 induces activation of 3 main mediators of apoptotic cell death; receptor-interacting protein 1 (RIP1), Fas-associated death domain protein (FADD) and TNFR1-associated death domain protein (TRADD) (Chen *et al.*, 2008; Hsu *et al.*, 1996; Hsu *et al.*, 1995; Tartaglia *et al.*, 1993). Concurrently, TNFR1 can activate TRAF2 through TRADD, which may coordinate with RIP1 to induce NF- κ B transmitting a pro-survival signal rather than apoptosis (Mahoney *et al.*, 2008; Micheau and Tschopp, 2003). Further pro-survival and proliferative signals occur when TNF α signalling induces TRAF2-JNK activation and AP-1 mediated transcription, leading to expression of inflammatory cytokines, cell growth and proliferative signals (Brach *et al.*, 1993; Dixit *et al.*, 1989; Rothe *et al.*, 1995a). The outcome of TNF α signalling (either activation of the apoptotic or anti-apoptotic arms) likely depends on a number of factors, be it the cell type, surrounding niche or additional signalling occurring, including co-activation of TNFR2. TNFR2 transduces through TRAF2, activating the NF- κ B and AP-1 transcription factors to promote survival (Rothe *et al.*, 1995b). Activation of both receptors is thought to invoke a functional crosstalk mechanism through the shared TRAF2 mediator. TNFR2 activation transduces through TRAF2 but also promotes its degradation (Arch *et al.*, 2000; Wu *et al.*, 2005). This causes depletion of TRAF2 from both TNFR1 and TNFR2 signalling pathways, causing preferential activation of the pro-apoptotic arm TNFR1 activation (Cabal-Hierro and Lazo, 2012; Rodríguez *et al.*, 2011).

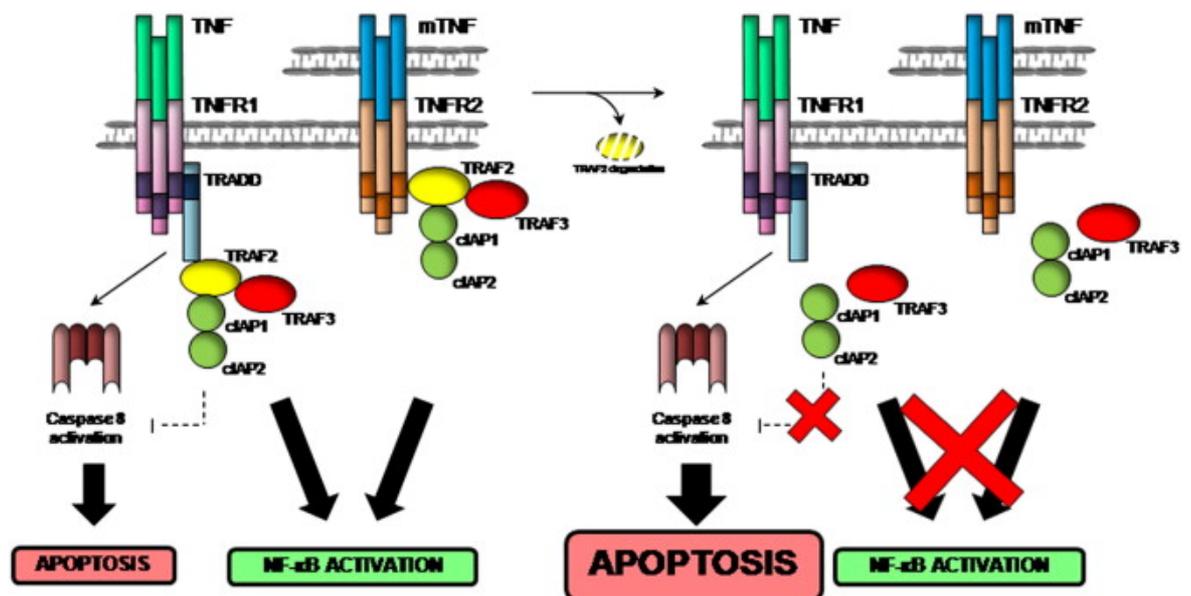


Figure 1.8: TNF α mediated signalling.

TNF α initiates signalling through trimeric TNFR1 or TNFR2. TNFR1 ligation results in the recruitment of TRADD to its cytosolic death domains (DD), which initiates signalling via recruitment and activation of Fas-associated DD protein (FADD) leading to activation of pro-caspase-8 and apoptosis. Alternatively, TRADD may activate signalling via TRAF-2 leading to NF- κ B activation, promoting cell survival. Co-activation of TNFR2 by TNF α results in TRAF2 activation (and NF- κ B) followed by TRAF2 degradation. Intracellular depletion of TRAF2 causes preferential activation of the TNFR1/FADD arm and apoptosis. Figure taken from : (Cabal-Hierro and Lazo, 2012)

1.9. **Cytokine signalling in PCa**

Due to the historical links of inflammation and tumourigenesis, there have been a number of cytokines that have been associated with the development, progression and survival of PCa cells (De Marzo *et al.*, 2007). These links have been supported by *in vitro* cell line experiments, *in vivo* xenograft studies and correlations of TME cytokine and receptor expression in patients with relapse and progression in many tumour types. In PCa IL-1, IL-6 and TGF- β are commonly linked with tumour progression. The physiological source of these cytokines has been elusive due to contradictory results and lack of reproducibility in different systems. It is conceivable that the differences in the inflammatory environment are a major contributing factor to these inconsistencies as many of these cytokines are regulated by other inflammatory signals. The human immune system is one of the multifaceted systems in biology and undeniably a powerful one. This attribute makes reproducing the same inflammatory environment incredibly difficult. Immune complexity additionally makes it difficult to separate homeostatic inflammatory responses contributing to resolving pathology from deregulation in inflammatory signals contributing to pathology. More often correlations made between inflammatory signals and disease progression are suggested as a good therapeutic targets, however many notorious complications have occurred and continue to occur when therapies are used to interrupt the immune system without fully understand the repercussions it might involve.

The IL-1 family encompasses eleven cytokines. IL-1 α and primarily IL-1 β are the most studied and both are produced as precursor proteins by a wide variety of cell types, including macrophages, fibroblasts and epithelial cells (Auron *et al.*, 1984; Lomedico *et al.*, 1984; Palomo *et al.*, 2015). While IL-1 β requires proteolytic cleavage (e.g. by caspase-1) for activity, IL-1 α can transduce signalling in both the immature and mature form (Guma *et al.*, 2009; Martinon *et al.*, 2002; Thornberry *et al.*, 1992). This second level of regulation means that IL-1 β cannot immediately initiate an inflammatory response, but instead requires stimulation that controls its maturation (e.g. via NF- κ B). IL-1 α on the contrary, functions as a damage-associated molecular pattern (DAMP) and can be released by necrotic or damaged cells, or secreted in either the immature or mature form (Afonina *et al.*, 2011; Cohen *et al.*, 2010). Cleavage of pro-IL-1 α by the Ca²⁺ activated calpain releases ppIL-1 α that can translocate to the nucleus and serve as a transcription factor, so fulfilling dual functions (Kobayashi *et al.*, 1990; Werman *et al.*, 2004). IL-1 α and IL-1 β bind to the receptor IL-1R1, which lacks a cytosolic domain and so requires recruitment of IL-1 receptor accessory protein (IL-1RAP; also known as IL-1RAcP) a co-activator that transmits activating signals downstream (Greenfeder *et al.*, 1995). IL-1 is known for initiating inflammation when damage occurs in the absence of pathogen infections (i.e. and lack of TLR ligation) (O'Neill, 2008). IL-1R1 ligation instigates signalling via MyD88, IRAK, TRAF6 and ultimately activation of NF- κ B and AP-1 (Muzio *et al.*, 1998). IL-1R1 therefore facilitates a feed forward loop by prompting transcription of pro-inflammatory cytokines including IL-1, IL-6, IL-8 and COX-2 (Tszuzaki *et al.*, 2003). Due to this potency, the IL-1 pathway requires several levels of

regulation. Both IL-1 α and IL-1 β can bind to IL-1R2, which lacking capacity to transmit signal, serves as a decoy receptor (Colotta *et al.*, 1993; McMahan *et al.*, 1991). IL-1R2 and IL-1RAP, as soluble proteins, can regulate IL-1 signalling in the extracellular space (Smith *et al.*, 2003; Symons *et al.*, 1995). Additionally, IL-1R1 can bind a third ligand IL-1 receptor antagonist (IL-1RN; also known as IL-1RA), an endogenous antagonist that competes with the actuating ligands, preventing signal activation (Dripps *et al.*, 1991).

Autoimmune pathology has been attributed, in part, to over activation of the IL-1 pathway. Consistent with this, therapeutic use of Anakinra (IL-1RN) in patients with autoimmune rheumatoid arthritis provides in part substantial clinical benefits (Bresnihan, 2002; Dayer *et al.*, 2001). *In vivo* studies of IL-1 signalling in cancer have indicated a cancer-promoting role, with associations with angiogenesis, growth and metastasis (Elaraj *et al.*, 2006). Polymorphisms in the IL-1 family have increased associated risk for PCa (Xu *et al.*, 2014). Inhibition of IL-1 α and loss of IL-1R1 reduces hepatocarcinoma burden (Sakurai *et al.*, 2008). Immunohistochemical staining of normal, BPH and PCa tissue revealed increased progression free survival in patients with high IL-1 expression (in both stroma and tumour) but low IL-1R1 expression, indicative of low reciprocal signalling (Torrealba *et al.*, 2017). To understand how this mechanistically might occur, *in vitro* and *in vivo* studies have investigated the effects of IL-1 on tumour cells. IL-1 β has been identified as a factor that promotes colon cancer cell epithelial to mesenchymal transition (EMT) and consequently metastasis and CSC transformation (Li *et al.*, 2012b).

IL-6 is a pleiotropic cytokine. It induces both pro- and anti- inflammatory outcomes, as well as direct effects on cell survival and differentiation (Scheller *et al.*, 2011). It can be stimulated in inflammatory responses via either TLR or IL-1 ligation (Nackiewicz *et al.*, 2014; O'Hara *et al.*, 2012). IL-6 can directly inhibit IL-1 signalling through the induction of IL-1RN, acting as a negative feedback regulator of IL-1 induction. As well as effects on IL-1, it induces IL-10 expression and inhibits TNF α , gaining rank as an immunosuppressive or anti-inflammatory cytokine more than a pro-inflammatory cytokine (Terai *et al.*, 2012). Through expression of anti-apoptotic molecules such as bcl-xl and direct inhibition of the “guardian of the genome” p53, the IL-6 pathway can support cell survival (Schwarze and Hawley, 1995; Yonish-Rouach *et al.*, 1991). For signal transmission, it binds to the ligand-binding component of the IL-6 receptor IL-6R α . Recruitment of the signal-transducing component IL-6ST (also known as gp130) permits signalling through the JAK-STAT pathway, primarily via STAT3 (Guschin *et al.*, 1995; Hibi *et al.*, 1990; Zhong *et al.*, 1994). As well as the classical cytokine signalling process, soluble IL-6R α -IL-6 complex can initiate signals in IL-6ST expressing cells (Jones *et al.*, 2001).

Expression of IL-6 is associated with pathology of numerous diseases including rheumatoid arthritis (RA), SLE and cancer; in fact, the first FDA approved anti-IL-6 drug was in the treatment of RA (Ishihara and Hirano, 2002). In cancer it is associated with virtually every step of cancer

development: malignant transformation, tumour growth and progression (Grivennikov and Karin, 2008; Santer *et al.*, 2010; Smith and Keller, 2001). Like IL-1, IL-6 is overexpressed in many tumours including melanoma and PCa (Royuela *et al.*, 2004; Shariat *et al.*, 2001; Valles *et al.*, 2013). In the TME, expression of IL-6 by endothelial cells is proposed to improve the tumourigenicity of CSCs in head and neck squamous cell carcinoma (HNSCC) (Krishnamurthy *et al.*, 2014). EMT is thought to be a direct consequence of IL-6 on tumour cells from a variety of tissues, with increases in vimentin expression (fibroblast marker), loss of E-cadherin (epithelial adhesion protein) and increased migration (Miao *et al.*, 2014).

1.10. Transforming growth factor- β (TGF- β)

Upon translation, a homodimer of TGF- β is bound to latency associated protein (LAP) and with/without latent TGF- β binding protein (LTBP) form an immature complex lacking biological activity (Gentry *et al.*, 1988; Gleizes *et al.*, 1997; Miyazono *et al.*, 1988; Wakefield *et al.*, 1988). LAP is required for the secretion of TGF- β ; together these molecules form the small latent complex (SLC) (Lopez *et al.*, 1992). The large latent TGF- β complex (LLC) is comprised of SLC bound to LTBP. Although a larger molecule, the LLC is secreted at a much faster rate than the SLC, indicating the involvement of LTBP in the secretion process (Miyazono *et al.*, 1991). In comparison, SLC lacking LTBP is largely retained in the golgi body (Miyazono *et al.*, 1992). Together this indicates that in the absence of LTBP, although there is some availability of TGF- β as part of the SLC, this is likely to be much lower than that if LTBP is expressed and the LLC can be formed (Olofsson *et al.*, 1992). LTBP also confers binding of LLC to the ECM (Olofsson *et al.*, 1995; Taipale and Keski-Oja, 1997; Taipale *et al.*, 1994). It is capable of directly binding to ECM proteins fibrillin-1 and fibronectin and can therefore provide a way to sequester TGF- β , particularly in an ECM rich organ such as the prostate (Dallas *et al.*, 2005; Isogai *et al.*, 2003).

Latent TGF- β activation (the release of TGF- β from LLC or SLC) can be achieved through a number of proteolytic enzymes (e.g. MMPs, plasmin), physiochemical perturbations within the microenvironment (e.g. pH or reactive oxygen species), or by binding of thrombospondin or integrins to the complex. Proteases that are known to specifically cleave LAP and so release TGF- β include MMPs (MMP-2 and MMP-9) and plasmin (Lyons *et al.*, 1990; Sato and Rifkin, 1989; Yu and Stamenkovic, 2000). Both of these MMP enzymes have been found to be increased in tumour stroma compared to normal, which may account for the increased TGF- β activity in these conditions. However, proteolytic cleavage may not be a major mechanism of TGF- β activation in vivo and instead significant evidence indicates nonspecific interactions with LLC/SLC in the ECM an important contributor to TGF- β activation (Bugge *et al.*, 1995; Munger *et al.*, 1999). Integrins are transmembrane proteins that allow the adhesion of cellular cytoskeleton to ECM proteins and are so involved in cell migration. Integrins bind to the RGD motif of LAP on the extracellular surface and upon a second interaction between the cytoskeleton and the cytoplasmic domain of the

integrin; TGF- β becomes released from the latent complex through a conformational change (Munger *et al.*, 1999). Thrombospondin-1 (THBS-1) is a homotrimeric glycoprotein that can mediate adhesion of cells to either neighbouring cells or ECM components through binding to an array of molecules including ECM components integrins, heparin, fibrinogen and collagen as well as the cell surface receptor CD36. THBS-1 has been shown to activate TGF- β by binding to LAP and liberating TGF- β for receptor interactions (Crawford *et al.*, 1998; Schultz-Cherry *et al.*, 1994a; Schultz-Cherry *et al.*, 1994b).

Of the 3 TGF- β isoforms (TGF- β 1, β 2 and β 3), TGF- β 1 is most studied (Bierie and Moses, 2006; Siegel and Massague, 2003). All 3 isoforms signal through the same receptors (TGF- β R1 and TGF- β R2) and the downstream class of signalling molecules SMADs (Figure 3.5) (Wrana *et al.*, 1994). It is not completely understood how the 3 isoforms confer different roles apart from differing spatially and temporally (Kubiczkova *et al.*, 2012). Prostate epithelial and stromal cells express TGF- β isoforms: while TGF- β 1 is expressed by epithelium and fibroblasts (and becomes upregulated in myofibroblasts), TGF- β 2 and - β 3 are expressed by the prostate epithelium.

TGF- β has paradoxical effects both on different cell types and on different stages of cancer (Roberts *et al.*, 1985; Roberts *et al.*, 1986). In healthy tissues TGF- β inhibits proliferation of epithelial cells, while having the opposite effect on stromal cells (Bottinger *et al.*, 1997; Clark *et al.*, 1997; Massague *et al.*, 2000; Xiao *et al.*, 2012; Zenzmaier *et al.*, 2015). These contradictory outcomes translate to TGF- β fulfilling both tumour suppressor and tumour promoting roles in malignancy. In malignant transformation, tumour cells become refractory to TGF- β mediated growth arrest and instead continue to proliferate in the presence of high levels of TGF- β , which corresponds to correlation of TGF- β R loss with PCa progression and bad prognosis (Bottinger *et al.*, 1997; Kim *et al.*, 1998; Levy and Hill, 2006; Wikstrom *et al.*, 1998; Zhao *et al.*, 2005). The change in TGF- β signalling in high grade tumour cells (but not normal and benign epithelium and low grade neoplastic cells) is thought to be due to a progressive shift in the tumour cell population and accumulation of tumour cells with inactivating mutations in TGF- β pathway, consistent with loss of the cytostatic effects of TGF- β (Kim *et al.*, 1998; Levy and Hill, 2006; Wikstrom *et al.*, 1998). This, consistent with clinical findings, is likely to result in upregulation of TGF- β ligands in the TME (Perry *et al.*, 1997). TGF- β is central to pathological fibrosis due to the stimulation of transdifferentiation of normal fibroblasts to myofibroblasts, this may also contribute to the acquirement of tumour stroma in PCa and other cancers (Evans *et al.*, 2003). True to the paradoxical effects of TGF- β , overactivating aberrations in the TGF- β signalling pathway resulting tumour promoting effects to include TGF- β mediated epithelial-mesenchymal transition (EMT) in tumour epithelial cells, a prerequisite for tumour progression to metastatic disease (Giampieri *et al.*, 2009; Mima *et al.*, 2013). In addition to the direct impact on tumour epithelial cells, TGF- β is an established immunosuppressive cytokine (as mentioned in the introduction to this chapter), an

attribute which likely accounts for the majority of TGF- β mediated pro-tumour consequences (Yamagiwa *et al.*, 2001). Treg cells are professional immune inhibitors, and fulfil many of these roles through the expression of TGF- β . This directly prevents CTL mediated killing, reduces the capacity of APCs to induce T cell activation and prevents T cell proliferation, so represents a prevalent molecule of the immunosuppressive arm of the anti-tumour immunity balance (Figure 3.1) (Chen *et al.*, 2005; Yang *et al.*, 2010).

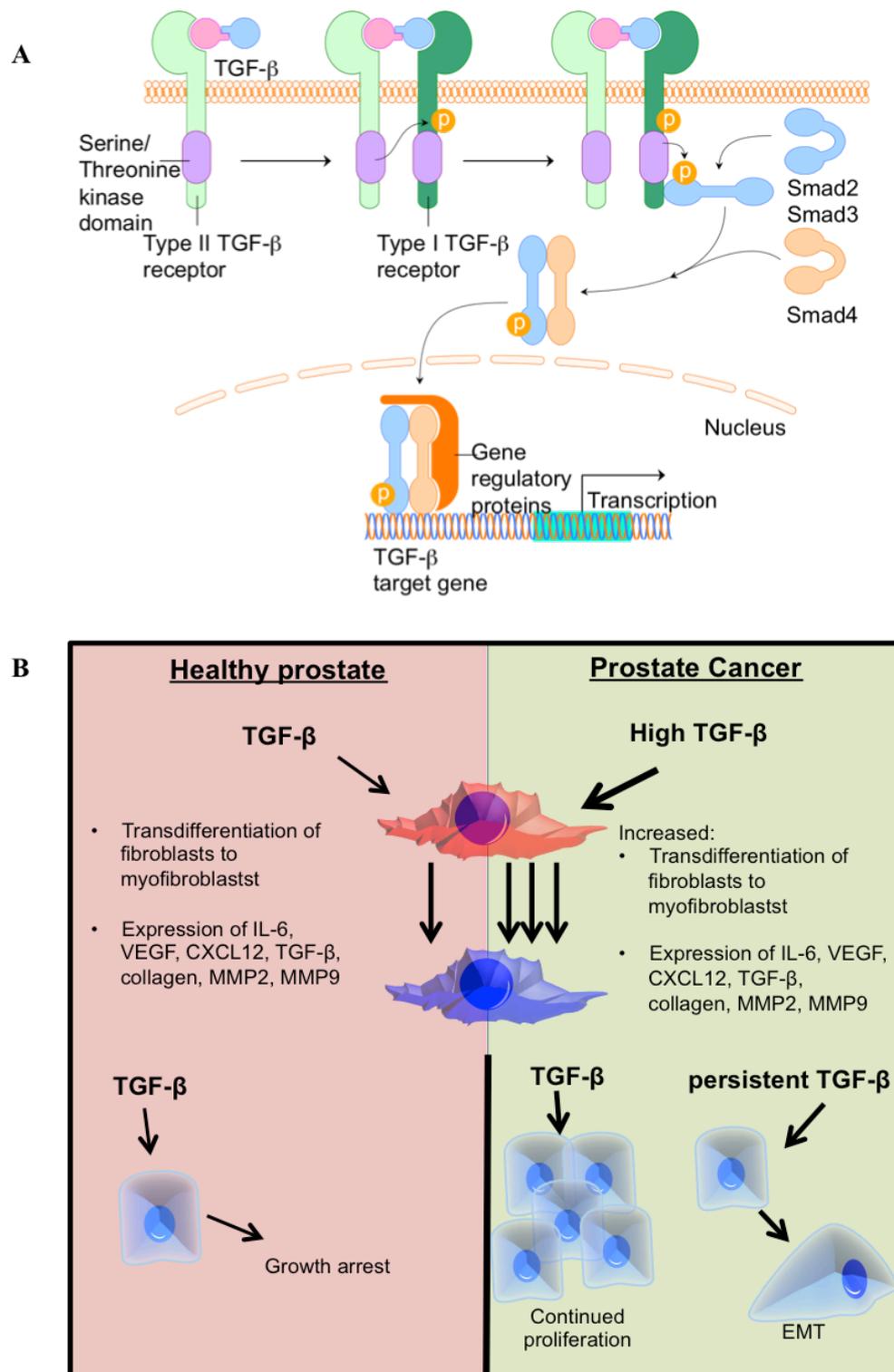


Figure 1.9: TGF- β mediated signalling in the prostate

(A) TGF- β ligation to a heterodimeric receptor of type I and type II TGF- β receptors initiates signal transduction by phosphorylation of SMAD2/SMAD3. pSMAD2/3 forms a heterodimer with SMAD4 which mediates transcription of TGF- β response genes. Schematic diagram made using motifolio ®.

(B) TGF- β signalling in the healthy prostate modulates growth of prostate epithelium. In prostate cancer TGF- β concentrations are elevated. Epithelial cells become resistant to TGF- β mediated growth arrest and undergo EMT. With amplified TGF- β signalling fibroblasts increase transdifferentiation to myofibroblasts and expression of IL-6, VEGF and MMP, which are often associated with tumour promoting properties.

1.11. **Major histocompatibility complexes class I and II**

T cells are activated when they recognise antigen presented on the MHC molecules on the cell surface of presenting cells (Rock *et al.*, 2016). In humans MHC is known as the human leukocyte antigen (HLA) and is one of the most highly polymorphic protein families in the human genome (S. Beck, 1999). The MHC class of proteins are both highly polygenic (there are more than 200 genes) and remarkably polymorphic. The inherited MHC variants are expressed equally, rather than in a dominant/recessive fashion, which allows thousands of allelic variants to be expressed in an individual. This polymorphism occurs in the region encoding the peptide binding groove, allowing MHC molecules to bind a very broad range of peptides and so provides an inherent mechanism to combat the variability existing in the pathogenic world (Falk *et al.*, 1991; 1994; Schmid *et al.*, 2010).

MHC molecules are ligands for the T cell receptors (TCR). During development, T cells become tolerised to all host proteins, which ensures that upon recognition of peptides derived from *mutated* self-proteins and *pathogenic* organisms they are activated (Klein *et al.*, 2014). CD4 and CD8 (used for characterising the TH and CTL subsets of T cells respectively) dictate whether MHC class I or MHCII molecules are recognised. MHC class I bound to intracellular protein fragments is expressed on all nucleated cells and are recognised by the CD8/TCR on CTLs. MHC I therefore provides CTLs with a window into cells to determine whether a threat exists (i.e. infection and mutation). In humans the MHC I molecules are transcribed from the *HLA-A*, *-B*, *-C*, *-E* and *-G* genes, classified as classical (*-A*, *-B* and *-C*) and non-classical (*-E* and *-G*) MHC I molecules. Whereas classical (MHC Ia) molecules are capable of initiating immune responses via presenting peptide to the TCR, non-classical (MHC Ib) are better known for inducing immune tolerance by interacting with inhibitory receptors on effector cells (Braciale, 1992; Le Bouteiller and Lenfant, 1996).

MHCII, on the other hand, is expressed mainly by professional APCs, presenting both intracellular and extracellular (via endocytosis) peptides. It is recognised by CD4/TCR on TH cells. The MHCII molecules that present antigens on the cell surface are transcribed from the genes *HLA-DP*, *-DQ* AND *-DR*. T cell activation is a tightly controlled process, requiring more than simple recognition of antigen and is explored in more detail in the upcoming chapter. T cells require co-stimulation to gain a license to kill, and are only to take action against cells expressing their cognate antigen in the absence of inhibitory signals. One of these inhibitory receptors expressed by T cells, belonging to the immune checkpoint family is the lymphocyte activation gene-3 (LAG-3) (He *et al.*, 2016). LAG-3 out-competes CD4 and upon binding to MHCII molecules delivers an inhibitory rather than an activation signal to the T cell (Triebel *et al.*, 1990).

1.12. Checkpoint Inhibition

Checkpoint inhibition provides a means of down-regulating the immune response in order to both promote self-tolerance and prevent collateral damage during on-going inflammation. T cells express a variety of checkpoint molecules on the cell surface involved in this response. The PD-1/PD-L1/PD-L2 axis plays an important part in down-regulation of T cells in peripheral tissues. For the purpose of this thesis the main focus was on PD-L1. To fully appreciate the relevance of this pathway it is necessary to review the physiological process of generating an immune response from T cell priming to effector activity. Simplified schematic representations of this are illustrated in Figures 4.1-4.3.

1.12.1. Checkpoint inhibition; physiological relevance

T-cell priming occurs in the secondary lymphoid tissue (Figure 4.1). Here, naive T cells encounter APC's expressing cognate antigenic peptide in the groove of a MHC molecule on its surface. Together with antigen recognition, T cells require co-stimulatory (e.g. CD28-CD80/CD86) and cytokine (IL-2) signals (termed signals 1, 2 and 3 respectively) producing fully active T cells that clonally expand and mount an antigen-specific response (Favero and Lafont, 1998; Goldrath and Bevan, 1999). Upon activation, T cells will: (i) secrete cytokines mediating their effector function e.g. IFN γ (ii) upregulate PD-1, IL-2R and chemokine receptors on the cell surface and (iii) enter the circulation to home to inflamed sites. Having upregulated PD-1, effector T cells become susceptible to PD-L1/PD-L2 mediated inhibition (Freeman *et al.*, 2000; Ishida *et al.*, 1992; Keir *et al.*, 2007; Latchman *et al.*, 2001). Alternatively, in Treg cells the PD-1: PD-L1/PD-L2 axis promotes proliferation and prevents cell death. It is further thought to promote the conversion of naive CD4⁺ T cells to inhibitory Treg, overall supporting tolerance over immune activation (Francisco *et al.*, 2009; Wang *et al.*, 2008). The general consensus has been that PD-1 co-inhibition was more important in the effector phase (i.e. at peripheral tissues) and a second checkpoint inhibitor CTLA-4 mediated inhibition only in the priming phase (Figure 4.2) (Fife and Bluestone, 2008; Keir *et al.*, 2006; Masteller *et al.*, 2000; Parry *et al.*, 2005). However, data has suggested that PD-1 ligation during the initial priming phase can have profound effects on the fate of T cell function during the effector phase (Goldberg *et al.*, 2007). Indications now suggest that both of these inhibition pathways are more complex than first thought, so better understanding of the basic immunology will help to progress the field.

Structurally, PD-1 (CD279) contains an IgV extracellular domain, a transmembrane domain and an intracellular domain. Upon ligation, the intracellular domain with an immunoreceptor tyrosine-based inhibitory motif (ITIM) and immunoreceptor tyrosine-based switch motif (ITSM) becomes phosphorylated and capable of recruiting the SHP-1 and SHP-2 phosphatases (Chemnitz *et al.*, 2004). SHP-2 dephosphorylates the CD3 ζ chain, hence mitigating further TCR signalling (Yokosuka *et al.*, 2012). Additionally, SHP-2 can inhibit co-stimulatory (CD28) mediated PI3K

activation and phosphorylation of signalling molecules downstream of the T cell receptor CD3 (e.g. ZAP70), effectively preventing further antigen recognition effector outcomes (IFN γ , TNF α and IL-2 secretion). PD-1 is upregulated on the surface of T cells upon antigen recognition, particularly in the absence of co-stimulation (Day *et al.*, 2006; Tewalt *et al.*, 2012). Therefore in chronic inflammation, antigen-specific T cells are repeatedly exposed to antigen inducing high PD-1 expression and are termed “exhausted” (Barber *et al.*, 2006; Day *et al.*, 2006). Due to this high PD-1 expression, there is a greater *capacity* for PD-1 mediated inhibition during chronic inflammation than initial antigen recognition. Although exhausted T cell activity only becomes impaired upon repeated PD-1 ligation i.e. exhausted (PD-1 high) T cells are only anergic (impaired activity) in the presence of ligands PD-L1 or PD-L2.

PD-L1 (B7-H1;CD274) can be induced by many cell types, whereas PD-L2 (B7-DC;CD273) expression is mainly restricted to professional APCs (Huber *et al.*, 2010; Kim *et al.*, 2005; Rozali *et al.*, 2012). *In vivo* chimera experiments demonstrate that, in peripheral tissue, PD-L1 expression by endothelial cells can maintain tissue tolerance in the absence of APC's (Rodig *et al.*, 2003). The IRF-1 response element on the PD-L1 promoter means that IFN γ can modulate its expression, so in the presence of IFN γ secreting CTL and TH1 cells, PD-L1 upregulation may induce tolerance (Lee *et al.*, 2006; Loke and Allison, 2003). Particularly in a milieu where co-inhibitory molecules are high and co-stimulatory molecules are low, immune cells are more likely tolerised to antigen and so less able to induce cell death (Harding *et al.*, 1992; Hawiger *et al.*, 2001). Although PD-L1 has dual specificity for both PD-1 and CD80, its affinity for PD-1 is greatest (dissociation constant $K_d=7.7\mu\text{M}$ for PD-1/PD-L1 vs $18.8\mu\text{M}$ for CD80/PD-L1) (Cheng *et al.*, 2013). PD-L2 has a higher affinity for PD-1 ($2.2\mu\text{M}$) than PD-L1 and no affinity for CD80. The physiological relevance of CD80 (B7-1): PD-L1 ligation is still being elucidated. Some reports identify CD80 as a PD-1 substitute and that PD-L1 can inhibit T cells through CD80 signalling as well as PD-1, potentially making PD-L1 inhibition a better therapeutic target than PD-1 (Park *et al.*, 2010). PD-L1/PD-1 inhibition occurs even in the absence of TCR ligation (i.e. T cell antigen recognition) though the level of inhibition may inversely correlate with the potency TCR signal. Kaiser et al demonstrate that in the presence of low levels of antigen (i.e. upon resolution of infection or in the case of a non-immunogenic tumour such as PCa) PD-1 high CD8+ T cells are most susceptible to PD-1/PD-L1 mediated inhibition (Kaiser *et al.*, 2012).

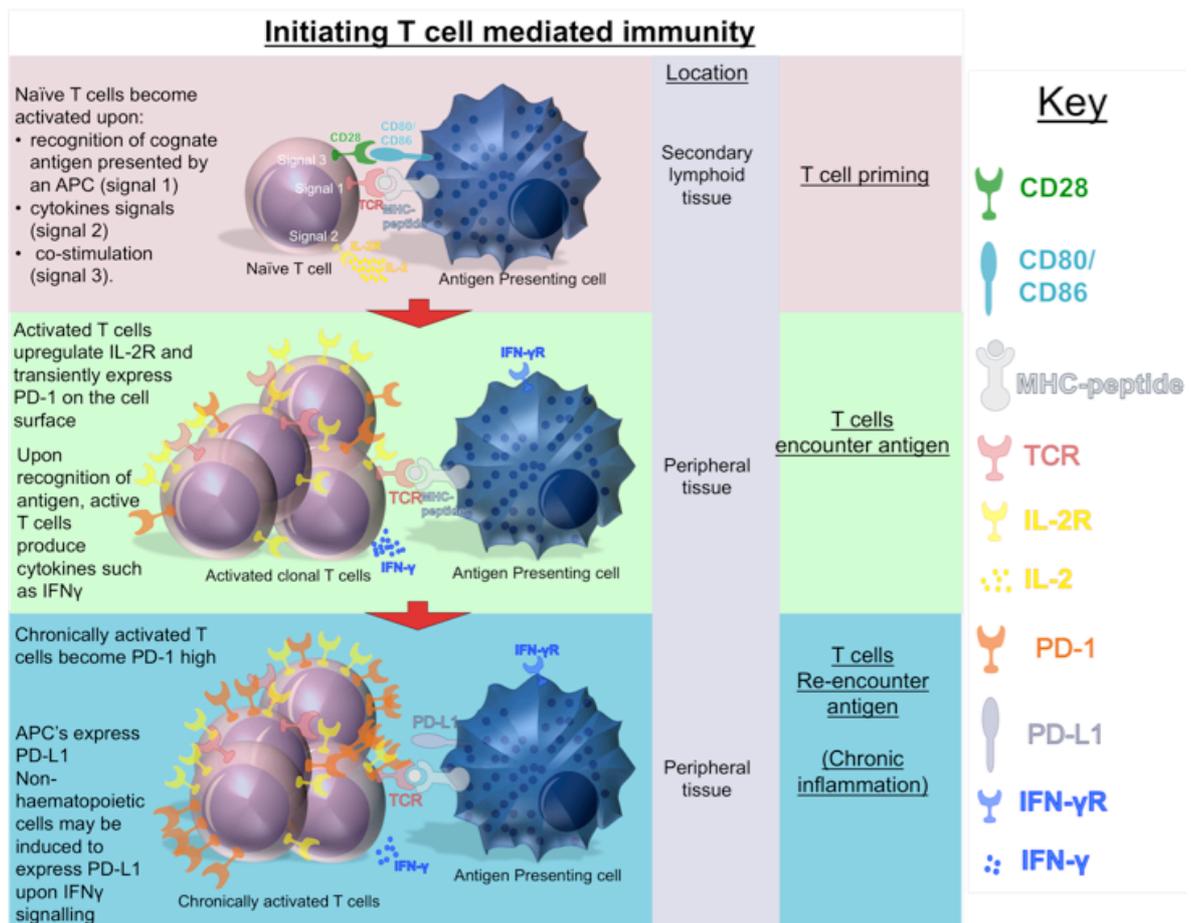


Figure 1.10: Simplified summary of T cell mediated immunity

T cells residing in T cell zones of lymph nodes encounter APCs presenting processed antigen on MHC molecules. When T cells encounter their cognate antigen, receive cytokine signals and co-stimulation they become activated, clonally expand and migrate to the periphery. Upon activation, T cells upregulate IL2R and transiently express PD-1 on the cell surface. Upon recognition of antigen in peripheral tissue, T cells release cytokines such as IFN γ . In conditions like chronic infections or unresolved inflammation where T cells continue to antigen, they become PD-1 high.

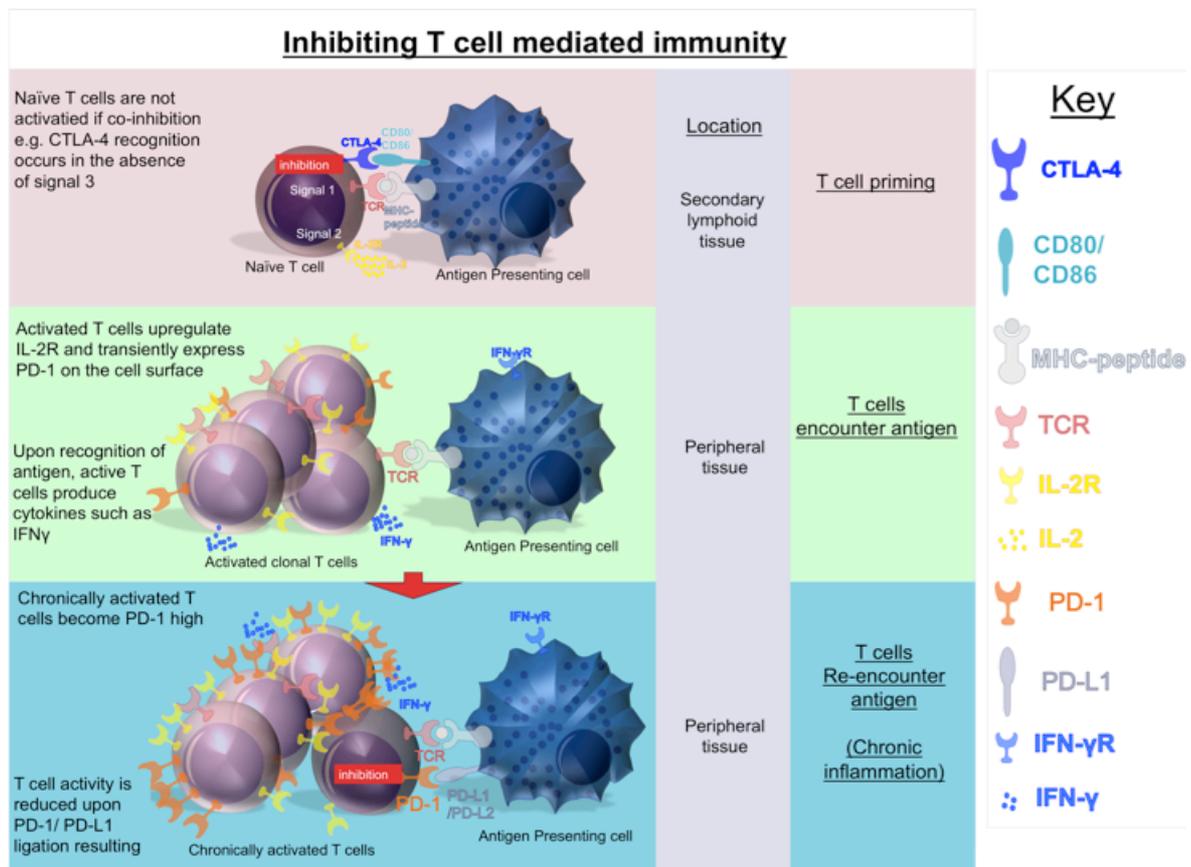


Figure 1.11: Simplified summary of T cell inhibition

T cells residing in T cell zones of lymph nodes encounter APCs presenting processed antigen on MHC molecules. T cells are inhibited upon ligation of CTLA-4 in lieu of co-stimulation. Activated T cells upregulate IL2R and express PD-1 transiently on the cell surface. Upon recognition of antigen in peripheral tissue T cells release cytokines such as IFN γ . In conditions such as chronic infections or unresolved inflammation, where T cells continue to be exposed to antigen, they become PD-1 high. PD-1 expression leaves T cells susceptible to PD-L1/PD-L2 mediated inhibition.

1.13. Summary and Aims

The predominant prostate stromal compartment drives prostate embryonic development and together with chronic inflammation, is heavily implicated in the progression of PCa. Stroma-immune interactions are well documented in lymphoid tissue and autoimmune disease, although are not fully explored in PCa. The impacts of immune cell mediators and normal/tumour prostate stromal cells have been investigated separately on epithelial cells. However the role of stromal cells in the context of an inflammatory prostate are less well understood, despite the prevalence of both in PCa. Recently with the emergence of immunotherapy and success rates in some cancers (e.g. melanoma) but not in PCa, there is a growing requirement to better understand the immune environment in the prostate, to which the stromal compartment will provide a fundamental backdrop.

Professor Norman Maitland's lab (Cancer Research Unit, University of York) has access to primary prostate tissue from patients undergoing a TURP or RP for BPH and PCa. The aims of this thesis are to utilise primary prostate epithelial and stromal cells to:

1. Analyse the response of stromal cells in inflammatory environments
2. Evaluate the role of prostate stromal cells in modulating local inflammation
3. Characterise morphology of BPH and PCa stromal cells in inflammatory environments and correlate to what is known about CAFs
4. Develop a method to characterise prostate infiltrating immune cells to understand the potential functional impact of these factors on anti-tumour immunity

1.14. Hypothesis

Prostate stromal cells, in addition to regulating the normal development of the prostate, and providing a supportive environment that allows tumour cells to thrive, may be important in the regulation of local immune activity. In response to inflammatory mediators such as cytokines derived from active immune cells, stromal cells may produce factors involved in either the propagation or inhibition of inflammation. An understanding of this will be an important factor for improving current therapeutic efficacies and stratifying patients based on the features of the stromal compartment. Further, most current treatment paradigms aim to initiate anti-tumour immunity that would improve the efficiency of treatment, therefore an appreciation of the impact this may have in the local environment will be important.

Chapter 2

Materials and Methods

2.1. Cell culture

2.1.1. Prostate stromal cells

Primary prostate stromal cells once extracted from human tissue were routinely cultured in Roswell Park Memorial Institute formulation 1640 (RPMI-1640; Life Technologies) supplemented with 2mM L-glutamine (Gibco, Life Technologies), 10% foetal calf serum (FCS; Hyclone) and 1% penicillin streptomycin (pen-strep; Gibco, Life Technologies). Complete media is termed as R10%.

2.1.2. Human Foreskin Fibroblasts

HFF cells were sourced from ATCC and cultured in Dulbecco's modified eagle medium (DMEM; Sigma-Aldrich) supplemented with 2mM L-glutamine, 15% FCS and 1% pen-strep. Complete media is termed as D15%.

2.1.3. Tonsil stromal cells

Primary tonsil stromal cells were extracted by Emily Taylor and routinely cultured in DMEM supplemented with L-glutamine, 10% FCS and 1% pen-strep. Complete media is termed as D10%.

2.1.4. Prostate epithelial cells

Primary prostate epithelial cells isolated from human tissue were cultured in keratinocyte serum free medium (KSFM; Gibco Invitrogen) supplemented with: recombinant human epidermal growth factor (5ng/ml; Gibco) bovine pituitary extract (BPE; Gibco) 50µg/m, 2ng/ml leukaemia inhibitory factor (LIF; Cambridge Bioscience), 2ng/ml stem cell factor (SCF; Preprotech), 100ng/ml cholera toxin (CT; SLS), 1ng/ml GM-CSF (Milteny Biotec LTD) and 1% glutamine, together herein termed complete KSFM.

All cells were cultured at 37C with 5% CO₂.

2.2. Extracting stromal and epithelial cells from human prostate tissue

Human prostate tissue was procured from Hull hospital (LREC 07/HI304/121), which was collected during TURP or channel TURP (chTURP) procedures and histologically examined. Tissue was freshly processed. A section of tissue was embedded in optimal cutting temperature (OCT; Merck), snap frozen in liquid nitrogen and stored at -80°C. Following rinsing in sterile phosphate buffered saline (PBS), the remainder of the tissue was chopped finely in collagenase solution (1000IU Worthington Collagenase Type I (Lorne Diagnostics) in 7.5ml/g of tissue). Minced tissue was then transferred to a Erlenmeyer flask and incubated overnight shaking (80rpm) on an orbital shaker at 37°C. Digested tissue was first triturated and then passed through a blunt

needle. The solution was centrifuged at 300g for 10 minutes, supernatant discarded and pellet resuspended in PBS. Centrifugation was repeated once and cells resuspended in 10ml RPMI 10% FCS. Differential centrifugation was used to separate stromal and haematopoietic cells from epithelia. The mixture was centrifuged for 1 minute at 800rpm to sediment epithelia, which could be collected using a pipette carefully avoiding the supernatant containing stromal and haematopoietic cells. Centrifugation and epithelia removal was repeated to enrich for individual epithelial and stromal fractions, which were processed further for culture as described in section 2.2.1 and 2.2.2, respectively.

2.2.1. Prostate epithelial cell culture

The combined pellet of epithelial cells was resuspended in PBS, centrifuged 3min 300g and trypsinised by resuspending in 5ml 1X Trypsin-ethylenediaminetetraacetic acid (EDTA; Gibco) and incubated 30mins at 37°C shaking at 80rpm to produce single cell epithelium. 10ml RPMI 10% FCS was added to epithelia to prevent further trypsin activity and the solution was vigorously shaken to mix. Epithelia were centrifuged 10min 300g and resuspended in RPMI 10% FCS. Centrifugation was repeated and pellet finally resuspended in 4ml warmed (37°C) complete KSFM and plated on collagen I coated 10cm plate (BD Biosciences) with 1-2ml irradiated (SIM)-derived 6-thioguanine- and ouabain-resistant (STO) feeder cells.

2.2.2. Prostate stromal cell culture

Stromal cells were resuspended in fresh R10 and added to a T75 tissue culture flask (Corning), left undisturbed for at least 2 days, until attached stroma could be observed (up to 2 weeks). At which point media was removed, replaced with PBS and gently shaken by hand to detach contaminating haematopoietic cells, erythrocytes and dead cells. After colonies of stromal cells were apparent and contaminating haematopoietic cells removed, stromal cells were allowed to become ~80% confluent at which point 1×10^6 cells (5×10^5 cells per vial) were frozen and the rest reseeded for experiments. If less than 1.5×10^6 cells, only 0.5×10^6 cells were frozen. Media (R10) was replaced twice weekly until suitable for subculturing (~80-90% confluent).

2.2.3. Cell subculture

Upon reaching confluency (~80-90%), media was removed and cells were washed liberally twice in sterile Dulbecco's-PBS (D-PBS; no CaCl_2 no MgCl_2 ; PAA) and 2ml or 5ml 1X Trypsin-EDTA added to a T75 flask or T175, respectively. Cells were incubated in trypsin up to a maximum of 7 minutes, although time varied depending on patient and passage number. Cells were monitored for rounding and detachment, once cells were observed to have detached, flasks were tapped to facilitate removal of most cells. Adding 5ml fresh warm R10 terminated trypsin activity and cells were collected, pelleted (centrifugation 5min 300g) and resuspended in 5ml for cell counts. Depending on specific experimental requirements, cells were seeded in 24, 12 and 6 well

plates (VWR). Table 2.1 was used for calculating number of cells to be seeding in different tissue culture plates. For routine passage cells were split 1:3. At least 1 vial of cells was frozen for each passage. All experiments were performed on primary prostate stromal cells below passage 5, most below passage 3.

Dish	Surface Area cm ²	Seeding density (~25%) (x10 ⁶)	Seeding density (~60%) (x10 ⁶)	Confluency (x10 ⁶)	Growth Media
T75	75	0.375	0.9	1.5 -20	10ml
T25	25	0.125	0.3	0.5-0.7	4ml
T160	162	0.8125	1.95	3.5-4.0	17ml
6-well	9	0.05	0.12	0.2	3ml
12-well	4	0.02	0.048	0.08	1ml
24-well	2	0.01	0.024	0.04	0.5ml
Stromal cells per cm²		0.005	0.012	0.02	

Table 2.1: Determining the approximate cell number for seeding at particular densities.

N.B. Cell numbers varied by patient due to inherent differences in typical cell size. Stated cell numbers are an approximation calculated from typical cell counts of cells retrieved from dishes of specified surface area and extrapolating accordingly.

2.2.4. Cryopreservation

Adherent cells were resuspended at 1×10^6 cells/ml in cell-specific culture media after detaching and quantifying cells. 500 μ l cells (0.5×10^6) were transferred to fully labelled 2ml cryovials (Corning). 500 μ l of freezing media (FCS, 20% dimethyl sulfoxide; DMSO; Sigma-Aldrich) was added drop-wise to cells before immediately transferring vials to a Mr Frosty (Nalgene) and storing at -80°C . For long-term storage vials were deposited in liquid nitrogen.

For revival of frozen cells, vials were retrieved from liquid nitrogen (LN_2) and transferred to a 37°C water bath until only a small portion of the cell mix remained frozen. Cells were added to 3ml pre-warmed media and pelleted, resuspended in 10ml warm media and transferred to a T75 flask. The next day media was replaced with fresh warm media so as to discard dead cells.

2.3. Treatment of cultured stroma and epithelia

Treatment media was prepared by diluting cytokines/agonists to the appropriate concentration in pre-warmed cell-specific media and vortexing to ensure even distribution. $\text{IFN}\gamma$ & $\text{TNF}\alpha$ treatment media contained human $\text{IFN}\gamma$ (Preprotech) at 12.5ng/ml and human $\text{TNF}\alpha$ (Preprotech) at 5ng/ml. IL-4 & $\text{TNF}\alpha$ treatment media contained 5ng/ml of both human IL-4 (Preprotech) and $\text{TNF}\alpha$. The stimulator of IFN genes (STING) agonist cGAMP (InvivoGen) was used at 20 μ M. The TLR1-9 agonist kit (InvivoGen) was used at concentrations instructed by the manufacturer. This kit contained - TLR1/2 Agonist: Pam3CSK4,- TLR2 Agonist: HKLM, TLR3

Agonist: Poly(I:C) (HMW), TLR3 Agonist: Poly(I:C) (LMW), TLR4 Agonist: LPS-EK standard, TLR6/2 Agonist: FSL1, TLR7 Agonist: Imiquimod, TLR9 Agonist: ODN1826. Recombinant Human CD14 protein (R&D Systems) and MPL-A (InvivoGen) were used at 1 μ g/ml and 100ng/ml, respectively.

Unless otherwise stated, cells were treated when ~70% confluent. Prior to treating, media was removed and cells rinsed with D-PBS. An appropriate volume (as stated in table 2.1) of treatment media was added gently to cells, which were then cultured for a time period depending on experimental requirements. Cytokine concentrations were chosen following experiments whereby cells treated with particular cytokines were titrated across a range of concentrations and expression of appropriate genes analysed by quantitative real time-PCR (qRT-PCR, section 2.6).

2.4. Clinical data from patients with prostatic disease

Patient code	Disease	Cell type	Age
H135/11	GI9	Stroma	56
H372/13	BPH	Stroma	66
H373/13	BPH	Stroma	82
H385/13	GI9	Stroma	82
H391/13	BPH	Stroma	75
H393/03	GI9	Stroma	55
H396/13	BPH	Stroma	65
H398/13	BPH	Stroma	66
H400/14	BPH	Stroma	81
H427/14	GI9	Stroma	69
H438/14	GI9	Stroma	68
H501/14	BPH	Stroma	59
H503/14	BPH	Stroma	77
H504/14	BPH	Stroma	66
H537/15**	BPH	Stroma	71
H225/12	BPH	Epithelium	63
Y070/09	BPH	Epithelium	86
H507/14	GI7	Epithelium	68
H239/12	GI9	Epithelium	50

**this sample was excluded from analysis as it was identified as an outlier based expression of stromal genes

Table 2.2: Clinical data of samples used throughout this thesis

2.5. RNA isolation

RNA was extracted using the RNAeasy Mini Kit (Qiagen). Media was removed and adherent cells were rinsed twice with sterile PBS before 350 μ L RLT buffer was added. Cells were observed to ensure lysis and transferred to a QIAshredder (Qiagen) for homogenisation. QIAshredders were centrifuged at full speed for 2 minutes, before column was removed, lid replaced and sample transferred to -20°C overnight. The following day the homogenate was thawed and processed using RNAeasy mini spin columns, which isolates and purifies total RNA. The concentration and the quality of purified RNA was determined using a nanodrop spectrometer, where 260/280 ratios indicate quality of purification. Ratios below 1.8 were considered contaminated. RNA samples were stored at -20°C and kept on ice during experiments. RNA was used for downstream analysis by qRT-PCR (section 2.6) and nanoString (section 2.7)

2.6. Quantitative Real Time PCR

2.6.1. Complementary DNA (cDNA) synthesis

RNA samples were diluted to a known concentration in nuclease free water to final volume of 10 μ L in a 0.2mL thin walled microcentrifuge tube. A master mix prepared using the high capacity cDNA reverse transcription kit (Applied Biosciences™) contained (per 10 μ L): 4.2 μ L nuclease free water, 2 μ L 10X Reverse Transcription buffer, 0.8 μ L 25X dNTPs, 2 μ L 10X RT random primers and 1 μ L Multiscribe® Reverse Transcriptase. Per sample 10 μ L of the master mix was added to the 10 μ L RNA solution of known quantity and transferred to a thermocycler PCR machine (SensoQuest) for reverse transcription. The cycle properties were: 25°C 10minutes, 37°C 2 hours, 85°C 5minutes and maintained at 4°C. cDNA was diluted to a known concentration by adding nuclease free water and stored at -20°C.

2.6.2. qRT-PCR reaction

Depending on the gene to be analysed (and the corresponding primer/probe), either Power SYBR® Green PCR Master mix or TaqMan Fast Universal PCR Master mix, no Amperase UNG (Applied Biosciences™) were used. For primers compatible with SYBR® Green a master mix containing: 12.5 μ L of Power SYBR® Green with 1 μ L each of Forward and Reverse Primers and 6.5 μ L nuclease free water was added to each well of a 96-well MicroAmp Optical reaction plate (Applied Biosciences™). Alternatively, for TaqMan probes a master mix contained: 10 μ L TaqMan Fast Universal PCR Master mix, 5 μ L nuclease free water and 1 μ L of appropriate gene expression assay probe. 4 μ L of cDNA (typically correlating to 1ng of original isolated RNA sample) was added to each well in duplicate. *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) expression was analysed and used as an endogenous control (EC) gene, to which genes of interest (GOI) were normalised. Control wells containing the appropriate master mix with either no cDNA or no RNA controls were included for each plate. Reactions were completed on either an Applied Biosystems 7300 Real-Time PCR System (SYBR® Green primers) or Applied Biosystems

QuantStudio 3 System (TaqMan probes). SYBR® Green PCR consisted of 50°C for 2minutes, 95°C for 10minutes and 40 cycles of 95°C 15seconds before 1minute 60°C during which data was collected. A meltcurve followed completion of the reaction in to assess specificity of amplicon production. This involves ramping the temperature from 50°C to 95°C during which the fluorescence is analysed. Upon separation of double stranded DNA fluorescence is reduced. Primers producing a single peak are considered specific.

Table 2.3 Primers and Probes

2.6.3. qRT-PCR analysis

Gene	Forward Primer Sequence	Reverse Primer Sequence
PD-L1	CATCTTATTATGCCTTGGTGTAGCA	GGATTACGTCTCCTCCAAATGTG
GAPDH	AAGGTGAAGGTCGGAGTCAA	AATGAAGGGGTCATTGATGG
Gene	Assay	Company
IDO1	Hs00984148_m1	Applied Biosystems
IDO2	Hs01589373_m1	Applied Biosystems
TGF-β1	Hs00998133_m1	Applied Biosystems
TGF-β2	Hs00234244_m1	Applied Biosystems
TGF-β3	Hs01086000_m1	Applied Biosystems
FAP	HS0090806_m1	Applied Biosystems
GAPDH	Hs02758991_g1	Applied Biosystems

On completion of the reaction an automatic threshold (fluorescence normalised to reference dye) of 0.2Rn was set in the linear phase of the curve so that threshold cycles (Ct) could be determined for GOI and EC genes. The Ct is the cycle number at which the fluorescence (from SYBR® Green of Taqman reporter fluorescence) passed the threshold. The mean Ct of the GOI for duplicate samples were calculated and normalised to mean Ct for the EC (termed the Δ Ct). From this, the $\Delta\Delta$ Ct was calculated by subtracting sample Δ Ct from the Δ Ct of a calibrating sample (e.g. an untreated control). Finally, $2^{-\Delta\Delta Ct}$ calculated the fold change, which were then plotted using Prism 6 (GraphPad). Table 2.4 demonstrates an example of raw data and the calculations used for data analysis.

	PDL1 mean ct (duplicate)	GAPDH mean ct (duplicate)	Relative to EC (dct)	Relative to untreated (ddct)	Fold change (2^{-ddct})
503 UNTREATED	22.025	15.13	6.895	0	1
398 UNTREATED	21.58	15.14	6.44	-0.455	1.3707828
391 untreated	20.655	14.525	6.13	-0.765	1.69937
396 untreated	21.225	14.74	6.485	-0.41	1.32868581
400 untreated	20.59	14.445	6.145	-0.75	1.68179283
503 TREATED	19.855	15.51	4.345	-2.55	5.85634278
398 TREATED	18.89	15.225	3.665	-3.23	9.38267959
391 treated	17.62	14.56	3.06	-3.835	14.2708563
396 treated	18.15	14.85	3.3	-3.595	12.0837807
400 treated	17.965	14.505	3.46	-3.435	10.8152867

Table 2.4 Exemplar raw data acquired from qRT-PCR and calculation

2.7. NanoString

NanoString was used to assess the expression of over 800 immune-related genes in a single reaction for each sample, using the PanCancer Immune panel (NanoString Technologies™).

2.7.1. Patient samples groups size

Prostate stromal cells were the principal cells to be investigated in this project. Stromal cells from 6 BPH patients and 5 G19 PCa patients were analysed in total. Of these patients, 3 BPH and G19 were treated with IL-4&TNF α or IFN γ &TNF α (8hours). This project primarily focused on the stromal response to IFN γ &TNF α , therefore as a comparison, stromal cells from SLO (tonsil) and skin fibroblasts (HFF) were used as controls and analysed in untreated and IFN γ &TNF α treated conditions. To compare prostate stromal cells to prostate epithelial cells, epithelial cells from 4 patients (2 BPH, 1 G17 PCa and 1 G19 PCa) were analysed, again in untreated and IFN γ &TNF α treated conditions. Dr Dominka Butler and Dr Robert Seed seeded prostate epithelial cells at 60% confluency in the Cancer Research Unit, York. Additional biological repeats could not be completed due to financial and sample availability restrictions. For a summary of the cohorts studied by nanoString, refer to Table 2.5, making note of the number of patients/lines included in each cohort. Due to the small number of patients, statistical analysis of individual genes has not been performed. However, genes that were significantly altered are noted in volcano plots.

Cell Type	Tissue source	Cytokine treatment	Number of patients
Stroma	BPH	Untreated	6
Stroma	BPH	IL-4&TNF- α	3
Stroma	BPH	IFN- γ &TNF- α	3
Stroma	G19	Untreated	5
Stroma	G19	IL-4&TNF- α	3
Stroma	G19	IFN- γ &TNF- α	3
Stroma	Tonsil control	Untreated	1
Stroma	Tonsil control	IFN- γ &TNF- α	1
Stroma	HFF control	Untreated	1
Stroma	HFF control	IFN- γ &TNF- α	1
Epithelium	BPH	Untreated	2
Epithelium	BPH	IFN- γ &TNF- α	2
Epithelium	G17	Untreated	1
Epithelium	G17	IFN- γ &TNF- α	1
Epithelium	G19	Untreated	1
Epithelium	G19	IFN- γ &TNF- α	1

Table 2.5: Cohort sizes used for nanoString analysis.

2.7.2. NanoString reaction

For nanoString experiments, RNA concentration was normalised to 20ng/ μ l, permitting analysis of 100ng when 5 μ l RNA was used. RNA was shipped on dry ice to Newcastle University and either immediately processed or stored at -80°C until processing. Kile Green and Anastasia Resteu of the Institute of Cellular Medicine, Newcastle University performed the nanoString reaction using the nCounter Analyser and the PanCancer Immune Profiling Panel. In total, 36 samples were analysed in 3 batches of 12 samples, the first of which I observed. Figure 2.1 is a schematic representation of nanoString processing. In summary, the Cancer Immune Reporter CodeSet and Capture ProbeSet (nanoString Technologies®) were thawed and gently mixed by inverting. These contain target specific sequences covalently bound to a biotin moiety on the 3' end (Capture probe) or a six position visible signal on the 5' end (Reporter probe). Each position on the

reporter probe signal can be one of four colours, a known sequence that corresponds to the target-specific sequence, so as to facilitate detection of specific mRNA molecules later. A mastermix containing 5 μ l hybridization buffer and 3 μ l Reporter CodeSet per sample was aliquoted into individual hybridization tubes (on a strip of 12 tubes), to which 5 μ l RNA (100ng) is added. To this, 2 μ l Capture ProbeSet was added, cap placed and inverted/flicked to ensure even dispersal throughout sample. The strip of hybridisation tubes now containing RNA, Reporter CodeSet and Capture ProbeSet was briefly spun and placed on a thermal cycler pre-heated to 65°C overnight (ramped to 4°C at 16hours) (Figure 2.1, Step 1). During this hybridization period the single stranded target RNA sequence binds by target-specific Capture and Reporter probes to form a double stranded target-probe complex.

The second day is an Automated Process whereby Step Two of Figure 2.1 is completed by magnetic bead-based purification. This involves:

1. Addition of magnetic beads, bound to complementary sequences to the Capture probes, which attach to the unbound portion of the Capture probe (i.e. all unbound Capture probes as well as target-probe complex, but not free Reporter probes). Free Reporter probes are washed away, as are cellular molecules from the RNA sample not bound to a probe.

2. Elution of Capture probes and target-probe complexes from magnetic beads, and addition of magnetic beads with sequence complementary to the free portion of the Reporter probe. In this step, target-probe complexes bind the beads, but Capture probes do not and are therefore washed away.

3. Finally, target-probe complexes are eluted from magnetic beads, leaving a purified solution without contaminating probes or RNA molecules. Target-probe complexes are immobilised and aligned on the cartridge.

On the third day, data was collected using an epifluorescence microscope on the nCounter Analyser. This facilitated counts of each individual Reporter probe (and therefore the corresponding mRNA molecule), which can be exported as a .csv document that can be analysed using software of choice.

2.7.3. Nanostring analysis: nSolver Analysis Software and programming using R

Using nSolver Analysis Software 3.0 counts in each sample were normalised by the geNorm algorithm, which assesses and normalises all samples to the 10 most stable housekeeper genes (included in the nanoString panel) across samples. The nSolver Advanced Analysis (PanCancer Immune) module was used to generate principle component analysis (PCA) and differential expression (a.k.a. Volcano) plots. Normalised counts were exported. R studio (version 0.99.9) was used to generate heatmaps (agglomerative clusters) of detected genes (where the maximum count across samples for each gene was above 20) and histograms of all genes. The distance metric used for hierarchical clustering was based on Pearson's correlation, which was chosen due to robustness to rescaling.

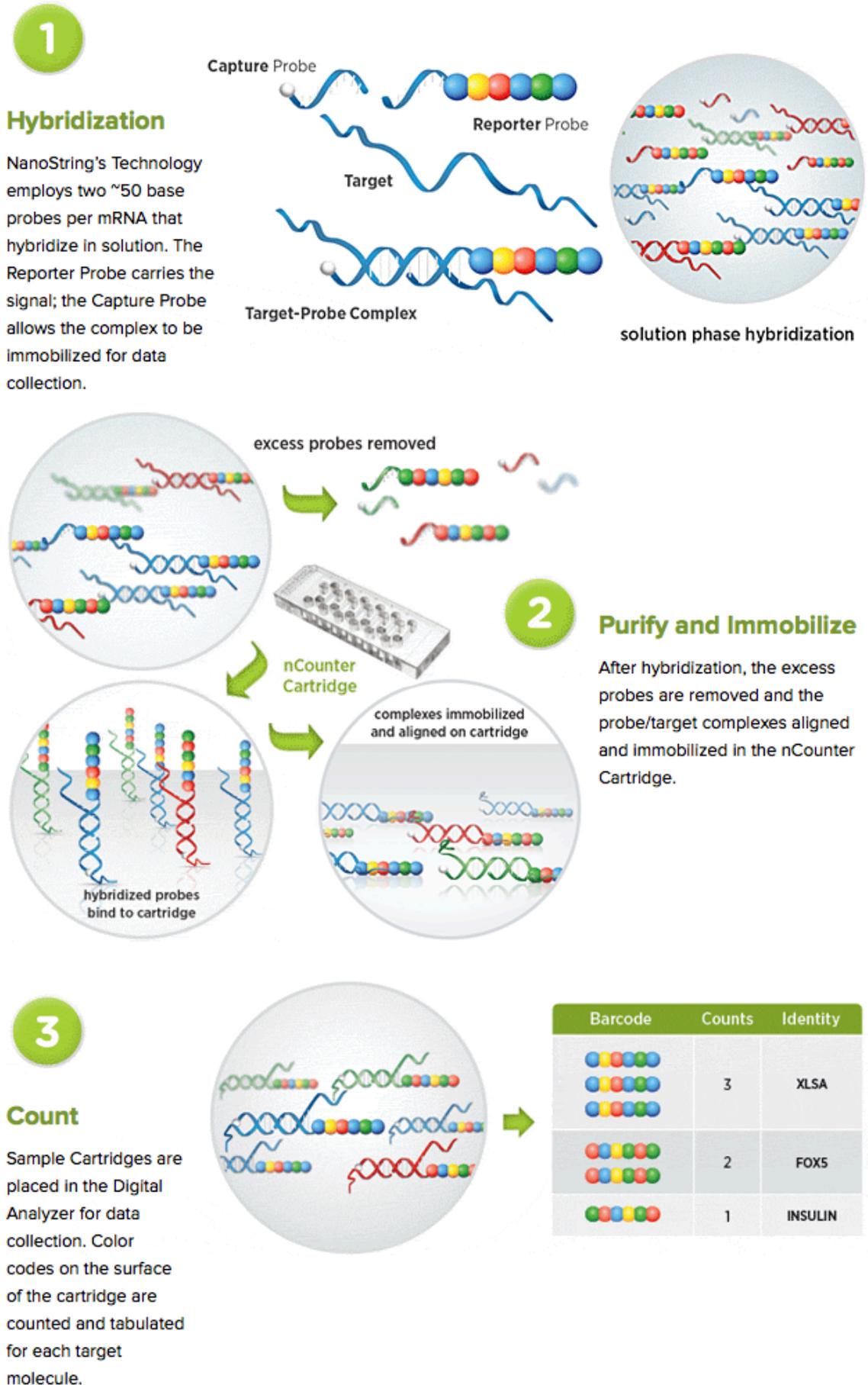


Figure 2.1 Schematic representation of nanoString reaction (section 2.7.2).

Figure taken from: <http://www.nanostring.com/applications/technology>

2.8. Flow cytometry

2.8.1. Isolation of prostate haematopoietic cells

A protocol was developed for the analysis of prostate infiltrating haematopoietic cells by flow cytometry (Chapter 6). Briefly, prostate tissue was chopped in 1X Hank's Balanced Salt Solution (HBSS; Life Technologies), cooled to 4°C. Chopped tissue was transferred to a C-Tube (Miltenyi Biotec) and enzyme solution added. A number of Liberase digestion enzymes (Liberase Test Kit; Roche) were tested, as detailed in Chapter 6, before Liberase Thermolysin Low (Roche) was chosen. Complete enzyme solution contained 0.25mg/ml Liberase TL, 1mg/ml DNase I (Sigma) diluted in 1X HBSS. The gentleMACs dissociator (Miltenyi Biotec) was used for mechanical disruption before and after incubation at 37°C for 15min. At each step, released cells were removed from enzyme solution (to prevent excessive exposure) and stored on ice in 1X HBSS. The cell solution was strained (70µm cell strainer; Corning) into a 50ml falcon tube (Corning) and hematopoietic cells isolated by density centrifugation (Histopaque-1077; Sigma) (400g at room temperature; 21°C, 20 min, no brake).

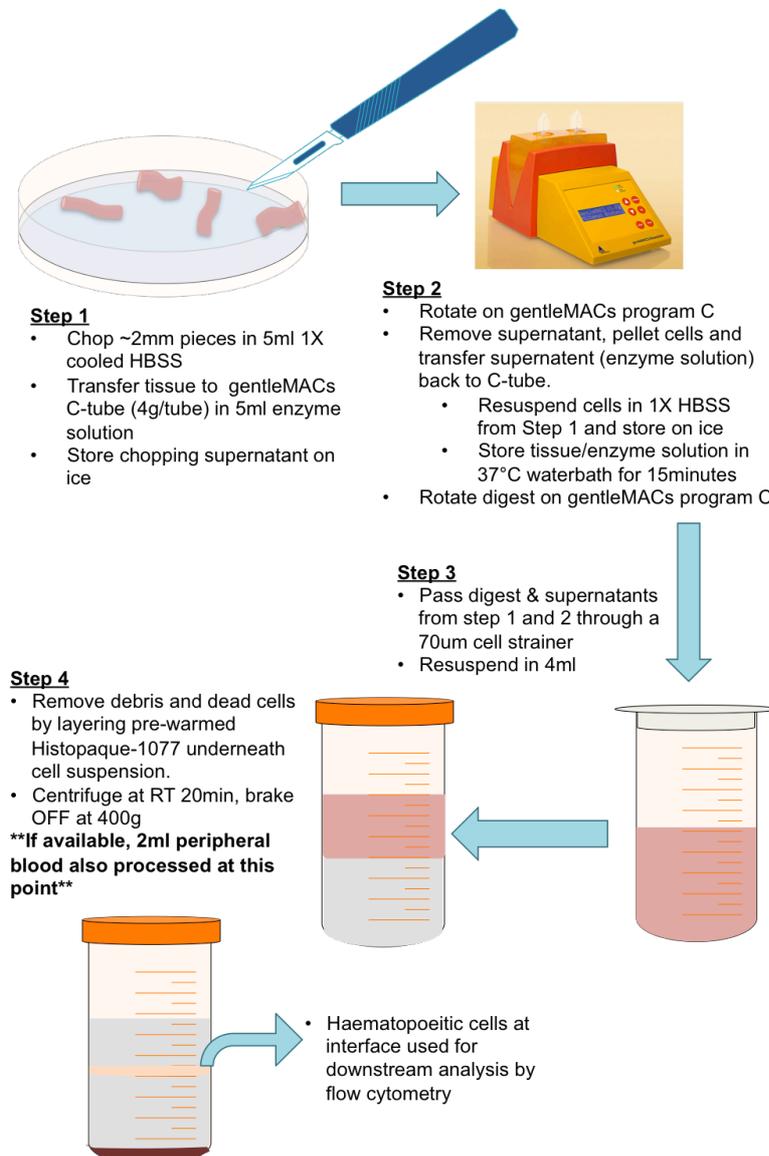


Figure 2.2 Isolating viable haematopoietic cells for analysis by flow cytometry

2.8.2. Staining of prostate infiltrating cells by flow cytometry

Haematopoietic cells collected using a Pasteur pipette (SLS) from the interface of 1XHBSS and Histopaque-1077 in Section 2.8.1 were pelleted and resuspended in cooled FACs wash (D-PBS, 2mM EDTA, 0.5% bovine serum albumin; BSA) for counting. Meanwhile an aliquot of tonsil mononuclear cells (MNCs) was thawed (according to section 2.2.4) and counted. Cells were resuspended in FACs wash at 1×10^6 cells/100 μ l and aliquoted into v-bottomed 96-well plate (VWR). Particular fluorescently conjugated antibodies were previously titrated on tonsil MNCs and an optimal concentration chosen. For each experiment, a mastermix containing a cell viability dye and specific fluorescently labelled/unlabelled antibodies at titrated concentrations (or corresponding isotype at the same concentration) were prepared. Due to low haematopoietic cell numbers extracted from prostate tissue, isotype and single colour controls were performed on tonsil MNCs and an unstained control was performed on prostate haematopoietic cells. Cells that were aliquoted in 96-well plate were centrifuged (@300g, 5min) and resuspended in 100 μ l antibody/isotype mastermix and stored on ice in the dark for 30min. 100 μ l FACs wash was then added to the cell/antibody solution and mixed before centrifugation at 300g, 5min, 4°C. To ensure complete removal of antibody, cells were washed 3 times by repeating the addition of FACs wash and centrifugation step. If unlabelled antibody was used, cells were further stained with an appropriate secondary antibody as described with primary antibody staining. For acquisition, cells were suspended in a final volume of 300 μ l and acquired immediately on a X-20 Fortessa Flow cytometer (BD). No antibody with a conjugate in the FITC fluorescence channel (530/30) was included when analysing prostate infiltrating haematopoietic cells, as this channel was kept clear to remove autofluorescent cells.

2.8.3. Detaching adherent stromal cells and staining for flow cytometric analysis

Stromal cells were seeded in 10cm plates and treated accordingly. For detachment, media was removed and 3ml of the stable Trypsin-like enzyme 1XTrypLE Express (Invitrogen) added to dishes (to prevent cleavage of cell surface molecules prior to staining). Cells were agitated at room temperature and periodically observed for detachment; time varied per patient, but was no more than 7 minutes. A cell lifter (Sarstedt Ltd) was used to completely detach remaining cells, which were collected with a 5ml pipette, transferred to a 15ml tube and triturated to minimise cell aggregation. The cell suspension was resuspended and live cells were counted using trypan blue dead cell exclusion dye. Cells were resuspended at 1×10^6 cells/ml, transferred to a v-bottomed 96 well plate (VWR) and later stained at 2.5×10^5 /100ul as in section 2.8.4.

2.8.4. Flow cytometry: acquisition and analysis

Cells labelled with fluorescent antibodies in Section 2.8.2 and 2.8.3 were acquired on low flow on a X-20 Fortessa Flow cytometer (BD) using BD FACS Diva software. The unlabelled controls were used to set voltages of photomultiplier tubes (PMTs) and positive gates were set

using isotype controls. Analysis was performed post-acquisition using FlowJo. Single colour controls were used to calculate a compensation matrix to correct for spillover fluorescence from a primary signal detected in a secondary channel. The median fluorescence of the positive population (of a single colour control) in a secondary channel is corrected to match the median fluorescence of the negative population. This process is repeated sequentially through all single colour controls until a complete compensation matrix is created and applied to all samples.

2.8.5. Normalised median fluorescence intensity (MFI) for prostate infiltrating lymphocyte samples

The background fluorescence in prostate infiltrating immune cell samples was additionally corrected for using the isotype and unlabelled controls, followed by the tonsil MNC control completed with every experiment according to the calculations below.

$$\begin{aligned} \text{Minus background MFI(Channel)} &= \\ \text{Prostate MFI(Channel)} - \text{Unstained MFI(Channel)} \end{aligned}$$

$$\text{Normalised MFI(Channel)} = \frac{\text{Minus background MFI(Channel)}}{\text{Tonsil MFI(Channel)}}$$

Channel; fluorescence channel

MFI; median fluorescence intensity

2.9. Western blotting

2.9.1. Protein isolation and quantification

Adherent cells were directly lysed on the plate post-treatment. Media was discarded and cells washed 3 times with D-PBS, ensuring to remove all remainder D-PBS. Cell lysis buffer (5ml 1% Triton-X-100, 150mM NaCl, 50mM Tris pH7.4, 50µl protease inhibitor cocktail; Sigma, Na₃Va₄, 50µl NaF) was added (volume depended on plate size, e.g. 100µl in a 10cm dish) and the plate rocked to ensure coating of all cells, before incubate on ice for 15mins. To ensure cell lysis occurred they were microscopically observed. A rubber cell scraper (Starstedt Ltd) was used to collect lysate and pipetting into a sterile 1.5ml eppendorf tube, which were kept on ice throughout isolation. Lysate was vortexed and cleared by centrifugation at 20,500rcf, 4°C, 15min. Supernatant was carefully collected into a new eppendorf tube and immediately stored at -20°C.

Protein content was measured using Pierce™ protein assay kit (Cat:23225), bicinchoninic acid assay (BCA; Fischer Scientific) by reverse pipetting 2.5µl protein lysate into a flat-bottomed 96-well plate in triplicate, adding 50µl BCA working reagent (50 parts Reagent A: 1 part Reagent B) and incubating 30min at RT. Absorbance was measured at 562nm on a plate reader, and sample protein concentration calculated relative to absorbance of standard samples of known concentration

(BSA ranging from 0 to 4µg/ml). Accuracy of pipetting was assessed by the linear regression (r^2) of the standard curve slope.

2.9.2. Loading protein and running SDS-PAGE gel

Protein was denatured by combining volume of protein lysate normalised to known concentration with lysis buffer and loading buffer (4X LDS sample Buffer; Fischer Scientific, with 10% 2-Mercaptoethanol; BME; Sigma) so the final solution contained 1X LDS and 2.5% BME. Final solution was then heated at 90°C for 10 minutes. Meanwhile, a resolving gel (12% acrylamide) was mixed, cast and allowed to polymerise. For 5ml of 12% resolving gel the following was combined: 1.6ml dH₂O, 2ml Acrylamide (ProtoFLOWGel; SLS), 1.3ml 1.5M Tris (pH8.8; Severn Biotech), 50µl sodium dodecyl sulphate (SDS; SLS), 50µl Ammonium persulfate (APS; Fischer Scientific) and 2µl Tetramethylethylenediamine (TEMED; Sigma) and immediately poured into a gel casting stand (OmniPAGE Mini; Cleaver Scientific) between sealed glass plates. The resolving gel was then layered on top with isopropanol to ensure that the gel sets linearly, which is removed fully before the addition of the stacking gel. 2ml stacking gel was mixed by combining: 1.4ml dH₂O, 330µl acrylamide, 250µl 1M Tris (pH6.8; Severn Biotech), 20µl SDS, 20µl APS and 2µl TEMED, which was layered on solidified resolving gel before 12-sample well comb is added and allowed to polymerise. The gel was transferred to a running tank filled with 1x SDS-PAGE Buffer (Geneflow Ltd) and protein samples (now denatured and cooled to RT) were loaded evenly, along with a SeeBlue® Plus2 Pre-stained Protein Standard (Fischer Scientific). Gels were run at 120V for 40min before gel was removed from glass plates and trimmed to prepare for transfer.

2.9.3. Transferring protein to a membrane and immunoblotting

Soaking a polyvinylidene difluoride (PVDF; Merck) membrane (0.45µm pore size) in methanol for 1min activated it, which was then rinsed well in transfer buffer (24mM Tris Base, 150mM Glycine, 20% Methanol in dH₂O). The gel and membrane were stacked in direction of current (cathode to anode) as follows: sponge, 2 layers of filter paper (Whatman™), gel, membrane, 2 layers of filter paper (Whatman™), sponge, all of which were pre-soaked in transfer buffer, ensuring no bubbles occur between layers. Protein from gels were transferred to the membrane by running at 110V for 60min. The membrane was blocked in 5% Milk (VWR) in TBS-T (10mM Tris Base, 100 mM NaCl, 0.1% Tween-20 at pH 7.6) for 30min at RT gently shaking (60rpm) before incubating overnight with primary antibody diluted in 5% Milk in TBS-T, gently shaking (60rpm) at 4°C. Membranes were then washed in TBS-T 1x15min, 3x10min at RT, gently shaking (60rpm). If primary antibody was unconjugated, the membrane was further incubated at RT for 1 hour in the appropriate secondary antibody, which was coupled to horse radish peroxidase (HRP). Washing steps were repeated as with primary and signal detected using GE Healthcare ECL Prime Detection reagent (SLS) and developed on UltraCruz™ Autoradiography Film (Santa Cruz)

in a dark room with a Xograph. For densitometry analysis, the density of the protein of interest is displayed relative to the density of the loading control (β -actin).

2.10. Ptychography- label free imaging of live prostate stromal cells

Ptychography was used to analyse various features of stromal cells including morphology, migration and cell division. Cells were plated at a density of 5×10^5 in 3ml R10 media per well in a glass bottomed 6-well plate (Cellvis). The lower seeding density ensured single cells could be distinguished. The plate was shaken laterally and longitudinally to ensure an even distribution of cells and cultured overnight. For each patient (6 in total, 3 each of BPH and PCa cohorts) cells were seeded in duplicate so that $\text{IFN}\gamma$ & $\text{TNF}\alpha$ treatment could be directly compared to untreated. Cells were treated as previously described (Section 2.3) and imaged over a 72hour period at 10min intervals on a VL21 inverted microscope (Phase Focus, Sheffield) contained in Solent Scientific environment chamber (Solent Scientific Limited) at 37°C with 5% CO_2 . Images were collected on an Olympus LMPlanFLN 20x/0.40 Objective and reconstructed according to the extended Ptychographic Iterative Engine (ePIE) algorithm (The Phase Focus Virtual Lens®, Phase Focus), which utilises a phase retrieval method to generate high contrast images without labelling. Data collected was analysed using the Cell Analysis Toolbox, V1.1.0 (Phase Focus) for cell segmentation at 6 stated time points, or the mTrackJ Plugin on ImageJ (Fiji) for manual cell tracking. Parametrics collected by cell segmentation were plotted using R Studio, whereby the given parametric (e.g. Area) were plotted against time in separate facet grids corresponding to disease and treatment and coloured by patient. Data collected by mTrackJ were plotted using Prism 6 (GraphPad Software). At the end of the time lapse imaging, cells were fixed and labelled as in section 2.11.1.

2.11. Immunohistochemistry

2.11.1. *In vitro* staining of cells in chamber slides

Stromal cells were seeded at 5×10^4 cells or 2.5×10^4 in 500 μl : 4- and 250 μl :8- well chamber slides (Lab-Tek, ThermoScientific), respectively. Cells were incubated overnight to allow attachment to slide before treated as before for 48hours. Media was removed and cells carefully washed in sterile PBS 3 times, fixed in 4% paraformaldehyde (PFA) before washing a further 3 times. 5% Goat serum (Sigma) was added to wells and incubated 30mins at room temperature (RT). For cell surface labelling, cells were incubated at 4°C overnight after 5% goat serum containing relevant antibody or isotype control was added at titrated concentration. The next morning, cells were washed 3 times and permeabilised in 0.5% Triton-X for 10 minutes (Sigma). Intracellular staining was performed as with surface staining, starting with a second blocking step in 5% Goat serum at RT. Intracellular label incubations were 30 minutes at room temperature rather than overnight. Cells were washed 3 times in PBS, and chamber well separator removed. A drop of Prolong gold anti-fade reagent with 4',6-Diamidino-2-Phenylindole, Dilactate (DAPI; Life

Technologies) was added to wells and slide mounted with a Coverslip (No 1.5 22x55mm, SLS) before storing overnight at 4°C and finally sealing with nail varnished. Slides and plates were stored at 4°C until imaged.

2.11.2. Confocal imaging

Cells were imaged using a Zeiss LSM880 inverted microscope controlled with the Zen 2.1 software, which benefits from 4 independent lasers and 6 laser lines; 405, 458, 488, 514, 561, and 633 nm. Typically, cells were labelled with antibodies and DAPI that could be detected in the 405, 488, 561 and 633nm channels. For comparability across experiments, laser power, pinhole and detector voltage gain & digital offset were kept constant. Each was imaged on separate tracks to minimise spillover and single colour controls were performed to ensure accuracy. Cells were imaged at x20 or x40 objectives, with tile scans and a z stack spanning total height of cells. Images were taken at 1024 x 1024 pixels and line averaging of 4. Images were processed using ImageJ (Fiji), where z stack was merged at maximum intensity.

2.12. Statistical analysis

Statistical analysis was done using GraphPad Prism or r Studio. When comparing the effect of disease and treatment a two-way ANOVA was done followed by a Tukey's multiple comparison test. Data was plotted on graphs with bars illustrating the mean values and error bars representing the standard deviation of the mean (SD) (GraphPad). On occasions where values were missing from the cohort, r Studio was used to determine statistical significance of trends by means of a generalised linear model (GLM).

Table 2.5 Antibodies

	Clone	Isotype	Conjugate	Company	Application
anti-human ICAM1	HCD54	mIgG1	A488	Biologend	FC, IHC
anti-human PDPN	NC-08	rat IgG2a	A488	Biologend	FC, IHC
anti-human VCAM1	IE10	mIgG2a	A488	R&D	FC, IHC
anti-human Fibroblast Activation Protein- α	427819	mIgG1k	APC	R&D	FC
anti-human CLIP	CerCLIP	mIgG1k	FITC	BD Biosciences	FC
anti-human HLA-dr	L243	mIgG2b	PE-Cy5	Biologend	FC
anti-humanHLA-E	3D12	mIgG1	PE	Biologend	FC
anti-human CD45	HI30	mIgG1k	BV605	Biologend	FC
anti-human PD1	EH12.2H7	mIgG1k	Biotin	Biologend	FC
anti-human CD8	RPA-T8	mIgG1k	BV711	Biologend	FC
anti-human CD4	OKT4	mIgG2b	PE-Cy5	Biologend	FC
anti-human CD3	HI30	mIgG2b	PE-Cy7	Biologend	FC
anti-human PD-L1	29E.2A3	mIgG2b	PE	Biologend	FC
anti-human PD-L1	5H1	mIgG1	Pur	Yale University	IHC
anti-human vimentin	V9	mIgG1k	e660	eBioscience	IHC
α -Smooth muscle actin (SMA)	1A4	mIgG2a	Cy3	Sigma	IHC
anti-human PD-L1	E1L3N	rabbit IgG	Pur	Cell Signaling Technologies	WB
anti-human β -actin	AC-15	mIgG2a	HRP	Sigma	WB
Other					
Viability Dye			e780	eBioscience	FC
recombinant human Lag-3 Fc Chimera Protein				R&D	FC
Secondary Antibodies					
Streptavidin			BV421	Biologend	FC
Anti-Human IgG (Fcy)	Polyclonal		PE	eBioscience	FC
Anti-Rabbit	Polyclonal		HRP	Cell Signaling Technologies	WB
Goat anti-mouse IgG	Polyclonal		A488	Invitrogen	IHC

Chapter 3

Transcriptional analysis of primary human prostate stromal cells

3.1. Introduction

Intricate balances of pro-/anti-inflammatory and stimulatory/inhibitory mechanisms maintain immune function to facilitate the destruction of pathogens and tumours without leading to overt inflammatory disease. In response to infection or aberrant host cells, early inflammation has a critical role in stimulating clearance of pathogens/tumour cells by phagocytic and lytic innate cells and stimulating the initiation of adaptive T and B cell responses. However, in addition to regulating the initiation of immune responses, inflammation profoundly effects gene transcription in epithelium and stromal fibroblasts, modulating epithelial structural integrity, function and repair by modulating proliferative capacity, function and migration. Thus to prevent localised tissue pathology the same set of signals that drive early inflammation also drive molecular and cellular processes that lead to downregulation of both the innate and adaptive immune responses. Regulation occurring through down regulation of inflammatory genes, regulatory cytokine and natural steroid secretion and expression of check-point inhibitors.

It is becoming an increasingly accepted paradigm that tumour microenvironments (TME) often harbour smouldering ineffective immune responses, which is thought to provide enough inhibitory signals to prevent effective tumour clearance, while delivering pro-inflammatory signals that has the potential to drive tumour cell survival, proliferation and metastasis (Balkwill *et al.*, 2005). In this chapter, the immune environment in PCa and examine how stromal cells and epithelial cells of primary patient prostate tissues may be involved in supporting a smouldering inflammatory environment.will be assessed

3.1.1. The cellular constituents of the effector phase in anti-tumour immunity

Complex interactions within the TME determine the outcome of anti-tumour immunity (Figure 3.1). Signals originating from the normal and transformed epithelium, normal and reactive stromal cells, and the infiltrating immune cells, which may either be regulatory or effector, impact on each of the other cellular components of the TME in a multifactorial network. Given that each signal is likely to affect the others and that the exact local concentrations cannot be well measured, this is a system that *in vitro* experimentation is unlikely to ever be able to fully recapitulate. Yet by utilising reductionist systems, a better understanding cellular phenotypes and interactions in a controlled environment can be achieved.

The cellular components of the effector phase of anti-tumour immunity are mainly reliant on APCs, CD4+ TH cells, CD8+ T killer cells (or CTL) and natural killer cells (NK). All of these immune cells are potent cytokine producers and are heavily regulated by soluble factors and cell-cell contact within the TME to conserve tissue homeostasis. For example, APCs (dendritic cells; DCs, macrophages) are powerful initiators of immune responses, but their efficacy in doing so depends on the inflammatory balance in the environment from which the antigen originates

(Pinzon-Charry *et al.*, 2005; Steinbrink *et al.*, 1999). However, this localised balance is forever evolving due to fluctuations in the concentrations and ratios of pro- and anti- inflammatory mediators and availability of antigen, hence the efficiency in anti-tumour immunity will oscillate. Each of the effector immune cell components secretes cytokines that mediate the effector outcomes, and are generally considered to belong to either the pro- or anti- immune ends of the balance. The T cell (TH and CTL) subsets are particularly important for mediating anti-tumour immunity (Frey, 2008). Antigen activated TH1, CTL and NK cells mediate responses through pro-inflammatory cytokines like IFN γ , which is often used *in vitro* to recapitulate pro-inflammatory environments and termed TH1 cytokines (Luheshi *et al.*, 2014). Oppositely, TH2 cells secrete anti-inflammatory/TH2 cytokines such as interleukin-4 (IL-4), which acts to further propagate anti-inflammatory signals in an autocrine matter by increasing the differentiation of naive TH cells to TH2 subtype (Ansel *et al.*, 2006; Swain *et al.*, 1990). Often the CD4:CD8 ratio (TH:CTL) is used as an indicator in cancer immunology research as to which pole of the balance the TME favours and so has associated prognostic value (Prall *et al.*, 2004; Sato *et al.*, 2005; Shah *et al.*, 2011; Toes *et al.*, 1999). As of yet though, the impact of the cytokines derived from these cells on stromal cells has not been fully elucidated.

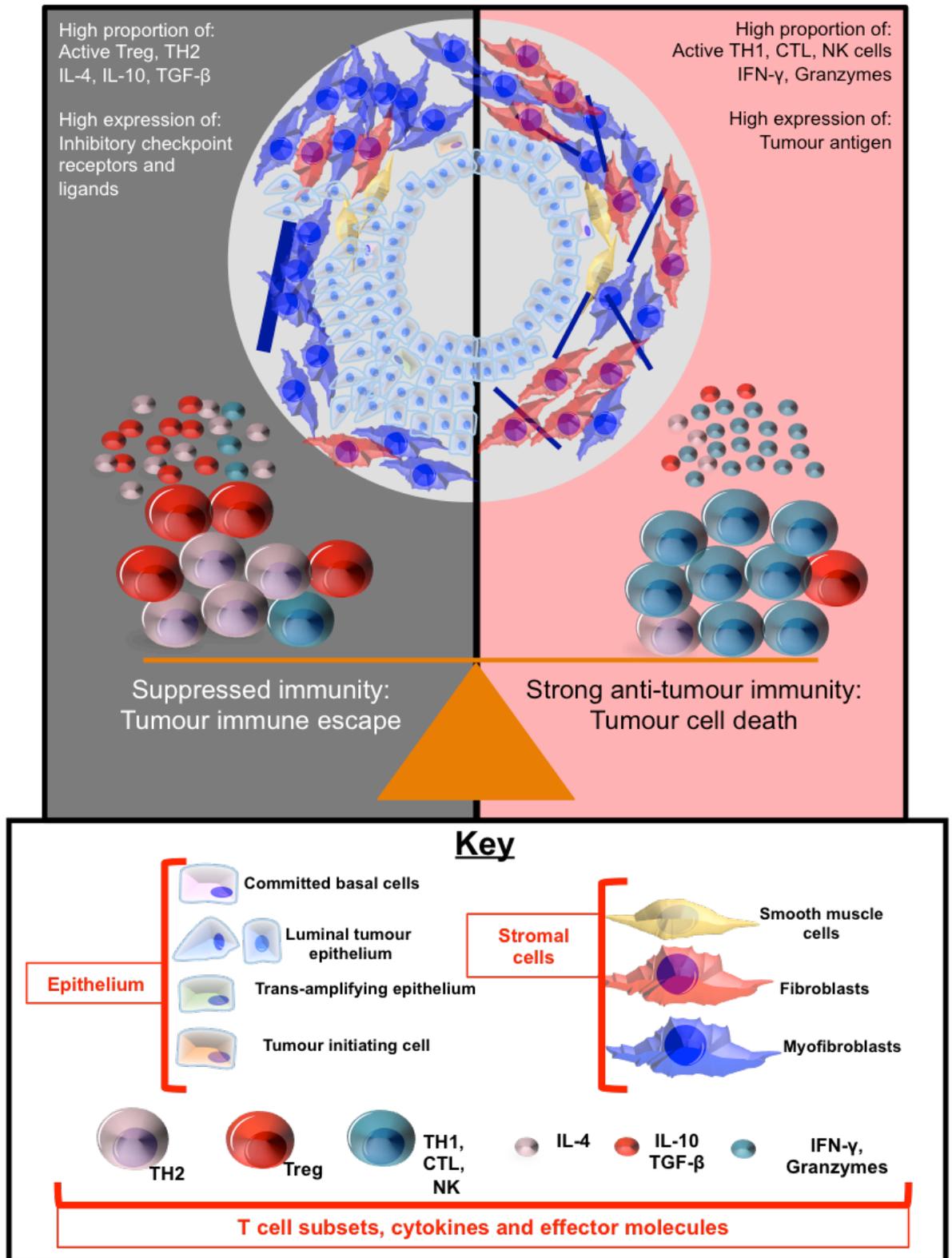


Figure 3.1: Effector T cells impact the balance of anti-tumour immunity

T cells are of particular important in upholding the balance between tolerance and immunity due to the powerful subtypes that maintain each extreme. Treg and TH2 cells increase local concentrations of IL-4, IL-10 and TGF-β and suppress TH1, CTL and NK cells; this is beneficial in preventing autoimmunity, but detrimental for the host in the case of a tumour as suppressive immunity permits tumour cell progression. For an effective anti-tumour immune response the opposite is required, dependent on increases in active TH1 and CTL with tumour killing capacity, facilitating tumour regress.

3.1.2. Summary and aims

For the purpose of this thesis, the roles of these two opposing ends of inflammatory extremes by using IFN γ and IL-4 as models are investigated. IFN γ is expressed potently by TH1 and CTL mediated immunity, T cells that are considered beneficial for anti-tumour immunity. TH2 cells are often associated with a poor prognosis in cancer patients and express IL-4 in large amounts. Knowing that these cytokines will not occur in isolation, TNF α in combination with these are used, due to evidential roles of synergistic activities. This is not to say that all inflammatory or anti-inflammatory cytokines will produce the same results, or that the presence of other cytokines in the milieu will not change the outcome in some way. However, investigate the effect of an overall imbalance in the localised inflammatory signals on the stromal compartment of the prostate, these combinations of cytokines are a suitable starting point.

The aims of this chapter were to:

- Understand the involvement of prostate stromal cells (derived from BPH and PCa) in responding to local inflammation.
- Determine the source of commonly PCa associated molecules and whether they are modulated by inflammatory signals in prostate stroma and epithelium (in BPH and PCa).

3.2. Results

3.2.1. Gene expression analysis of prostate epithelial and stroma cells

Epithelial and stromal cells cultured from patient prostate tissue were either treated with $\text{IFN}\gamma$ & $\text{TNF}\alpha$ or untreated. Control stroma (HFF cells and tonsil stroma) is included to compare as both BPH and G19 PCa are states of disease. Prostate stroma was also treated with IL-4 & $\text{TNF}\alpha$, although prostate epithelium and control stroma were not. Gene expression was analysed by nanostring whereby actual counts of mRNA molecules per gene are returned. All expressed genes were used to present data with sample clustering as an overview using *t*. Genes are scaled across samples (Row Z score). Figure 3.2 demonstrates all detected genes in all samples. Two principal clusters exist, one containing epithelia and second containing stroma. Within these two clusters, $\text{IFN}\gamma$ & $\text{TNF}\alpha$ treated samples cluster separately from untreated samples (and IL-4 & $\text{TNF}\alpha$ treated stroma). To explore individual cohorts in more detail, each are displayed as heatmaps. In each case, genes that weren't detected within the cohort were excluded.

Genes detected in epithelial cells are demonstrated in Figure 3.3. While $\text{IFN}\gamma$ & $\text{TNF}\alpha$ treatment primarily defines clustering, within each of these clusters, BPH was separately clustered from G17 and G19 epithelial samples. This is evident by subsets of genes that are either upregulated (bottom central green cluster) or downregulated (top central red cluster) in G17 or G19 epithelium compared to BPH. There is also a clear subset of genes that were upregulated by $\text{IFN}\gamma$ & $\text{TNF}\alpha$ and a smaller subset downregulated by $\text{IFN}\gamma$ & $\text{TNF}\alpha$.

Further inspection of stromal samples (Figure 3.4) reveals a similar trend with $\text{IFN}\gamma$ & $\text{TNF}\alpha$ treatment as with epithelium, containing a major upregulated gene cluster in the $\text{IFN}\gamma$ & $\text{TNF}\alpha$ treated, and a minor downregulated cluster. In the case of stroma however, disease did not appear to significantly impact on gene expression. Analysis of IL-4 & $\text{TNF}\alpha$ treated prostate stroma in comparison to untreated (Figure 3.5) demonstrates IL-4 & $\text{TNF}\alpha$ did not impact on stroma significantly enough to result in differential clustering as $\text{IFN}\gamma$ & $\text{TNF}\alpha$ does. However there is a small subset of genes that were upregulated in treated stroma over untreated stroma. Due to the ambiguity of this cluster, a bracket has been added to highlight it. Again, disease did not made a significant impact on hierarchical clustering.

To further explore the impact of disease on stromal cells, principle components analysis (PCA) and differential expression represented by volcano plots are demonstrated (Figure 3.6 and 3.7). PCA allows clustering of samples based on, in this case, its transcriptional profile, by compressing all the data onto a single plot. This is achieved by plotting the data onto axis that describe the principle components of the data. PC1, the first principle component explains the direction in which most of the variation within the data set occurs. PC2, explains the second most variation, and so on. More similar cell types will cluster together, due to similar transcriptional

profiles. The PCA plots included in Figure 3.6 and 3.7 display the first to fourth PC plotted against each other. Untreated stromal samples separated by disease in the first 2 PC, which capture 35% of the variation in the data in total (PC1 and PC2, which explain 20% and 15% of the variation respectively) (Figure 3.10A). As presented in the volcano plot, *IL-13R α 2* was the most differentially expressed gene between disease cohorts (demonstrated by x axis) and the most statistically significant (y axis; Figure 3.10B), which is discussed further later in the chapter. *PTGS2*, *VEGF α* , *IL-1RN* and *LT β R* are among the subset of genes that were significantly downregulated in G19 stroma (-fold change) compared to BPH stroma. Of note, fewer genes were upregulated in G19 stroma compared to BPH (+fold change). Included in these genes are *STAT6*, *TICAM* and *PSMB9*. Analysing the effect of treatment in the same way (Figure 3.7) is consistent with analysis by heatmaps. IFN γ &TNF α treated stroma separated distinctly from both untreated and IL-4&TNF α treated stroma by PC1 vs. PC2, in which 52% of variation is explained (Figure 3.7 A). Nevertheless, both IFN γ &TNF α and IL-4&TNF α produced potent and statistically significant changes in gene expression (Figure 3.7 B&C). Consistent with what is known about cellular responses to these cytokines, genes that are known to be modulated by IFN γ or IL-4 are included in the most upregulated and significant changes.

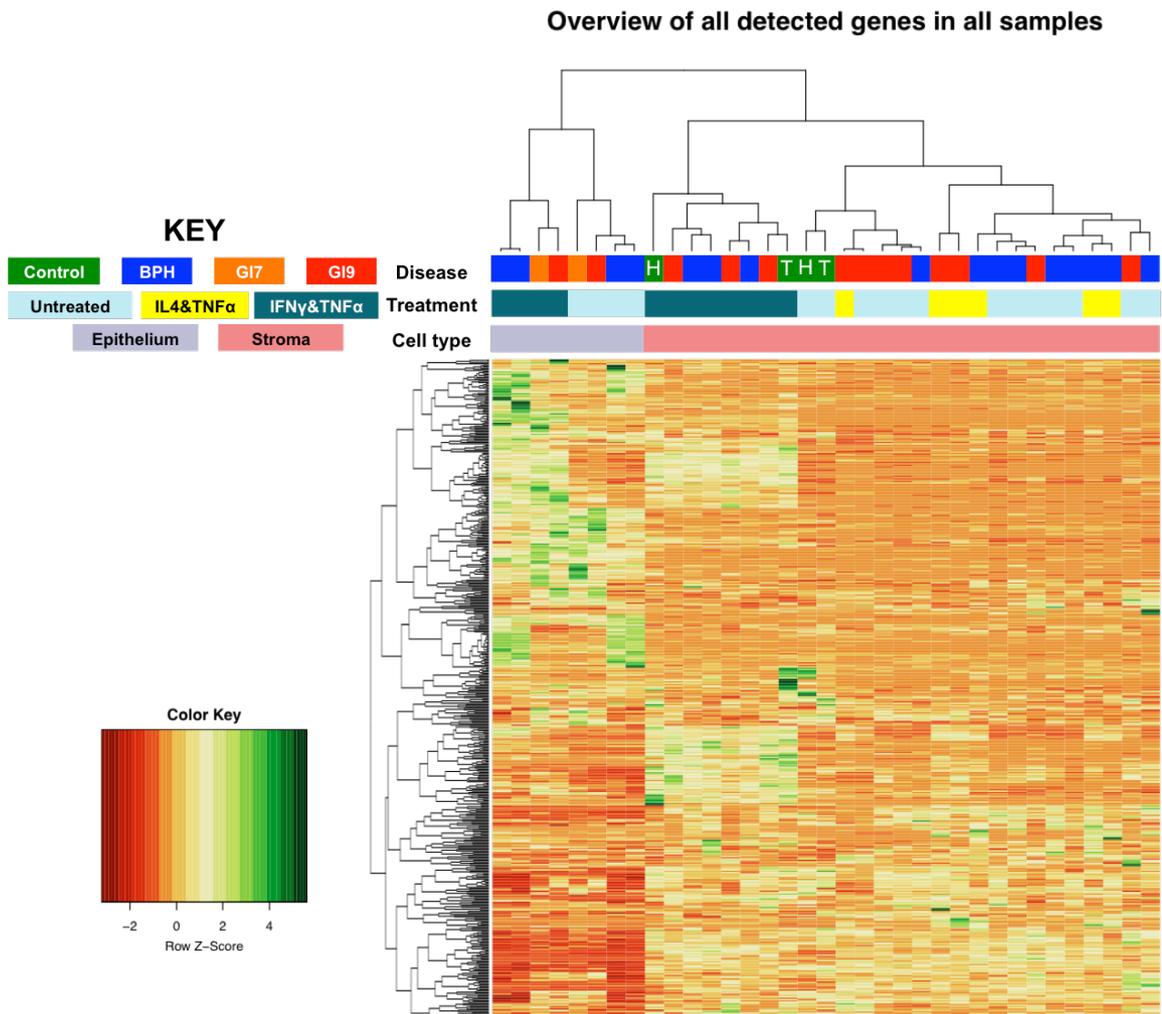


Figure 3.2: Gene expression analysis of prostate stroma in comparison to prostate epithelium and control stroma

Normalised counts obtained from nanoString analysis and detected above background are plotted. For hierarchical clustering, distance between samples (columns) and genes (rows) was calculated according to Pearson's correlation. Counts are scaled by row (i.e. across samples) and coloured by row Z-score, where green indicates high expression and red indicates low expression relative to other samples. Above the plot, colours indicate the disease, treatment and cell type of the corresponding sample below. Within the control samples (coloured green), "H" and "T" indicate HFF and Tonsil control stroma, respectively. n=35

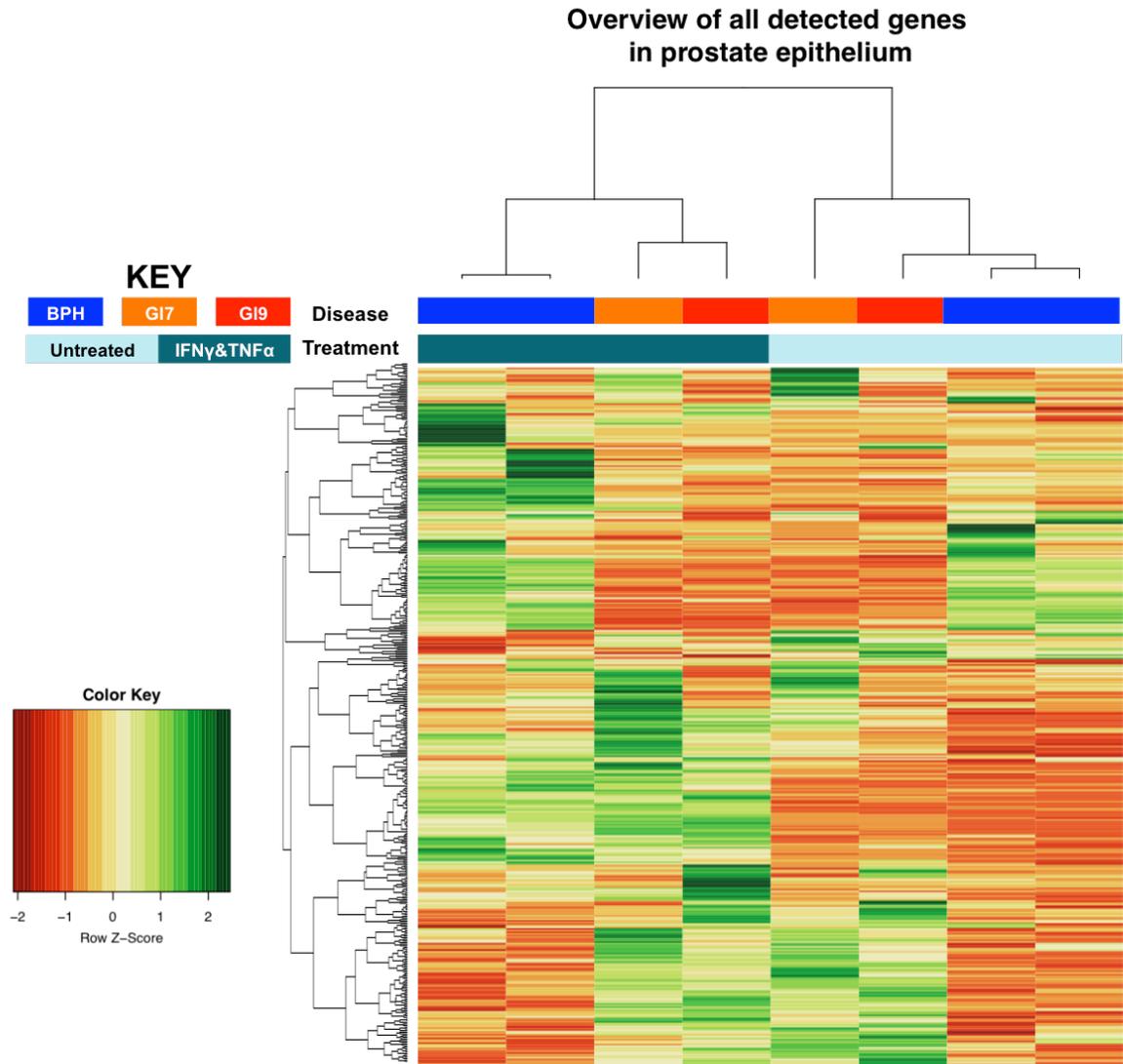


Figure 3.3: Gene expression analysis of prostate epithelium

An enlarged plot of the epithelial cluster evidenced in Figure 3.2, with undetected genes (that are detectable in stromal cells) removed. For hierarchical clustering, distance between samples (columns) and genes (rows) was calculated according by Pearson's correlation. Counts are scaled by row (i.e. across samples) and coloured by row Z-score, where green indicates high expression and red indicates low expression relative to other samples. Above the plot, colours indicate the disease and treatment of the corresponding sample below. n=8

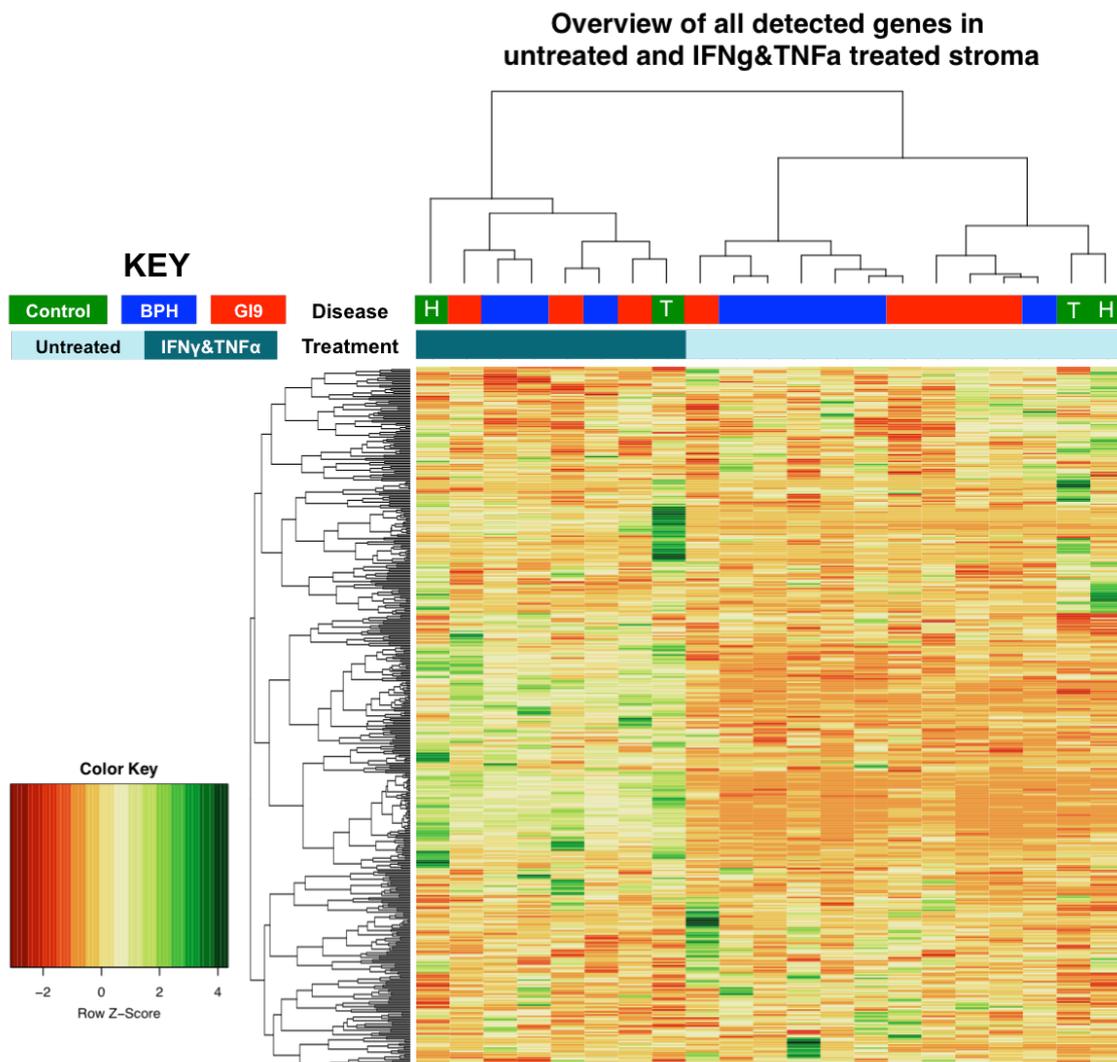


Figure 3.4: Gene expression analysis of untreated and IFN γ &TNF α treated stroma

An enlarged plot of stromal cells from Figure 3.2, with undetected genes removed (which are detectable in epithelial or IL-4&TNF α treated cells). For hierarchical clustering, distance between samples (columns) and genes (rows) was calculated according by Pearson's correlation. Counts are scaled by row (i.e. across samples) and coloured by row Z-score, where green indicates high expression and red indicates low expression relative to other samples. Above the plot, colours indicate the disease and treatment of the corresponding sample below. Within the control samples (coloured green), "H" and "T" indicate HFF and Tonsil control stroma, respectively. n=22

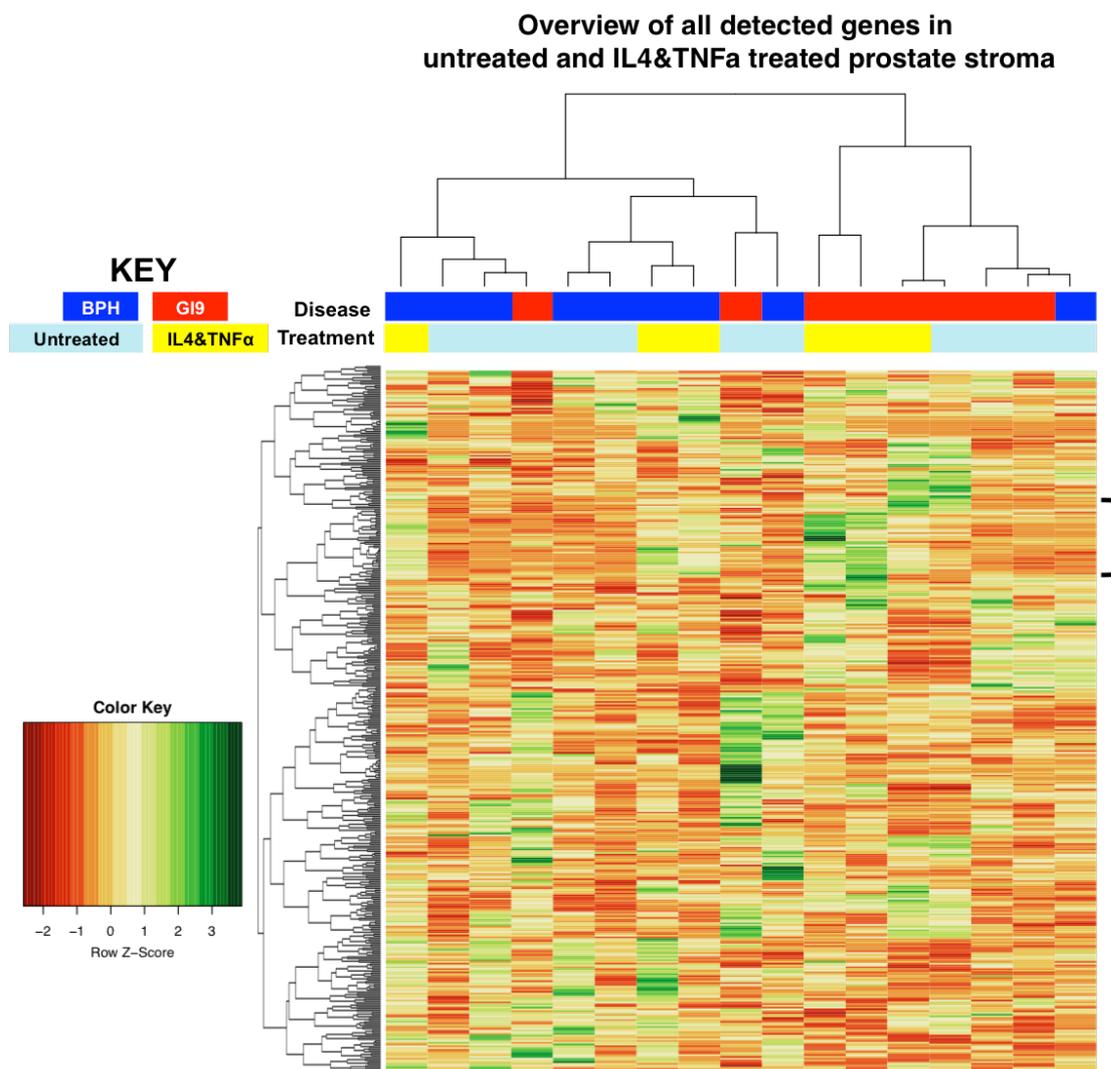


Figure 3.5: Gene expression analysis of untreated and IL-4&TNF α treated prostate stroma

An enlarged plot of stromal cells from Figure 3.2, with undetected genes removed (which are detectable in epithelial or IL-4&TNF α treated cells). For hierarchical clustering, distance between samples (columns) and genes (rows) was calculated according by Pearson's correlation. Counts are scaled by row (i.e. across samples) and coloured by row Z-score, where green indicates high expression and red indicates low expression relative to other samples. Above the plot, colours indicate the disease and treatment of the corresponding sample below. The bracket illustrates a subset of genes regulated by IL-4&TNF α treatment. n=17

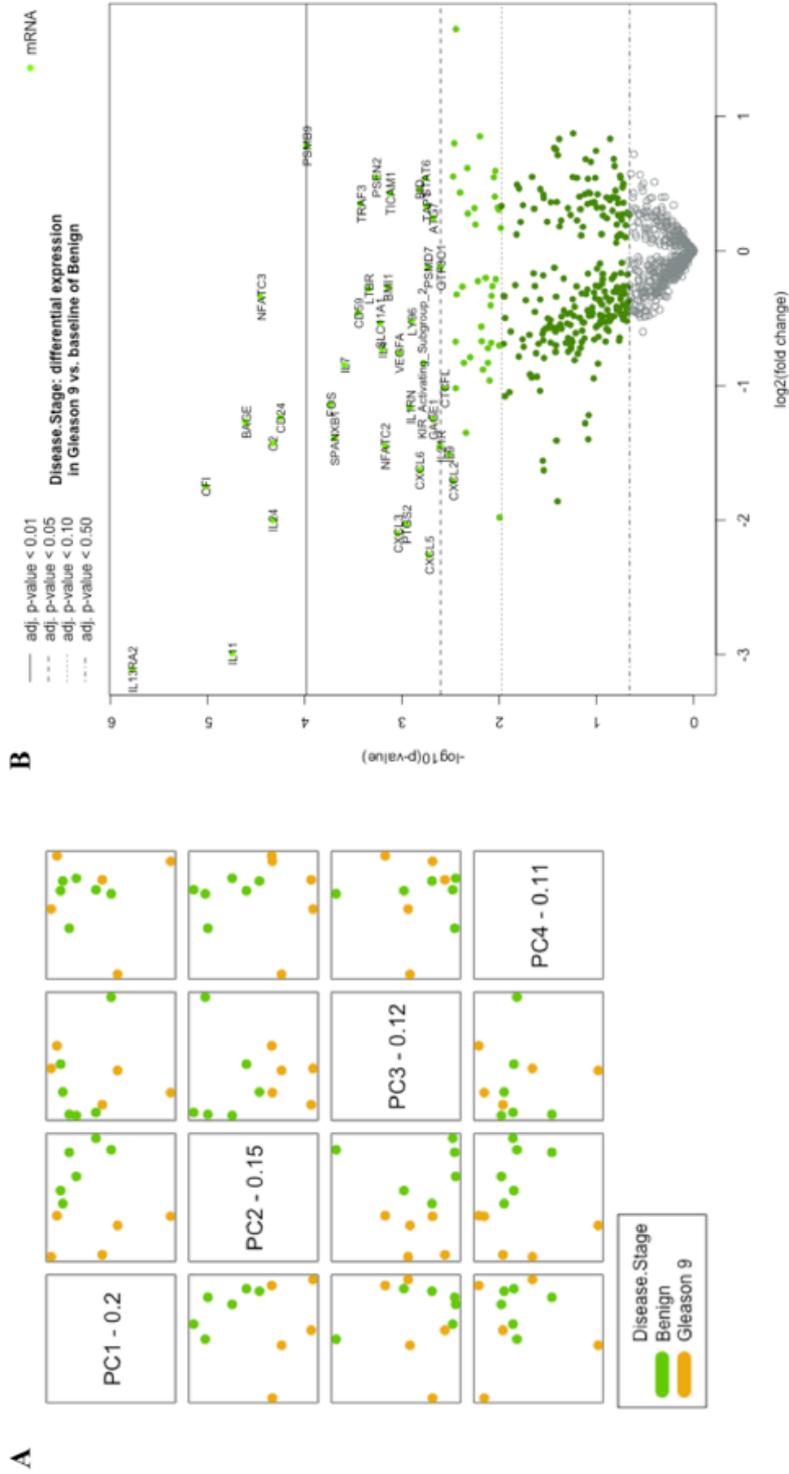


Figure 3.6: Differential transcriptional profile of prostate stroma between disease cohorts

Using the gene expression data from nanoString analysis PCA (A) and volcano (B) plots were generated using nSolver Analysis Software 3.0. In A the first to fourth PC are plotted against each other (PC1-PC4). The variance explained by the stated PC is highlighted on the plot, i.e. PC1 -0.2 indicated the first PC explains 20% of the variance in the cohort. Each point on the plot is a patient sample, all are untreated and are coloured by disease. In B the log₂(fold change) is plotted against the log₁₀(adj p-value), where the p-value is adjusted by the Benjamini–Hochberg method to control for the false discovery rate (FDR) when making multiple comparisons. Each point on the plot is a detected gene in the samples and change in gene expression in G19 stroma compared to BPH stroma is indicated by the position on the x axis. A negative fold change indicates reduced expression in G19 compared to BPH (log₂(fold change)=0). Horizontal lines indicate the adjusted p values as shown in the key. Benjamini and Hochberg (1995)

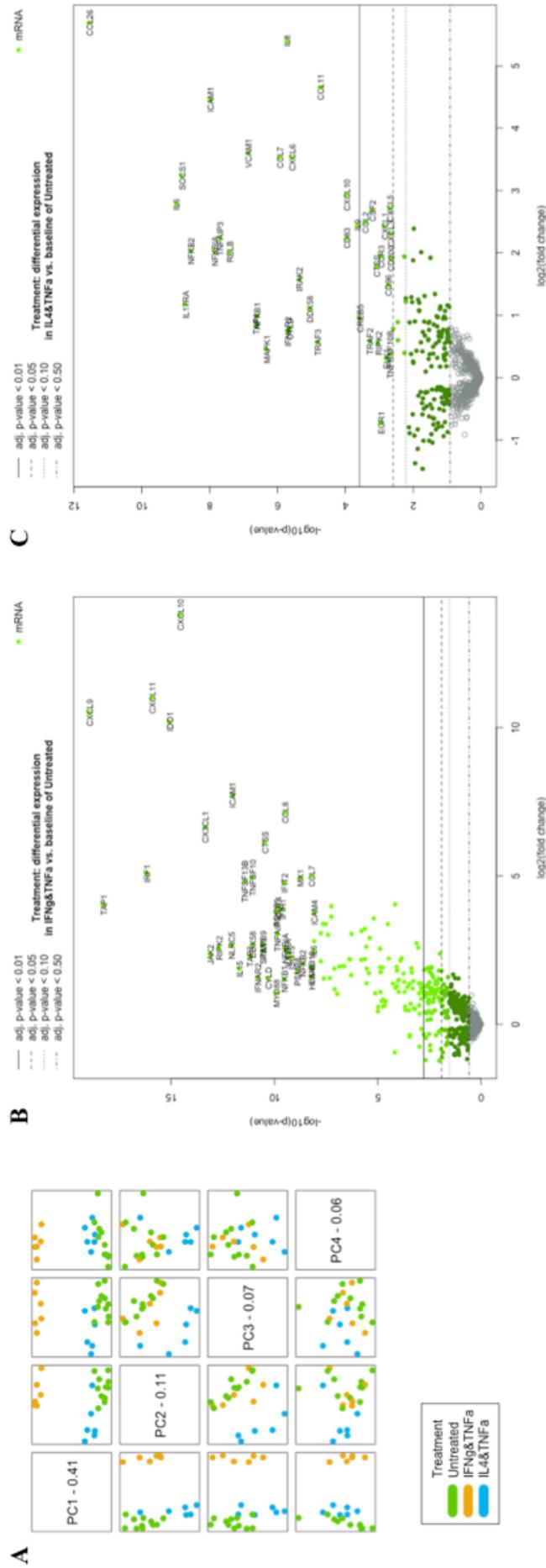


Figure 3.7: Differential transcriptional profile of prostate stroma between treatment groups

Using the gene expression data from nanostring analysis PCA (A) and volcano (B, C) plots were generated using nSolver Analysis Software 3.0. In A the first to fourth PC are plotted against each other (PC1-PC4). The variance explained by the stated PC is highlighted on the plot, i.e. PC1 -0.41 indicated the first PC explains 41% of the variance in the cohort. Each point on the plot is a patient sample and are coloured by treatment group. In B and C Each point on the plot is a detected gene in the samples and change in gene expression in IFN γ &TNF α (B) or IL-4&TNF α (C) treated stroma compared to untreated stroma is indicated by the position on the x axis. Horizontal lines indicate the adjusted p values as shown in the key. Benjamini and Hochberg (1995)

3.2.2. Quantifying cell subsets marker expression in patient cell cultures confirms cell types

The expression of markers restricted to certain cell subsets were analysed to confirm that immune, endothelial or epithelial cells do not contaminate prostate and control stromal cultures (Figure 3.8 and 3.9). It was confirmed that immune cell markers such as *CD19*, *CD163* and *CD3* were not expressed in any of the cultures; counts fall below 20 in all cases, which is within the range of background counts. Prostate and control stromal cultures express high levels of *COL3A1*, *COLEC12* and *THY1*, which were not expressed in the patient epithelial cultures. Endothelial cell genes *CD34* and *PECAMI* were not detected in any cultures, indicating their absence. Likewise, epithelial cell markers *EPCAM* and *CEACAM1* were expressed in epithelial cultures (albeit at a lower level than expected) and undetectable in stromal cultures. Altogether these data indicate that neither stromal nor epithelial cultures contain detectable amounts of contaminating immune or endothelial cells and epithelial cultures do not contain stromal cells or vice versa.

3.2.3. Type I and Type II IFN receptors were expressed in all cultures, while the ligands were not

The ability of prostate stromal cells to respond to immune cell derived cytokines relies on the expression of the corresponding receptors. To analyse the capacity of cultures to participate in IFN signalling, the ligand and receptors of Type I and Type II IFN were examined (Figure 3.10). Type I IFNs (*IFNA1*, *IFNA2*, *IFNA7* and *IFNB1*) were not expressed, though the receptors (*IFNAR1* and *IFNAR2*) were. Similarly, *IFNG* was not expressed and the receptor *IFNGR1* was (N.B ifngr2 was not present on the nanostring panel). In both cases the receptor was upregulated upon treatment with IL-4&TNF α or IFN γ &TNF α in some but not all cultures. *IFNAR1* was expressed to a higher degree in stromal cultures compared to epithelium and upregulated marginally in stroma and BPH epithelium, but not G17 or G19 epithelium. PCa epithelium appears to express a higher level of *IFNAR2* than BPH epithelium and stroma, although all responded similarly to IFN γ &TNF α . PCa epithelium expresses a higher level of *IFNGR1*. While control stroma, BPH stroma, BPH epithelium and G17 epithelium upregulated *IFNGR1* in response to IFN γ ligation, the G19 stroma and G19 epithelium appeared to have lost this ability. These data are an indication that tumour cells upregulate *IFNAR1*, *IFNAR2* and *IFNGR1* and may provide a mechanism for tumour cell to respond to low levels of local IFN. It is also evident from these data that stromal cells are highly capable of responding to IFN signalling. This is of particular importance given the microanatomical structure of the prostate; where stromal cells usually dominate the ratio of epithelium to stroma and are spatially more relevant to prostate infiltrating lymphocytes since the microvessels they extravasate from are situated in the stromal compartment.

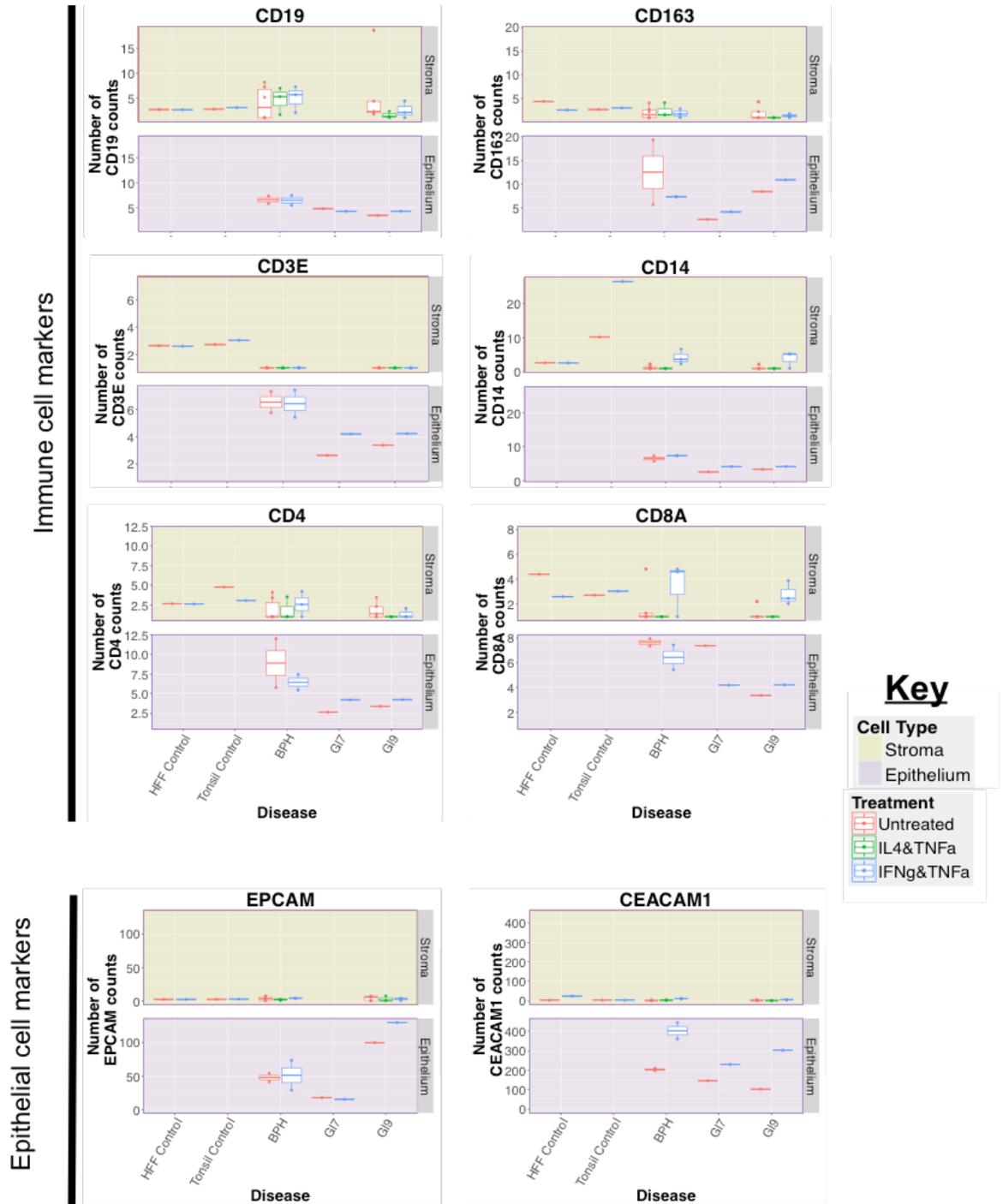


Figure 3.8: mRNA counts of genes corresponding to cell markers in all samples

Immune and epithelial cell markers are plotted as boxplots. Each point is a sample and are coloured by treatment group. Epithelial and stromal samples are plotted in separate facet grids with a scaled y-axis, which are coloured accordingly. Counts below 20 are considered below background and negative controls.

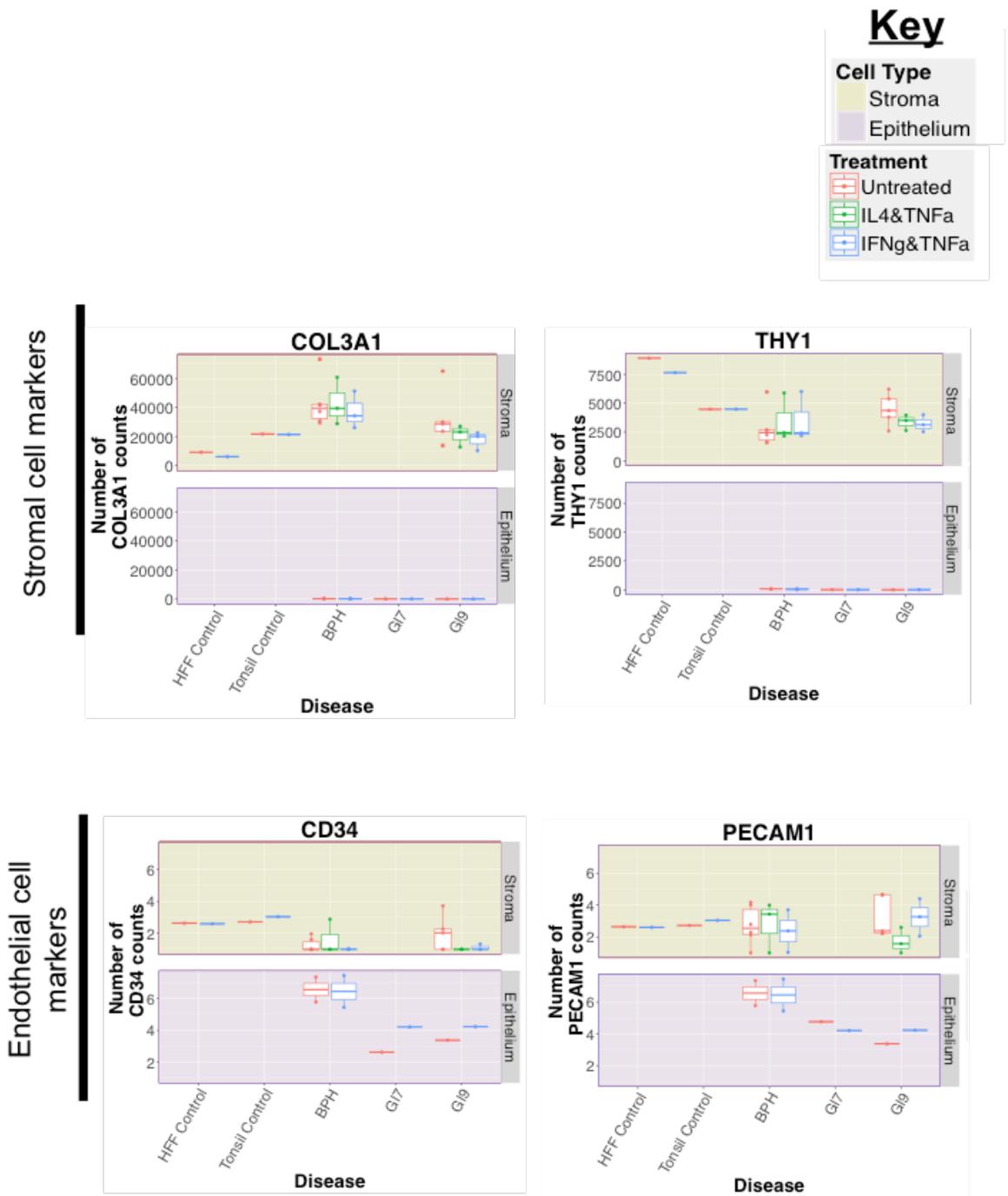


Figure 3.9: mRNA counts of genes corresponding to cell markers in all samples

Stromal and Endothelial cell markers are plotted as boxplots. Each point is a sample and are coloured by treatment group. Epithelial and stromal samples are plotted in separate facet grids with a scaled y-axis, which are coloured accordingly. Counts below 20 are considered below background and negative controls.

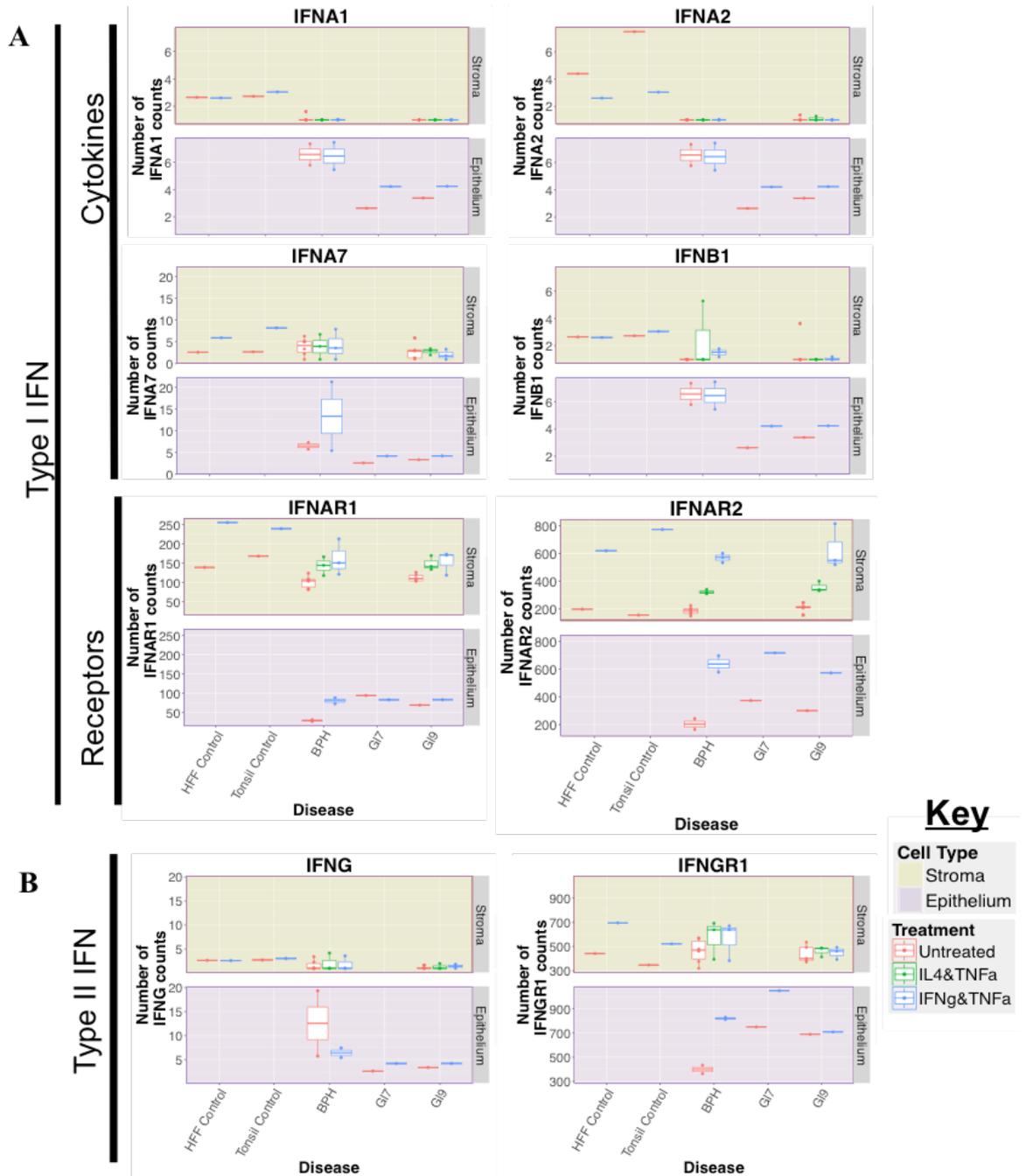


Figure 3.10: mRNA counts of genes belonging to the Type I and Type II IFN signalling pathways

Counts of genes belonging to the Type I (A) and Type II (B) IFN ligands and receptors are plotted coloured by treatment group. In each case, ligands are expressed below the negative threshold, while receptors are expressed and inducible in both cell types. Epithelial and stromal samples are plotted in separate facet grids with a scaled y-axis, which are coloured accordingly.

3.2.4. Differential expression of Type II IL-4 signalling molecules in G19 prostate stroma

Both IL-4 and IL-13 can signal through the type II IL-4 receptor (IL-4R α & IL-13R α 1) (diagram of IL-4 signalling in Figure 3.12). Neither cytokine were expressed by stromal or epithelial cells, nor is the *IL2RG*, typically restricted to haematopoietic cells (Figure 3.11). Both *IL-4R* and *IL-13RA1* were expressed in stromal and epithelial cells. While *IL-4R* was upregulated in prostate stroma compared to control stroma, and upregulated in PCa epithelium compared to BPH, the opposite is true for *IL-13RA1*. This may indicate that IL-4 can better stimulate prostate stroma and PCa epithelium than control stroma and BPH epithelium, and that IL-13 is more effective in the alternative settings. Of the type II IL-4 signalling molecules, only *TKY2* was differentially expressed. It was upregulated in treated G19 stroma, indicating an increased propensity to respond to IL-13R α 1 ligation. IL-13R α 2 has controversial roles in IL-13 signalling and is developing more established associations with cancer progression and metastasis. Many have described it as a decoy receptor, since it lacks a cytoplasmic domain (Orchansky *et al.*, 1997). It has specificity for IL-13 but not IL-4, and is suggested to deplete local IL-13 availability and so preventing downstream IL-13 signalling. Examination of *IL-13RA2* in control stroma and prostate stroma and epithelium revealed that it was expressed to a considerably higher degree in BPH stroma than any other cultured cells.

3.2.5. Receptors of the TNF ligand family are expressed by stroma and epithelium

TNF α , *TNF β* (also known as TNF and lymphotoxin- α ; LTA, respectively) and *LT β* were not expressed in any tested cultured cells at the mRNA level (Figure 3.17). As previously discussed, *TNF α* has specificity for both TNFR1 and TNFR2 (a.k.a. TNFRSF1A and TNFRSF1B). It is evident from this data that stromal cells expressed both *TNFRSF1A* and *TNFRSF1B*, indicating a capacity for *TNF α* signalling through either receptor. Interestingly, only when treated with IFN γ &*TNF α* and not IL-4&*TNF α* were the receptors upregulated. To understand whether this was due to an IFN γ specific response or whether IL-4 signalling in some way inhibits *TNF α* receptor upregulation, each cytokine should be used in isolation and receptors analysed. Interestingly, prostate epithelium did not express *TNFRSF1B*, indicating an inability to signal through TNFR2.

Tumour necrosis factor alpha inducible protein 3 (TNFAIP3, a.k.a A20) is an inhibitor of TNF mediated apoptosis, as well as a number of other pathways including NF-kB and IL-1. Mice deficient in TNFAIP3 succumb prematurely to significant inflammatory diseases (Lee *et al.*, 2000). In all cultures except PCa epithelium, *TNFAIP3* was induced by both cytokine treatments. If activity correlates with mRNA expression, the lack of upregulated *TNFAIP3* would indicate tumour cell susceptibility to *TNF α* mediated apoptosis, consistent with other reports, particularly when combined with the lack of TNFR2 expression (Malynn and Ma, 2009). Consistent with this, *FADD* (fas associated via death domain; FADD) is higher expressed in PCa epithelium compared to BPH, but was not upregulated with treatment. *TRAF2* was upregulated in response to cytokine

treatment in all cell cultures except G19 epithelium, despite the absence of *TNFSF1B* expression in prostate epithelium. Lymphotoxin- β (LT β) as discussed in chapter 1, is required in lymphoid organogenesis, where LT β R on stromal cells facilitates recruitment of early immune cells. Interestingly, while it was expressed consistently highly on stromal cells from all settings and BPH epithelium, it was greatly upregulated on PCa epithelium, and inducible with IFN γ &TNF α . This may provide a mechanism for immune cell recruitment by tumour cells.

3.2.6. Expression of TLR in the cellular components of the prostate

The expression of toll like receptors (TLRs) in control and prostate stromal cells and prostate epithelium were analysed (Figure 3.14). TLRs play a key role in the innate immune response and ligand binding tends to induce expression of pro-inflammatory cytokines (typically IL-1, IL-6 and IL-8) and chemokines. Since the prostate often harbours acute and chronic infections, it is surmised that TLR expression might provide a mechanism for establishing the chronic inflammation that often causes clinical symptoms. *TLR5-TLR10* were not expressed in any of the cultured cells. While *TLR1* was expressed, it was possibly not at a high enough level to be physiologically significant. *TLR3* displayed a consistent pattern of expression across cell type and disease, indicating a shared ability to detect and respond to dsRNA.

The expression of *TLR2* and *TLR4* is interesting due to the distinct expression between cell types. Epithelial cells expressed *TLR2*, which was inducible upon treatment but this inducibility appeared to decline with disease progression, while stromal cells from all settings show no capacity to express this receptor. The opposite is true of *TLR4*, which was expressed and inducible in stromal cells but not in epithelium. TLR2 recognises an array of pathogen associated molecular patterns (PAMPs) (e.g. lipoproteins, porins and haemagglutinin). TLR4, on the other hand, has specificity for just a few PAMPs including lipopolysaccharide (LPS) and its derivatives, which are expressed on the outer membrane of Gram-negative bacteria. Endogenous ECM molecules such as hyaluronin and fibronectin are also able to bind and activate TLR4. It is unclear why TLR4 should be expressed on stromal cells but not epithelium, but may simply be another mechanism whereby stromal cells modulate and maintain homeostasis in the microenvironment. This is particularly relevant since its expression was increased upon IFN γ &TNF α treatment, and so might synergise with existing inflammation to promote an additional inflammatory response. The response of TLR4 to normal ECM components may instead be more relevant, and provide the prostate stromal compartment with a mechanism to detect aberrant remodelling and induce an inflammatory response.

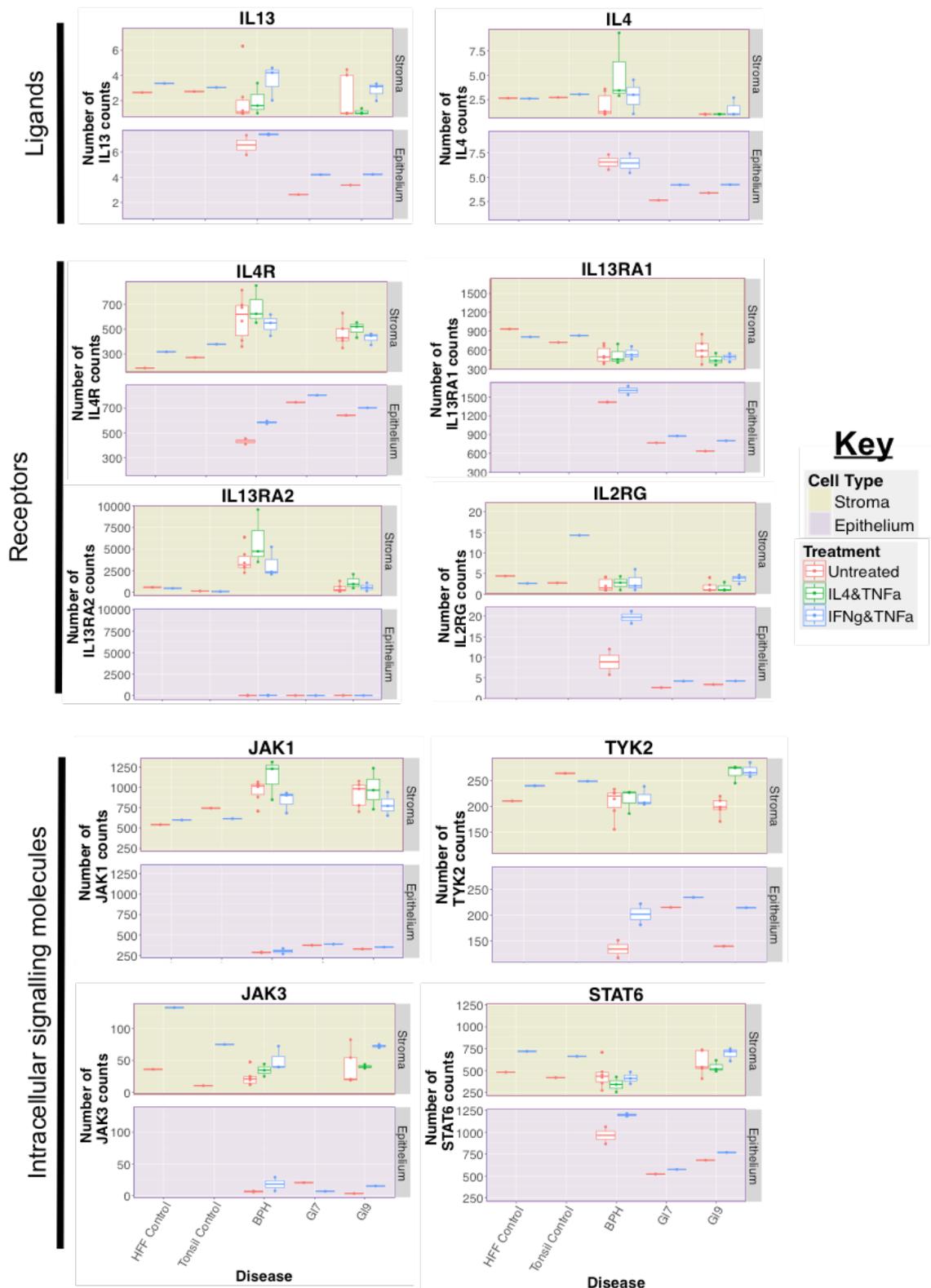


Figure 3.11: mRNA counts of genes belonging to the IL-4 signalling pathway

Counts of genes corresponding to IL-4 ligands, receptors and intracellular signalling molecules are plotted coloured by treatment group (refer to Figure 3.12 for a diagrammatic representing of IL-4 signalling). In each case, ligand counts are below the negative threshold. IL-4 receptors IL-4R, IL-13RA1, and IL-13RA2 but not the common γ chain IL2G and intracellular signalling molecules are expressed. Epithelial and stromal samples are plotted in separate facet grids with a scaled y-axis, which are coloured accordingly.

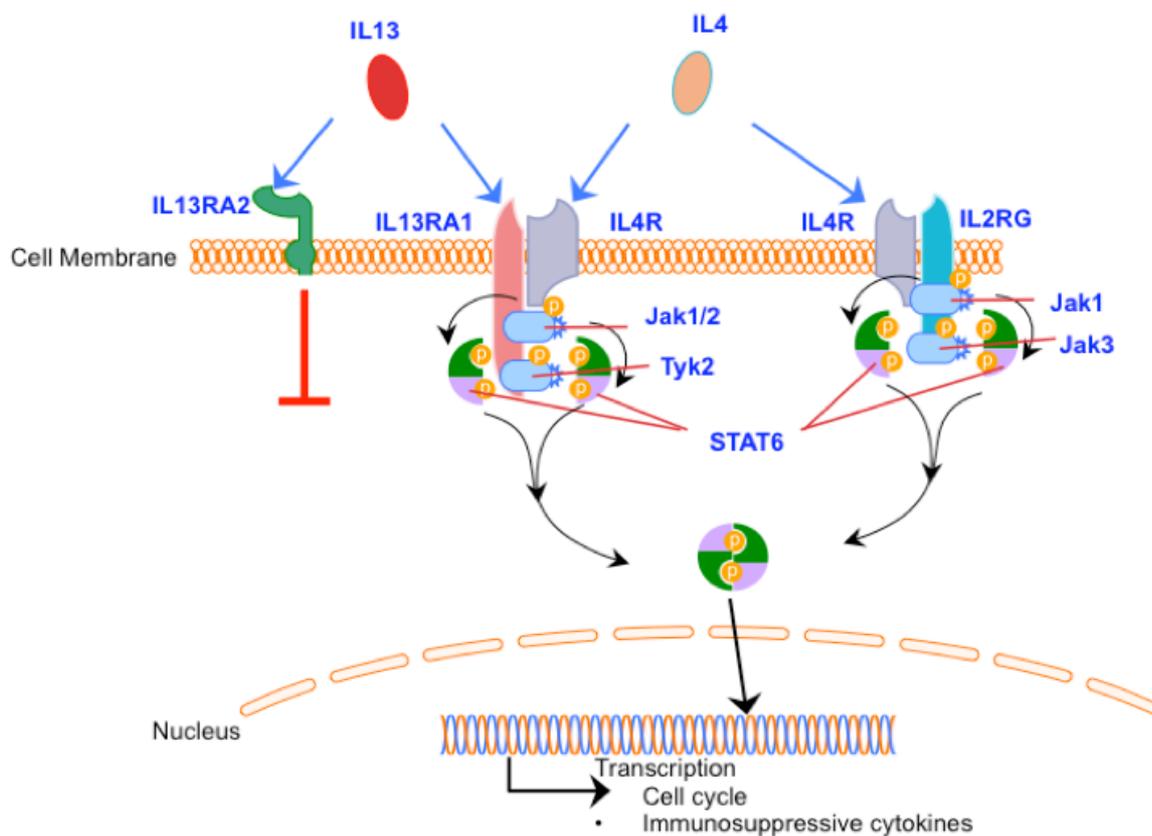


Figure 3.12: Schematic diagram of IL-4 signalling

A diagrammatic representation of IL-4 signalling is demonstrated as a reference to figure 3.11, created using motifolio®. IL-4 or IL-13 can signal through a complex of IL-13 α 1-IL-4R. The inhibitory or “decoy” receptor IL-13 α 2 can sequester only IL-13 but cannot transmit signal. IL-4 may also transmit signal through a heterodimeric receptor of IL-4R-IL2R γ (a.k.a. the common γ chain; γ c), when IL2R γ is expressed (normally restricted to haematopoietic cells). IL-13 α 1 transmits signalling through JAK1 or JAK2 and Tyk2, while IL2R γ signals via JAK1 and JAK3. Either signalling results the formation of a phosphorylated STAT6 homodimer.

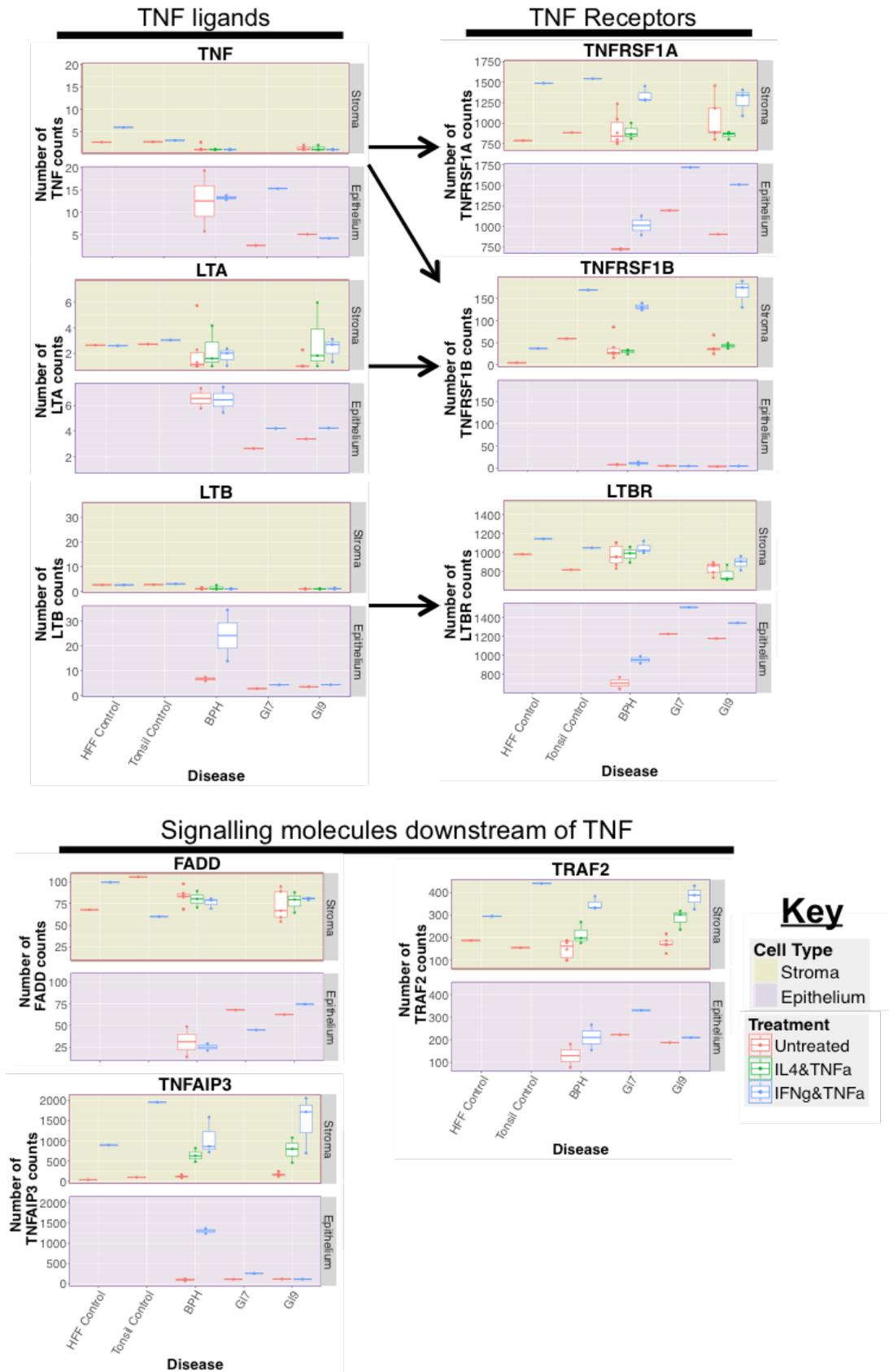


Figure 3.13: mRNA counts of genes belonging to the TNF signalling pathway

Counts of genes corresponding to TNF ligands, receptors and intracellular signalling molecules are plotted coloured by treatment group. In each case, ligand counts are below the negative threshold or very low. TNF receptors TNFRSF1A, TNFRSF1B and LTBR and intracellular signalling molecules are expressed. Epithelial and stromal samples are plotted in separate facet grids with a scaled y-axis, which are coloured accordingly.

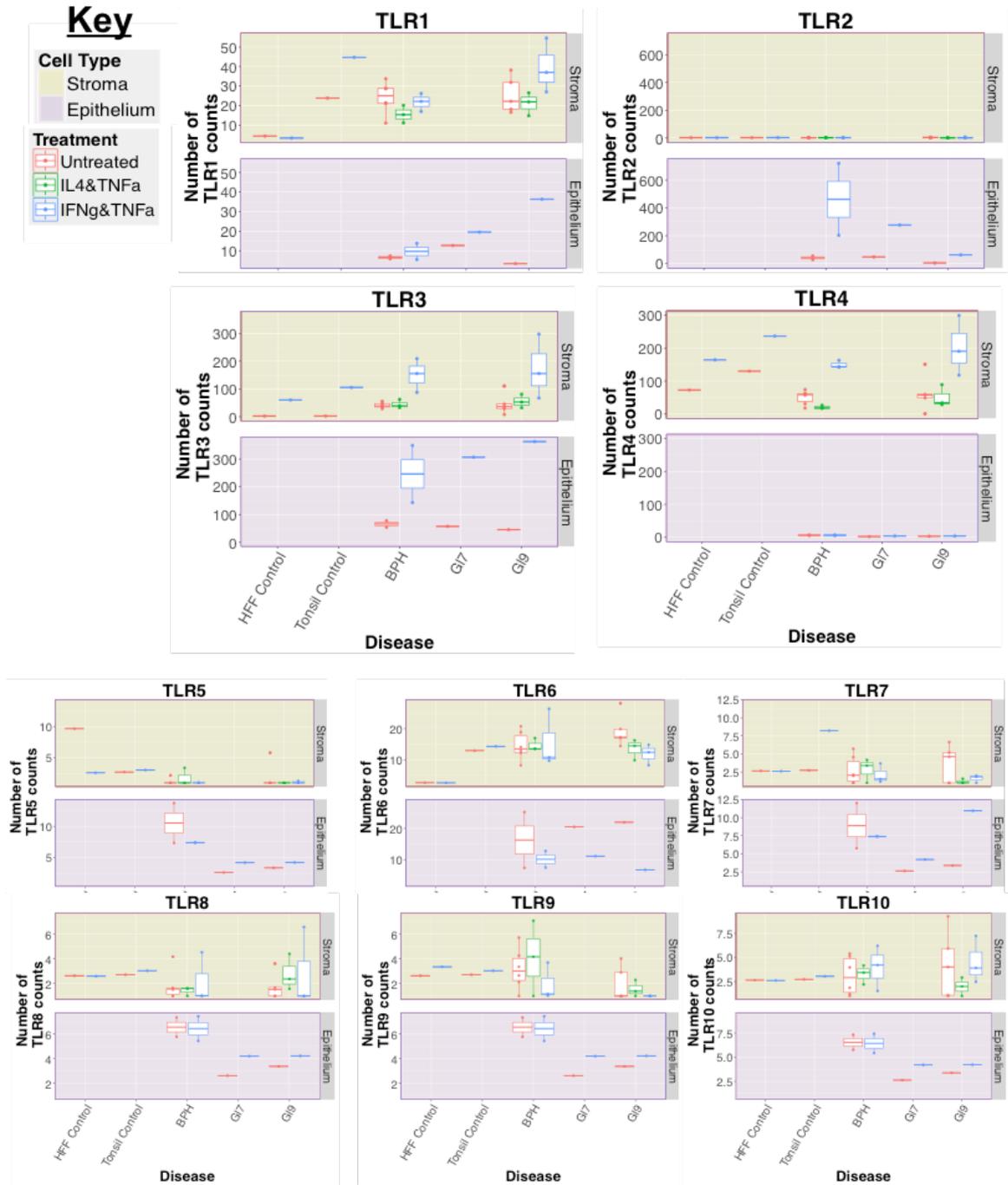


Figure 3.14: mRNA counts of genes corresponding to Toll like receptors

Counts of genes corresponding to TLRs are plotted coloured by treatment group. TLR5-TLR10 counts are below the negative threshold. TLR1-TLR4 was detected in either stromal or epithelial cells. Epithelial and stromal samples are plotted in separate facet grids with a scaled y-axis, which are coloured accordingly.

3.2.7. Investigating expression of cytokines associated with PCa

Knowing that both IL-6 and IL-1 have been associated with increased survival and proliferation of prostate tumour cells the mRNA expression of the signalling molecules of each pathway were examined.

Analysis of the upstream IL-1 signalling molecules demonstrated that *IL-1 α* & *IL-1 β* were expressed at the mRNA level to a greater extent in prostate epithelium, and inducible with IFN γ &TNF α (Figure 3.15 A). The activating receptor *IL-1R1* was expressed to a greater extent in prostate stromal cells (and inducible in control stroma), while *IL-1RAP* was expressed to a minor degree in prostate stroma and increased in epithelium (Figure 3.16 B). Since *IL-1RAP* is required for downstream signalling, this indicates while there is expression of *IL-1RAP*, there may be a greater capacity to respond to IL-1 in prostate epithelium than stroma. Interestingly, *IL-1RN*, which acts to inhibit IL-1R1 mediated signalling, was upregulated in IFN γ &TNF α treated BPH epithelium but no other cells. This suggests a mechanism to reduce local inflammation in BPH. The expression of *IL-1R2* is likely not enough to be physiologically significant.

The IL-6 pathway is mainly facilitated by JAK-STAT signalling and through both STAT3 mediated transcription of survival genes and by inhibition of p53 (or TP53) prevents apoptosis by 2 mechanisms (Figure 3.16 B). Cytokine treated stromal cells mainly expressed IL-6 whereas expression of the receptor *IL-6R* was greater in prostate epithelium, and consistent with other reports, upregulated in G19 epithelium (Figure 3.16 A). Stromal cells, particularly from the prostate, expressed consistently high levels of *IL-6St*, which is required for activation of cytosolic IL-6 signalling. Prostate epithelium still expressed significant levels of IL-6st expected to support IL-6 signalling in these cells. STAT3 is expressed in both cell types suggesting capacity to transduce IL-6 signalling. Of interest, *P53* was reduced in G19 epithelium at the transcriptional level, consistent with the tumourigenic associations of this transcription factor. IL-6R can facilitate IL-6 response in neighbouring cells as a secreted form. Taken together, these data indicate a capacity of IL-6 signalling in both stromal and epithelial cells, with stromal cells as a substantial source in the inflamed prostate. It is conceivable that IL-6 signalling in the inflamed prostate may therefore contribute to increased survival and p53 mediated inhibition of apoptosis, which if sustained has the potential to contribute to mutational burden and tumourigenesis.

TGF- β is associated with PCa due to the observation that tumour cells becoming refractory to TGF- β mediated cell cycle inhibition, the high levels of activated TGF- β ligands detected in tumour tissues and the strong association with immune inhibition. Therefore, the TGF- β ligands contained within the nanoString panel were analysed. Since the TGF- β receptors were not present in the panel, whether the loss of receptors in PCa cells could be attributed to TGF- β refraction could not be addressed. *TGF- β* isoforms 1 and 2 mRNA was expressed in all cell types and diseases analysed (Figure 3.17). While *TGF- β 1* is upregulated at the transcriptional level in tumour

cells with disease progression, it was expressed at particularly high levels in stromal cells regardless of disease. However, TGF- β is a cytokine with multiple levels of regulation at the post-translational stages of production, some of which were outlined (section 3.1.6). Some of these mechanisms are mediated through proteolytic cleavage by plasmin and MMP, or the conformational change in the latent complex facilitated by integrins and THBS1. Only *THBS1* could be analysed by nanoString, which was found also to be expressed to excess in the stroma. Interestingly, *THBS1* was upregulated in the tumour cells derived from the patient with G17 disease.

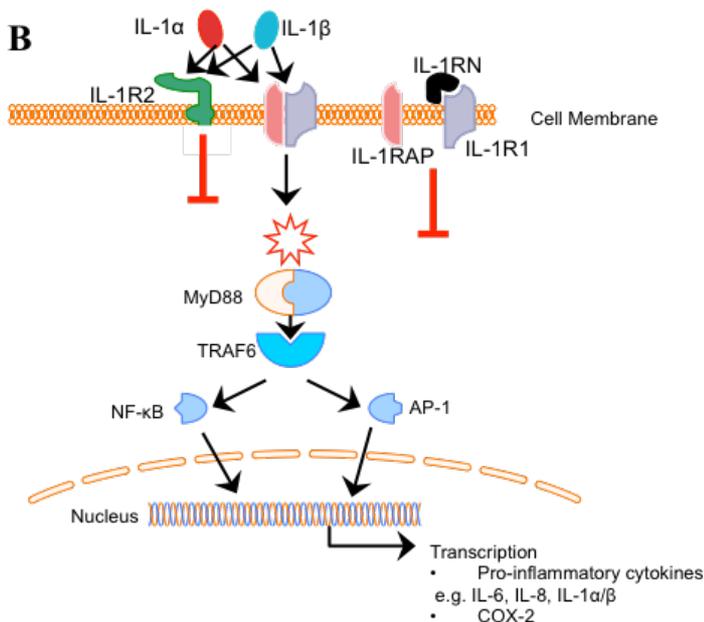
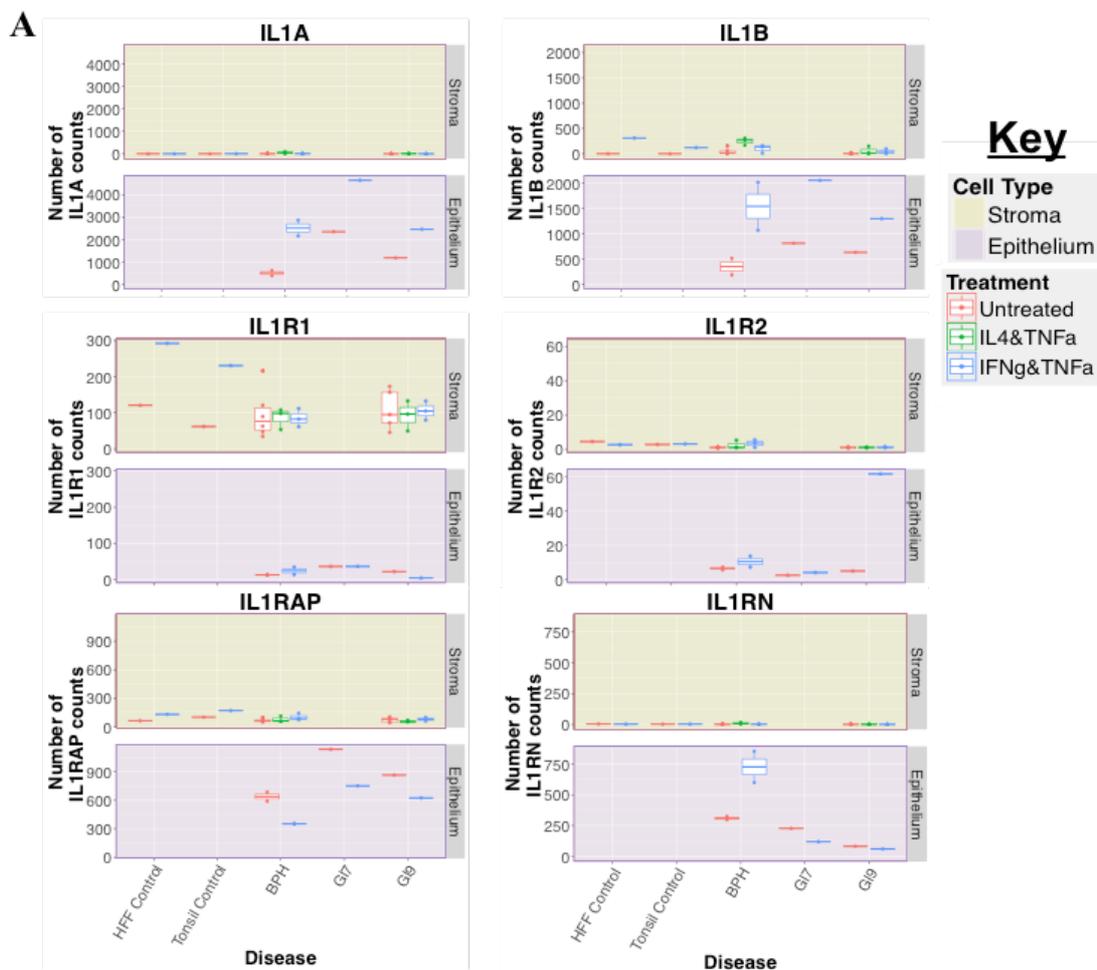


Figure 3.15: mRNA counts of genes belonging to the IL-1 signalling pathway

(A) Counts of genes corresponding to IL-1 ligands and receptors are plotted, coloured by treatment group. Epithelial and stromal samples are plotted in separate facet grids with a scaled y-axis, which are coloured accordingly. (B) A diagrammatic representation of IL-1 signalling, created using motifolio®. IL-1 α and IL-1 β can signal through a heterodimeric receptor of IL-1RAP and IL-1R1. The inhibitory or “decoy” receptor IL-1R2 can sequester only IL-1 α /IL-1 β but cannot transmit signal. IL-1RN is an inhibitory ligand, which blocks and prevents signalling through the IL-1R/IL-1RAP.

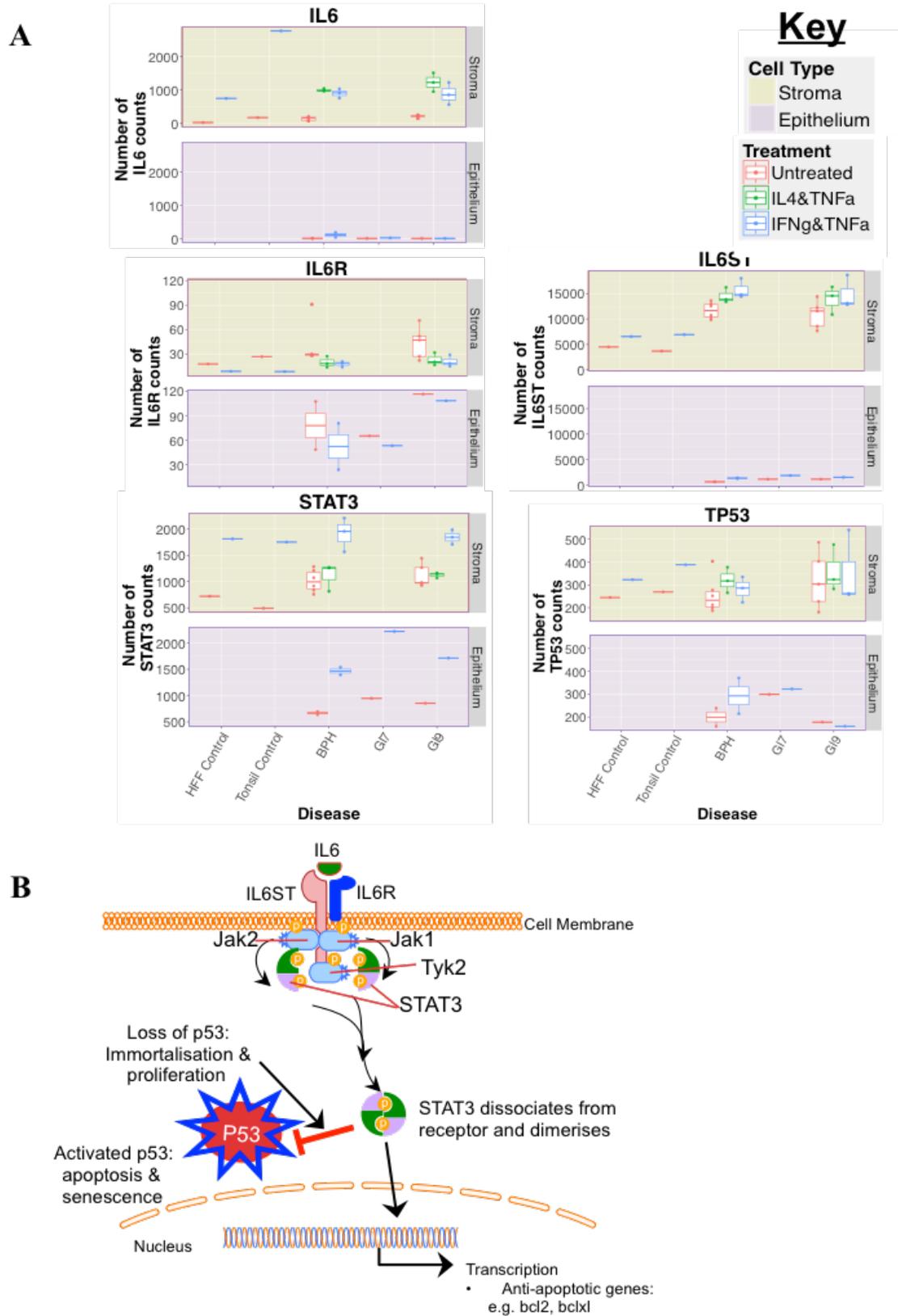


Figure 3.16: mRNA counts of genes belonging to the IL-6 signalling pathway

(A) Counts of genes corresponding to IL-6 ligands, receptors and intracellular molecules are plotted, coloured by treatment group. Counts below 20 are considered below background and negative controls. Epithelial and stromal samples are plotted in separate facet grids with a scaled y-axis, which are coloured accordingly. (B) A diagrammatic representation of IL-6 signalling, created using motifolio®. IL-6 signals through a heterodimeric receptor of IL-6R and IL-6ST. IL-6ST transmits signal through activation of STAT3, which forms a homodimer and mediates transcription. STAT3 homodimer also prevents P53 activation, so inhibiting cell death.

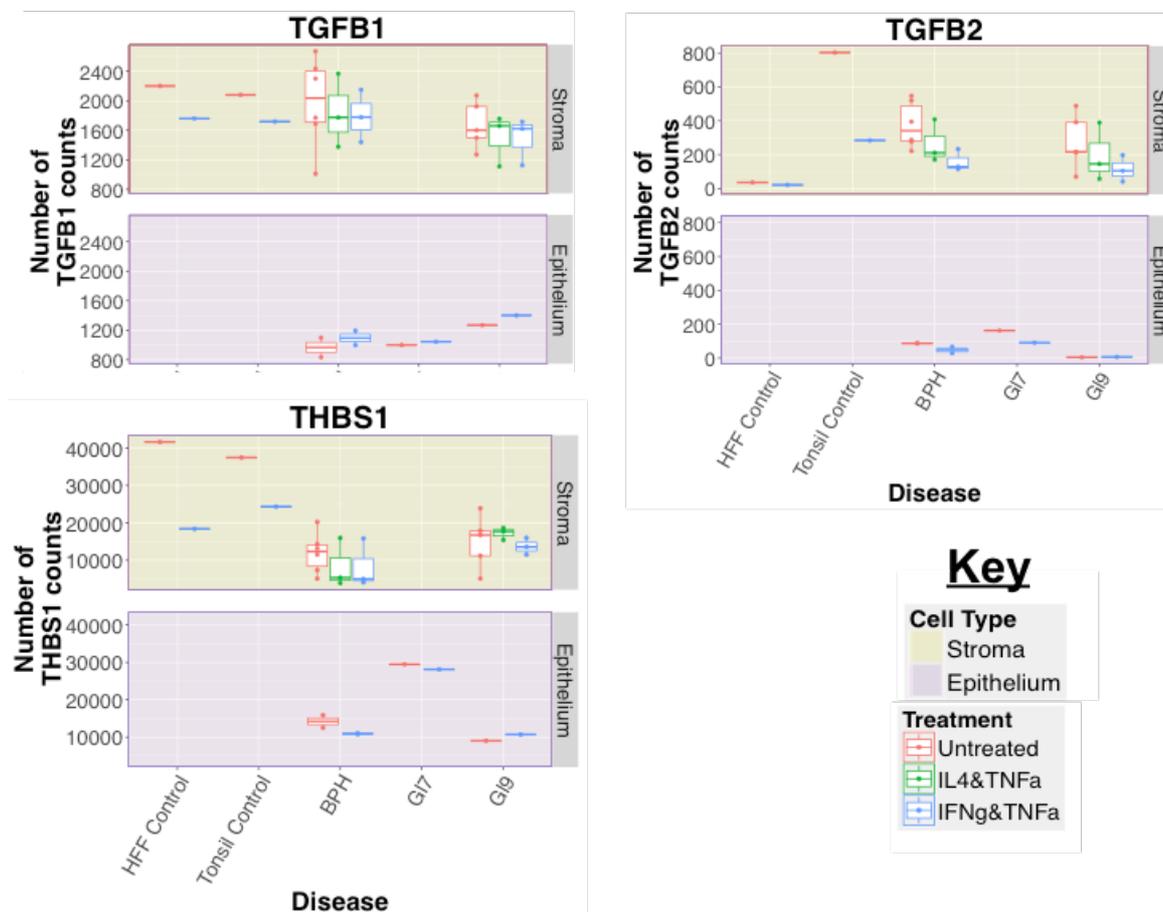


Figure 3.17: mRNA counts of genes corresponding to TGF- β associated molecules

Counts of genes corresponding to TGF- β ligands and THBS1 (a latent TGF- β activator) are plotted, coloured by treatment group. Epithelial and stromal samples are plotted in separate facet grids with a scaled y-axis, which are coloured accordingly. Counts below 20 are considered below background and negative controls.

3.2.8. Expression of AM

Cellular AM are important for many processes, not limited to: homeostatic maintenance of cellular polarity and tissue architecture, EMT and cellular communication. These processes are often implicated in cancer development and immune cell-host cell interactions, so whether changes occur in disease progression and cytokine treatment in prostate stroma and epithelial were analysed. Consistent with the literature, carcinoembryonic antigen-related cell adhesion molecules (CEACAM) *CEACAM1* and *CEACAM6* were expressed in epithelial cells but not stroma (Figure 3.18). These AM were initially discovered to be upregulated in the tumour epithelium of colorectal cancer and expressed in embryonic tissues at high levels and are used as a prognostic indicator in colorectal cancer (CRC) and breast cancer (BCa) (Beauchemin and Arabzadeh, 2013; Gold and Freedman, 1965). Overexpression has been associated with aggressive disease through involvement in migration and invasiveness (Ebrahimnejad *et al.*, 2004). *CEACAM1* and *CEACAM6* (also known as NCA) epithelial expression in the prostate is normal. *CEACAM1* is positively correlated with tumour progression in gastric cancers, though the opposite is true for PCa, where *CEACAM1* has been shown to be lost in human PCa tissues (Busch *et al.*, 2002; Shi *et al.*, 2014). This loss is thought to have implications in the regulation of cell proliferation and polarity, and reintroduction of *CEACAM1* in prostate cancer cells delayed tumour growth (Busch *et al.*, 2002; Hsieh *et al.*, 1995; Kleinerman *et al.*, 1995). While *CEACAM6* expression has not been widely investigated in PCa, it was found not to be upregulated with disease progression compared to other tumours (Blumenthal *et al.*, 2007). Here it is demonstrated that both *CEACAM1* and *CEACAM6* were decreased in epithelial tumour cells compared to BPH. It may be that the loss of CEACAM in the tumour cells of PCa is indicative of epithelial-mesenchymal transition (EMT), since they are not expressed by prostate stroma.

ICAM-1 and *VCAM-1* are best documented for the role in transmigration of immune cells across the vascular endothelial barrier into tissues. Endothelial cells in inflamed tissues upregulate *ICAM-1* and *VCAM-1*, which bind to leukocyte function associated antigen-1 (LFA-1) and very late antigen-4 (VLA-4) respectively, expressed on activated immune cells to facilitate immune extravasation to inflamed tissues. However, both *ICAM-1* and *VCAM-1* also mediate stromal-ECM interactions and were previously shown to have increased cell surface expression in high Gleason stroma and in inflammatory conditions, which correlated with the propensity of stromal cells to mediate PCa cell invasion (Lakins, 2012). Upon analysing *ICAM1* and *VCAM1* it's confirmed that both were expressed by prostate stroma and upregulated when treated with cytokines. Both prostate epithelium and stroma upregulated *ICAM1* when treated with $IFN\gamma$ & $TNF\alpha$. The expression was greater in prostate stroma, and moderately upregulated by $IL-4$ & $TNF\alpha$. In contrast, *VCAM-1* was upregulated to a greater degree when treated with $IL-4$ & $TNF\alpha$ than $IFN\gamma$ & $TNF\alpha$. As prostate epithelium was not treated with $IL-4$ & $TNF\alpha$ it cannot be confirmed whether this is a consistent response. At the mRNA level differential expression by disease grade was not detectable.

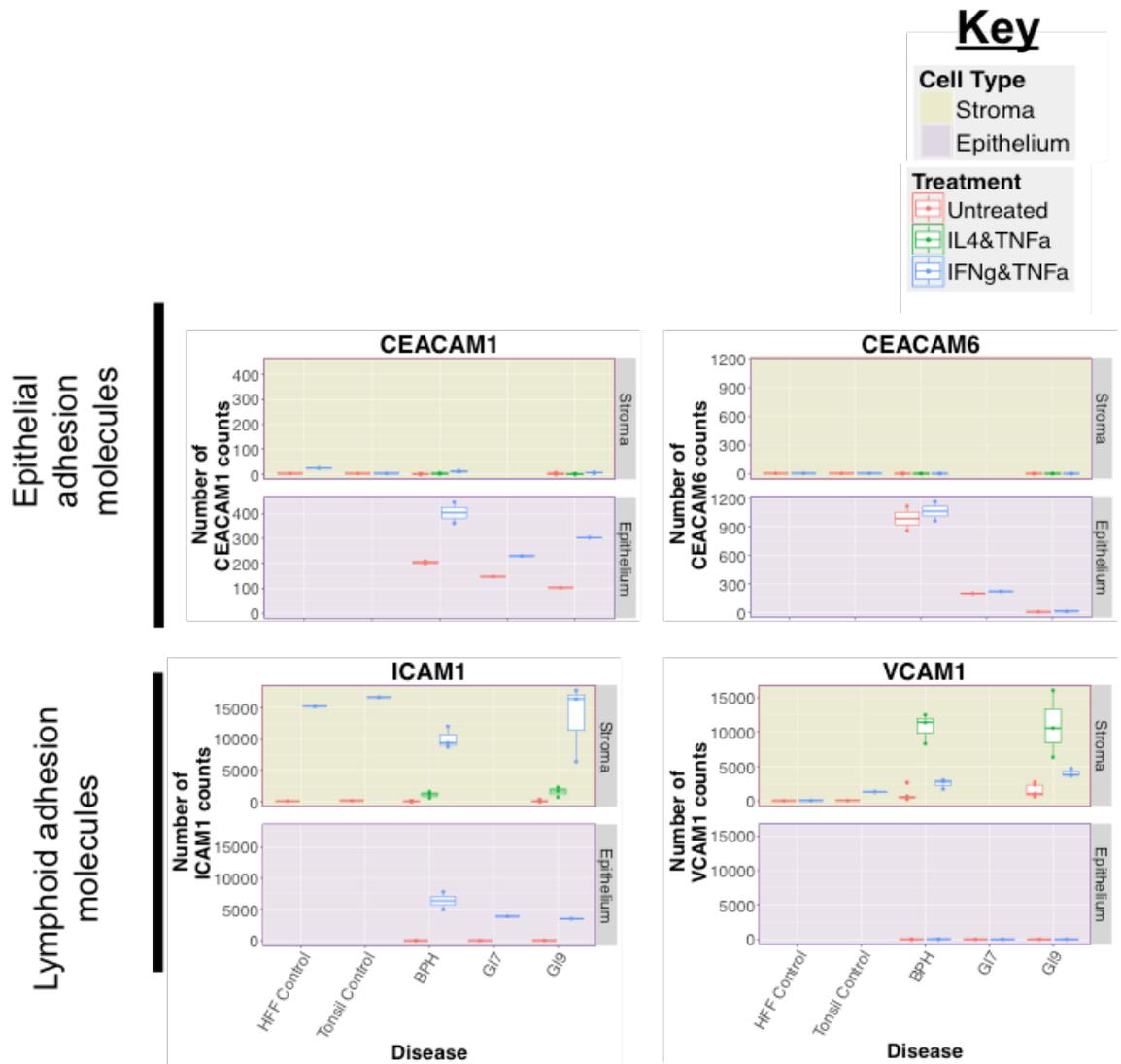


Figure 3.18: mRNA counts of genes corresponding to AM

Counts of genes corresponding to AM are plotted, coloured by treatment group. Epithelial and stromal samples are plotted in separate facet grids with a scaled y-axis, which are coloured accordingly. Counts below 20 are considered below background and negative controls.

3.2.9. Immunomodulatory roles of stroma in the prostate

When MHC Ia is expressed on the cell surface they can bind to CTL TCR and NK cell killer cell inhibitory receptors (KIRs). The expression of MHC Ia molecules and upregulation upon cytokine signalling indicates a propensity for stromal and epithelial cells to present internal antigens for surveying CTL and ability to inhibit NK cells (Figure 3.19). The expression of MHC Ib molecules has been implicated in preventing host rejection of transplant tissue and as a mechanism of tumour immune escape (Kochan *et al.*, 2013). HLA-E is a known high affinity ligand for the NK cell and CTL inhibitory receptor CD94/NKG2A and can to a lesser degree bind to the NK activating receptor CD94/NKG2C. HLA-G delivers an inhibitory signal to interacting with a range of receptors expressed on different immune cells (T cells B cells, NK cells and APCs). MSCs co-express HLA-E and HLA-G on the surface, which is hypothesised to, in part, provide these cells with the weak immunogenicity they exhibit (Stubbendorff *et al.*, 2013). HLA-G was expressed in prostate epithelium and consistently above 500 counts in stroma, both were found to upregulate *HLA-G* when treated with IFN γ &TNF α . This indicates that both prostate stroma and epithelial cells are capable of delivering an inhibitory signal to infiltrating immune cells, particularly in inflammatory environments. In light of these data, it is conceivable that co-expression by these cells may confer resistance to CTL-mediated killing, as in similar reports (Malmberg *et al.*, 2002).

To understand whether stromal cells have the capacity to present antigen on MHCII molecules those that were included in the nanoString panel were analysed. Antigen processing on MHCII is demonstrated in Figure 3.21. Given that *HLA-DM* α/β and *HLA-DO* were expressed at low levels in stromal cells even under IFN γ &TNF α treatment, it is indicative a reduced capacity to cleave the invariant chain (or CD74), which is expressed at high levels (Figure 3.20). The MHCII molecules that present antigens on the cell surface are transcribed from the genes *HLA -DP*, *-DQ* and *-DR*, which were as expected expressed at variable levels across patients but generally become upregulated with IFN γ &TNF α treatment.

While it is possible stromal and epithelial cells can present antigen, it is unlikely they can stimulate T cell activation without signal 1 and signal 2, therefore co-stimulatory molecules were analysed (Figure 3.22). *CD80*, *CD86*, *CD70* and *ICOSLG* were not expressed at levels above background in stromal or epithelial cells. Interestingly *CD40* was expressed upon IFN γ &TNF α in both stromal and epithelial cells, as was *TNFRSF14* (best known as HVEM or LIGHT receptor). CD40 expression has been previously documented in human fibroblasts and upon ligation has been shown to deliver an activating signal to the fibroblasts to induce expression of cytokines (IL-6 & IL-8), hyaluronan and COX-2 (Sempowski *et al.*, 1998; Wassenaar *et al.*, 1999; Yellin *et al.*, 1995). Although, the CD40 expression is not able to induce TH cell activation alone, pre-activating fibroblasts with anti-CD40 induces TH cell proliferation, most likely through production of inflammatory molecules by fibroblasts (Nakayama *et al.*, 2015; Willermain *et al.*, 2000).

In light of the described data the expression of known immune inhibitory molecules expressed by prostate stroma and epithelia were analysed, bearing striking results (Figure 3.23). In some cases, for example *IDO1*, molecules are upregulated by both cell types under inflammatory conditions, however the level expressed by stroma far exceeds that of epithelium. KIR ligands MICa and MICb are both upregulated in prostate tumour cells compared to BPH. The expression of MICb in stroma was similar to that of PCa cells. MICb expression was upregulated by stroma treated with IFN γ &TNF α to a greater degree than in epithelium. IFN γ &TNF α treated cells expressed both *CD274* (PD-L1) and *PDCD1LG2* (PD-L2). The expression by treated stroma was greater than that of treated epithelium, whereas the opposite was true for *PDCD1LG2*. *CD276* (B7-H3) is hypothesised to deliver an inhibitory signal to T cells, however its interacting partner and the mechanism behind this have not been fully unveiled. *CD276* (B7-H3) was expressed consistently in stroma, but interestingly was upregulated by PCa cells. *PTGS2* (better known as COX-2) is responsible for the production of prostaglandins, potent mediators of inflammation. Non-steroidal anti-inflammatory drugs (NSAIDS) such as aspirin target COX-2 (and COX-1 to a lesser extent) to reduce consequences of inflammation. Interestingly, although expressed by stroma in BPH and PCa, *PTGS2* was expressed moderately higher in BPH stroma than G19 stroma both basally and upon cytokine treatment. Moreover, the expression was elevated in IFN γ &TNF α treated epithelium and control stroma. Altogether these data indicate a high propensity of stromal cells to inhibit the immune system, particularly under inflammatory conditions. Prostate epithelium share this capacity to an extent, although expression was often higher in stroma than epithelium. When considering an environment such as the prostate this information is highly relevant, given the relative frequency of prostate stroma to epithelium. It also indicates that in a stroma heavy prostate tumour, the threshold for immune activity is likely required to be much higher than that containing a lower population of stroma.

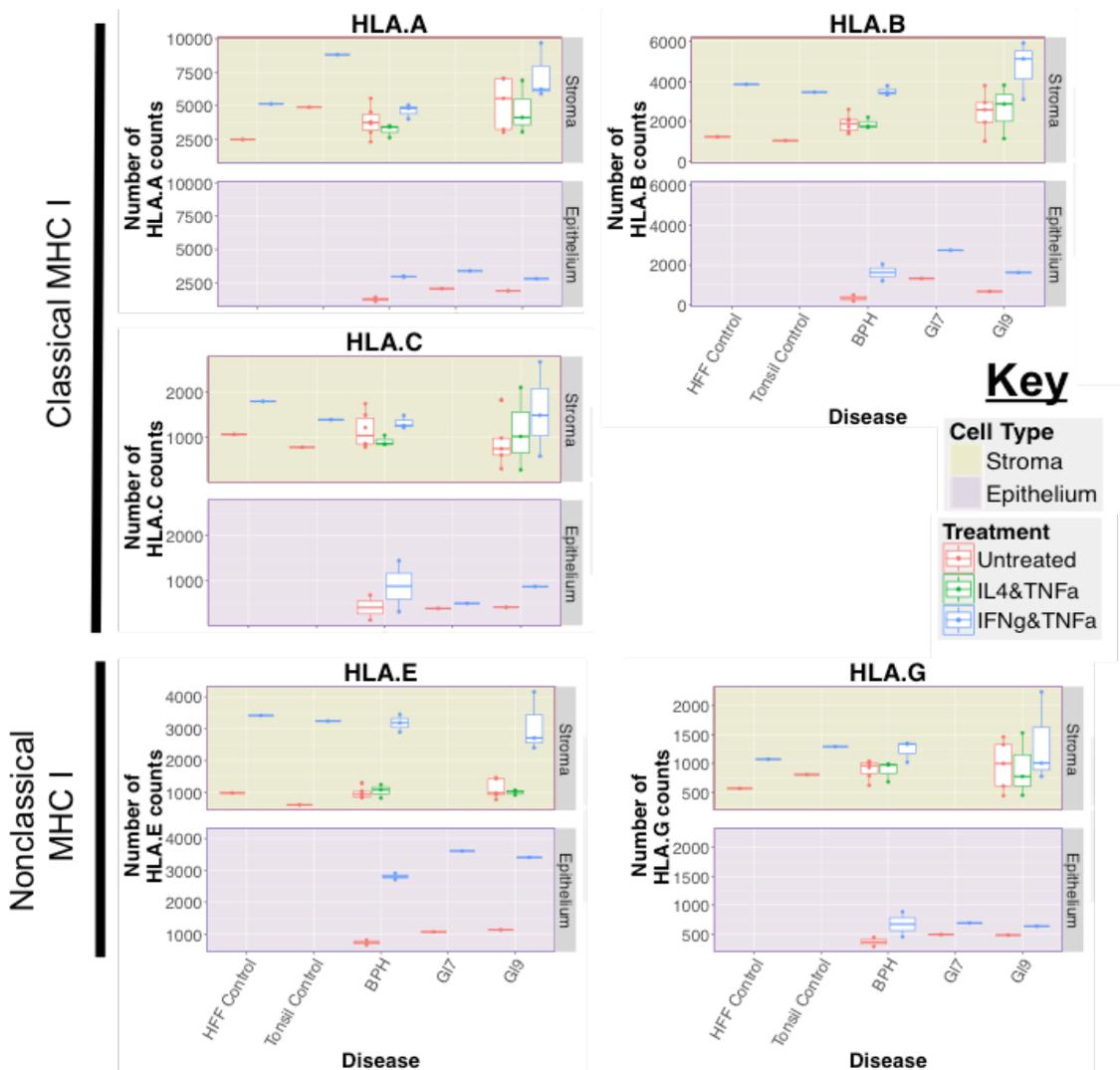


Figure 3.19: mRNA counts of genes corresponding to MHC class I molecules

Counts of genes corresponding to classical (MHC Ia) and nonclassical (MHC Ib) MHC I molecules are plotted, coloured by treatment group. Epithelial and stromal samples are plotted in separate facet grids with a scaled y-axis, which are coloured accordingly. Counts below 20 are considered below background and negative controls.

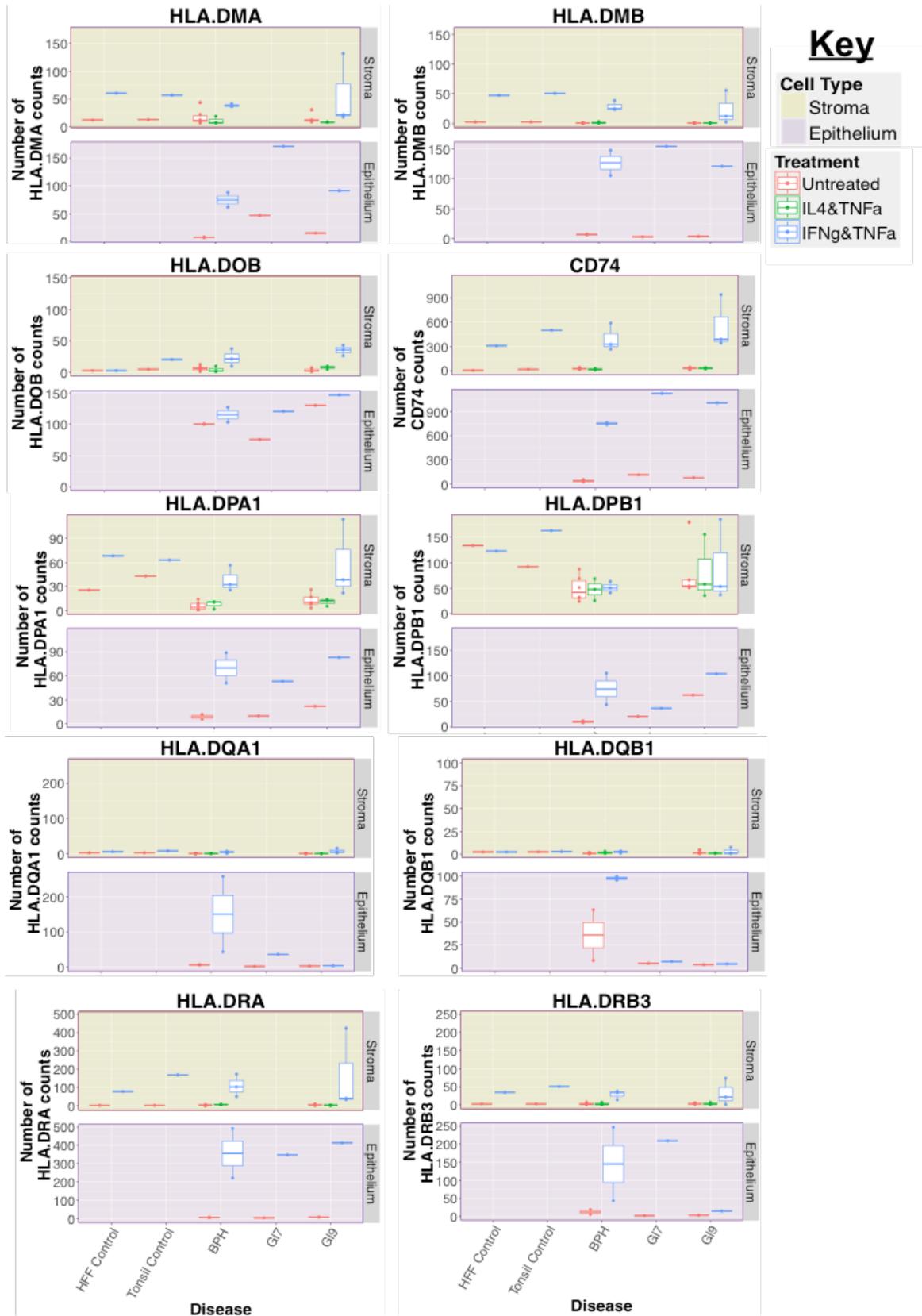


Figure 3.20: mRNA counts of genes belonging to MHC class II molecules

Counts of genes corresponding to MHCII molecules are plotted coloured by treatment group (refer to **Figure 3.21** for a diagrammatic representing of MHCII processing). Epithelial and stromal samples are plotted in separate facet grids with a scaled y-axis, which are coloured accordingly. Counts below 20 are considered below background and negative controls.

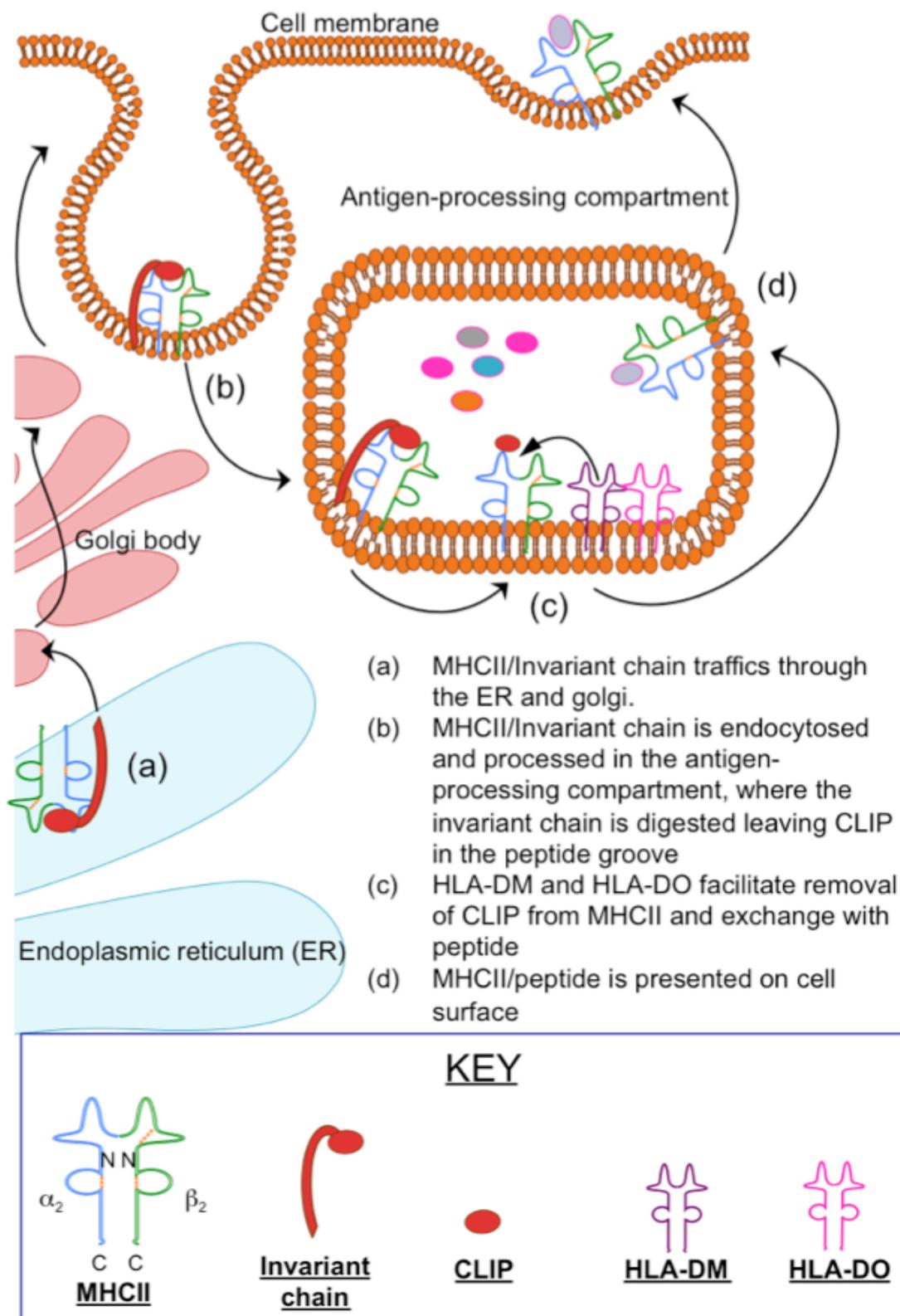


Figure 3.21: Schematic diagram of MHCII processing

A diagrammatic representation of MHCII processing is demonstrated as a reference to Figure 3.20, created using motifolio®. MHCII molecules (HLA DP, DQ and DR) are bound to the invariant chain (CD74) blocking the peptide groove in the ER. The complex is trafficked to the cell surface via the golgi body, where it is endocytosed and processed for antigen binding. The invariant chain is cleaved, leaving CLIP in the peptide groove. HLA-DM and HLA-DO facilitate removal of CLIP for exchange with peptide. The MHCII/peptide complex is trafficked back to the cell surface for antigen presentation.

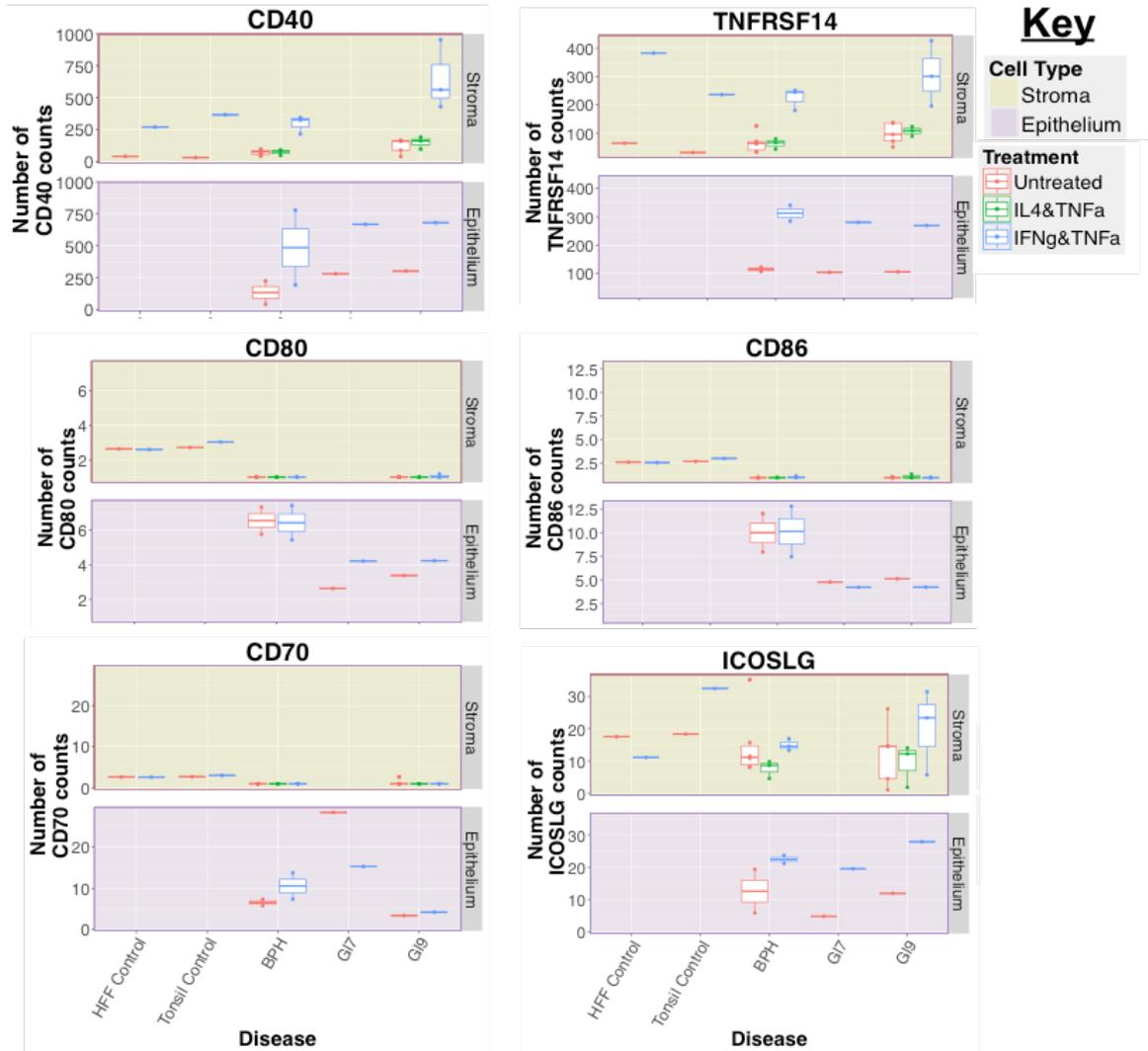


Figure 3.22: mRNA counts of genes involved in immune cell stimulation

Counts of genes corresponding to co-stimulatory molecules are plotted coloured by treatment group. Epithelial and stromal samples are plotted in separate facet grids with a scaled y-axis, which are coloured accordingly. Counts below 20 are considered below background and negative controls.

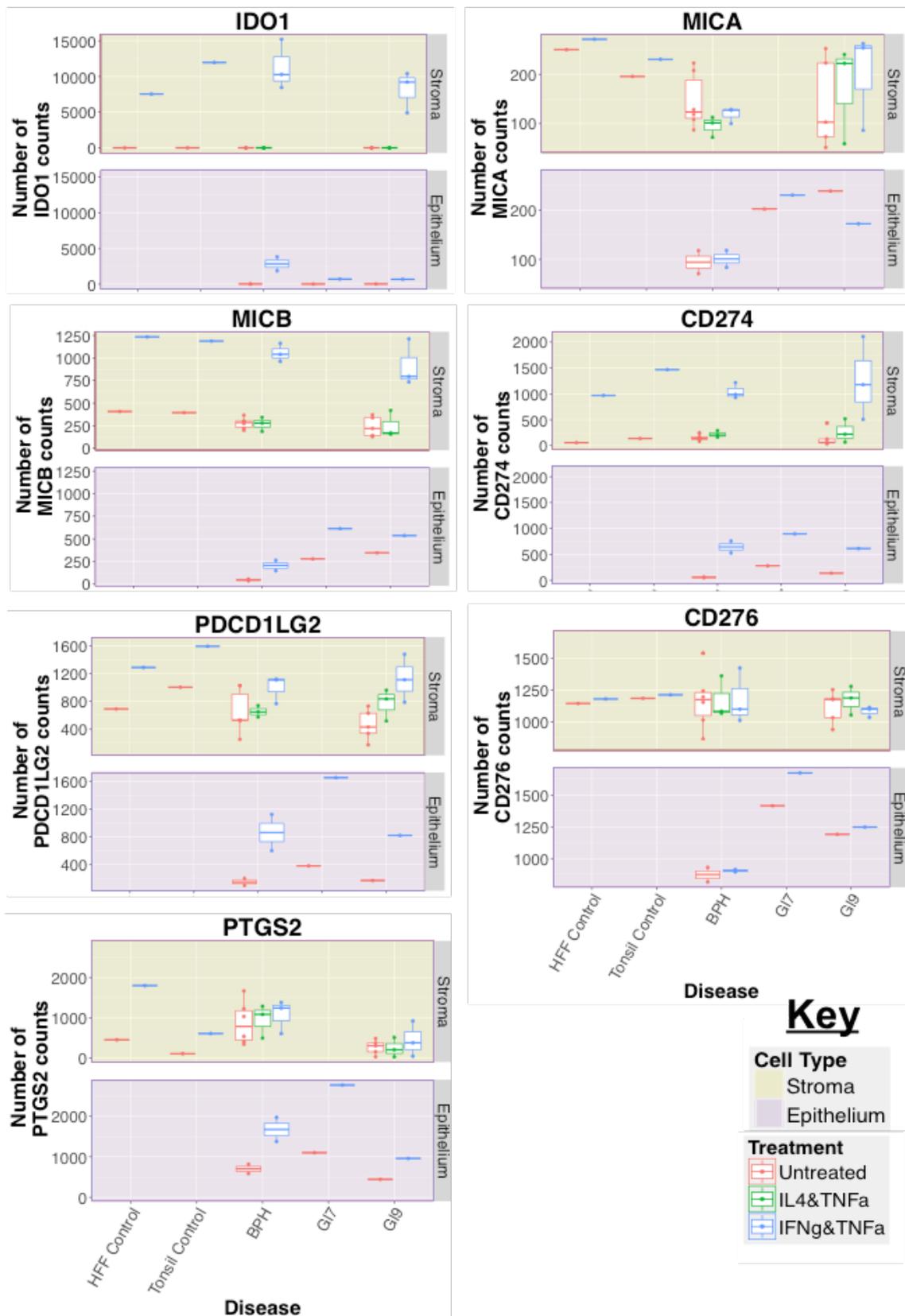


Figure 3.23: mRNA counts of genes involved in immune cell inhibition

Counts of genes corresponding to inhibitory molecules are plotted coloured by treatment group Epithelial and stromal samples are plotted in separate facet grids with a scaled y-axis, which are coloured accordingly. Counts below 20 are considered below background and negative controls.

3.2.10. Summary of results

- Stromal cultures express markers consistent with fibroblast phenotype but lack endothelial and epithelial markers. Stromal cells do not contaminate epithelial cultures. This permits the use of *in vitro* models to separate these prostate populations to understand their interactions with infiltrating immune cells and their relative contribution to common PCa associations.
- Stromal and epithelial cells express type I and type II IFN receptors and TNF receptors but not the ligands, indicating the capacity to respond to local inflammation.
- IL-4 signalling appears to be modified in G19 stromal cells. They lack the inhibitory *IL-13RA2* receptor and display increased levels of *TYK2*, the intracellular signalling molecule. This may indicate an increased capacity for G19 stroma to respond to local IL-4. This may be particularly relevant in PCa metastasis as reports demonstrate increased 3D migration when treated with IL-4, which is imparted on neighbouring tumour cells.
- Epithelial cells expressed *IL-1*, whereas stroma expressed the receptors, potentially indicating a paracrine signalling mechanism between stroma and epithelium in the prostate. *IL-6* is inducibly expressed in stromal cells while the receptors are expressed by both stroma and epithelium, which may support anti-apoptotic mechanisms. Prostate stromal cells constitutively expressed *TGF- β* at a higher level than prostate epithelium.
- *ICAM1* is inducibly expressed in both stromal and epithelial cells, though the expression by stroma far exceeds that of epithelium. *VCAM1* is upregulated to a greater extent under IL-4&TNF α treated conditions in stroma, IFN γ &TNF α treated stroma upregulated *vcam1* but epithelium do not.
- Stromal cells express and upregulate MHCIIb inhibitory molecules.
- Stromal cells do not express *HLA-DM* or *HLA-DO*, required for the removal of CLIP from the peptide groove of MHCII (which is expressed and cytokine inducible), consistent with inability to present antigen like classical APCs. Similarly, co-stimulatory molecules (*CD80*, *CD86*, *CD70* and *ICOSLG*) are not expressed, although stromal cells may be able to induce immune cell activation upon CD40 ligation.
- Stromal cells express many immune inhibitory molecules, including IDO1, PD-L1 and PD-L2.

3.3. Discussion

Histological studies give context in 3D space cell specific expression, though observations often vary between patients making it difficult to draw conclusions. This, in part, may be due to differences in unidentified signals within the environment and temporal variability. An alternative method has been LCM mediated extraction of cell types from tissue and downstream transcriptional analysis. Again this is highly dependent on signals in the local environment and further discounts temporal and spatial relevance of molecules. Another caveat of this kind of method is incomplete understanding of the source of signals detected since immune cells infiltrating tissue can be extracted alongside tissue specific cell types from their designated microanatomical space. By culturing patient prostate cells and confirm cell types, better control of the environment can be achieved and used to understand the response of cells to specific signals. Knowledge from studies like this can be used to inform histological studies about the importance of certain inflammatory states.

3.3.1. Influence of disease on transcriptional profile

Primary epithelial cells displayed a distinct and compelling demarcation in the transcriptional profile with disease progression, though their stromal counterparts did not. Tumour epithelial cells, though not the focus of this thesis, exhibit a clear loss of a number of immune related molecules. Similarly, a distinct panel of molecules become over expressed. Tumour cells display a number of features consistent with neoplastic transformation, including loss of P53 and epithelial associated AM (Beauchemin and Arabzadeh, 2013; Brady and Attardi, 2010; Busch *et al.*, 2002). Unsurprisingly, stromal cells cluster completely separately from epithelial cells. Stroma separates only subtly by disease, which is more apparent by PCA than hierarchical clustering. Direct comparison of untreated prostate stromal cells shows only a few differentially expressed genes (of those that are included in the nanoString panel), explaining this marginal separation. Interestingly, within these genes is the inhibitory receptor belonging to the IL-4/IL-13 pathway, IL-13R α 2. This may be of particular clinical significance since IL-4 has been previously shown to induce a migratory phenotype in prostate stromal cell (Lakins, 2012). Though IL-13 was not directly tested, the documented redundancy of IL-4 and IL-13 suggests a similar response may be induced by IL-13 (Hallett *et al.*, 2012). The loss of the inhibitory receptor suggests in the context of a high IL-4/IL-13 environment, G19 stroma will be less able to sequester IL-13 preventing its signalling in stromal and epithelial cells. Moreover, the elevated IL-4/IL-13 signalling (exacerbated by the lack of *IL-13R α 2* and increased *TYK2*) will induce a migratory phenotype in stromal cells, which has been shown to confer invasiveness of tumour cells (Lakins, 2012). Another included in this category is *PTGS2* (better known as COX-2). The current study identifies a G19 stromal specific loss in expression of *COX-2*. This is contrary to the literature, as COX-2 upregulation is frequently associated with cancer progression (Gupta *et al.*, 2000; Kirschenbaum *et al.*, 2000). Consistent with this pro-tumour role aspirin (a COX-2 inhibitor of the class of NSAIDs) treatment contributes to men having a reduced risk of PCa (Jafari *et al.*, 2009; Salinas *et al.*, 2010).

Physiologically, COX-2 is responsible for the production of the inflammatory mediators prostaglandins. Prostaglandins are thought to be involved in tumour progression through the induction of cytokines like IL-6 and T cells immunosuppression (Chinen *et al.*; Hinson *et al.*, 1996; Li *et al.*, 2015; Mahic *et al.*, 2006). The clinical significance of this finding is unclear and merits further investigation. Although, this may be an artefact of comparing G19 stroma to BPH rather than normal, or mRNA expression may not be representative of protein expression.

3.3.2. Cytokine signalling in prostate cancer with links to infection

Cytokine signalling has been attributed to tumour survival in many tissues. In PCa this has been mainly IL-1, IL-6, TGF- β and their related molecules, which are often found elevated in the serum or tissue of PCa patients (Culig and Puhr, 2012; Diener *et al.*, 2010; Ivanovic *et al.*, 1995; Rodriguez-Berriguete *et al.*, 2013; Shariat *et al.*, 2001). Though the cellular source has been disputed, so determining this can reveal mechanisms underpinning TME signalling required for tumour survival. IL-1 expression by PCa cell lines and increased detection in PCa compared to healthy prostate tissue has pushed associations with IL-1 signalling and cancer progression (Abdul and Hoosein, 2000; Ricote *et al.*, 2004). This is supported by studies demonstrating IL-1 mediated proliferation and differentiation to a more aggressive neuroendocrine phenotype in PCa cells (Liu *et al.*, 2013). In the current study, epithelial cells expressed IL-1 α and IL-1 β . The receptors were expressed on epithelia and stroma, evidence for autocrine and paracrine signalling initiated by epithelium. The reduced expression of IL-1 inhibitory molecules (IL-1RII and IL-1RN) by tumour epithelia indicates a potential mechanism of increased IL-1 signalling in cancer. IL-1 in part mediates its pro-tumour functions through induction of IL-6 and COX-2, which may account for increased detection of these molecules in high grade PCa (Li *et al.*, 2012a; Tsuzaki *et al.*, 2003).

IL-6 has been detected in prostate tumour epithelial cells *in vitro* and in the prostate stromal compartment by histology, supporting both autocrine and paracrine growth signals in PCa (Giri *et al.*, 2001; Sung *et al.*, 2013). In contrast to many *in vitro* studies of PCa cell lines, this study found IL-6 was expressed and inducible by both IL-4 and IFN γ in prostate stromal cells but not primary epithelial cells. This finding corroborates a recently published histological study (Yu *et al.*, 2015). They demonstrate a stromal specific expression of IL-6, which was increased in areas of inflammation and postulate that IL-6R or IL-6RAP expressing epithelial/tumour cells can respond to stromal derived IL-6 (Yu *et al.*, 2015). Interestingly, they also demonstrate how discrepancies between theirs and earlier studies that suggested epithelial expression of IL-6 could be explained by the use of an unspecific batch of commercial anti-IL-6 (Morrissey *et al.*, 2010; Yu *et al.*, 2015). *In vitro* and *in vivo* studies have identified IL-6 as a regulator of prostate cancer cell proliferation by transactivation of the AR (Hobisch *et al.*, 1998; Malinowska *et al.*, 2009). Therefore, recognising stromal cells as potent IL-6 producers provides additional evidence for stromal mediated cancer progression in response to inflammatory signals.

TLR expression is upregulated upon inflammatory stimulus and inflammatory cytokines IL-1 and IL-6 are inducible by TLR ligation (Ozato *et al.*, 2002). This relationship has reinforced the association of infection with cancer initiation and progression (Sato *et al.*, 2009). It has been of particular interest in the prostate due to the prominence of infection and chronic inflammation. The current study highlights an IFN γ &TNF α mediated response of TLR1-4 in either stroma or epithelium, which is not present in IL-4&TNF α treated cells. PCa TLR expression has associated prognostic value and trials investigating the use of TLR agonists show some significance (Davis *et al.*, 2011; Gonzalez-Reyes *et al.*, 2011; Yamazaki *et al.*, 2014). Gonzalez-Reyes *et al.* found increased TLR3, 4 & 9 in PCa tissue compared to BPH (Gonzalez-Reyes *et al.*, 2011). The data presented in the current study implies this increase may be simply secondary to local inflammatory stimuli. While epithelia and stroma differentially expressed TLR2 and TLR4 (and TLR5-9 were not detected), publications with results to the contrary indicate that some signals (whether inflammatory or otherwise) may induce different responses. For example, TLR4 expression was detected on both stroma and epithelium, suggesting epithelia have the capacity to express TLR4 under certain conditions (Gatti *et al.*, 2009). TLR2/4 ligation and activation of the signalling pathway by high-mobility group box 1 protein (HMGB1) released upon cell death is beneficial for APC activation (Abe *et al.*, 2014; Rovere-Querini *et al.*, 2004). Interestingly, here HMGB1 was significantly downregulated in PCa epithelia (supplementary data), a characteristic documented elsewhere and found detrimental for initiation of anti-tumour immunity (termed tolerogenic as opposed to immunogenic cell death) (Kroemer *et al.*, 2013; Shen *et al.*, 2009; Yamazaki *et al.*, 2014). Notably though, elevated HMGB1 expression has conversely been shown to promote tumour cell survival (Jube *et al.*, 2012; Wu *et al.*, 2008). Thus, TLRs (and their ligands) have dual roles in tumour progression. On one hand they promote immune activation whilst on the other promoting tumour cell survival through IL-1/IL-6 dependent mechanisms or otherwise.

3.3.3. The influence of stromal cells in the TH1/CTL vs. TH2 immunity balance

As discussed in section 3.1.1 and demonstrated in Figure 3.1, TH1/CTL (IFN γ) vs. TH2 (IL-4) immune balance has different connotations in anti-tumour immunity. A high proportion of IFN γ producing cells in the TME is considered indicative of strong anti-tumour immunity. Alternatively, a high proportion of IL-4 producing TH2 cells are associated with tumour immune escape. However, this is based largely on the types of immune cells present and their effects on tumour cells but does not consider the impact these cells have on resident cells in the TME. Our lab has previously demonstrated an IL-4 mediated pro-metastatic effect on prostate stromal cells. A comparison of IL-4 and IFN γ (both in combination with TNF α) on stromal cells in this chapter has demonstrated a much more significant transcriptional impact on immune related classes of molecules from IFN γ treatment than IL-4. While a balance in favour of TH1/CTL immunity is considered beneficial for anti-tumour immunity, the influence on stromal cells, at least in the prostate, seems to indicate a significant reprogramming to an immune regulatory phenotype. This involves an upregulation in a number of chemokines, AM and immune inhibitory molecules.

Consistent with previous data, IL-4 induces an upregulation in AM that were associated with the migratory phenotype (ICAM1 and VCAM1). Of note though, some of these are also upregulated by IFN γ and at a much more significant level (e.g. ICAM1). So, by either inducing a migratory phenotype that promotes metastasis, or an immune inhibitory phenotype that promotes tumour immune escape, either side of the TH1/CTL vs. TH2 balance reprograms prostate stromal cells producing a phenotype favourable for tumour progression.

The clinical impact of the immune inhibitory phenotype by stromal cells in response to IFN γ is significant, especially in light of the surge in checkpoint immunotherapy and lack of efficacy with mCRPC patients (discussed in detail in Chapter 4). T cells receiving an inhibitory signal from stroma is of particular significance. Upon infiltration, tumour activated T cells recognise tumour antigens, expressed by tumour epithelia but not stroma. Stromal cells lacking tumour antigen are therefore not the targets of anti-tumour immunity. This study also demonstrated prostate stromal paucity in stimulatory molecules. Both antigen recognition and co-stimulatory molecules are capable of overpowering engagement of inhibitory molecules in effector T cells. Therefore lack of tumour antigen and stimulatory molecules makes stromal cells in TME vital players in inhibiting immunity. Of course, three-cell-communication may occur between tumour, stromal and immune cells allowing recognition of tumour antigen and engagement of inhibitory molecules. Though in the context of a stroma heavy TME, tumour antigen concentrations will be slight, making it challenging for T cells to engage target cells. Furthermore the inhibitory phenotype of stroma raises the threshold required to activate T cells, a threshold unlikely to be achieved in a TME with low tumour antigen.

3.4. Concluding remarks

This chapter aimed to understand the involvement of prostate stromal cells (derived from BPH and PCa) in responding to local inflammation. Transcriptional analysis of prostate stroma under conditions representing TH1 (IFN γ &TNF α) and TH2 (IL-4&TNF α) dominated environments reveals a more powerful response to TH1 cytokines. Though transcriptional expression does not guarantee representative of protein expression and activity, it does provide insights and avenues for further research. By using this technique a number of prospective pathways that could be further researched were identified. The potential interplay of the IL-1, IL-6, TLR and COX-2 are particularly interesting, though are not addressed in this thesis. The IFN γ &TNF α mediated response in immune inhibitory molecules was of particular interest and are the main focus herein.

Chapter 4
Immune Inhibitory Roles of
Prostate stroma

4.1. Experimental rationale

Prostate cancer is considered to be immunologically “cold” owing to lower levels of immunogenicity. This is most likely due to a combination of the slow growing nature of the disease and low mutagenic load, restricting the potency of and ability to generate immune responses. Yet, analysis of prostate infiltrating lymphocytes (PILs) has indicated that clonally expanded T cells occur at high numbers in PCa (Sfanos *et al.*, 2009). Although this study found a common antigen across these patients, the identity of the antigen itself is yet to be elucidated. Data suggests that PCa can potentially be targeted by the immune system, but that (i) lack of efficient killing by CTL, (ii) immunosuppression in the microenvironment, (iii) or low expression of tumour antigen, prevents effective tumour clearance. In chapter 3, data identified transcriptional upregulation of key immune inhibitory molecules in prostate stroma treated with TH1/CTL cytokines. In light of this, potential mechanisms of immune inhibition by prostate stroma were investigated. It was considered that in the context of PCa, where reactive stroma can substantially overshadow tumour cells and that PILs likely are intimately associated with stroma while invading the prostate tissue, these mechanisms could provide a significant impediment for anti-tumour immunity.

4.2. Immunotherapy and prostate cancer

Immunotherapy aims to rejuvenate immune responses that have failed to eliminate tumour growth. This is comprised by 7 key stages in a cancer-immunity cycle, described in Chapter 1 (Figure 1.5) (Chen and Mellman, 2013). These stages are: (1) Release of cancer cell antigens, (2) Cancer antigen presentation, (3) Priming and activation, (4) Trafficking of T cells to tumours, (5) Infiltration of T cells into tumours, (6) Recognition of cancer cells by T cells and (7) Killing of cancer cells. Impairment at any stage in the cycle hinders effective anti-tumour immunity and immunotherapy is intended to restore this.

One of the only FDA approved immunotherapies for the treatment of prostate cancer is the cellular vaccine Sipuleucel-T (also known as Provenge®) (FDA, 2010). To generate vaccines autologous APCs are incubated with recombinant human prostatic acid phosphatase (PAP) fused to granulocyte-macrophage colony-stimulating factor (GM-CSF) *ex vivo*, aiming to activate patient immune cells, with PAP as a target antigen (Small *et al.*, 2000). So, it is theorised to improve immune cell targeting of prostate cells expressing the PAP antigen. Sipuleucel-T provides only a modest 4-month improvement in median overall survival (mOS) in patients compared to placebo treated (Flanigan *et al.*, 2013). Sipuleucel-T is currently approved only for the treatment of patients with incurable mCRPC, but presumably patients with earlier stages of the disease, free from metastasis, would have an improved benefit from an immunotherapy treatment. Clinical trials treating patients with localised PCa with Sipuleucel-T are on-going and provide evidence for improved immune infiltration (Fong *et al.*, 2014).

Prostvac-VF® is a vector based vaccine in clinical trials for the treatment of mCRPC. A plasmid containing PSA and 3 immune co-stimulatory molecules (CD80, ICAM1 and LFA-3) is transfected into a mammalian host cell line. Viral vectors infect the host cell line to produce viral vectors containing recombinant DNA encoding PSA and co-stimulatory molecules (Madan *et al.*, 2009). Prostvac-VF is proposed to stimulate a natural immune response upon subcutaneous injection. The viral vectors are phagocytosed by APCs leading to presentation of PSA on MHCI and MHCII molecules and expression of co-stimulatory molecules, which go on to activate PSA specific T cells. Upon activation, T cells target PSA expressing cells resulting in lysis of tumour cells and release of further tumour antigens. Clinical trials so far have highlighted a potential benefit for mCRPC patients treated with Prostvac-VF, with an approximate 8.5-month median OS benefit in one phase II trial (Kantoff *et al.*, 2010).

The use of conditional replicating adenoviruses (CRADs) for cancer treatment is appealing, since if true tumour specificity is achieved it would minimise the off-target effects typical of common cancer treatments like radiotherapy and chemotherapy (Alemany *et al.*, 2000). Ad[I/PPT-E1A] is an oncolytic adenoviruses under investigation for the treatment of PCa patients. It aims to selectively infect and replicate in prostate tumour cells resulting in both tumour cell death and an inflammatory environment capable of supporting a PCa specific immune response. It incorporates the use of 3 prostate associated genes (**1.**Prostate specific membrane antigen; PSMA, **2.**PSA and **3.**T cell receptor γ -chain alternate reading frame protein; TARP) that controls activity of the viral E1A protein required for virus replication and it is for this reason it is termed “PPT-E1A” (Cheng *et al.*, 2006). Both *in vitro* and preclinical *in vivo* experiments have confirmed oncolysis and tumour regression (Adamson *et al.*, 2012; Cheng *et al.*, 2006; Schenk *et al.*, 2014).

However recently, the most notable immunotherapeutics in the treatment of cancers have undoubtedly been checkpoint inhibitors. To date though, of all the clinical trials with checkpoint inhibitors in prostate cancer, none have met primary end points or provided any survival benefit for patients.

4.2.1. Checkpoint inhibition; therapeutic relevance in cancer

The PD-1/PD-L1 pathway can regulate T cell responses to both acute and chronic infections, the latter of which has historically been investigated in more detail (Barber *et al.*, 2006; Day *et al.*, 2006). Inhibiting the PD-1/PD-L1 pathway can benefit survival and the ability of mice to clear infections in an antigen specific T cell dependent manner. It is of no surprise then that the emerging field of cancer immunotherapy has ventured to PD-1/PD-L1 (Fig 4.1). Immunotherapies targeting checkpoint molecules first began with CTLA-4 antibody mediated inhibition (Ipilimumab) (Egen *et al.*, 2002; Krummel and Allison, 1995). It was shown to provide clinical benefits in the mouse and later in the clinic, however can cause significant off target effects through autoimmune mechanisms (Leach *et al.*, 1996; Peggs *et al.*, 2009; Robert *et al.*, 2011). Ipilimumab is approved

for the treatment of metastatic melanoma at a dosage of 3mg/kg and has been demonstrated to provide significant OS benefits in a range of solid tumours in clinical trials. At this dose, Ipilimumab treated patients had a mOS of 10months, compared to 6.4months in vaccine treated patients (Hodi *et al.*, 2010). Furthermore, of the Ipilimumab treated patients 45.6% and 23.5% survived at 1 and 2 years respectively, in comparison to 25.3% and 13.7% in vaccine treated patients. Treatment related deaths occurred in 3% of the Ipilimumab cohort and 1.5% of the vaccine cohort. Importantly, these clinical benefits appear to be prolonged even after withdrawal from treatment, providing evidence for generation of immunological memory (Prieto *et al.*, 2012). Such clinical responses have earned Ipilimumab (and immunotherapy by extension) headlines as the new cancer wonder drug. There is no question that CTLA-4 inhibition renews anti-tumour immunity in this setting, although the significant side effects that patients exhibit indicate that self-reactive T cells are inappropriately given a licence to kill. Off-target effects are mainly attributed to CTLA-4 acting in the T cell priming phase. Since it is largely thought to contribute to the generation of primed T cells in SLO, blocking this may lead to generating a larger pool of antigen specific T cells primed against self-antigens as well as tumour antigens.

Theoretically, off-target effects would be minimised by targeting the PD-1/PD-L1 pathway, due to narrowing or improving the function of antigen specific T cells in periphery. Tumour cells have been demonstrated (in both tissues and cells lines) to have upregulated PD-L1 and are speculated to provide a means of tumour immune escape. Nivolumab is a human IgG4 anti-PD-1 monoclonal antibody developed by Bristol-Myers Squibb that is FDA approved for clinical use in the treatment of advanced/ metastatic melanomas and non-small cell lung cancer (NSCLC). Clinical trials are on-going in various other cancers, however to our knowledge, there has been no benefit for patients with PCa. Brahmer and colleagues have published clinical studies investigating the efficacy of Nivolumab in the treatment of patients with advanced solid tumours. In an initial dose escalation study, tumour regression (including complete and partial responses) was detected in all disease groups (melanoma, CRC, NSCLC and renal cell carcinoma; RCC) but not CRPC, even at the highest dosage of 10mg/kg (Brahmer *et al.*, 2010; Topalian *et al.*, 2012). In targeting PD-L1 (BMS-936559), objective responses were observed in patients with melanoma, NSCLC, RCC and ovarian cancer, but not in CRC, pancreatic or BCa (Brahmer *et al.*, 2012). Of note, PCa was not investigated in this study. In both of these studies, immune related adverse effects occurred, but were not as frequent or severe as in studies with Ipilimumab treated patients. In advanced NSCLC, Nivolumab (@3mg/kg) provided a benefit of 9.2months mOS compared to 6months mOS in those treated with Docetaxel as a second line therapy (Brahmer *et al.*, 2015). Overall survival at 1 year was almost doubled in the Nivolumab treated patients than Docetaxel (42% vs 24%), whereas progression free survival was 21% and 6% in Nivolumab and Docetaxel treated patients, respectively. The response rate of patients on Nivolumab earned it FDA approval for the use as a second-line therapy for NSCLC (@3mg/kg) in 2015 and is additionally approved in the treatment of metastatic melanoma and RCC (FDA, 2015a; 2015b; 2015c; 2016).

In the treatment of PCa, checkpoint inhibition has proved much less successful. Importantly, the use of PSA in evaluating patient responses may influence checkpoint inhibitor efficacy in PCa patients since its suitability as a biomarker has been widely disputed. Secondly, response is likely confounded by prior treatments such as ADT, radiotherapy and chemotherapy, which impact (either negatively or positively) on immune cell activity (Onyema *et al.*, 2015; Roden *et al.*, 2004; Wirsdorfer *et al.*, 2014). Another important factor is patient age, since the immune response declines with age (immunosenescence) (Weinberger, 2017). In one Phase II study treating mCRPC patients with Ipilimumab ± radiotherapy, biochemical regression of >50% was observed in only 15% treated with Ipilimumab (10mg/kg), 1 patient (4% of cohort) had a complete response, 4% had partial responses, 21% had stable disease and 29% had progressive disease (Slovin *et al.*, 2013). Most patients suffered some degree of treatment related adverse events (AE) including 46% with grade 3/4 AE (e.g. hepatitis) in the 10mg/kg cohort, requiring in some cases corticosteroid treatment or withdrawal from the trial. Strikingly, one death occurred as a direct result of the treatment, when treated with a lower dosage of 5mg/kg (Slovin *et al.*, 2013). An interesting observation in this trial is that, while there was no increased tumour regression in combination treated patients, there was a higher proportion of AE in patients not receiving radiotherapy compared to those in combination. This may indicate radiotherapy induced TAA release, increasing immune targeting of the tumour and as a result reducing off target effects.

Similar observations were noted in other checkpoint inhibition trials of PCa patients. For example, no improved OS was observed with Ipilimumab 10mg/kg treatment (vs. placebo; 46.8% vs. 40.4% at 1-year, mOS 11.2months and 10.0months) after radiotherapy in docetaxel-experienced patients in a phase III trial, although there was some indication of PSA response (13.1% vs. 5.2%) (Kwon *et al.*, 2014b). Again, similar AE occurred in most patients, including 4 deaths (1%) due to Ipilimumab toxicity (Kwon *et al.*, 2014b). In chemotherapy-naive patients treated with Ipilimumab 10mg/kg vs. placebo, mOS was 28.7months vs. 29.7 months and progression free survival (PFS) was 5.6months vs. 3.8months (Beer *et al.*, 2017). As before, AE were common and Ipilimumab caused 9 (2%) deaths (Beer *et al.*, 2017). Further Ipilimumab studies are on-going and intend to better understand the mechanisms underlying the responses in PCa and relevance with combination therapy (e.g. NCT01194271). Investigating Nivolumab (or similar PD-1/PD-L1 targeting therapies) in the treatment of PCa is still in the immature phases and clinical trials are on-going (e.g. NCT00730639, NCT02601014, NCT02933255).

It is still unclear what makes immunotherapy more effective in a subset of patients or tumours in certain tissues. As of yet, response to checkpoint inhibition has been associated with high tumour expression of the checkpoint molecule prior to therapy, consistent with it “jamming” the cancer immunity cycle (Herbst *et al.*, 2014). However, responses to Nivolumab are often seen in patients who are PD-L1 negative. The results presented in Chapter 3 of IFN γ &TNF α treatment on a range of cell types and disease stages indicates that PD-L1 can be expressed by all cell types regardless of disease, so it is likely to occur in all patients upon local immune activation.

Mutational burden is also linked to predicting patient responses, an indication of tumour immunogenicity. As previously described in Chapter 1, prostate tissue contains a high proportion of stromal cells relative to epithelium in contrast to both melanoma and NSCLC, which typically have less stroma (Zhang *et al.*, 2015). Given that tumour associated stroma have well described roles in supporting the progression and migration of tumour cells, it would not be a leap of faith to consider they also impact on anti-tumour immunity. Having observed the response of stroma to IFN γ &TNF α in upregulating immune inhibitory molecules, it was hypothesised that upon infiltration of the prostate, T cells would be overwhelmed by the inhibitory mechanisms employed by stroma. And although epithelia are also able to respond similarly, it was not to the same extent. Moreover, even though there would be an expansion of the epithelial population in cancer, they are unlikely to increase beyond that of the stroma until advanced high grade PCa.

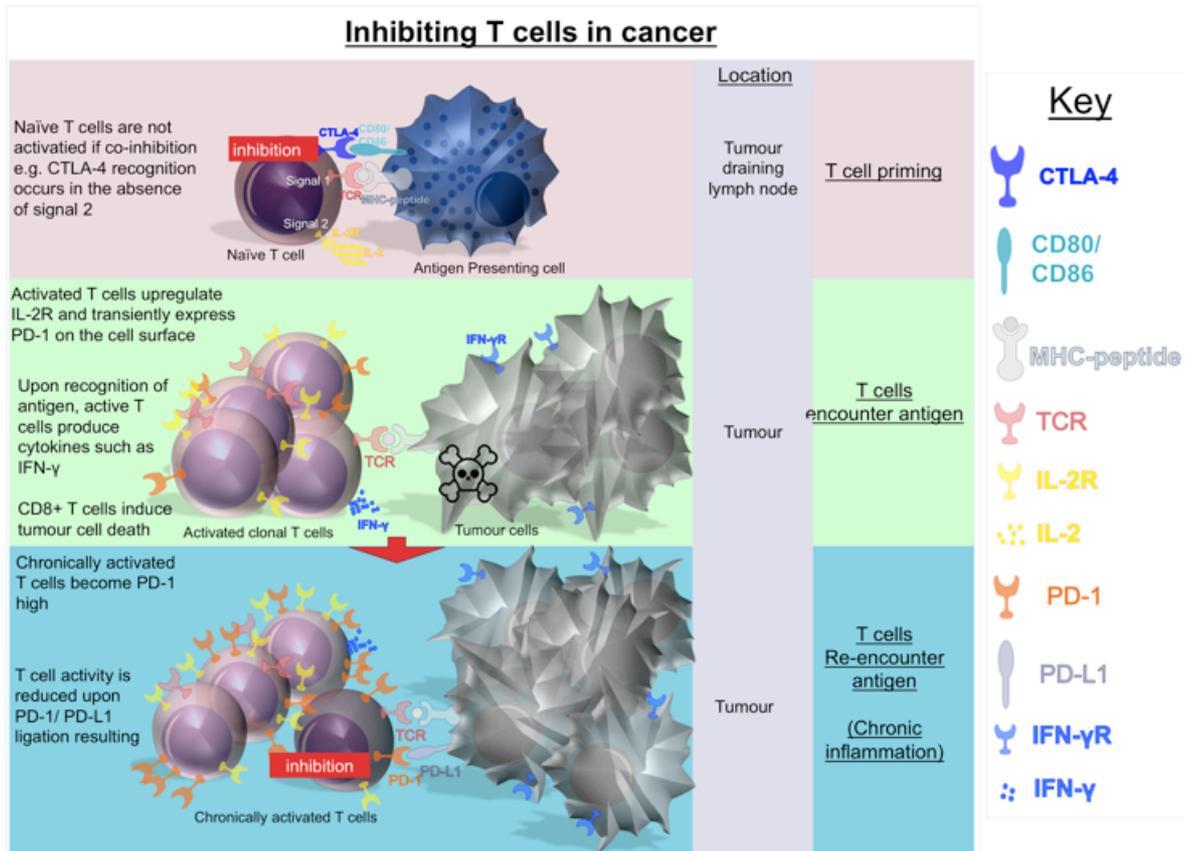


Figure 4.1: Simplified summary of T cell inhibition in the context of cancer

T cells residing in T cell zones of lymph nodes encounter APCs presenting processed antigen on MHC molecules. T cells are inhibited upon ligation of CTLA-4 in lieu of co-stimulation. Activated T cells upregulate IL2R and express PD-1 transiently on the cell surface. Upon recognition of antigen in peripheral tissue, T cells release cytokines such as IFN γ , which may induce tumour cells to express PD-L1. CD8+ T cells are capable of inducing tumour cell death by releasing perforin and granzymes. T cells that are continually exposed to antigen, they become PD-1 high. PD-1 expression leaves T cells susceptible to PD-L1/PD-L2 mediated inhibition by tumour cells.

4.3. Other mechanisms of T cell inhibition

Expression of checkpoint inhibitors is not the only documented mechanism of direct immune inhibition by tumour cells. There has also been evidence of expression of IDO and LAG-3 ligands, both of which were upregulated by prostate stroma when treated with IFN γ & TNF α in Chapter 3.

IDO is an enzyme, which through diminishing the bioavailability of tryptophan, impedes effector T cell activity. Physiologically, IDO expression is important for controlling the maternal immune response in pregnancy, preventing immune mediated foetus rejection (Munn *et al.*, 1998). Expression in pathological conditions contributes to excessive immune suppression (Soliman *et al.*, 2010; Sucher *et al.*, 2010). It has been incriminated as an immune evasion tactic in many cancers, urging investigation of IDO inhibitors in cancer treatment. Two isoforms of IDO exist (IDO1 and IDO2), of which IDO1 is more commonly investigated and significant in the context of cancer (Ball *et al.*, 2007; Metz *et al.*, 2007). Various immune related signals induce IDO expression, including cytokines such as IFN γ and LPS (Dai and Gupta, 1990; Takikawa *et al.*, 1988; Yoshida and Hayaishi, 1978; Yoshida *et al.*, 1981). IDO metabolises the essential amino acid tryptophan to kynurenine. Reduction in tryptophan availability activates a nutritional stress response, a mechanism whereby cells protect themselves from “starvation”. In the absence of tryptophan, the proportion of uncharged tRNA (tRNA lacking cognate amino acid) and tryptophan catabolites increases. The stress response kinase general control non-derepressible protein 2 (GCN2) preferentially binds uncharged tRNA and becomes activated, initiates eIF2 α mediated translation of activating transcription factor 4 (ATF4) and repression of cell growth (Dong *et al.*, 2000; Harding *et al.*, 2000; Munn *et al.*, 2005). While this pathway is highly conserved in all cells, cancer cells modulate and mutate signalling pathways to regulate their response to amino acid depletion more efficiently than T cells, making T cells more susceptible to IDO mediated growth inhibition than tumour cells (Timosenko *et al.*, 2016). Activation of this pathway in T cells induces cell cycle arrest, reduces activation and increases susceptibility to apoptosis (Lee *et al.*, 2002; Munn *et al.*, 1999). IDO mediated increases in tryptophan catabolites has been shown to induce naive T cell differentiation to Treg cells in the tumour draining lymph node, increasing systemic tolerance of TAA (Fallarino *et al.*, 2006; Mezrich *et al.*, 2010; Munn *et al.*, 2004). Furthermore, the catabolites are directly toxic to the IFN γ producing T cells (CTL and TH1) belonging to the aforementioned anti-tumour arm of the anti-tumour immunity balance, but not on TH2 cells (Figure 3.1 pg70) (Frumento *et al.*, 2002). These attributes means IDO directly contributes to tipping the balance in favour of suppressed immunity and prohibiting strong anti-tumour immunity. Tumour expression of IDO1 is linked to worse prognosis across many cancers, including PCa (Brandacher *et al.*, 2006; Feder-Mengus *et al.*, 2008; Ferns *et al.*, 2015; Ino *et al.*, 2006; Liu *et al.*, 2009; Pan *et al.*, 2008; Suzuki *et al.*, 2010; Weinlich *et al.*, 2007). Inhibition of IDO *in vitro* and *in vivo* contributed to an introduction of inhibitors into clinical trials, which are still in the immature phases and few have released results (Friberg *et al.*, 2002; Koblisch *et al.*, 2010; Uyttenhove *et al.*, 2003). Of those that

have, inhibition of IDO in patients appears to induce similar AE as those treated with checkpoint inhibitors, consistent with an immune mediated mechanism of action (Beatty *et al.*, 2017; Iversen *et al.*, 2014; Soliman *et al.*, 2014; Soliman *et al.*, 2016; Vacchelli *et al.*, 2014). Disease stabilisation has been detected and objective responses occur. Although promising, further research is required to determine if this approach can provide any advantage compared to standard treatments.

Checkpoint molecules work in synergy (Woo *et al.*, 2012). LAG-3 is a second immune checkpoint receptor expressed on the surface of antigen activated T cells and through binding to MHCII molecules, inhibits T cells at tumour sites. Like PD-1, LAG-3 appears to be less potent at producing autoimmune disorders when deleted in mice than CTLA-4, an indication that while both may be involved in T cell priming, they likely are more relevant in the effector phase (Miyazaki *et al.*, 1996; Nishimura *et al.*, 2001; Waterhouse *et al.*, 1995). Similarly, the inhibition of LAG-3 increases tumour specific T cell activation, which is increased when in combination with loss of the PD-1 pathway (Foy *et al.*, 2016; Grosso *et al.*, 2007; Huang *et al.*, 2015; Turnis *et al.*, 2012; Woo *et al.*, 2012). This is particularly important given the co-expression of PD-1 and LAG-3 on TILs (Grosso *et al.*, 2009; Matsuzaki *et al.*, 2010a). As a result, cancer immunotherapy is moving toward more targeted combinatorial approaches, leading to an exciting new era in the cancer-immunology field.

4.4. Summary and Aims

PD-L1, IDO and MHCII expression by professional APCs and non-haematopoietic cells such as endothelium, inhibits T cell mediated immunity and is important for the resolution of inflammation and controlling self-reactive T cells. In the case of cancer, this inhibitory signal may be provided by the neoplastic cells, infiltrated APC's or the surrounding tumour microenvironment. Not much is known about the role of stromal cells in this pathway. Since there is a high density of stromal cells in the prostate and proportions remain high in even high grade PCa, the aim was to better understand the impact of inflammatory cytokines on expression of immune inhibitory molecules, in light of Chapter 3.

1. Investigate the expression of inhibitory molecules by patient-derived stromal cells.
2. Understand differential expression of inhibitory molecules between stromal cells derived from patients diagnosed with BPH and G19 PCa.

4.5. Results

4.5.1. Prostate stromal cells upregulate programmed death- ligand 1 (PD-L1) in response to IFN γ

To understand the stromal PD-L1 response to IFN γ , prostate stromal cells derived from a patient with Gleason 9 PCa were cultured in the presence of increasing concentrations of IFN γ for 48hours before mRNA and protein was collected. As shown in Figure 4.2, PD-L1 is significantly upregulated even at the lowest concentration used (12.5ng/ml), at both the mRNA level (Figure 4.2 A) and the protein level (Figure 4.2. B). The expression is IFN γ dose dependent and at the concentrations used there was no evidence of a plateau in the response. These data together indicate that stromal cells in the prostate play a role in controlling local immune cell activity, through expression of the inhibitory PD-L1 molecule.

4.5.2. TNF α in the presence of IFN γ amplifies the IFN γ dependent upregulation of PD-L1 in prostate stroma

In the literature, TNF α and IFN γ have typically been used in combination to induce PD-L1 expression. To understand whether TNF α provides any additional or detrimental effects on the PD-L1 response induced by IFN γ , the combinatorial effects of both cytokines were investigated. Increasing concentrations of TNF α and IFN γ (Figure 4.3 A left and right respectively) were supplemented in the media of BPH derived stromal cells, and PD-L1 expression analysed 48hours later. TNF α alone does not induce PD-L1 expression at the concentrations used (up to 20ng/ml; concentrations are cited in summary Figure 4.4). However, consistent with Figure 4.2, IFN γ (at lower concentrations than Figure 4.2) increases PD-L1 expression. The chosen concentrations for each cytokine (12.5ng/ml of IFN γ and 5ng/ml of TNF α) were then used in combination with increasing concentrations of the other (Figure 4.3 B). IFN γ raises the fold change in PD-L1, however the addition of TNF α does not appear to have a linear effect (left). The presence of 5ng/ml of TNF α with increasing IFN γ produced a linear response at a higher level than when TNF α is absent (B right and A right respectively). This indicated that TNF α amplifies the IFN γ dependent response. To investigate whether different combinations of cytokines would have an impact on the upregulation of PD-L1, the balance of IFN γ and TNF α concentration was altered (Figure 4.3 C). This suggested, unsurprisingly, that PD-L1 upregulation is more dependent on IFN γ than TNF α . These data are summarised in a 3D graph (Figure 4.4). For all further experiments TNF α and IFN γ were used in combination at 5ng/ml and 12.5ng/ml.

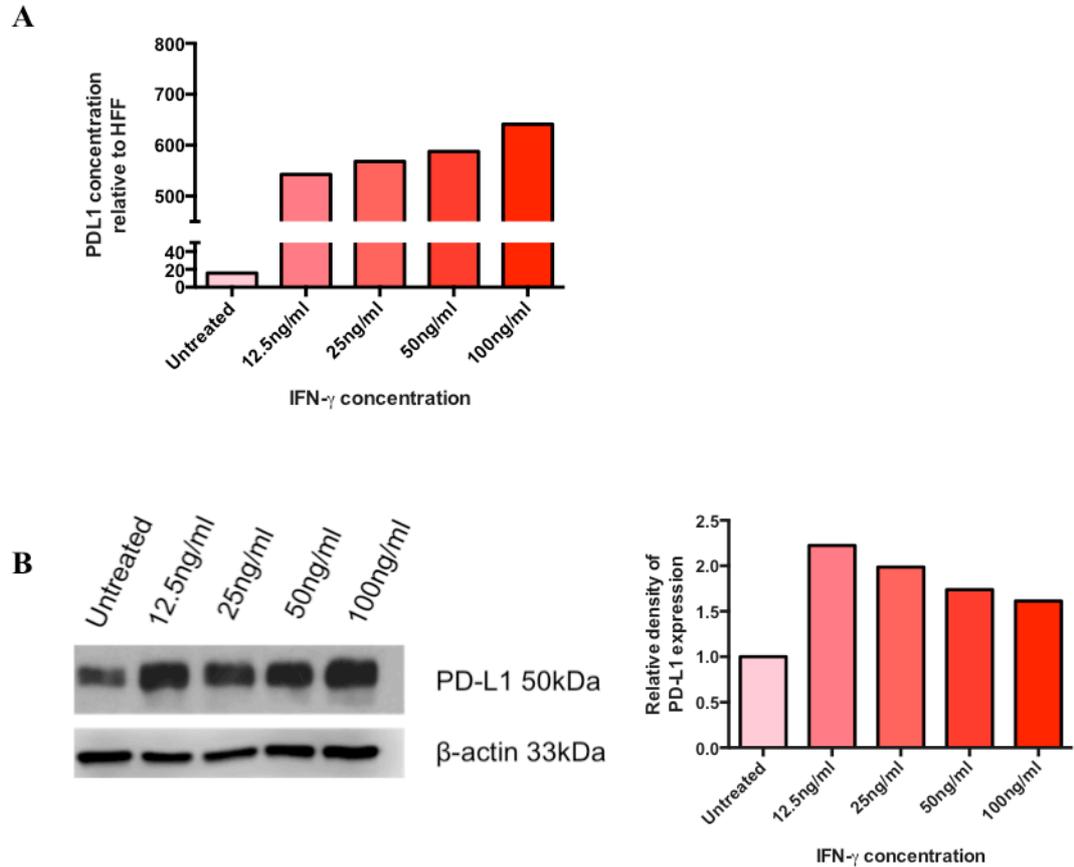


Figure 4.2: IFN γ treatment upregulates PD-L1 in cultured patient derived patient stromal cells in a dose-dependent manner

Stromal cells were cultured in the presence of increasing concentrations of IFN γ for 48 hours. **(A)** PD-L1 mRNA expression was analysed. Stromal cells were lysed, mRNA isolated, before retro-transcription and analysis by RT-qPCR. PD-L1 expression was normalised to internal control gene GAPDH and is presented as the relative concentration compared to Human foreskin fibroblasts (HFFs) cDNA. **(B)** Protein expression was analysed by Western blotting. Cells were lysed, cleared and 5ng of lysate loaded on a gel before transferred and probing for PD-L1 and loading control β -actin.

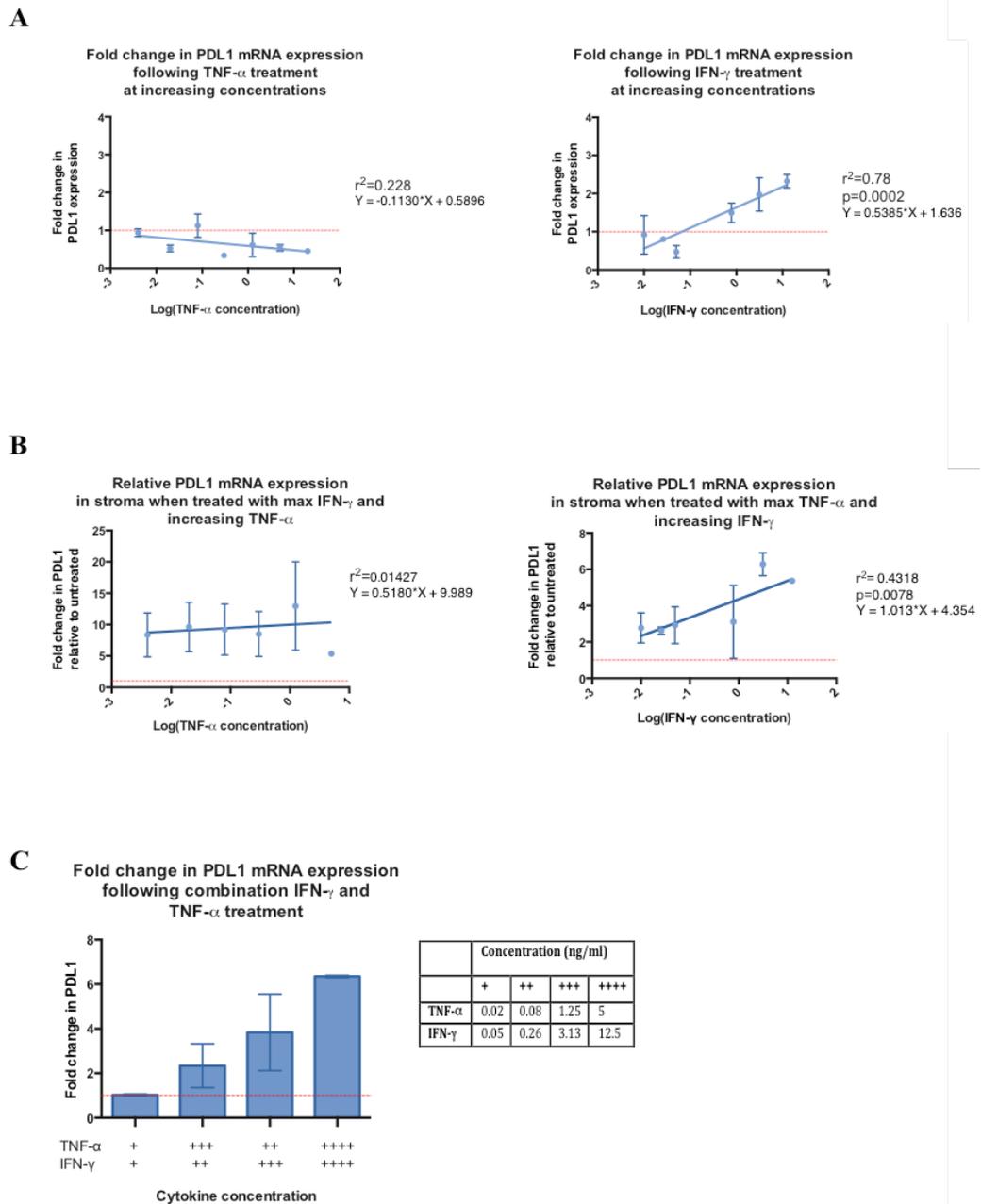


Figure 4.3: TNF α amplifies the IFN γ dependent upregulation of PD-L1 in prostate stroma.

Prostate stromal cells were cultured in media supplemented with varying concentrations of IFN γ and TNF α where indicated. After 48hours media was removed and mRNA extracted as described in Figure X. cDNA was analysed by RT-qPCR and PD-L1 expression was normalised to GAPDH. Data is presented as the mean fold change in PD-L1 (relative to untreated control) \pm SD of duplicate experiments, versus log(cytokine concentration) in order to analyse the linear relationship of PD-L1 response explained by cytokine concentration. The equation, r^2 and where appropriate the p value corresponding to the significance of the slope fitting a non-zero regression given by linear regression analysis is presented alongside the plots. (A) Initially PD-L1 mRNA response was considered when treated with increasing concentrations (TNF α ;0.004-20ng/ml and IFN γ ;0.01-12.5ng/ml) of TNF α (left) and IFN γ (right) (B) Secondly the chosen concentrations of TNF α (5ng/ml) and IFN γ (12.5ng/ml) used in all other experiments were combined with the same increasing concentrations of the additional cytokine as in (A). (C) Finally, variable combinations of IFN γ and TNF α were supplemented in the media as indicated in table (C).

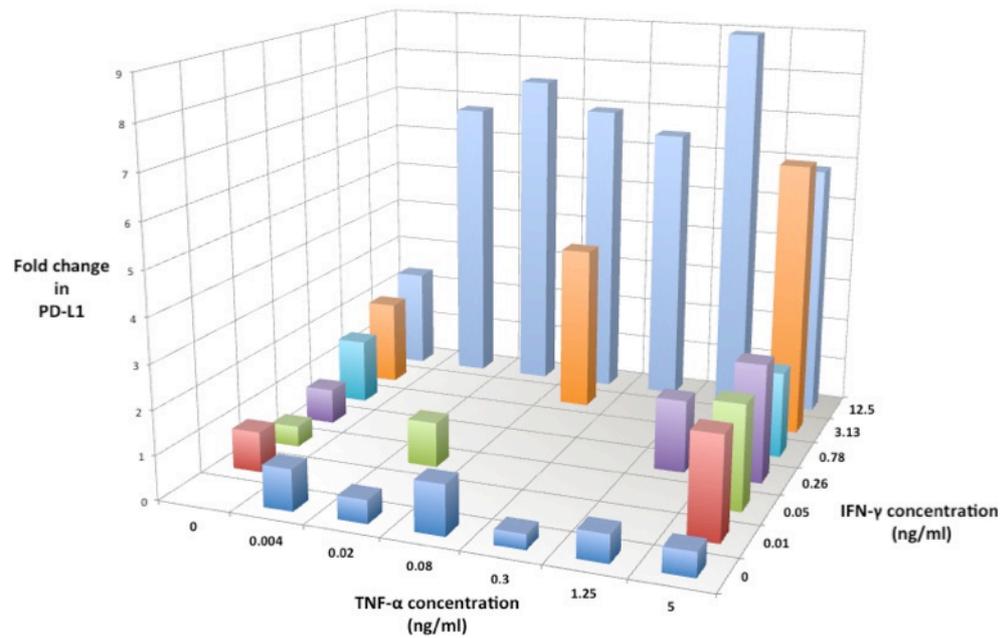


Figure 4.4: TNF α amplifies the IFN γ dependent up-regulation of PD-L1 in prostate stroma.

Figure 4.5 summarised in a 3D graph, data is the mean of duplicate technical replicates is shown.

4.5.3. Stromal cells upregulate PD-L1 rapidly when exposed to IFN γ and TNF α and continue to express high levels after removal

HFF, BPH and PCa derived stromal cells were treated with IFN γ and TNF α and mRNA PD-L1 expression was analysed to determine whether disease had an impact on the time to respond to cytokines (Figure 4.5 A). The peak of PD-L1 gene expression occurs at 8 hours of treatment for all and declines at similar rates. PD-L1 expression in the lysate was analysed for HFF and PCa stroma. For both, PD-L1 protein expression increases over time to a maximum at between 24-48hours (Figure 4.5 B and C). To understand for how long local immune cell activity could affect the immunosuppressive state of stromal cells, HFF cells were treated for 24hours. At this point the supplemented media was removed and cells washed to ensure complete removal of cytokines. Unsupplemented tissue culture media was added back to the cells, which were further incubated for the indicated time points. This data demonstrates that 8 days after removal of cytokines, HFF cells continue to express an increased level of PD-L1 mRNA. While a 24hour IFN γ &TNF α treated control was not included, the data from Figure 4.5 A suggests that an approximate decline from 40-fold upregulation to 3-fold occurred in the initial 24hours. If representative of the *in vivo* mechanism this data is highly relevant-especially in the context of a tumour where extended periods of immunosuppression may allow for tumour immune escape.

4.5.4. PD-L1 upregulation is a conserved response to IFN γ & TNF α in patients with prostatic disease

To investigate the expression of PD-L1 by prostate stroma, a number of patients with BPH (6) and PCa (5) were treated with IFN γ &TNF α . One patient with BPH was excluded from analysis, as he was an outlier in expression of classical stromal markers by nanoString (Chapter 3). Every patient-derived stromal culture increased PD-L1 gene expression (Figure 4.6 A, N.B. mRNA at 48hours) and protein expression (Figure 4.6 B), when treated with IFN γ &TNF α . It is evident from Figure 4.6B that basal PD-L1 protein expression varies across patients, however the upregulation is preserved. This baseline variability may be indicative of the level of immunological activity in the prostate at the time of surgery, or different phenotypic mixtures. It was also clear that PD-L1 expression by stroma did not differ between disease groups. Given that PD-L1 functions at the cell surface to inhibit local T cells, stromal cells were labelled with a PD-L1 specific antibody and fluorescent secondary antibody before analysis by confocal imaging, which confirmed localisation to the cell surface (Figure 4.6 C). This also appeared to highlight population heterogeneity in the PD-L1 expression on untreated stroma. For flow cytometry (Figure 4.6 D), a PD-L1 specific PE conjugated antibody labelled the surface of unfixed, unpermeabilised stromal cells. Both confirm an increase in PD-L1 on the cell surface when treated with IFN γ &TNF α . In the context of prostate tissue, T cells infiltrating through PD-L1 high stroma will be negatively regulated activity if expressing PD-1. This may provide the vital shift in the tumour-immune balance required to allow tumour immune escape and consequently tumour progression.

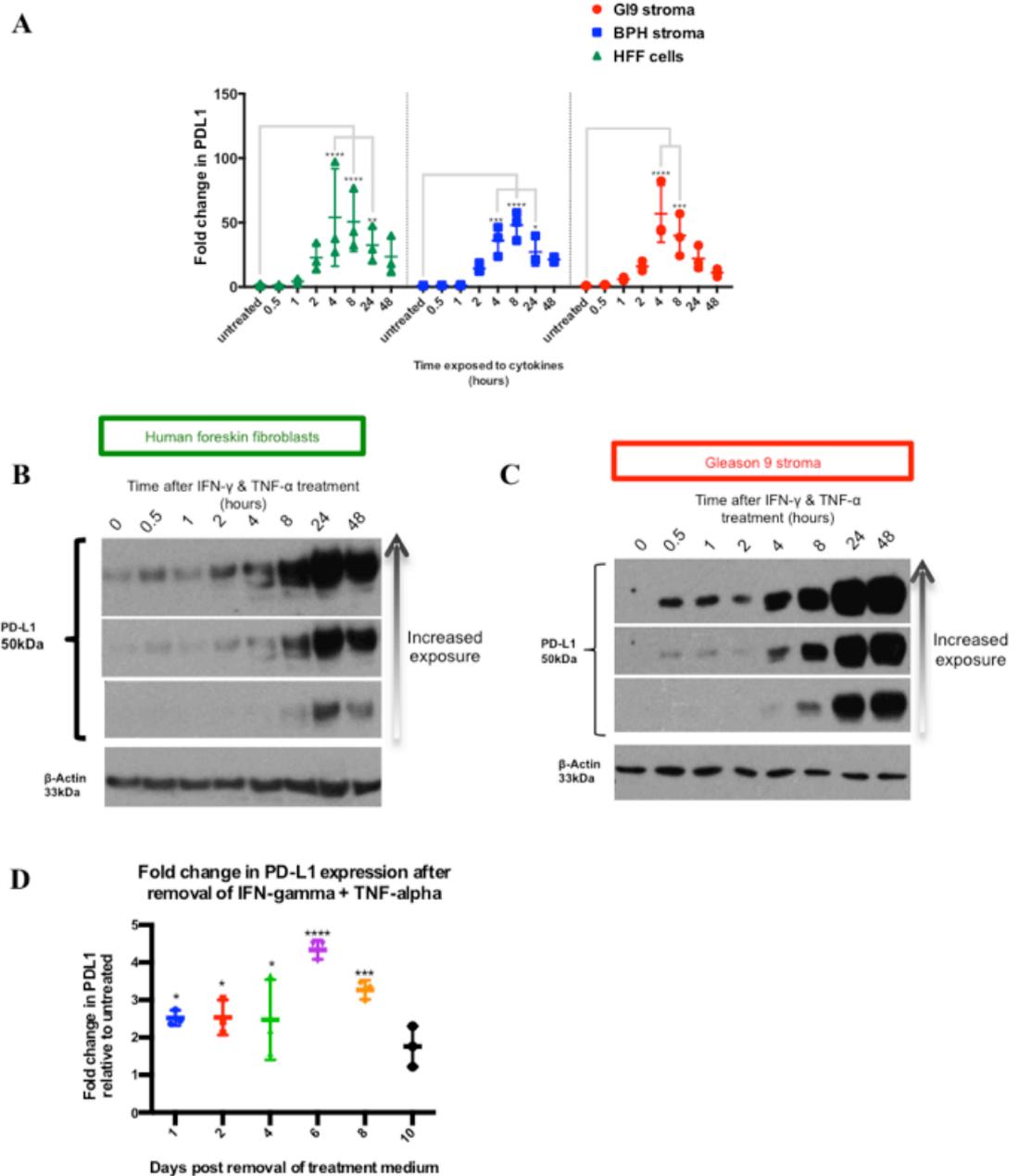


Figure 4.5: IFN γ and TNF α effect on stromal cell PD-L1 expression over time

Stromal cells (HFF, prostate cancer or benign prostatic disease stroma) were cultured in the presence of IFN γ & TNF α before RNA (A) or protein lysate (B-C) was collected at indicated time points. (A) PD-L1 mRNA expression was analysed by RT-qPCR, mRNA was isolated as previously described and PD-L1 expression measured, normalised to GAPDH and is represented as fold change compared to untreated control. Data is mean \pm SD of three technical replicates. (B-C) PD-L1 protein expression was analysed by Western blotting. Stromal cells were lysed, protein lysate collected as previously described and PD-L1 or β -actin presence measured. (D) PD-L1 mRNA expression was analysed by RT-qPCR, HFF cells were cultured in the presence of IFN γ and TNF α for 24 hours before washing to remove supplement cytokines and further cultured for the indicated time points. At these times, cells were lysed and analysed as in (A). Data is mean \pm SD of two technical replicates

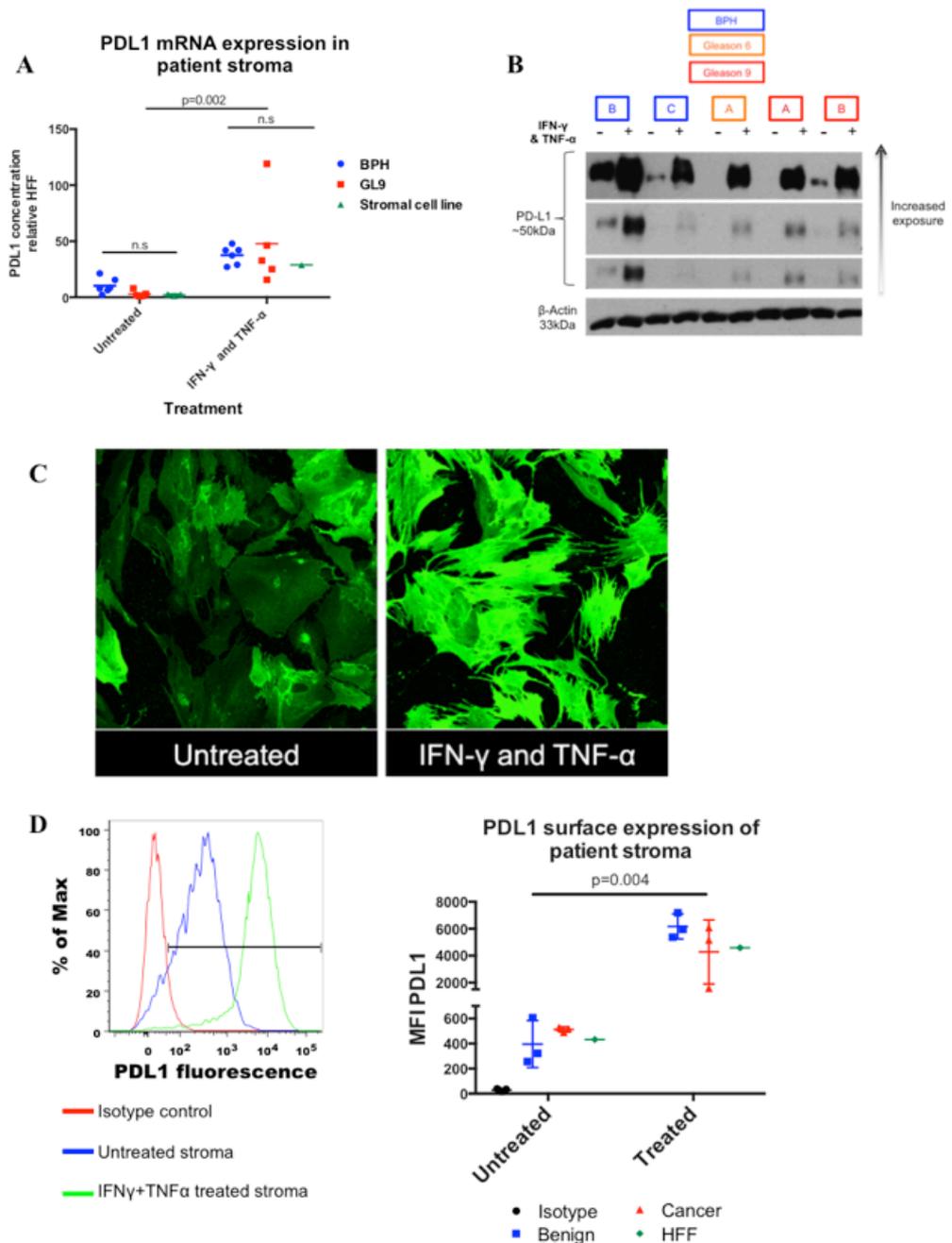


Figure 4.6: PD-L1 expression in patient prostate stroma with either cancer or benign disease

Patient derived prostate stromal cells were cultured with or without IFN γ and TNF α for 48 hours. **(A)** PD-L1 mRNA expression was analysed. Stromal cells were lysed, mRNA isolated, before retro-transcription and analysis by RT-qPCR. PD-L1 expression was normalised to internal control gene GAPDH and is presented as the relative concentration compared to HFF cDNA. Data shown is the mean \pm SD of triplicates for 6 BPH patients, 5 Gleason 9 prostate cancer patients and 3 stromal cell lines untreated (HFF, ADSC and tonsil derived stromal cells) and treated HFF cells. **(B)** Protein expression was analysed by Western blotting. Cells from 5 patients with prostatic disease were lysed, cleared and 5ng of lysate loaded on a gel before transferred and probing for PD-L1 and loading control β -actin. Data presented is a representative example of 3 separate experiments. **(C)** Confocal image of prostate stromal cells fixed and labelled with anti-PD-L1 and anti-mouse A488 fluorophore **(D)** Flow cytometric analysis of cell surface PD-L1 expression by prostate stroma. Cells were detached and labelled (unpermeabilised) with an anti-PD-L1-PE antibody before analysis by flow cytometry. The left panel demonstrated an exemplar histogram, with MFI for each patient plotted on the right. Statistical significance was measured by a RM 2Way ANOVA with a *post hoc* Tukey's multiple comparisons test.

4.5.5. Stromal cells respond to IFN γ & TNF α by upregulating a number of immune inhibiting molecules

Having comprehensively explored the expression of PD-L1 by prostate stromal cells, the potential role that prostate stroma may have in the regulation of immune activity through other well described mechanisms was analysed. Taking 8 hours as the peak of gene expression resulting from IFN γ &TNF α exposure, the expression of PD-L1, IDO1 and IDO2 were examined. In agreement with earlier data (Figure 4.5 & 4.6), PD-L1 is consistently increased in response to IFN γ &TNF α , as are IDO1 and IDO2 (Figure 4.7 A,C, D). Together this data indicates redundant mechanisms of immune cell inhibition.

TGF- β (particularly isoform 1) expression is also strongly associated with reactive stroma; therefore the impact of disease and cytokine treatment on the gene expression of all three isoforms was investigated (Figure 4.9). Unexpectedly, TGF- β 3 was consistently downregulated in G19 stroma compared to BPH, while TGF- β 2 expression was slightly reduced by IFN γ &TNF α at the mRNA level. TGF- β 1 was unchanged by disease or cytokine treatment. However, the high level of TGF- β in all patient-derived stromal cultures is supported by nanoString counts in chapter 3.

Analysis of molecules on the cell surface of stromal cells revealed that HLA-DR, an MHCII molecule capable of binding LAG-3 on T cells, is upregulated in response to IFN γ &TNF α (Figure 4.9). For presenting antigens to immune cells CLIP should be cleaved from MHCII molecules. With upregulation of HLA-DR, CLIP is also present at an increased level on the surface of stromal cells. Correspondingly, there is evidence of an upward trend in binding of a recombinant LAG-3-Fc molecule in IFN γ &TNF α treated cells, although this is quite minimal. Simultaneously, HLA-E is expressed and becomes upregulated, when stromal cells are treated. HLA-E has been associated with the inhibition of T cells and NK cells. Altogether these data indicate that in addition to PD-L1 stromal cells may be able to downregulate inflammation in the local environment by a multiple and well-characterised mechanisms.

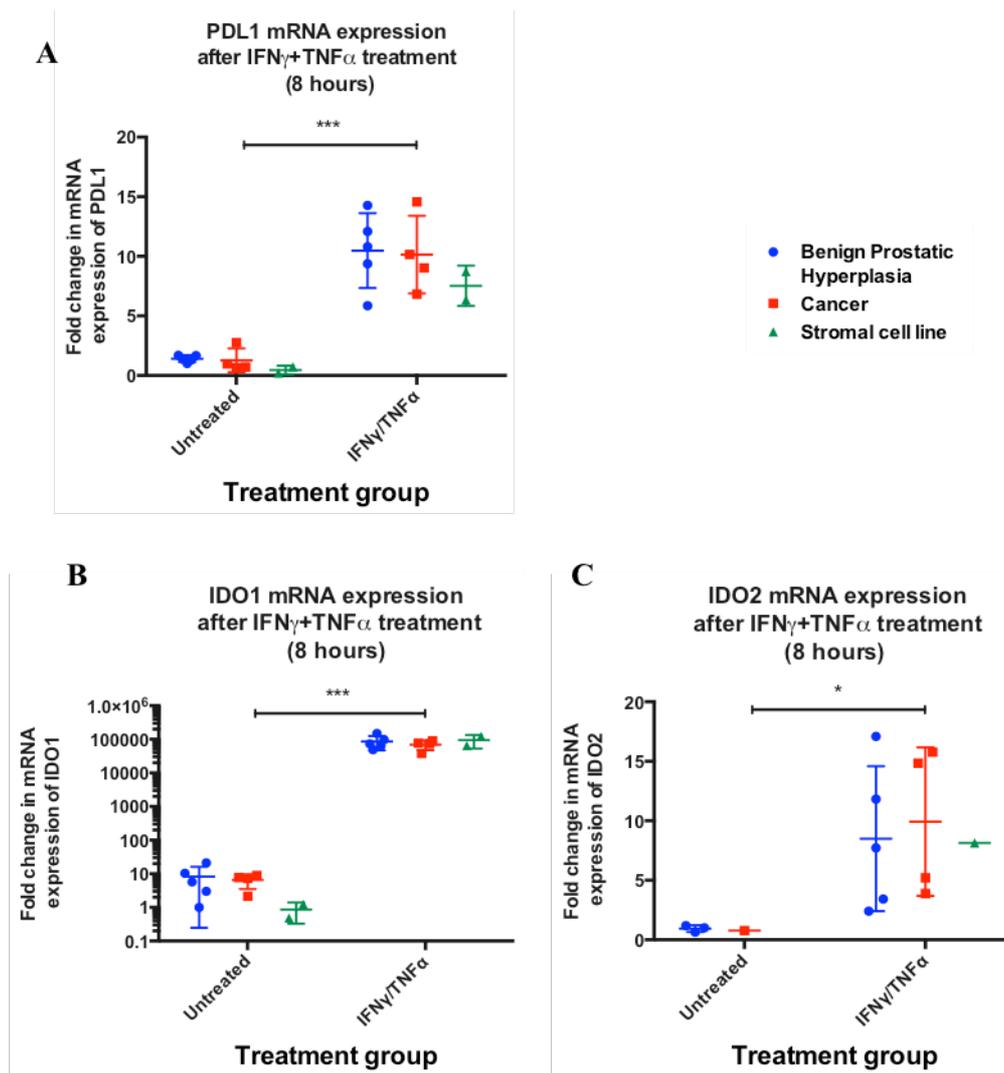


Figure 4.7: Analysis of stromal cell gene expression after 8 hours of IFN γ and TNF α supplementation

As determined in Figure 4.5 8 hours was found to be the peak PD-L1 gene expression response time to IFN γ and TNF α , therefore a panel of molecules of interest were further investigated in 5 BPH and 4 G19 patients, as well as HFF and tonsil derived stromal cells. Cells were supplemented with IFN γ and TNF α (12.5ng/ml and 5ng/ml respectively) for 8 hours before mRNA extraction and gene expression analysis as previously described. PD-L1 (A), IDO1 (B) and IDO2 (C) expression was measured by RT-qPCR, normalised to GAPDH and is presented as the mean fold change \pm SD. Statistical significance was measured by a RM 2Way ANOVA with a Tukey's multiple comparisons test (A&B). Due to missing values (not detected in tonsil stroma and untreated HFF), a generalised linearised model (GLM) was used to determine significance for IDO2 expression (Signif. codes: p= 0:***, 0.001:**, 0.01:*).

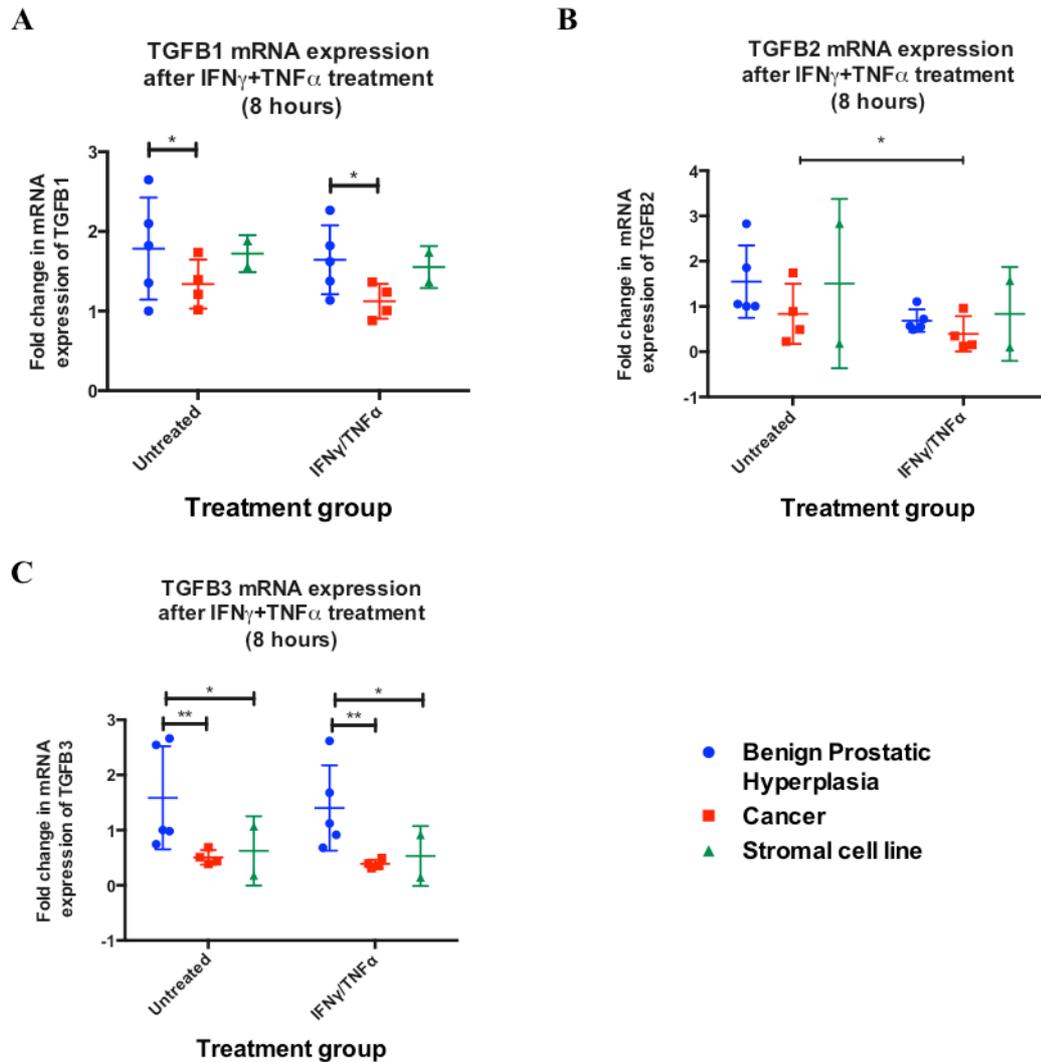


Figure 4.8: Analysis of stromal cell TGF- β expression after 8hours of IFN γ and TNF α supplementation

As determined in Figure 4.5 8hours was found to be the peak gene expression response time to IFN γ and TNF α , therefore a panel of molecules of interest were further investigated in 5 BPH and 4 G19 diagnosed patients as well as HFF and tonsil derived stromal cells. Cells were supplemented with IFN γ and TNF α (12.5ng/ml and 5ng/ml respectively) before mRNA extraction and gene expression analysis as previously described. TGF β 1 (**A**), TGF β 2 (**B**) and TGF β 3 (**C**) expression was measured by RT-qPCR, normalised to GAPDH and is presented as the mean fold change \pm SD. Statistical significance was measured by a RM 2Way ANOVA with a Tukey's multiple comparisons test.

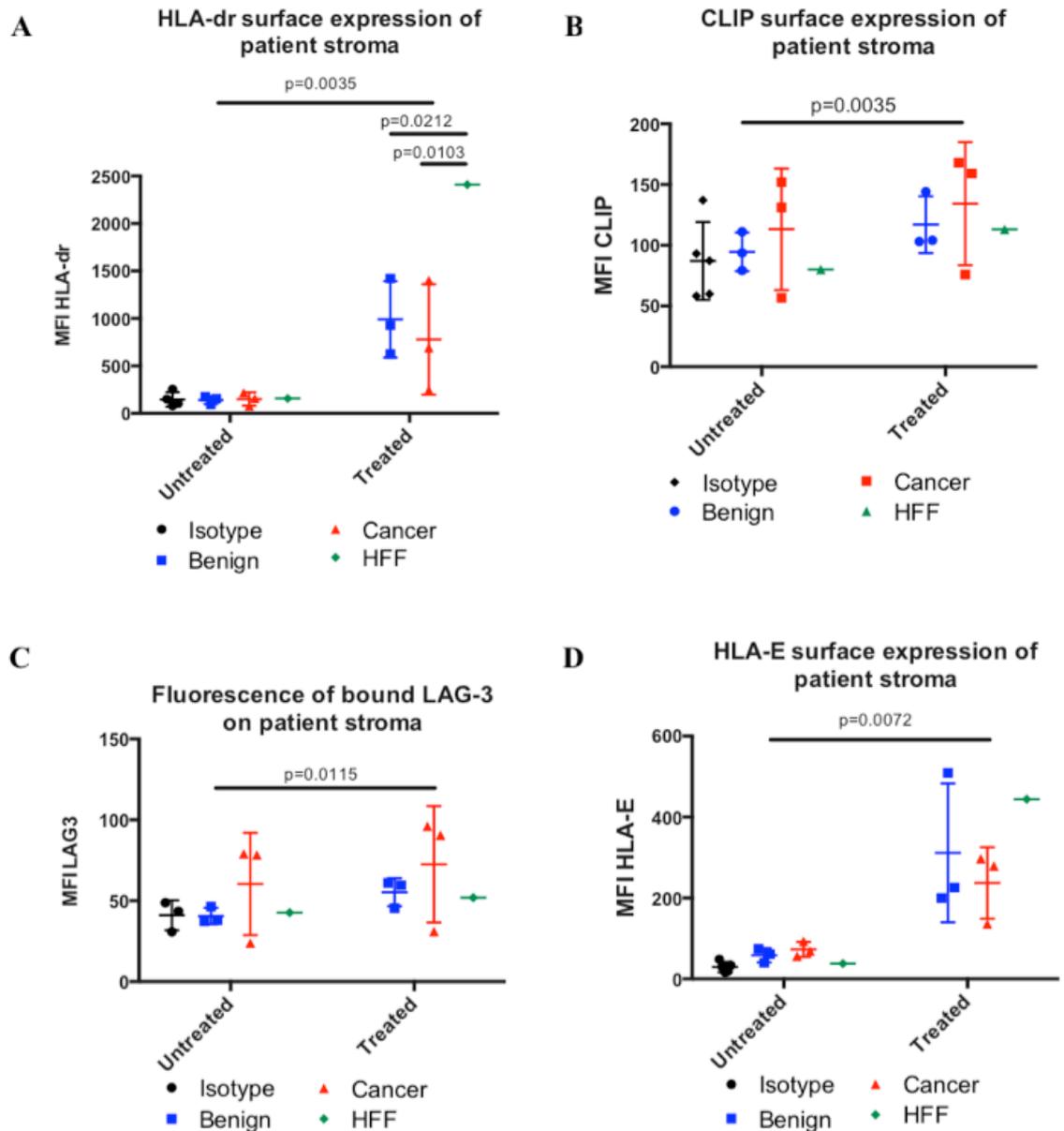


Figure 4.9: Flow cytometric staining of patient stroma and HFF cells that were IFN γ & TNF α treated or untreated

Stromal cells from 3 patients with either BPH or PCa were supplemented with or without cytokines for 48hours before collecting for analysis by flow cytometry. Cells were gated to exclude dead cells and debris before analysing surface expression of HLA-dr (A), CLIP (B), LAG-3 binding (C) and HLA-E (D). At least 10,000 events were collected for analysis and the relevant isotype control was performed in parallel. Statistical significance was measured by a RM 2Way ANOVA with a Tukey's multiple comparisons test.

4.5.6. TLR activation does not influence expression of PD-L1 in prostate stroma

Incidence of BPH and PCa is associated with chronic infections of the prostate. The effects of TLR agonists on prostate stromal cells' expression of PD-L1 was considered, in order to investigate whether stromal TLR-mediated PD-L1 expression could account for unresolved infections of the prostate. Of all the agonists investigated, initially on cancer associated stroma, the TLR4 agonist LPS-EK was the only one which upregulated PD-L1 (Figure 4.11 A). Therefore, this was investigated further in stroma derived from 4 BPH patients and 3 cancer patients. LPS-EK was not found to consistently mediate the upregulation of PD-L1 in these patients (Figure 4.11 B). MPL-A is a more effective human TLR4 agonist than LPS-EK and coordinates with rCD14 for its function. Due to conflicting results in A&B, MPL-A was used in stromal treatments, which did not affect PD-L1 expression (Figure 4.11 C).

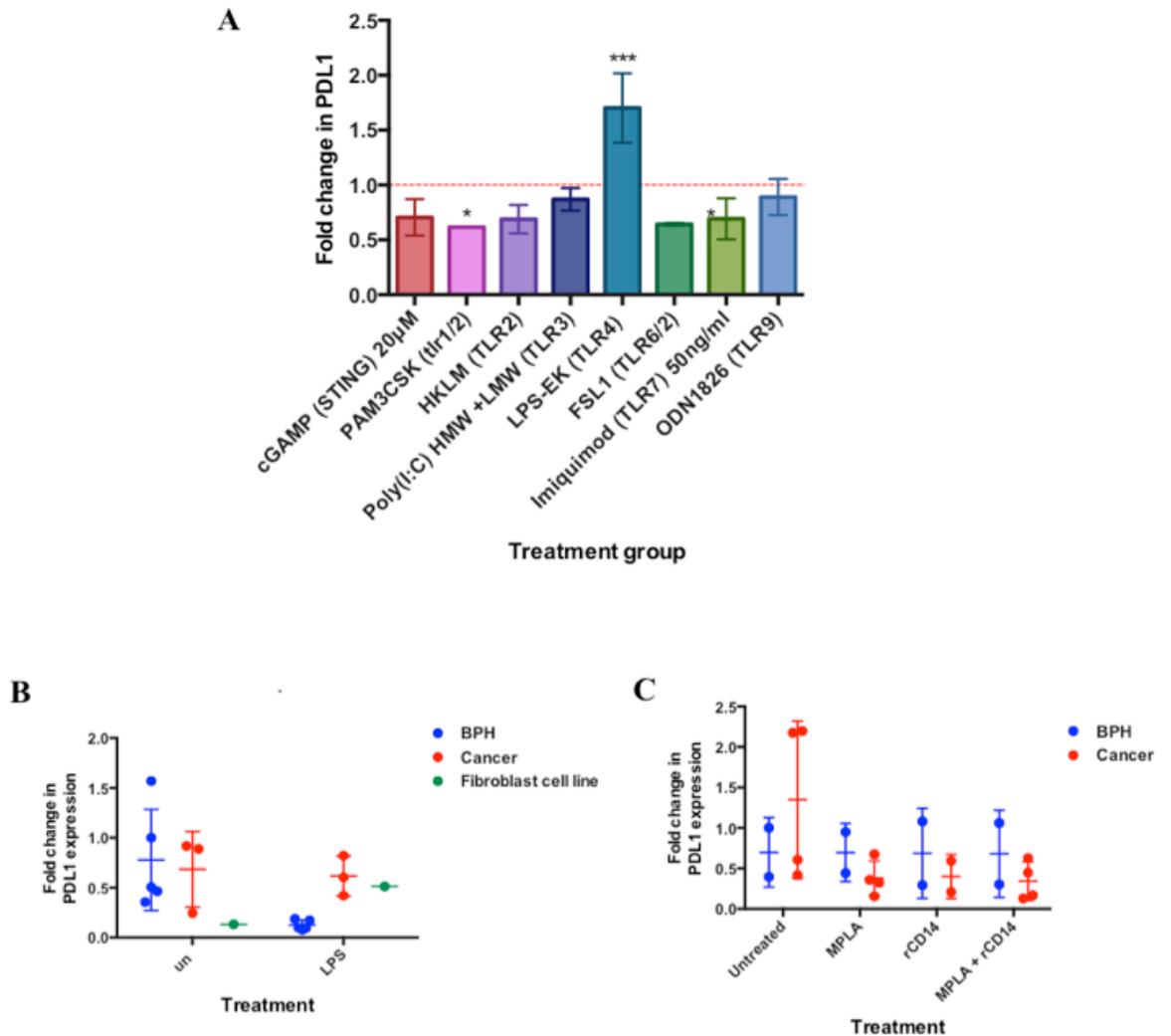


Figure 4.10 RT-qPCR analysis of PD-L1 expression in prostate stroma treated with LPS or MPLA±rCD14

Prostate stromal cells were supplemented with TLR agonists (A), LPS (B) or MPLA±rCD14 and (C) for 24 hours. As previously described mRNA was extracted and RT-qPCR used to analyse expression of PD-L1. Data presented is the mean fold change \pm SD in PD-L1 of patients diagnosed with BPH and G19 prostate cancer.

4.6. Summary of Results

- IFN γ induces a dose dependent upregulation in PD-L1 by stroma, which is amplified in the presence of TNF α and peaks for mRNA at 8hours, or protein at 48hours. This expression remains upregulated even 10 days after removal of the cytokines.
- Stroma derived from patients with both BPH and G19 PCa upregulate PD-L1 in response to IFN γ at similar levels, as do stromal cell lines.
- PD-L1 is localised to the surface of the cell, where it functions as a ligand for the co-inhibitory receptor PD-1 on T cells
- Prostate stromal fibroblasts express many other inhibitory molecules that can either be induced or are constitutively expressed: IDO1, IDO2 and 3 isoforms of TGF- β . Expression of MHCII molecules (HLA-DR) on the cell surface is upregulated on treated stromal cells and LAG-3 binding is correspondingly upregulated to a minimal but statistically significant level.
- TLR4 ligation does not consistently upregulate PD-L1 in patient stroma.

4.7. Discussion

4.7.1. Stromal mediated immune regulation contribution to tumour immune escape

Given the involvement of stromal cells in the progression of cancers as well as the association of inflammation and cancer progression, it is important to understand how prostate stromal cells regulate inflammation of tumours. Research in PCa has paid particular attention to the stromal compartment in the past, yet the interplay of immune cells and stroma needs further investigation. The data presented in this chapter indicates BPH and G19 PCa stroma share the same potential to inhibit infiltrating immune cells. However, that is not to say that this should not be a meaningful consideration in PCa. Prostate stromal cells may express this immunosuppressive ability in early stage PCa, potentially stimulating local immune cell anergy. It may also provide an alternative or additional explanation for the inflammatory cancer theory, and the association of recurring UTIs and PCa development. Infections likely result in local TH1 and CTL cell mediated immunity that establishes immune inhibitory stroma (primarily aiming to restore homeostasis). If recurrent infections occur in the prostate and promote inhibitory stroma, it may consequently provide an **indirect** mechanism for tumour immune escape. During the initial development of PCa, the prostate microanatomy remains unaffected, so a dense stromal compartment surrounds the early tumour, providing a barrier between infiltrating immune cells and tumour cells.

The clinical trials of checkpoint inhibitors in PCa have indicated some clinical activity, however no improved survival has been documented. The existence of a substantial prostate stromal compartment indicates a higher threshold of immune activation is required to overcome local inhibition and target tumour cells. This may occur as a cyclic process or in waves of immunity where immune activity rises locally, causing antigen release, stromal mediated immune suppression and accumulation of infiltrating immune cells (Figure 4.14). Additionally, the inflammation may also promote the development of a reactive stromal compartment, indicated by the occurrence of reactive stroma in wounds and prostatitis. Reactive stroma has established roles in promoting cancer progression. Through facilitating migration of PCa cells (when treated with inflammatory cytokines) and inhibition of immune cells, reactive stroma provides a fundamental environment for promoting PCa development (Lakins, 2012). The data presented here indicated the high proportion of stromal cells in the prostate likely contributes to a highly immunosuppressive and tolerogenic environment. This will require a stronger immune response to surpass the threshold and prevent T cell anergy, which would be provided by immunogenic antigens, low levels of inhibitory molecules and high levels of co-stimulatory molecules. The prostate lacks all 3 of these traits, impacting on the efficacy of APCs at generating T cell mediated immunity as well as the efficacy of T cell effector function in the prostate environment. Crucially, it seems the clinical activity generated with checkpoint inhibition is not sufficient to overcome this inhibitory environment in the prostate. Importantly, this research is not only relevant in the context of checkpoint inhibitor therapeutics, but also in Sipuleucel-T and similar vaccine approach as well as chronic infections of the prostate.

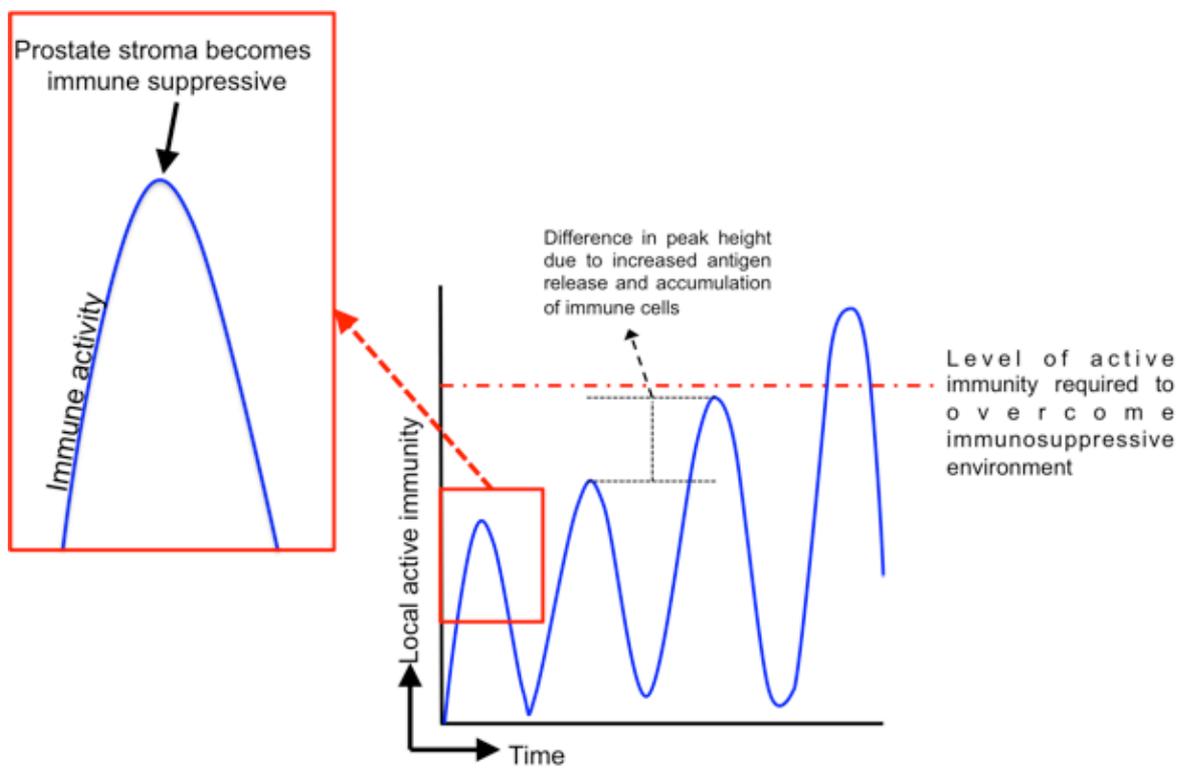


Figure 4.11. A schematic representation of immunological waves resulting in generation of an immunosuppressive environment.

4.7.2. Concluding remarks

In this chapter the aim was to investigate the expression of immune inhibitory molecules by patient-derived stromal cultures and determine if the expression differed between disease groups. The presented data indicate an important role for prostate stroma in establishing an immunosuppressive environment that prevents effective immunity required in targeting infection and neoplastic transformation. An important factor is the utilisation of a number of well characterised mechanisms described as adaptive resistance by tumour cells, namely PD-L1, IDO1, MHCII and HLA-E expression. Additionally, the constitutive expression of TGF- β isoforms will contribute to an immunosuppressive background in the prostate. This will negatively impact on two crucial stages of the cancer-immunity cycle. APCs activated in an inhibitory environment are less able to generate effective T cells and instead produce tolerogenic T cells. Effector T cells in the prostate are unable to efficiently kill due to the high expression of co-inhibitory molecules and cytokines. This environment is additionally more favourable to Treg cells than T cells able to induce tumour cell death. Altogether, prostate stroma provides an environment detrimental to effective anti-tumour immunity.

Chapter 5

Phenotypic Analysis of Prostate

Stromal cells

5.1. Experimental rationale

While there were a few distinctions between BPH and PCa stromal fibroblasts by nanoString analysis (Figure 3.6 pg78), there were no differences in checkpoint inhibitor expression by disease group (Chapter 4). There is a great body of evidence documenting functional stromal changes with cancer development. In PCa, increased proportions of myofibroblasts are detected and considered an “activated” fibroblast phenotype. These cancer myofibroblasts have over time become synonymous with CAFs. It was an interest, given the findings of Chapter 4, to determine whether the inhibitory phenotypes could be attributed to certain stromal phenotypes.

5.1.1. Stromal cell phenotypes

As discussed in Chapter 1, stromal cells may conform to various stromal subsets. Identification of reactive stromal cells becomes difficult by specific marker expression, due to the inherent plasticity. Instead fractionation is predominantly done by a combination of markers (Barron and Rowley, 2012). For example smooth muscle cells express α -smooth muscle actin (α SMA) but not vimentin. Fibroblasts express vimentin but not α SMA, and myofibroblasts are an intermediate type cell, expressing both α SMA and vimentin. Myofibroblasts are also typically characterised by a larger flattened morphology, due to an expansion of the endoplasmic reticulum to facilitate increased protein production (Figure 5.1). Prostate stromal cultures contain mainly fibroblasts and myofibroblasts. Although all experiments were performed on prostate stromal cultures within five passages (the majority within three), SMC and endothelial cells are lost as early as passage one due to overgrowth of the other subsets so are not analysed in this chapter (Hall *et al.*, 2002; Lakins, 2012). The absence of endothelial cells in cultures is supported by the lack of endothelial marker expression by nanoString (Chapter 3, Figure 3.5). Therefore vimentin and α SMA should be sufficient to discriminate between fibroblasts and myofibroblasts.

Many pathological mechanisms can be attributed to either defective myofibroblast activity/recruitment or persistence of myofibroblasts. In older mice, scar formation is defective due to reduced myofibroblast numbers and activity (Bujak *et al.*, 2008). Conversely, development of fibrotic conditions such as idiopathic pulmonary fibrosis is attributed to overactive or persistent myofibroblasts (Huang and Horowitz, 2014). This is well understood in the context of aberrant wound healing. Cells at wound sites increase TGF- β 1 production, which acts to recruit local fibroblasts and circulating MSCs to the wound where they are “activated” to acquire the myofibroblast phenotype (Desmouliere *et al.*, 1993; Pakyari *et al.*, 2013). This is typified by the gain of α SMA stress fibre expression; a characteristic of true SMCs (Darby *et al.*, 1990; Desmouliere *et al.*, 1993). Expression of cytoskeletal α SMA facilitates increased contractile force and consequently wound closure. Following the formation of scar tissue, most myofibroblasts undergo apoptosis and are cleared, restoring homeostasis after wound healing (Desmouliere *et al.*, 1995; Dobaczewski *et al.*, 2006; Jugdutt, 2003). As well as increased contractility, myofibroblasts

display increased deposition/remodelling of ECM components and increased expression of ECM degradation enzymes such as MMPs, which are both characteristics of PCa (Desmouliere and Gabbiani, 1994; Krušlin *et al.*, 2015; Stearns and Stearns, 1996). Paradoxically, this could both permit tumour invasion (as MMPs are often observed increased at the invasive front), and provide a physical barrier (ECM) against infiltrating immune cells and tumour cells (Adachi *et al.*, 2001; Hall *et al.*, 2002; Sentani *et al.*, 2014). The increased contractility exhibited by myofibroblasts is facilitated in part by increased expression of α SMA, a cytoskeletal filamentous fibre. The rapid restructuring of α SMA within the cytoplasm facilitates cell movement, therefore providing myofibroblasts with increased migratory capacity, as well as the contractility required for wound closure. Therefore, the presence of myofibroblasts in the context of wound healing can be beneficial by promptly facilitating wound closure, but equally, persistence can be detrimental to tissue architecture.

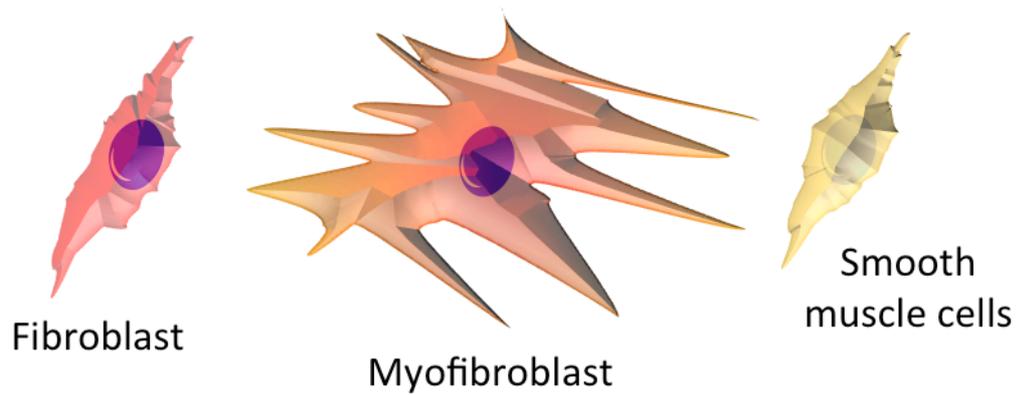
5.1.2. Cancer associated fibroblasts (CAFs)

The presence of CAFs in many types of tumours has been investigated and regularly associated with bad prognosis (De Wever *et al.*, 2008; Saigusa *et al.*, 2011; Wikberg *et al.*, 2013). CAFs are thought to provide the tumour microenvironment with a rich source of secretions (e.g. growth factors) as an attempt to promote resolution of homeostasis. For survival, tumour cells must be able to exploit the effects of myofibroblast-mediated wound healing actions to their advantage, so CAFs become pro-tumour rather than pro-healing. Myofibroblasts can promote angiogenesis in order to promote immune cell access to the tumour, though consequently provides the nutrients tumour cells require for survival (Hughes, 2008). In lung adenocarcinoma, fibroblasts adjacent to the tumour were described as podoplanin positive compared to normal lung fibroblasts (Kawase *et al.*, 2008). Similarly, podoplanin positive CAFs in melanoma were associated with worse prognosis (Kan *et al.*, 2014). BCa contains similar stroma:tumour cell ratios as in PCa (~80%, identified by α SMA positivity) (Sappino *et al.*, 1988). Whereas in colorectal cancer (CRC) <20% of tumours contain CAFs, although the presence of them is associated with bad prognosis (Tsujino *et al.*, 2007). Moreover, early stage but not high grade CRC expresses high levels of fibroblast activation protein- α (FAP) (Henry *et al.*, 2007).

FAP has been described as a marker of CAFs, however has also been identified on myofibroblasts in granulation tissue and other pathological sites (Jacob *et al.*, 2012). It is a membrane bound serine protease, containing a catalytic domain on the extracellular surface. Upon dimerization, it can act as a dipeptidase (hydrolysing pairs of amino acids) or as a gelatinase/collagenase (degradation of gelatin and collagen fibres; belonging to the same family as MMP enzymes) (Park *et al.*, 1999). In addition to facilitating local invasion, FAP may also enable accumulation of immune cells through release of cytokines/chemokines bound on ECM fibres. FAP expression is PCa stroma compared to normal prostate stroma by IHC and qPCR of immortalised cultured cells (Jia *et al.*, 2016). While it was expressed to a higher degree in stroma

associated with a range of malignant epithelial tumours, it was also expressed in benign tumours, but not normal tissues (Garin-Chesa *et al.*, 1990).

Attempts to utilise CAFs as a therapeutic target have to date been largely unsuccessful. Inhibition of MMP activity was not found to benefit patients (Coussens *et al.*, 2002). Identification of FAP as a CAF marker led to it being utilised as a therapeutic target. Using an immunoconjugate therapy (FAP5-DM1; FAP targeting monoclonal antibody conjugated to the cytotoxic agent DM1) did demonstrate the potential to target CAFs as a method to reduce tumour volume through specific cell death in dividing FAP⁺ cells, without off target effects in other tissues (Ostermann *et al.*, 2008). Similarly, stimulating an anti-FAP specific immune response prophylactically prior to tumour challenge suppressed tumour growth, when used in combination with chemotherapy (Loeffler *et al.*, 2006). Moreover, this therapeutic had no detrimental effect on wound healing. Together these data highlight the cancer-supportive benefits of reactive stroma, but does not provide evidence that FAP specifically contributes to the pro-tumour effects of reactive stroma.



	Vimentin	α SMA
Fibroblasts	+	-
Myofibroblasts	+	+
Smooth Muscle Cells	-	+

Figure 5.1: Prostate stromal phenotypes' morphology and marker expression

Traditional fibroblasts adhere to the classic spindle morphology and express high levels of the intermediate filament vimentin. Myofibroblasts are typically larger than fibroblasts and display increased quantity and size of protrusions together with expression of α SMA. SMCs are the smallest of the 3, have a spindle morphology and express only α SMA.

5.2. Summary and Aims

Stromal cells derived from PCa and BPH have been shown to differ functionally however, data presented thus far have not elucidated many differences in the context of interactions with immune cells. Various molecules have been associated with CAFs, but a specific marker has not been identified. Therefore, in this chapter the aim was to:

- Determine whether stromal cultures from BPH and PCa could be differentiated, based on morphology, expression of stromal antigens and CAF associated markers.

5.3. Results

5.3.1. Immunofluorescence and morphological characterisation of prostate stromal cultures

Myofibroblasts are typically considered to express both vimentin and α SMA compared to fibroblasts, which only express vimentin. To fit with the literature, stroma derived from patients with G19 PCa, should have a high proportion of myofibroblasts (double positive for vimentin and α SMA), compared to normal prostate stroma. This expression profile together with a loss of the classic “spindle” shape of fibroblast should distinguish myofibroblasts. Access to sufficient normal prostate tissue to establish an *in vitro* culture was not possible, therefore fibroblasts derived from human foreskin (HFF's) was used as a comparison. Thus, prostate stroma derived from BPH and G19 PCa at low passage was labelled intracellularly (after permeabilisation) with antibodies specific for vimentin and α SMA under untreated and cytokine treated conditions. These markers were paired with 3 other stromal antigens ICAM1, VCAM1 and podoplanin, which are expressed on the cell surface. The cell surface markers had been previously characterised by our lab, where they were found to be important in migration of prostate stroma and consequently PCa cells. Marker expression was also influenced by IL-4&TNF α (Lakins, 2012).

Overall, both patient cohorts (BPH; Figures 5.2-5.4 and PCa; Figures 5.5- 5.7) exhibited a mixed population of cells by both marker expression and morphology. In comparison to prostate fibroblasts, HFF cells (Figures 5.8-5.10) consistently demonstrated the typical spindle shape associated with fibroblasts. G19 stroma contained a higher percentage of cells double positive for vimentin and α SMA (so fitting the myofibroblasts category) in the field of view (FOV). However, double positive cells also occurred at a low rate in the BPH cultures. Even in the HFF culture, some cells weakly expressed α SMA. In all cell types ICAM1 was upregulated on the cell surface after treatment with IFN γ &TNF α , but not IL-4&TNF α . VCAM1 was increased to the greatest degree when prostate stroma was treated with IL-4&TNF α but mildly increased under IFN γ &TNF α conditions. However, VCAM1 stimulation was not conserved in the HFF cultures. Podoplanin expression was not impacted by treatment conditions, and was expressed at consistent levels by prostate stroma, but not HFF cells.

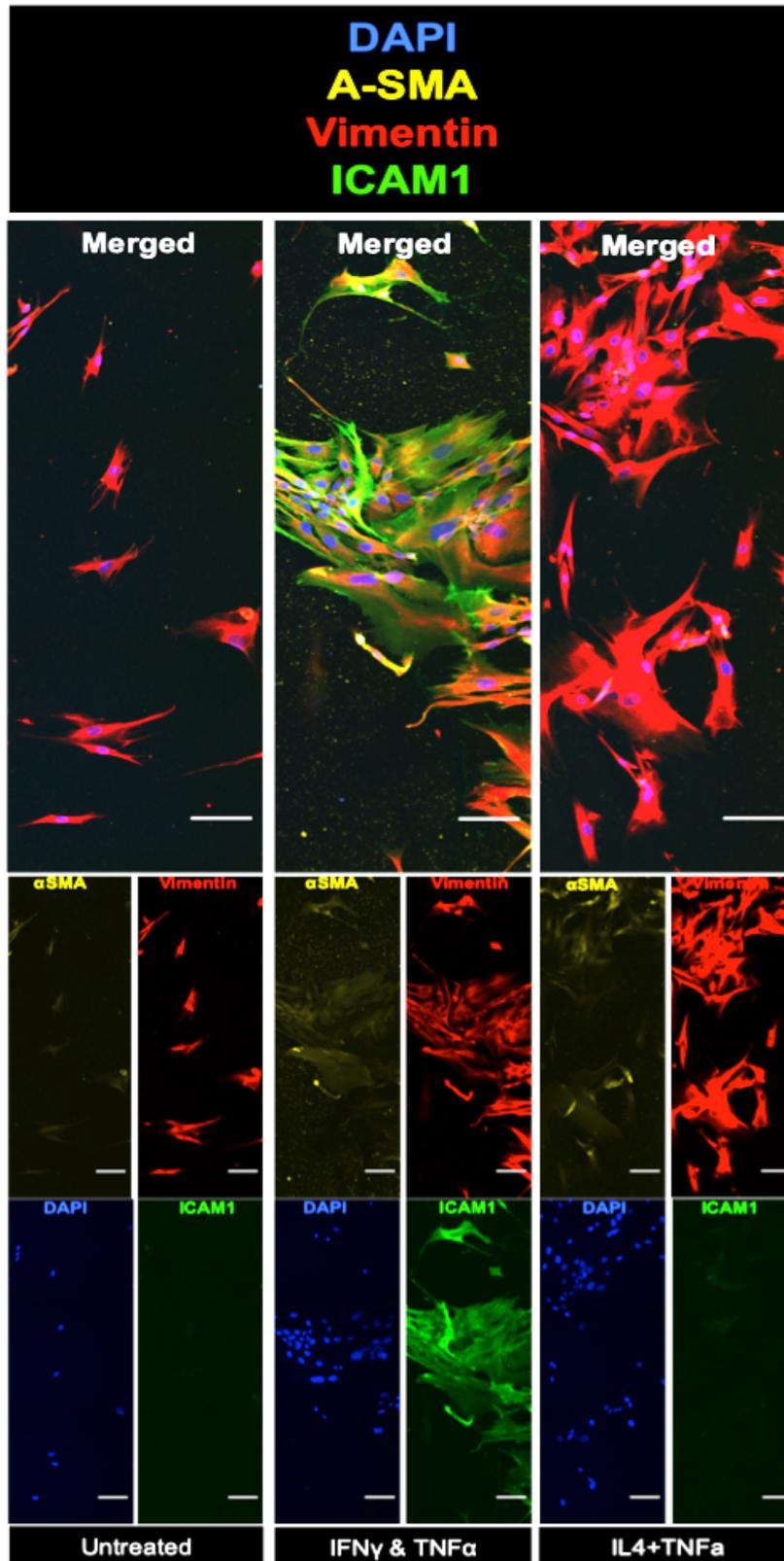


Figure 5.2: Immunofluorescent labelling for changes in ICAM1 in benign stroma after supplementation with IFN γ or IL-4 with TNF α .

Benign stromal cells were seeded on 4-well chamber slides and cultured in media containing the indicated cytokines. Untreated is on the left, IFN γ and TNF α (12.5ng/ml and 5ng/ml) in the centre and IL-4 and TNF α (5ng/ml for both) on the right hand side. Cells were incubated for 48hours before fixing in 4% PFA and labelling with surface molecule targeting fluorophore-conjugated anti-ICAM1. Intracellular labelling was performed following permeabilisation, with fluorophore-conjugated Vimentin and α SMA specific antibodies. Tile scan (1x4) images were taken on a confocal microscope on x20. Scale bar is 100 μ m.

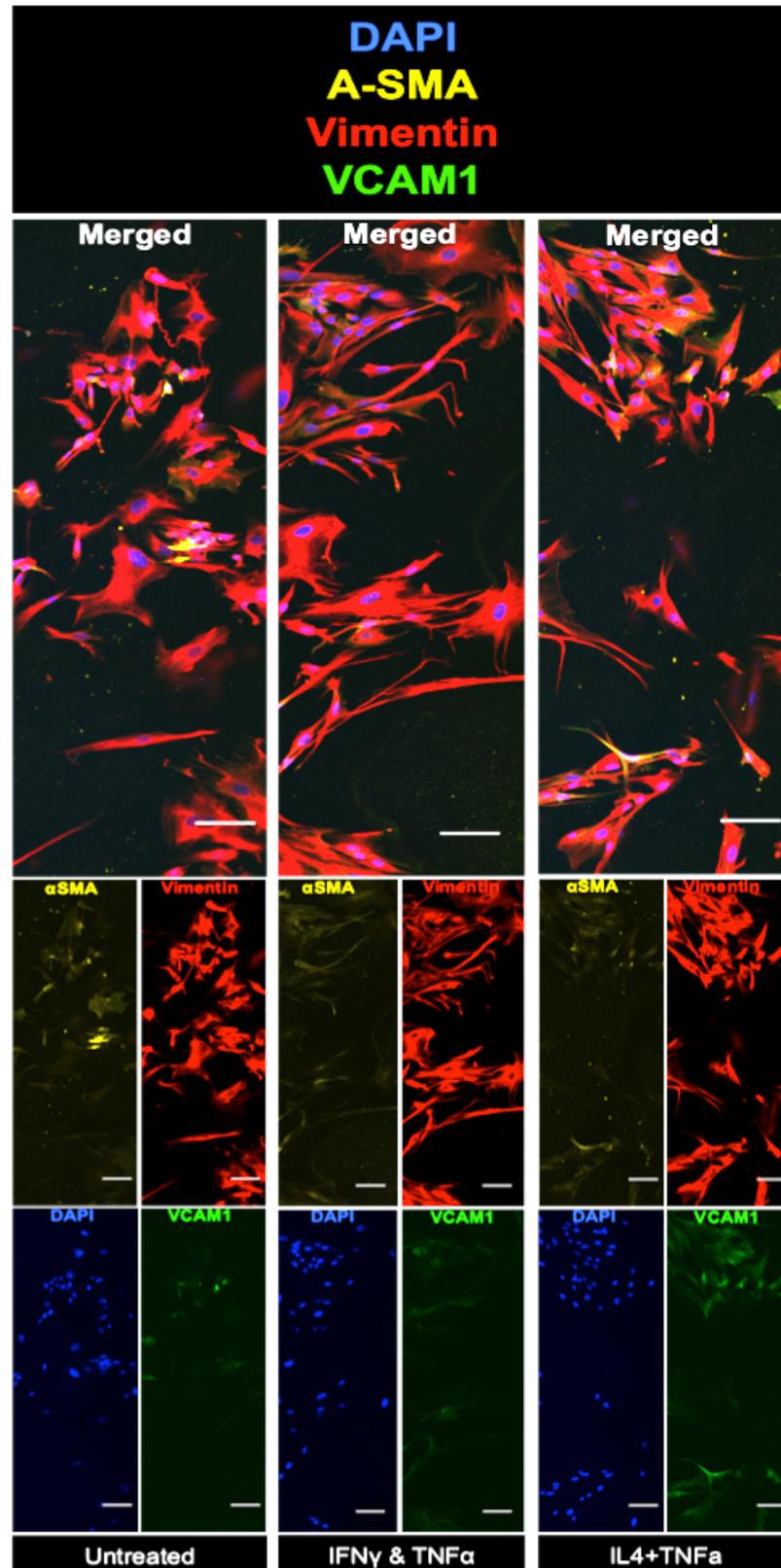


Figure 5.3: Immunofluorescent labelling for changes in VCAM1 in *benign* stroma after supplementation with IFN γ or IL-4 with TNF α

Benign stromal cells were seeded on 4-well chamber slides and cultured in media containing the indicated cytokines. Untreated is on the left, IFN γ and TNF α (12.5ng/ml and 5ng/ml) in the centre and IL-4 and TNF α (5ng/ml for both) on the right hand side. Cells were incubated for 48hours before fixing in 4% PFA and labelling with surface molecule targeting fluorophore-conjugated anti-VCAM1. Intracellular labelling was performed following permeabilisation, with fluorophore-conjugated Vimentin and α SMA specific antibodies. Tile scan (1x4) images were taken on a confocal microscope on x20. Scale bar is 100 μ m

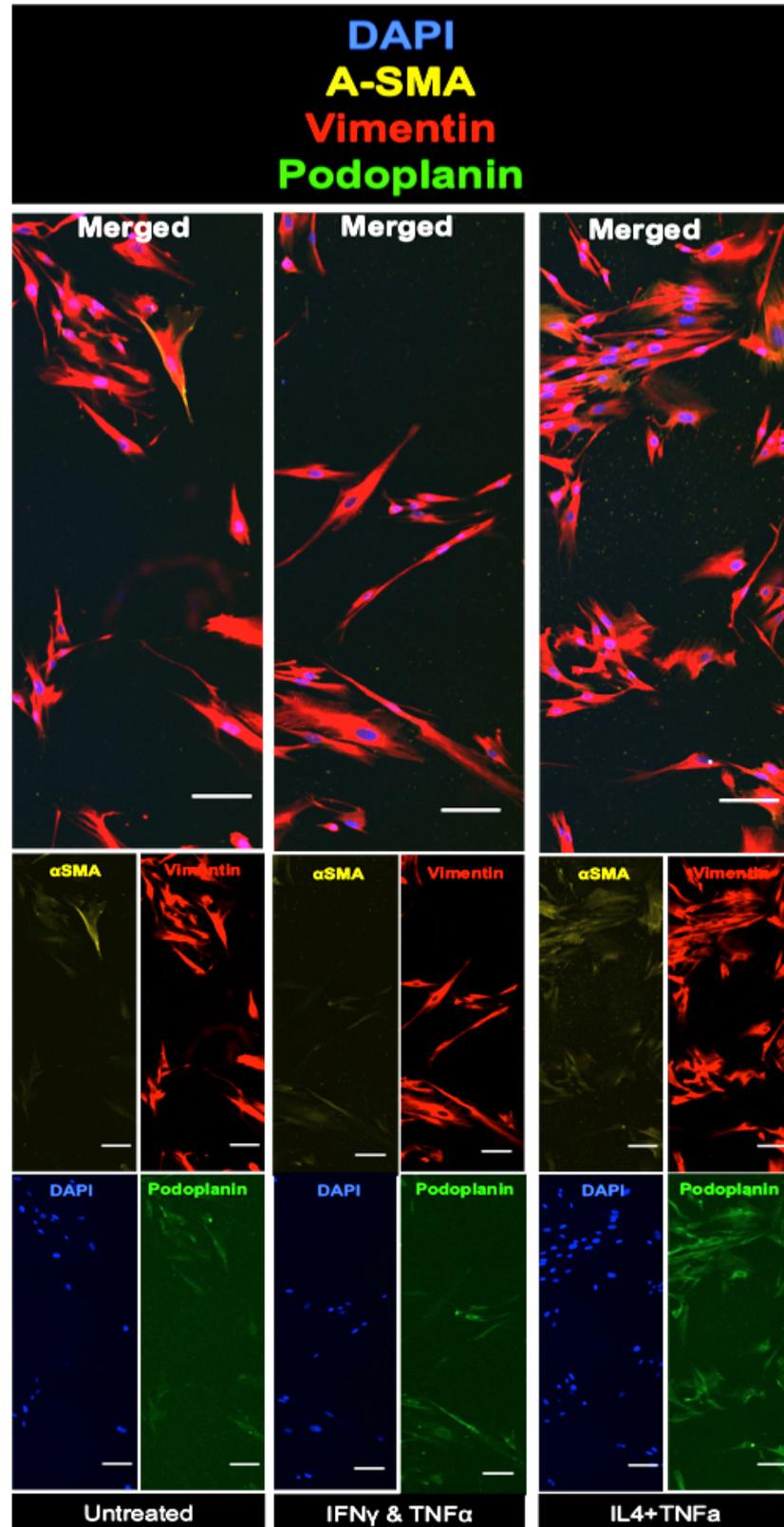


Figure 5.4: Immunofluorescent labelling for changes in podoplanin in *benign* stroma after supplementation with IFN γ or IL-4 with TNF α

Benign stromal cells were seeded on 4-well chamber slides and cultured in media containing the indicated cytokines. Untreated is on the left, IFN γ and TNF α (12.5ng/ml and 5ng/ml) in the centre and IL-4 and TNF α (5ng/ml for both) on the right hand side. Cells were incubated for 48hours before fixing in 4% PFA and labelling with surface molecule targeting fluorophore-conjugated anti-Podoplanin. Intracellular labelling was performed following permeabilisation, with fluorophore-conjugated Vimentin and α SMA specific antibodies. Tile scan (1x4) images were taken on a confocal microscope on x20. Scale bar is 100 μ m

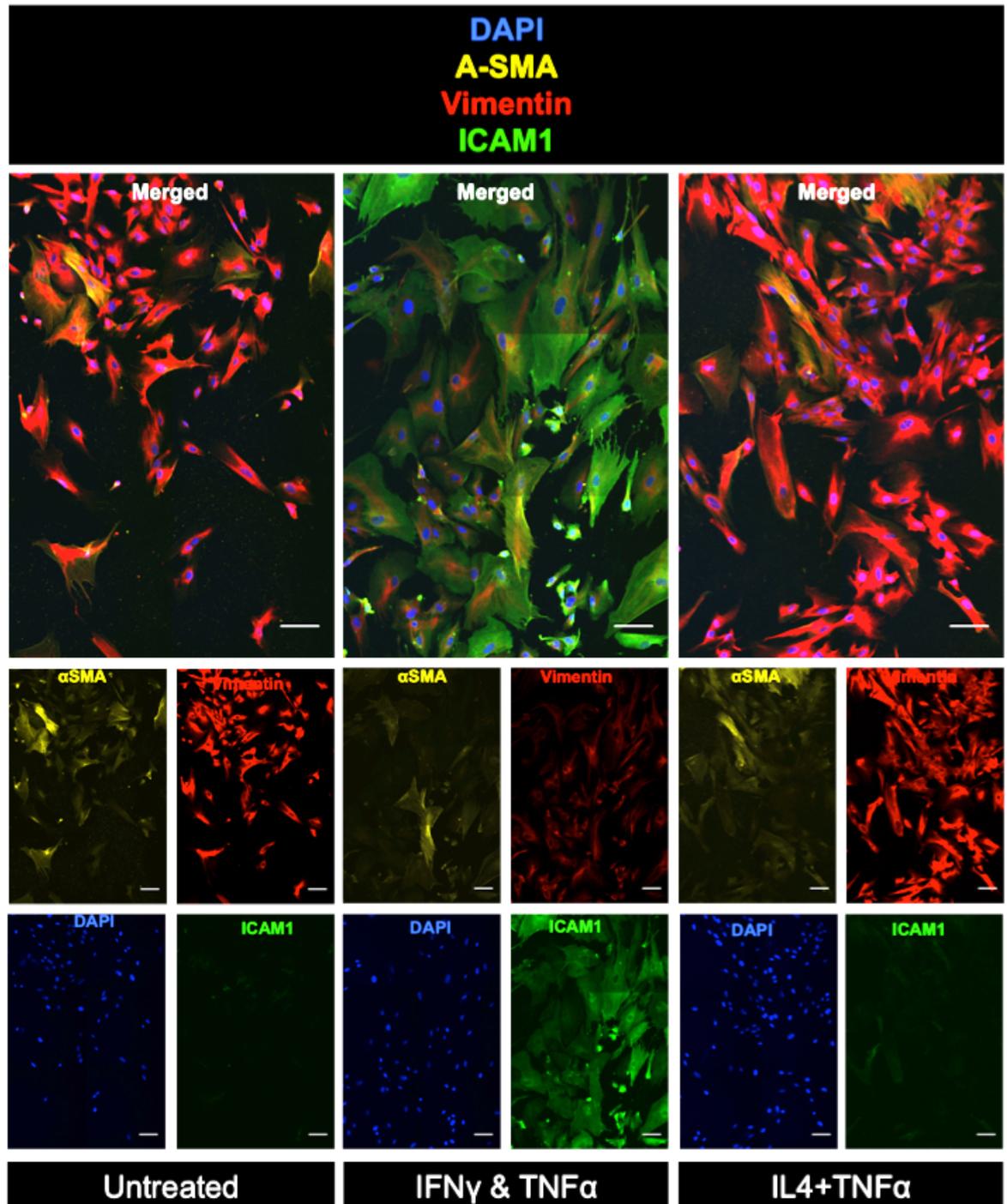


Figure 5.5: Immunofluorescent labelling for changes in ICAM1 in cancer stroma after supplementation with IFN γ or IL-4 with TNF α

Cancer stromal cells were seeded on 4-well chamber slides and cultured in media containing the indicated cytokines. Untreated is on the left, IFN γ and TNF α (12.5ng/ml and 5ng/ml) in the centre and IL-4 and TNF α (5ng/ml for both) on the right hand side. Cells were incubated for 48hours before fixing in 4% PFA and labelling with surface molecule targeting fluorophore-conjugated anti-ICAM1. Intracellular labelling was performed following permeabilisation, with fluorophore-conjugated Vimentin and α SMA specific antibodies. Tile scan (3x4) images were taken on a confocal microscope on x20. Scale bar is 100 μ m

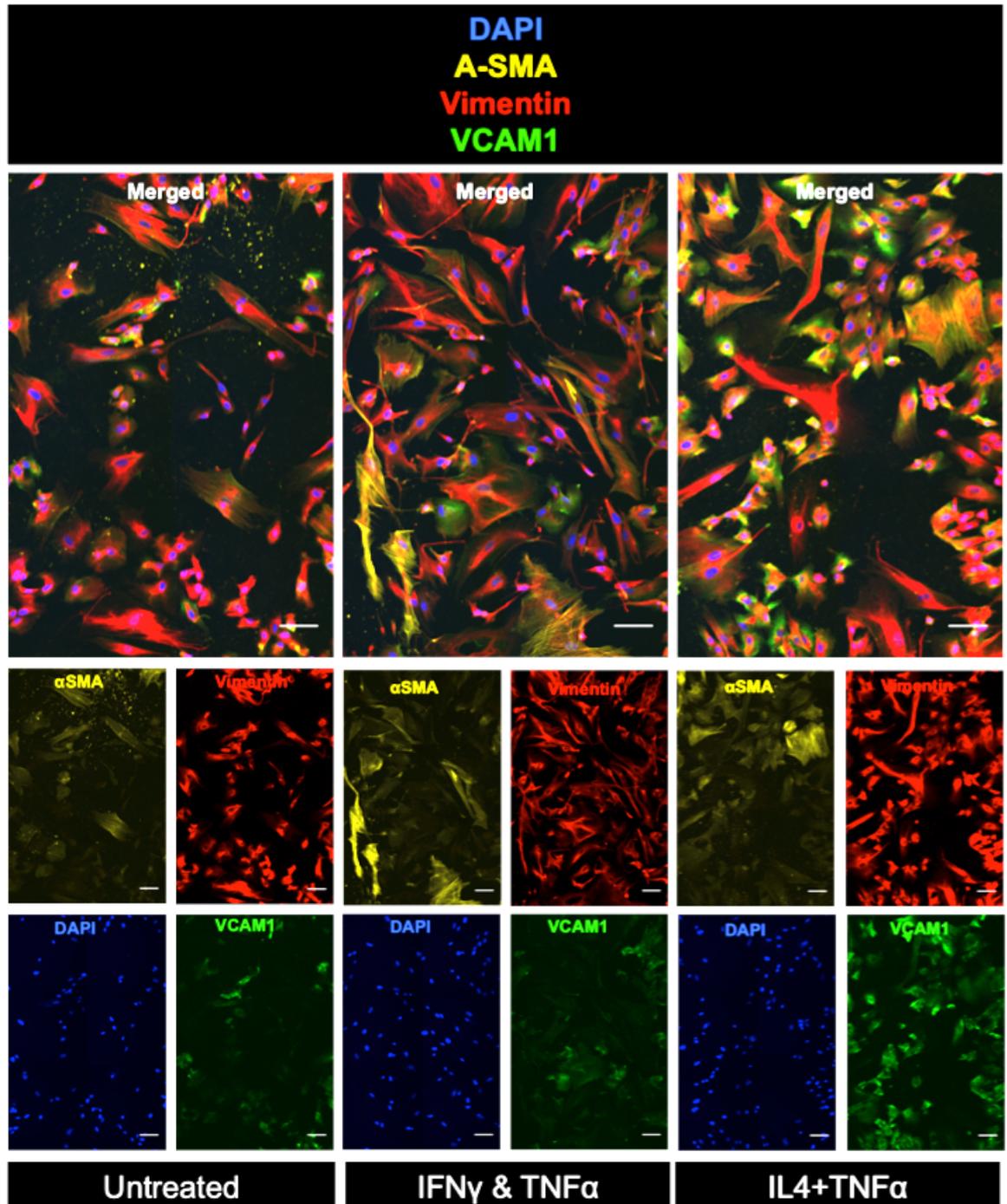


Figure 5.6: Immunofluorescent labelling for changes in VCAM1 in cancer stroma after supplementation with IFN γ or IL-4 with TNF α

Cancer stromal cells were seeded on 4-well chamber slides and cultured in media containing the indicated cytokines. Untreated is on the left, IFN γ and TNF α (12.5ng/ml and 5ng/ml) in the centre and IL-4 and TNF α (5ng/ml for both) on the right hand side. Cells were incubated for 48hours before fixing in 4% PFA and labelling with surface molecule targeting fluorophore-conjugated anti-VCAM1. Intracellular labelling was performed following permeabilisation, with fluorophore-conjugated Vimentin and α SMA specific antibodies. Tile scan (3x4) images were taken on a confocal microscope on x20. Scale bar is 100 μ m

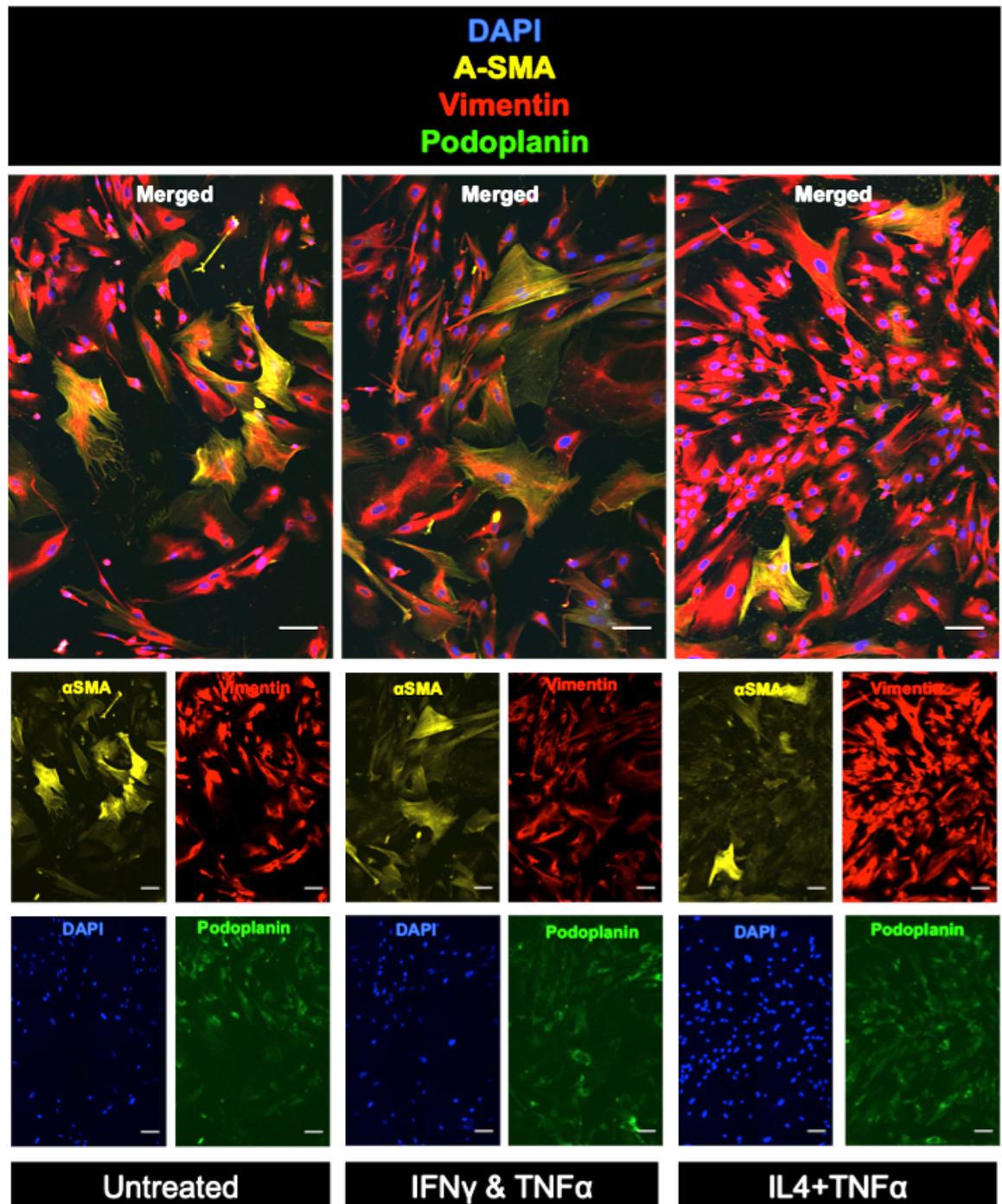


Figure 5.7: Immunofluorescent labelling for changes in podoplanin in *cancer* stroma after supplementation with IFN γ or IL-4 with TNF α

Cancer stromal cells were seeded on 4-well chamber slides and cultured in media containing the indicated cytokines. Untreated is on the left, IFN γ and TNF α (12.5ng/ml and 5ng/ml) in the centre and IL-4 and TNF α (5ng/ml for both) on the right hand side. Cells were incubated for 48hours before fixing in 4% PFA and labelling with surface molecule targeting fluorophore-conjugated anti-Podoplanin. Intracellular labelling was performed following permeabilisation, with fluorophore-conjugated Vimentin and α SMA specific antibodies. Tile scan (3x4) images were taken on a confocal microscope on x20. Scale bar is 100 μ m

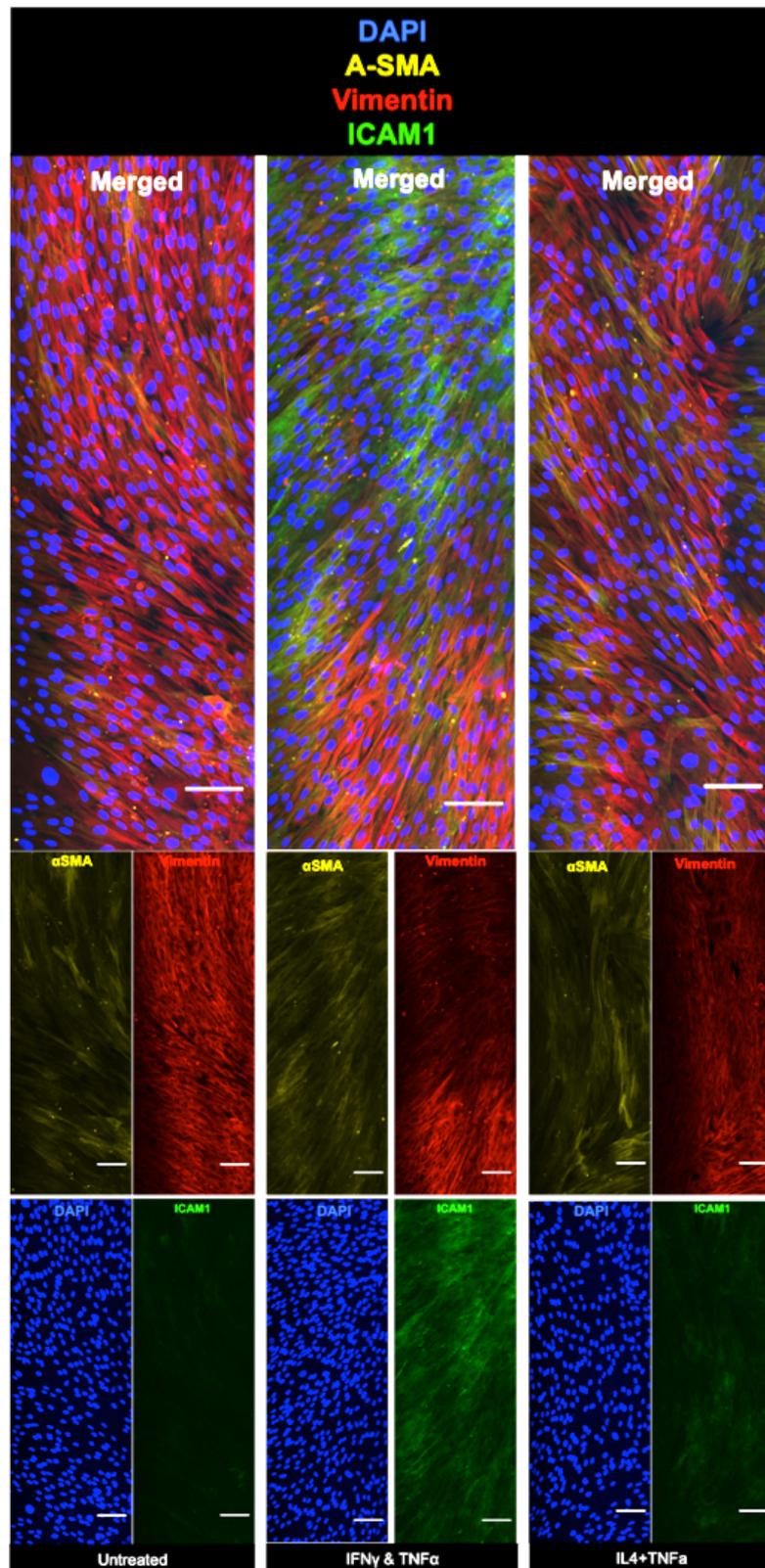


Figure 5.8: Immunofluorescent labelling for changes in ICAM1 in HFF after supplementation with $\text{IFN}\gamma$ or IL-4 with $\text{TNF}\alpha$

HFF cells were seeded on 4-well chamber slides and cultured in media containing the indicated cytokines. Untreated is on the left, $\text{IFN}\gamma$ and $\text{TNF}\alpha$ (12.5ng/ml and 5ng/ml) in the centre and IL-4 and $\text{TNF}\alpha$ (5ng/ml for both) on the right hand side. Cells were incubated for 48hours before fixing in 4% PFA and labelling with surface molecule targeting fluorophore-conjugated anti-ICAM1. Intracellular labelling was performed following permeabilisation, with fluorophore-conjugated Vimentin and αSMA specific antibodies. Tile scan (1x4) images were taken on a confocal microscope on x20. Scale bar is 100 μm

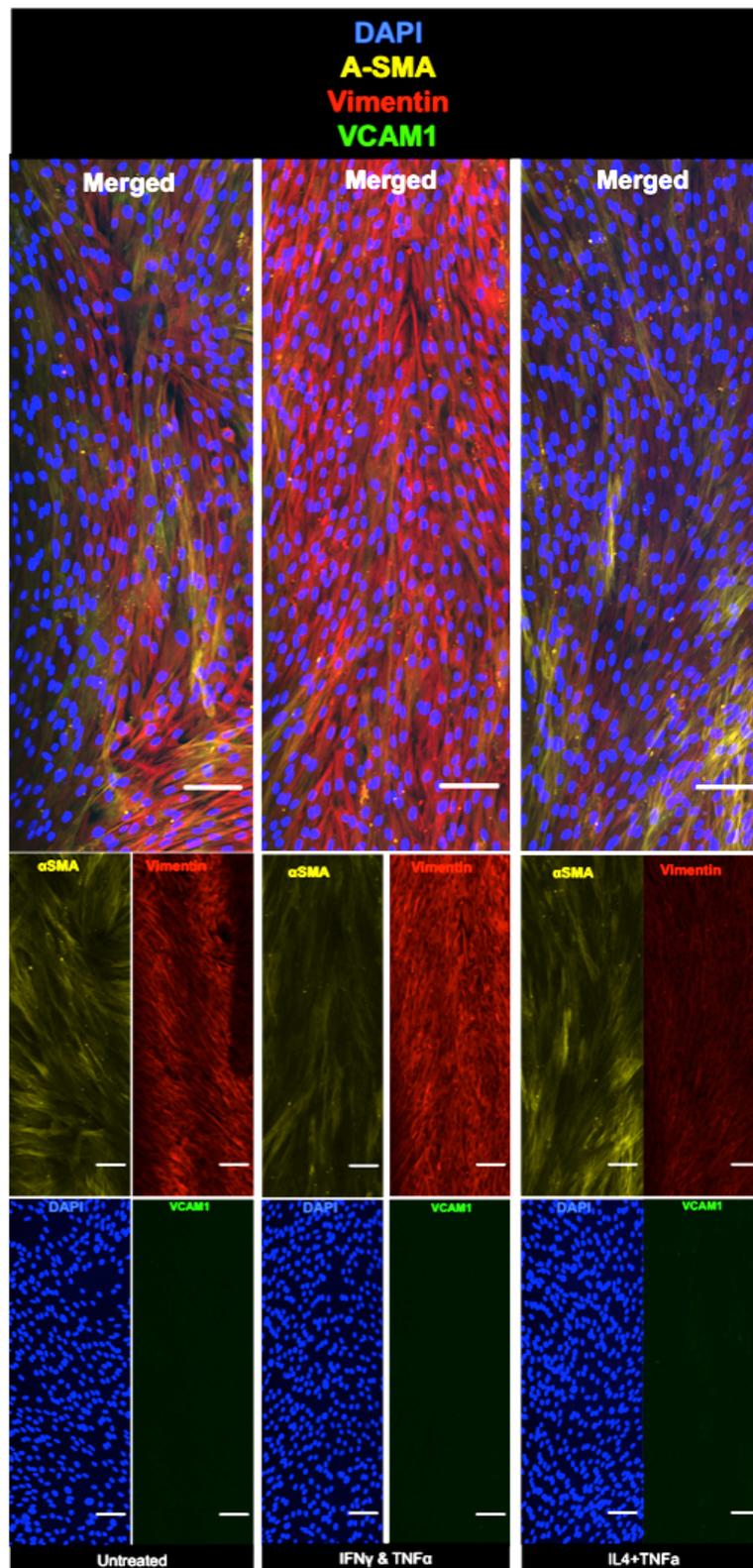


Figure 5.9: Immunofluorescent labelling for changes in VCAM1 in HFF after supplementation with IFN γ or IL-4 with TNF α

HFF cells were seeded on 4-well chamber slides and cultured in media containing the indicated cytokines. Untreated is on the left, IFN γ and TNF α (12.5ng/ml and 5ng/ml) in the centre and IL-4 and TNF α (5ng/ml for both) on the right hand side. Cells were incubated for 48hours before fixing in 4% PFA and labelling with surface molecule targeting fluorophore-conjugated anti-VCAM1. Intracellular labelling was performed following permeabilisation, with fluorophore-conjugated Vimentin and α SMA specific antibodies. Tile scan (1x4) images were taken on a confocal microscope on x20. Scale bar is 100 μ m

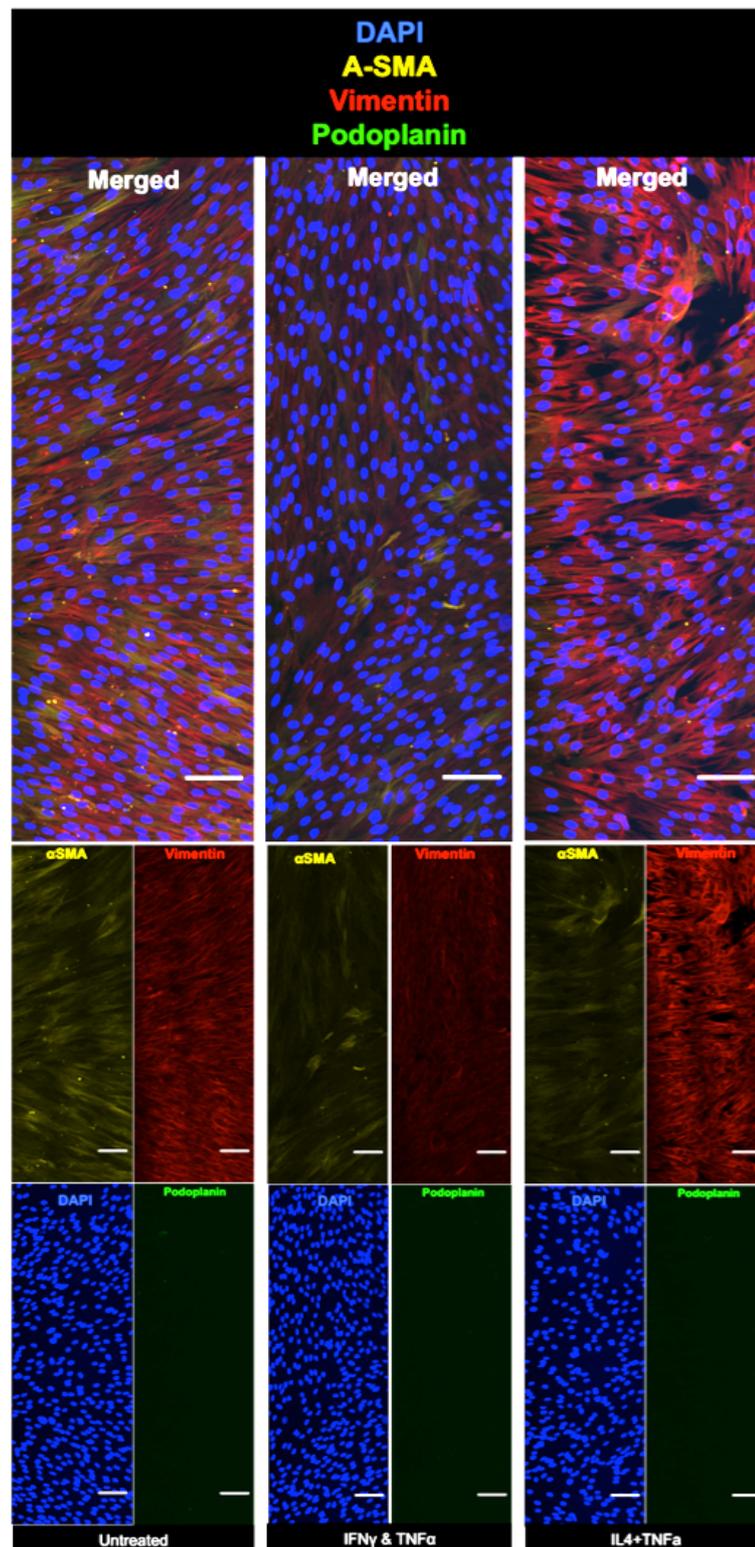


Figure 5.10: Immunofluorescent labelling for changes in podoplanin in *HFF* after supplementation with IFN γ or IL-4 with TNF α

HFF cells were seeded on 4-well chamber slides and cultured in media containing the indicated cytokines. Untreated is on the left, IFN γ and TNF α (12.5ng/ml and 5ng/ml) in the centre and IL-4 and TNF α (5ng/ml for both) on the right hand side. Cells were incubated for 48hours before fixing in 4% PFA and labelling with surface molecule targeting fluorophore-conjugated anti-Podoplanin (C). Intracellular labelling was performed following permeabilisation, with fluorophore-conjugated Vimentin and α SMA specific antibodies. Tile scan (1x4) images were taken on a confocal microscope on x20. Scale bar is 100 μ m

5.3.2. Flow cytometric analysis confirms expression profile of ICAM1, VCAM1 and podoplanin in a group of patients

To confirm the expression of the AM (ICAM1 and VCAM1) and podoplanin, cell surface staining of these molecules and analysis by flow cytometry was performed in an expanded number of patients (Figure 5.11). As with *in vitro* staining, ICAM1 is upregulated in all cytokine treated stromal cells. VCAM1 was expressed by prostate stromal cells (but not HFFs) and was not upregulated when treated with IFN γ &TNF α (N.B IL-4&TNF α treated stromal cells were not analysed by flow cytometry). Likewise, prostate stromal cells, but not HFFs, express podoplanin and there is an upward trend when treated with IFN γ &TNF α .

5.3.3. FAP cannot be associated exclusively with cancer associated stroma over normal or benign stroma *in vitro* and is upregulated by IFN γ &TNF α on the cell surface.

FAP has long been associated with stroma typically classified as CAFs. Having briefly considered that untreated PD-L1 expression may correlate with a phenotypic difference between cultures, the expression of FAP in patient cultures was investigated. The hypothesis was that FAP may be upregulated by IFN γ &TNF α , indicating that immune activation causes a switch in the localised stromal cell phenotype resulting in the CAFs classification in the context of tumours. Surprisingly, FAP mRNA was neither increased in cancer-derived stroma (contrary to the literature), nor in IFN γ &TNF α treated stroma (Figure 5.12A). The FAP cell surface expression in low passage stroma derived from BPH and PCa patients, in comparison to low passage HFF cells was analysed (Figure 5.12B). HFFs express the highest FAP levels on the cell surface. It is unchanged between BPH and PCa untreated stroma. It is, however, upregulated on the cell surface when stroma is treated with IFN γ &TNF α .

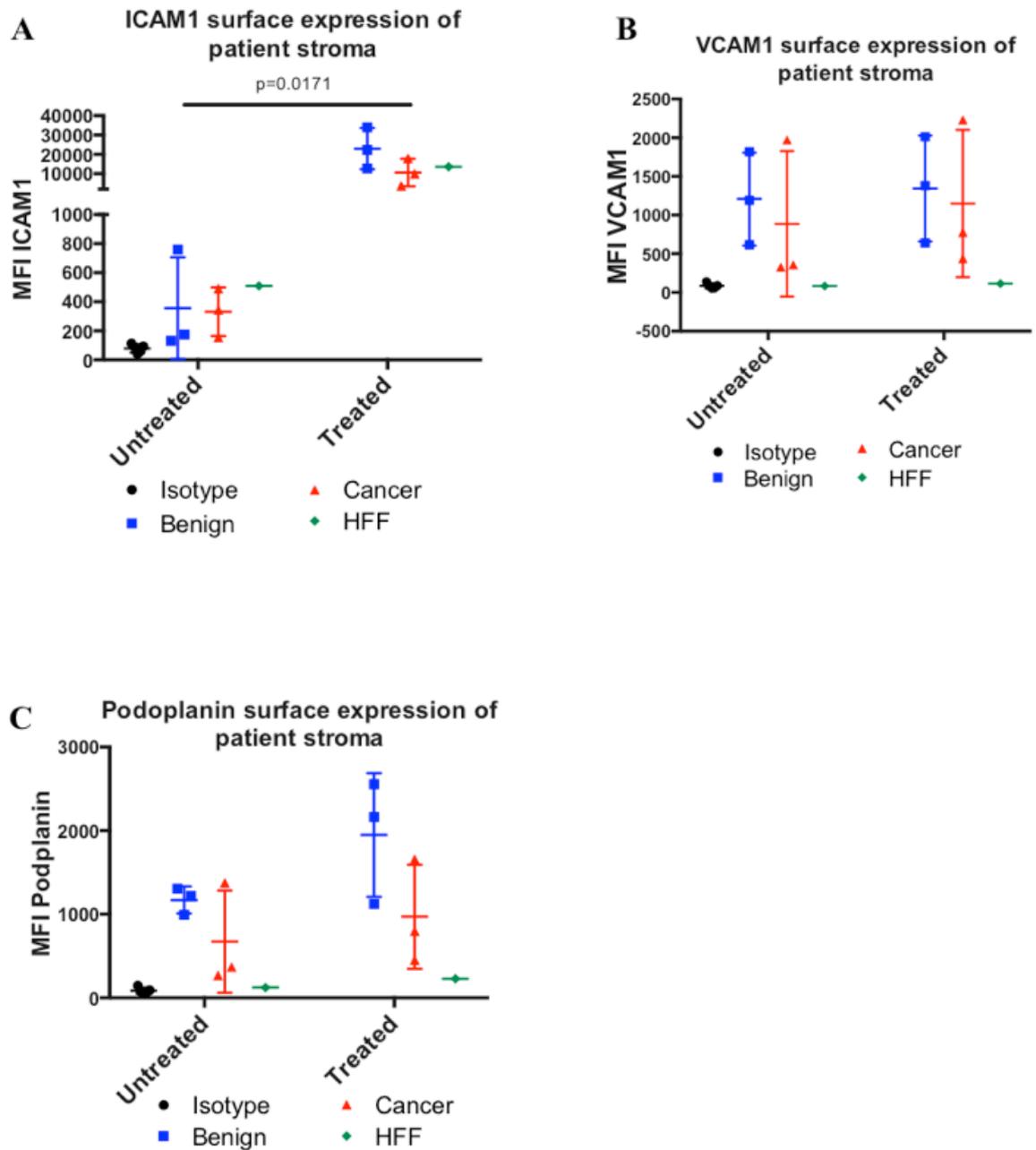


Figure 5.11: Flow cytometric staining of patient stroma and HFF cells that were IFN γ & TNF α treated or untreated

Stromal cells from 3 patients with either BPH or PCa were supplemented with or without cytokines for 48 hours before collecting for analysis by flow cytometry. Cells were gated to exclude dead cells and debris before analysing surface expression of ICAM1 (A), VCAM1 (B) and Podoplanin (C). At least 10,000 events were collected for analysis and the relevant isotype control was performed in parallel.

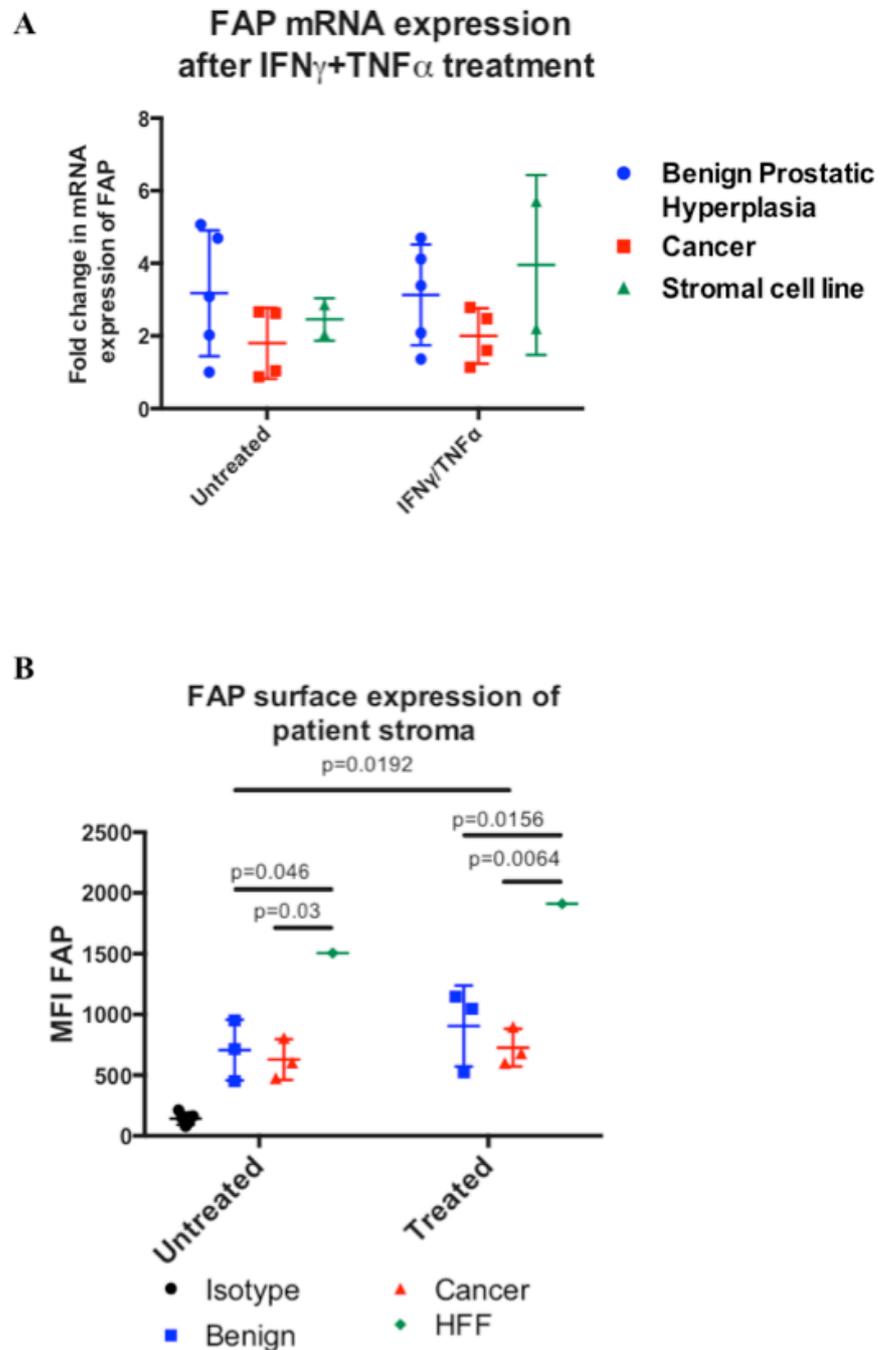


Figure 5.12: FAP expression by patient prostate stroma

Stromal cells from 3 patients with either BPH or PCa were supplemented with or without cytokines for 8hours before mRNA collection (**A**) and 48hours before collecting for analysis by flow cytometry (**B**). FAP expression was measured by RT-qPCR, normalised to GAPDH and is presented as the mean fold change \pm SD (**A**). Cells were gated to exclude dead cells and debris before analysing surface expression of FAP, at least 10,000 events were collected for analysis and the relevant isotype control was performed in parallel (**B**). Statistical significance was measured by a RM 2Way ANOVA with a Tukey's multiple comparisons test.

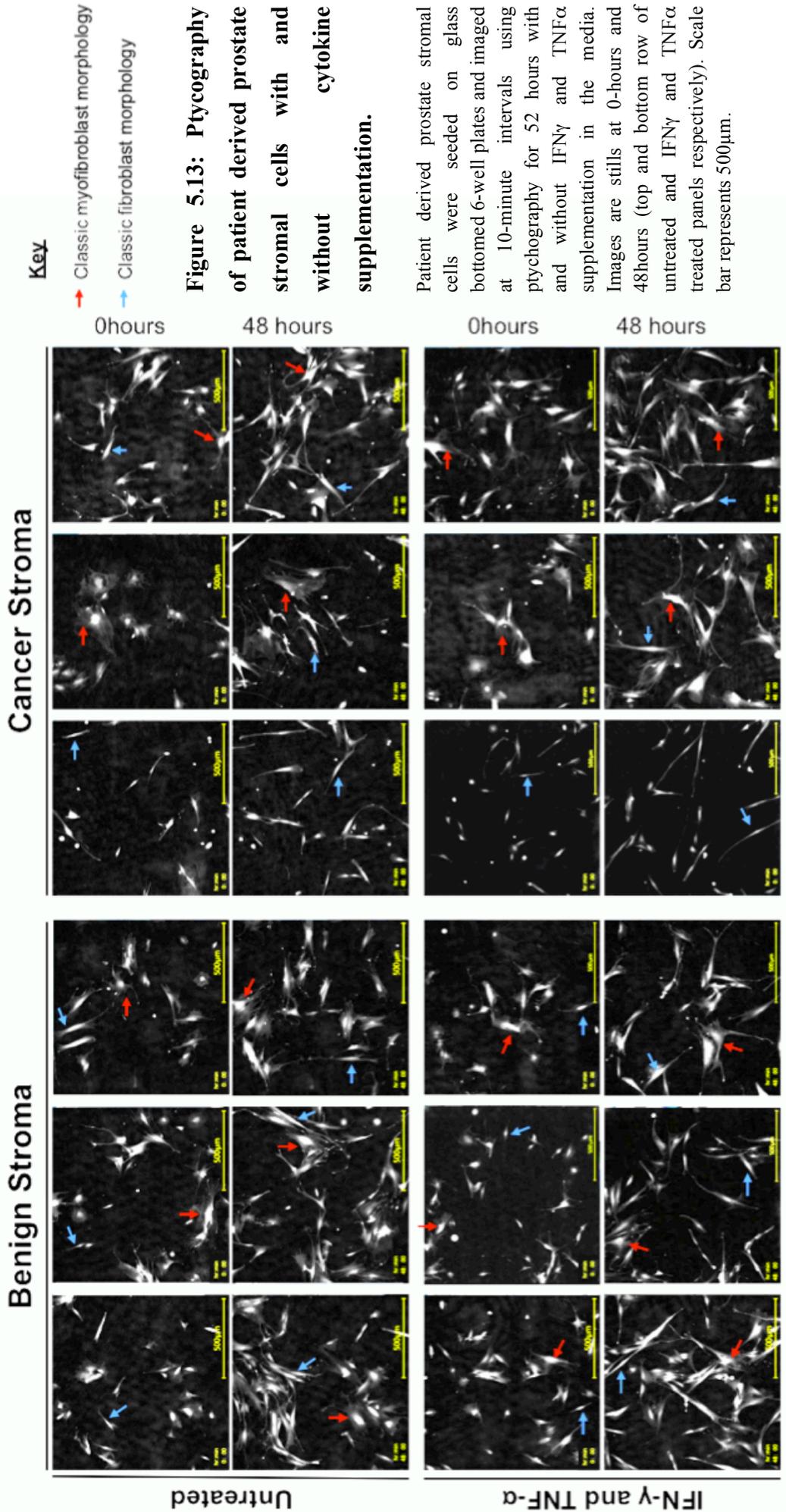
5.3.4. Analysis of prostate stromal cells by ptychography

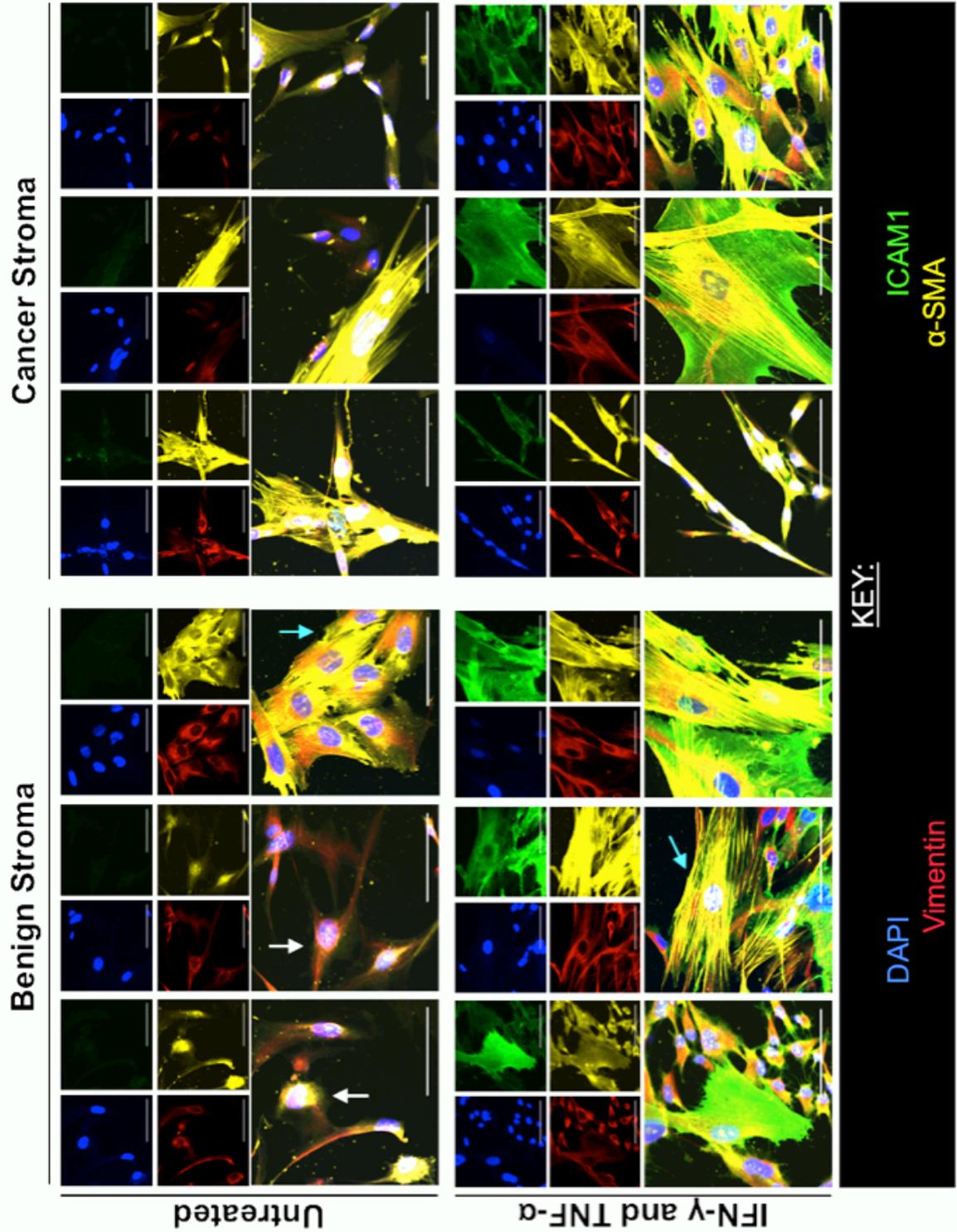
Fixation of cells *in vitro* induces morphological change. Therefore, to get a more representative characterisation of prostate stroma, cultures derived from 3 patients of each disease group were analysed by label-free time lapse imaging (Marrison *et al.*, 2013) in untreated and IFN γ &TNF α treated conditions, to determine if cytokine treatment caused changes in the proportions of myofibroblasts and fibroblasts in culture. Figure 5.15 illustrates the cells at 0 and 48 hours by phase contrast. It was evident during the time course, and displayed in these images, that cell morphology varies greatly both within patients and between patients. Cell shape was fluid over time. Cells adapted to both the spindle shape and a large flattened morphology with many dendrites that would be considered as a myofibroblast phenotype, over time. Disease groups could not be associated with one phenotype over another, and treatment could not be seen to impact on cell shape by phase contrast microscopy.

Following completion of the time lapse images, cells were again fixed and labelled for vimentin, α SMA and ICAM1, given that ICAM1 was previously shown to be upregulated in response to IFN γ &TNF α (Figure 5.2 and 5.5). In this case, the classic stromal antigens vimentin and α SMA did not provide any evidence of an enrichment of either fibroblasts or myofibroblasts favoured in either disease group nor with treatment, though treatment did induce the expected ICAM1 upregulation. This is in contrast to the previous examples, where an increased proportion of double positive cells was present in G19 samples. However, here, 3 patients of each disease were analysed in comparison to one of each previously. All cells stained staining for α SMA, however the dispersion did appear to differ between treatments. For example, the first 2 BPH derived cultures contained cells with weak nuclear/peri-nuclear positivity of α SMA and spindle morphology. Similar cells were present in the 3rd PCa culture untreated and 1st PCa treated culture. The staining becomes clearly localised to a network of cytoplasmic fibres in the alternate images (culture 1&2 BPH treated, culture 1 PCa untreated and culture 3 PCa treated), considered to be a myofibroblast phenotype. As a whole therefore, it could be concluded that fibroblasts and myofibroblasts exist in both BPH and PCa cultures, but that these phenotypes are likely not to be static and stromal cells may be influenced to fit either grouping.

5.3.5. Cell segmentation of stromal cells allows quantification of cell size

Cell segmentation was used to compare cell shape and size in different cultures and treatment conditions (e.g. Figure 5.15). As the Phase Focus software could not segment cells automatically, time point images (0, 2, 4, 8, 24, 48 hours) were taken of all conditions and segmentation optimised for each individually. Relevant data from this process were then plotted (Figure 5.16) which allowed the cell area, mass length width and thickness to be considered in untreated and treated BPH and PCa stroma. These data suggested that a linear relationship does not occur between any of the parameters and time. When comparing the untreated and treated cultures within disease groups, it becomes evident that treatment did not impact cell size.





Key
 ↓ α-SMA- Vimentin+
 ↓ α-SMA+ Vimentin+

Figure 5.14:
Immunofluorescent
imaging of patient derived
prostate stromal cells with
and without cytokine
supplementation.

Following completion of imaging in Figure 4.19, cells were immediately fixed in 4% PFA before cell surface labelling with anti-ICAM1-A488 (green) and intracellular staining (after permeabilisation with 0.5% Triton) with anti-vimentin-A647 and anti-αSMA-cy3 (red and yellow respectively). Cells were then imaged on a confocal microscope at x40. Scale bar represents 100µm.

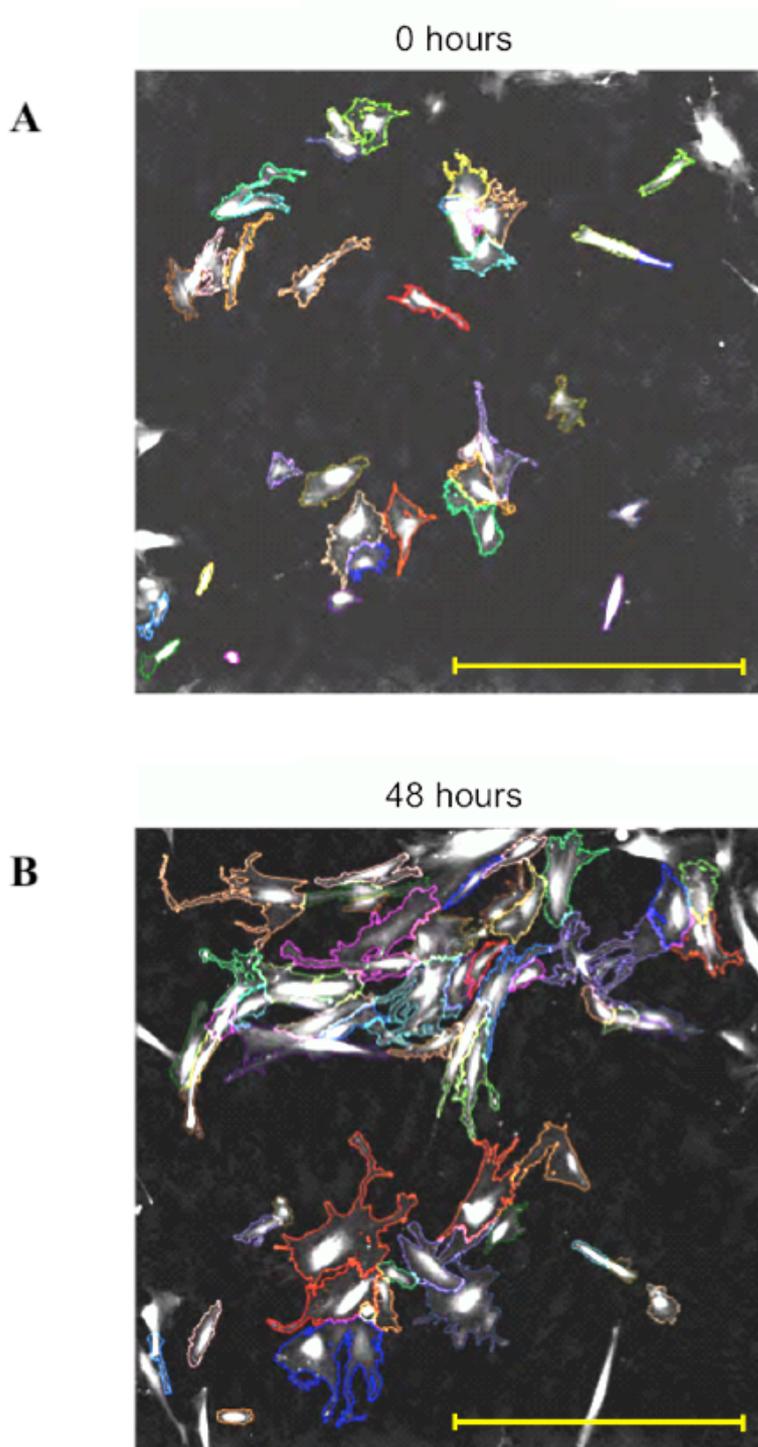


Figure 5.15: Analysis of stromal cell morphology after IFN γ and TNF α treatment in cultured stromal cells derived from patients with prostatic disease.

Ptychography was used to capture images of prostate stromal cells over 52hours at 10 minute intervals. Images taken at 0, 2, 4, 8, 24 and 48 hours were used for segmentation as demonstrated at 0h (A) and 48hours (B). Scale bar represents 500 μ m.

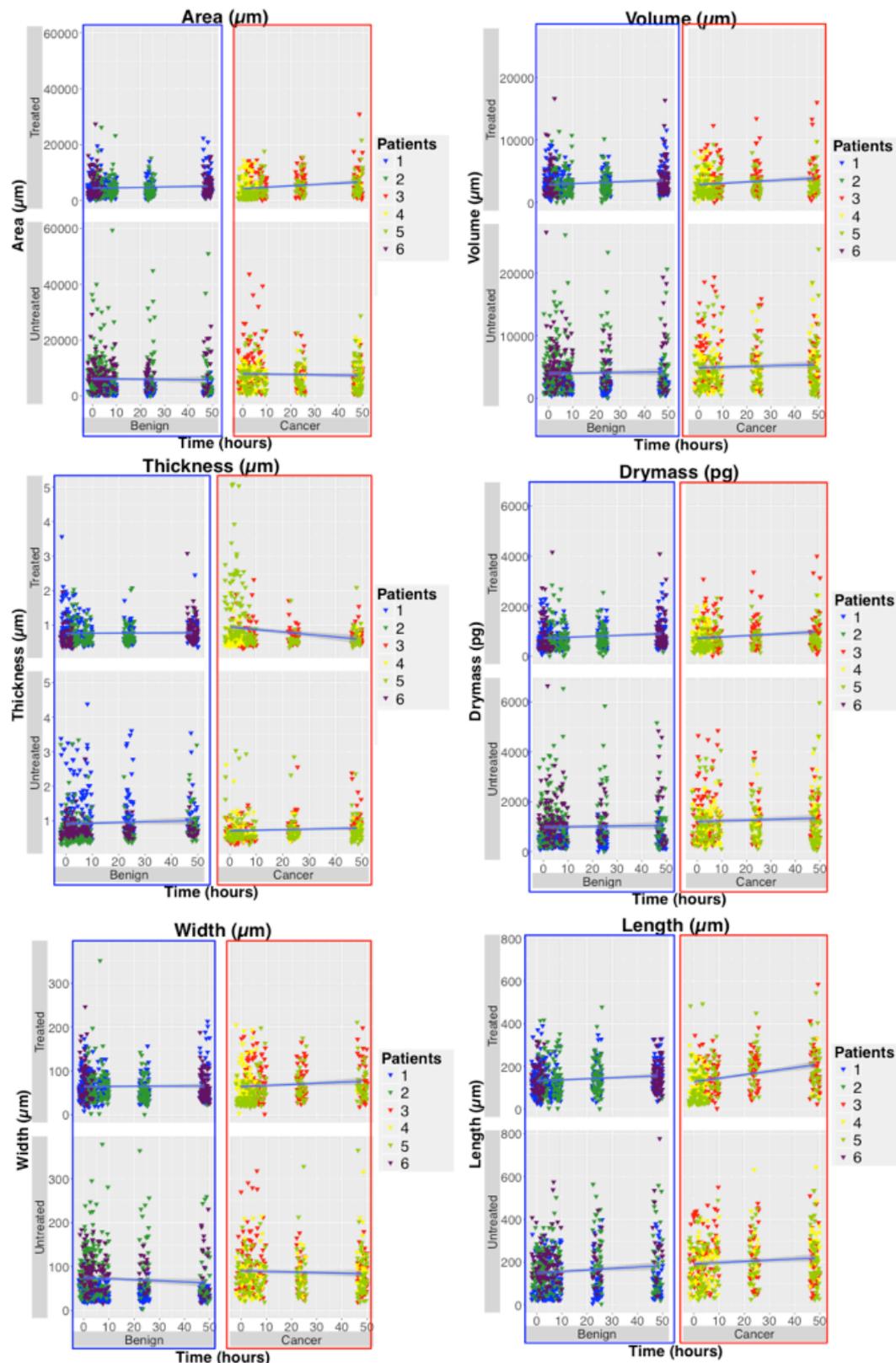


Figure 5.16: Analysis of stromal cell morphology after IFN γ and TNF α treatment in cultured stromal cells derived from patients with prostatic disease.

Phase focus microscopy was used to capture images of prostate stromal cells over 52 hours at 10 minute intervals. Images taken at 0, 2, 4, 8, 24 and 48 hours were segmented (demonstrated in figure 5.15) to quantify morphological differences between diseases and treatments. Parameters were plotted against time (hours) and linear regression was used to understand whether a relationship existed. Data is coloured by patient and each point indicates 1 cell. Area (μm^2), Volume, Thickness, Drymass (pg), Width (μm) and Length (μm) were plotted for patients with BPH and PCa for untreated and treated conditions.

5.3.6. Stromal cell lineage analysis highlights the importance of population enrichment over time in culture

Potential population differences between disease groups and treatment conditions could be due to specific enrichments of populations within cultures. To investigate this possibility, cells in the FOV imaged during ptychography were manually tracked in FIJI mTrackj® to identify enhanced proliferation of cells, either within lineages or an increase in the number of lineages (Figure 5.17 and 5.18 BPH and G19 respectively). In each well, every cell that remained in FOV for more than 1 hour was followed over time and given a designated track; in the figure, a horizontal line of a single colour illustrates this. Upon proliferation, where the original cell divides and gives rise to 2 daughter cells, the track diverges; this is illustrated by branching of the original track at the time point mitosis occurred, keeping the colour consistent to signify a lineage cluster. All cells were analysed this way and a complete cell lineage tree produced for each well (Figures 5.17 and 5.18 A). Of the clusters that undergo more than 1 cycle of mitosis it is clear that daughter cells undergo the 2nd and 3rd cycles in synchrony with each other (indicated by a red arrow at each cycle in Figure 5.17A; untreated patient 1). In the cases where tracks are not completed cells have either gone out of frame or have undergone cell death (e.g. blue arrow Figure 5.17 A; untreated patient 1). Cells continued to proliferate in the presence of IFN γ &TNF α .

The number of cycles identified in each well is also plotted as pie charts (Figure 5.17 and 5.18 B) to more easily illustrate cell proliferation. There is no conserved trend evident from this data due to variation between patients. Conclusions could be more confidently made with a greater number of cells analysed for each patient. This was not possible to do in the current project due to the large size of stromal cells, though advances with technology may allow greater FOV to be assessed. It is clear however from this data that a greater degree of proliferation can occur over time, leading to an enrichment of specific lineages and a potential loss of heterogeneity. This may account for the variation observed across patients and is likely the reason some characteristics are lost in culture, especially with prolonged passage.

Plots of the mean cell speed and distance travelled for each patient's cells demonstrated that cancer stroma have increased mobility (Figure 5.19 A&B) in culture compared to BPH stroma. There is also an indication that with treatment cancer stroma becomes slower and total BPH stroma migration is reduced, although this does not reach statistical significance. This resulted in cells frequently moving in and out of frame in the cultures, as shown by the number of unfinished tracks (Figure 5.19 A). Further to consideration of the number of cycles cells undergo in culture, the frequency of proliferation is also plotted as a histogram against time (Figure 5.19 C&D; benign and cancer respectively), which does not reveal a conserved or clear trend with regards to effect disease and cytokines have on proliferation. There is an indication that an increase in the number of cells that don't divide within the time frame in the cancer stroma cultures treated with cytokines, similar to untreated and treated BPH stroma, compared to untreated cancer stroma.

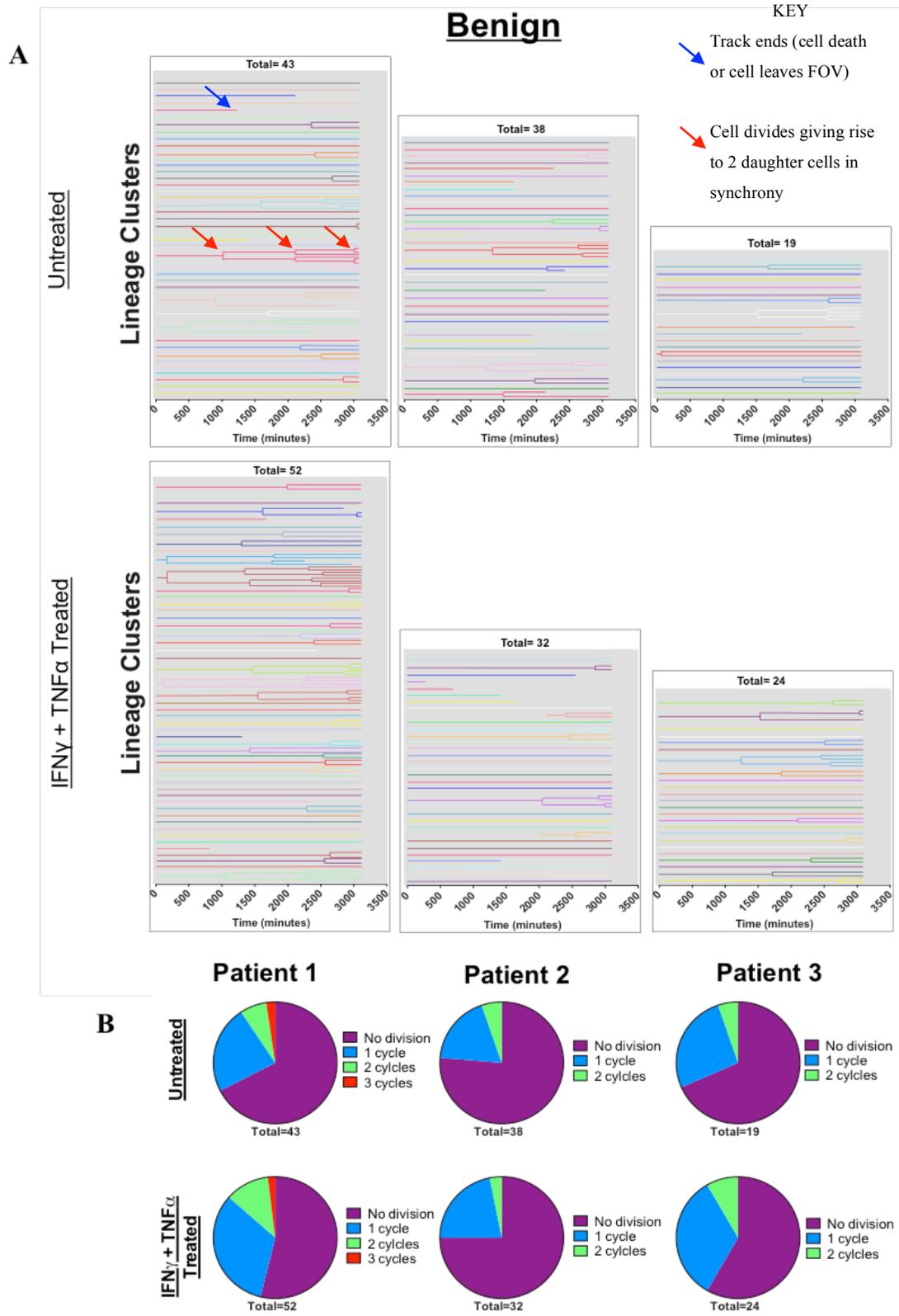


Figure 5.17: Analysis of cell lineage progression with IFN γ and TNF α treatment in stromal cells derived from patients with benign prostatic disease.

Images captured during ptychography (Figure 5.15) were used to manually track cells in FIJI mTrackj over 52hours. Lineages were then clustered based on cells of origin in order to determine whether IFN γ and TNF α affect frequency of (1) proliferation (2) proliferative stromal cells. The top and bottom panels of (A) and (B) corresponds to 3 separate patients without and with cytokine supplementation respectively. (A) A single line corresponding to a cell in the field of view extends horizontally over time. Separation of the line into 2, 4 and 8 secondary tracks correspond to cell division, giving rise to daughter cells. Tracks are coloured by the cell of origin. (B) Pie charts of the number of mitosis cycles each lineage in (A) undergoes.

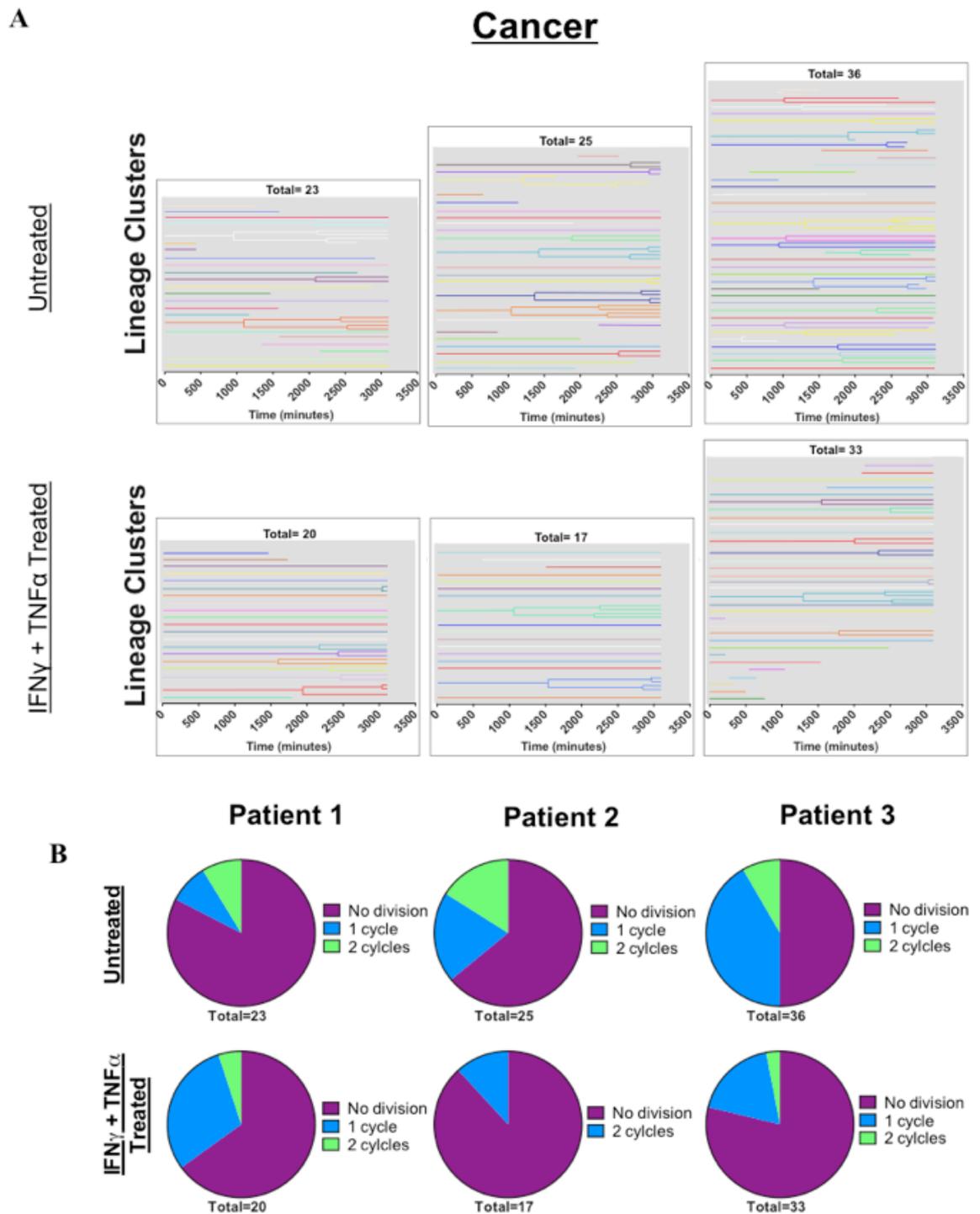


Figure 5.18: Analysis of stromal cell lineage after IFN γ and TNF α treatment in cultured stromal cells derived from patients with prostate cancer.

Images captured during ptychography (Figure 5.15) were used to manually track cells in FIJI mTrackj over 52hours. Lineages were then clustered based on cells of origin in order to determine whether IFN γ and TNF α affect frequency of (1) proliferation (2) proliferative stromal cells. The top and bottom panels of (A) and (B) corresponds to 3 separate patients without and with cytokine supplementation respectively. (A) A single line corresponding to a cell in the field of view extends horizontally over time. Separation of the line into 2, 4 and 8 secondary tracks correspond to cell division, giving rise to daughter cells. Tracks are coloured by the cell of origin. (B) Pie charts of the number of mitosis cycles each lineage in (A) undergoes.

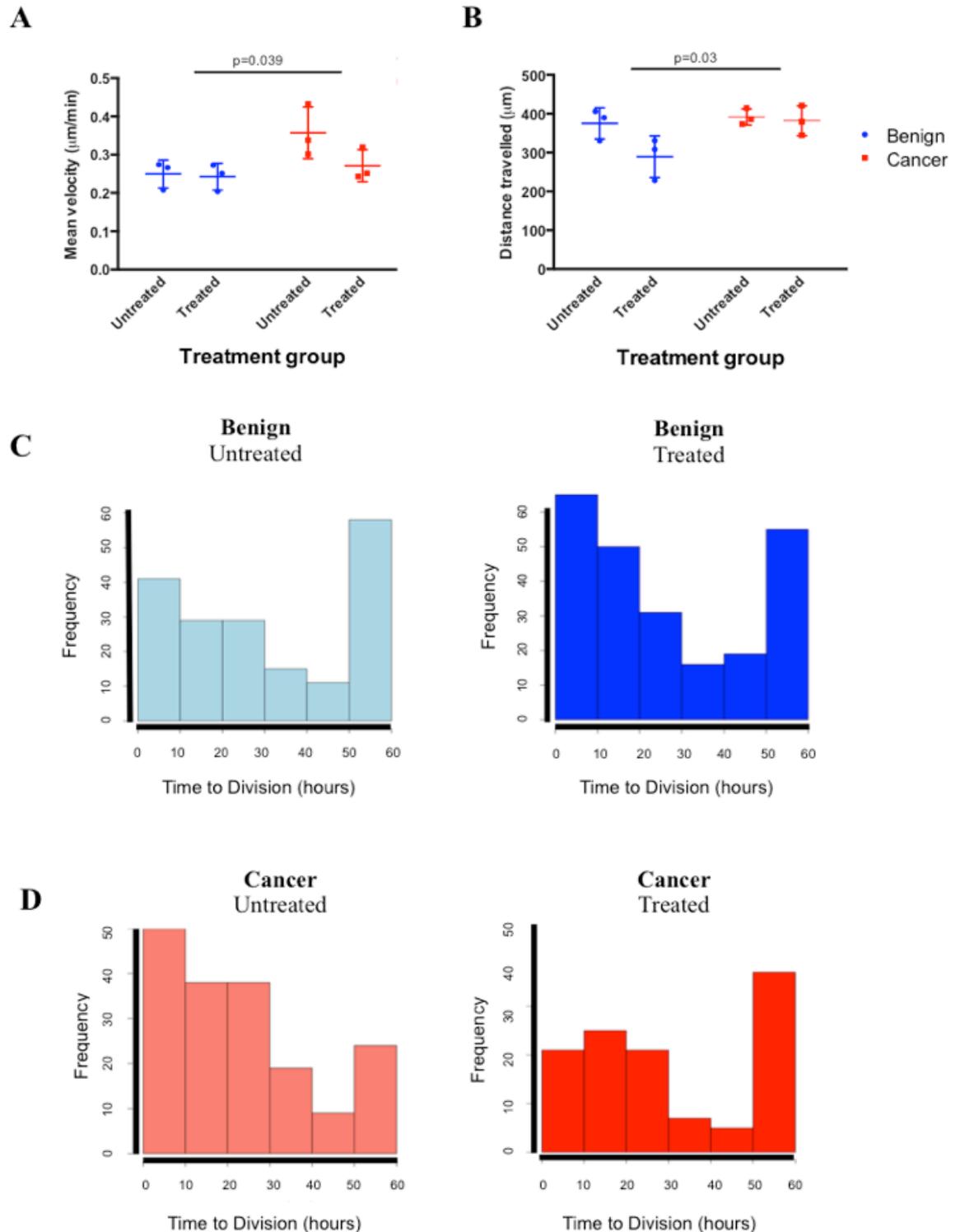


Figure 5.19: Analysis of IFN γ and TNF α effect on cell speed and proliferation in stromal cells derived from patients with prostatic disease.

Images taken during ptychography were used to track cells in mTrackJ and cell speed, distance travelled and time between mitosis cycles was analysed. For (**A** and **B**) the mean velocity or total distance travelled for each patient untreated or treated is plotted. Data is the mean of this \pm SD. Significance was considered by means of a two-way repeated measures ANOVA, which finds disease has a significant effect on both velocity of cells and total distance travelled. The time between mitosis cycles was plotted as histograms for benign (**C**) and cancer (**D**) stroma, taken from data presented in Figures X&Y. For each the frequency (y-axis) of cells that divide at the given times on the x-axis is plotted.

5.4. Summary of Results

- Analysis of stromal cell phenotypes reveals that both IFN γ &TNF α and IL-4&TNF α induce changes in AM ICAM1 and VCAM1. Prostate stroma express podoplanin, which was mildly upregulated by IFN γ &TNF α . This is consistent in both BPH and Cancer stroma. HFF cells neither express podoplanin nor are induced to express VCAM1, but do upregulate ICAM1.
- Stroma cultures from PCa and BPH contain both myofibroblasts (vimentin+ & α SMA+) and fibroblasts (vimentin+ & α SMA-). Morphology alone did not distinguish between myofibroblasts and fibroblasts, without additional markers.
- FAP expression on the cell surface was increased to a small degree in response to IFN γ & TNF α .
- Daughter stromal cells born from the same cell of origin divide in synchrony. BPH and Cancer stroma both proliferate in the presence of IFN γ &TNF α . Without analysing more cells of each patient it cannot be determined if differences in proliferation occur between disease or cytokine treatment.
- Cancer stromal cells migrate faster and farther than BPH.

5.5. Discussion

5.5.1. Myofibroblasts/CAFs in culture

In culture, the myofibroblast phenotype (vimentin& α SMA double positive cells) or morphology (large flattened cells with dendrite-like extensions) cannot be consistently associated with cancer stroma relative to BPH, since they were present in cultures derived from both patient groups. Moreover, spindle morphology does not guarantee lack of myofibroblast phenotype by marker expression. HFF cultures conversely, as expected, do not contain cells with either the myofibroblast phenotype or morphology, at least in the conditions used here. This suggests signals that occur in both BPH and PCa encourage myofibroblast growth. Due to the shorter doubling time of HFF cells compared to prostate stroma within the treatment period of 48hours, the HFF cultures reach monolayer confluency, compared to 60-70% in prostate stroma (cell loss also occurs during antibody labelling). Contact inhibition and spatial restriction is likely to affect morphology of stromal cells, as they extend to fill the free space. This may induce the differences observed between HFF and prostate cultures. However, even at low confluency, HFF cells maintain their spindle morphology, so it is unlikely this could account for the absence of myofibroblasts in these cultures. Others have demonstrated that it is possible to differentiate HFF cells into myofibroblasts after culture with TGF- β 1. To accurately determine whether prostate myofibroblasts are a disease, inflammation or a prostate associated stromal phenotype; normal prostate stroma should be investigated (since both PCa and BPH are proliferative diseases). IFN γ &TNF α does not appear to induce the myofibroblast phenotype or morphology in stromal cultures within the treatment periods (maximum 52hours), although, prolonged exposure to IFN γ &TNF α may change fibroblast phenotype. TGF- β 1 can induce myofibroblasts in culture. After just 72 hours in culture, fibroblasts, human adipose stem cells and *in vivo* change phenotype (Midgley *et al.*, 2013; Tuxhorn *et al.*, 2002). This TGF- β 1 mediated differentiation is dependent on epidermal growth factor receptor (EGFR), CD44 and can be influenced by cell-cell and cell-ECM contact (Midgley *et al.*, 2013). Due to a loss of EGFR expression in high passage fibroblasts, they lose the capacity to differentiate to a myofibroblast phenotype. Data presented in the previous two chapters demonstrated a high endogenous expression of TGF- β 1 in the prostate, regardless of disease, which may account for the myofibroblasts found in culture.

Fibroblasts and fibroblast-like cells are heterogenous (hence the use of the stroma terminology). Stroma extracted from different tissue types across the body and even within the same tissue, exhibits heterogeneity in both function and phenotype. These stromal variations can be due to fluctuations in microenvironmental pressures (e.g. mechanical forces, signalling molecules or inflammation). Crucially, these stromal subtypes might be just that; fluctuations of the cell state. Maintenance of a phenotype may depend on the microenvironment they originate; one that can never be fully recapitulated *in vitro*. Indeed, the scientific value of *in vitro* investigations on stroma from pathological tissues relies on altered stroma being a committed, rather than fluid, phenotype. Nevertheless, these cells may retain the same competence to fulfil different functional roles when

in a different microenvironment (such as culture). The capacity and proclivity of stromal cells to adjust in response to the local milieu is likely to be similar within tissues types, but influenced by neighbouring cells. So while BPH and PCa stroma fulfil different functions within the context of their specific microenvironment (inflammation and epithelial signals), they may both respond in a similar manner when taken out of this environment. Analysis of heterogenous populations by qRT-PCR (or similar techniques) and western blotting means amalgamating and presenting heterogenic variables as a representative of a culture as a whole, which may be erroneous and mask subtle but significant changes.

5.5.2. FAP as a tumour stroma marker

FAP expression has been associated with CAFs (Jia *et al.*, 2016; Liu *et al.*, 2012), however in culture stromal FAP gene expression did not differ between the BPH and G19 derived stroma. This could indicate that the stromal cultures derived from PCa did not contain CAFs. Although to manage this, samples with high grade G19 disease was selected for choosing PCa stroma. These particular samples were used due to the high content of cancer in the tissue collected. Previous publications from the Maitland lab, employing the same technique for stromal culture establishment have demonstrated functional distinctions between BPH and PCa stroma. Lastly, all experiments were conducted on low passage stroma to lessen the outgrowth of particular lineages. Taking this all into consideration, it leaves the conclusion that the reported increased FAP expression in cancer stroma is not detectable at the mRNA level in cultured stroma. It may be that CAFs taken out of the local environment are not so different from BPH stroma, although *in vitro* experiments by others and us have displayed differences in migration and gene expression (Eiro *et al.*, 2016; 2017; Hall *et al.*, 2002; Lakins, 2012; Yang *et al.*, 2005). Given that surface FAP expression is increased upon IFN γ &TNF α , it indicates that FAP expression in tumour stroma is in part due to local inflammation as a *consequence* of tumour presence, rather than tumour-derived signalling (Brokopp *et al.*, 2011; Tillmanns *et al.*, 2015). Since inflammation is associated with both BPH and PCa stroma, this could account for the similarities. It would also explain FAP expression in malignant (and to a lesser degree benign) tumour tissue but not normal. If this were the case it would be expected that HFFs express relatively little FAP. Though, HFFs express increased surface FAP, in both untreated and IFN γ &TNF α treated conditions, relative to BPH and PCa stroma. Since expression is still increased after treatment, it is possible that this elevated expression is due to different culture conditions of HFF and prostate stromal cells. HFF cultures have been grown in D15% (compared to R10% for prostate stroma) as per commercial recommendations, therefore the increased serum concentration likely results in this irregularity. DMEM and RPMI have distinct compositions that may also have effected FAP expression. For example, calcium and L-Isoleucine contents are doubled in DMEM compared to RPMI (2001). To test this, HFF cells should be equilibrated in R10% and comparisons made.

The observation of a potential IFN γ &TNF α mediated FAP upregulation is interesting (though this increase was of a small magnitude) and may explain some of the earlier discussed implications of targeting FAP as a cancer therapeutic. Importantly, targeted cytotoxicity of FAP+ stromal cells is likely also to eradicate a substantial immunosuppressive compartment of the tumour, since stromal cells also upregulate a range of immune inhibitory molecules in response to TH1/CTL immune activity (Chapter 4). This would be consistent with recently emerging data demonstrating synergy between checkpoint inhibition and FAP+ cell depletion (Wen *et al.*, 2016; Zhang and Ertl, 2016).

5.5.3. Proliferative capacity of prostate stroma

Analysing living populations of prostate stroma by ptychography highlighted the potential for stroma subtype selection *in vitro*, supporting the importance of using low passage samples. This selection likely occurs even in the first subculture stages resulting in a shift of subtype population densities and differences in the heterogeneity between *in vivo* and *in vitro*. This phenomenon has been demonstrated by others and will be discussed in more detail in Chapter 7.

5.5.4. Concluding remarks

This chapter aimed to determine whether stromal cultures from BPH and PCa could be discriminated by morphology, expression of stromal antigens and CAF associated markers. It was found that disease did not impact on these parameters. Myofibroblasts occurred in cultures from both disease groups. Consistent with previous data from the Coles and Maitland lab, stromal cell migration and adhesion molecule expression was influenced by addition of cytokines. Interestingly, IFN γ &TNF α treatment increased FAP expression. Questions remain as to whether stromal phenotypes (and associated characteristics) are *attributable to* or a *result of* disease pathology. Are these phenotypes a result of permanent differentiation or can they be reversed? It is likely that to understand these dynamic cells, a better understanding of their lineage and response to environment should be understood, but mainly functional phenotypes should be separated by expression profile. Only then, can cell types be separated when isolated from tissue and the influence of the microenvironment fully tested.

Chapter 6

Analysing prostate infiltrating lymphocytes in patients with prostatic disease

6.1. Introduction

The infiltration of effector T cells into the tumour is it critical for the initiation and efficacy of the anti-tumour immune responses. Data presented in the previous chapters demonstrate a significant role for stromal cells in modulating *local* T cell function. Prostate stromal fibroblasts express a number of immune inhibitory molecules including PD-L1, IDO and TGF- β , upregulated by IFN γ &TNF α ; a model of TH1/CTL mediated immunity. Many studies have attempted to analyse tumour infiltrated T cell (TIL) activity using IHC, though while this method can provide spatial relevance, it is limited to only a few molecules and a small portion of tissue. To analyse functionality of the T cells, flow cytometry provides a means of labelling cells with a larger number of antigen specific antibodies, and therefore gives better distinction of immune subtypes. As well as defining the populations of immune cells within the tissue, it would permit analysis of activation and exhaustion phenotypes. Furthermore, extraction of live TILs means that the actual capacity to accomplish tumour killing could be assessed by T cell cytotoxicity assays.

Flow cytometry has been used in the characterisation of TILs from a number of tissues. In ovarian cancer, phenotypic analysis of intratumoural lymphocytes identified infiltration of active tumour specific CD8+ CTLs in patients seropositive for the TAA NY-ESO-1 and that PD-1 and Lag-3 were potential mechanisms of inhibiting these cells in the TME (Matsuzaki *et al.*, 2010b). Similarly, in hepatocellular carcinoma patients, TILs were proportionally skewed toward a Treg dominated T cell population, which varied depending on tumoural location (i.e. intratumoural, peritumoural and periphery) (Wu *et al.*, 2013). Furthermore, the Treg populations extracted from the different locations within the tumour also displayed proportionally impaired function correlating with distance from the tumour.

However, few studies have analysed flow cytometric data of PILs and a well-characterised method for doing has not been established. Instead, many studies have evaluated the functionality of circulating lymphocytes, assuming the characterisation of peripheral T cells will be representative of those infiltrating tissue. However, a study of 20 patients comparing PILs to patient matched peripheral blood T cells demonstrated a significant upregulation of PD-1+ CD8+ T cells from the tissue compared to blood, indicating this is not the case (Sfanos *et al.*, 2009). An earlier study from the same group demonstrated an increased propensity of IFN γ production (upon stimulation with phorbol 12-myristate13-acetate and ionomycin) in selected TH1 cells isolated from prostate tissue compared to patient matched peripheral blood (Sfanos *et al.*, 2008). Although, in neither case the authors stated the quantity of events in each gate, whether a viability delineator had been used, and the use of contour plots rather than dot plots made it difficult to assess the immune populations. A second group has recently published papers employing methods of analysis of viable PILs (Norström *et al.*, 2016). T cells infiltrating BPH tissue were extracted and phenotypically analysed using a protocol published almost two years earlier (Norstrom *et al.*, 2014). On comparing the median frequency of immune cell subsets, they found significant

proportional changes of T cell subsets between peripheral blood and TILs. The TIL fractions had increased proportions of CD8⁺ T cells and a reduction in CD4⁺ T cells, resulting in an overall reduced CD4:CD8 (blood: 1.7 compared to tissue: 0.6). They also documented reduced proportions of NK cells and B cells, with an increased in Treg frequency. These T cells displayed differential expression of regulatory receptors compared to circulating T cells. In all instances PD-1, LAG-3 and CTLA-4 were increased in the TILs. These data support an overall immunosuppressive environment within BPH tissue. By histological analysis, lymphocytes phenotypically consistent with Treg (CD4⁺CD25⁺FOXP3⁺) were a substantial proportion of lymphocyte clusters in the region of tumour tissue and were PD-1⁺ (Ebelt *et al.*, 2009). The authors describe these cells as embedded within a “dense stromal compartment”. Notably, PD-L1⁺ cells were present but not identified by marker expression and presumed APCs. Moreover, tumour cells were PD-L1⁻ in all PCa tissue from each of the 17 patients.

6.2. Summary and Aims

An established protocol that would permit isolation of live TILs from prostate tissue for phenotypic and functional analysis has been absent. Given the inhibitory roles of stroma presented in previous chapters the aim was to:

1. Establish a protocol for extracting viable PILs
2. Analyse immune cell subsets and determine expression of phenotypic exhaustion and activation markers from BPH and PCa tissue

6.3. Results

6.3.1. Extraction of prostate infiltrating immune cells from patient tissue for analysis by flow cytometry requires a short digestion

A protocol that permitted the analysis of freshly isolated viable lymphocytes from human prostate tissue was required in order to understand the activity and subtypes of T cells infiltrating prostate tissue. Initially the methodology used for isolation of epithelium and stroma for culture (described in methods) was tested. This protocol released cells with a high degree of autofluorescence and the proportion of CD45+ cells were not viable. The hypothesis was that lymphocytes, unlike stroma and epithelium, would not have strong connections to the ECM or surrounding cells and would therefore not require an overnight collagenase digestion. With this in mind, short digestions with liberase blends were compared to gentleMACs dissociation and overnight digestion (Figure 6.1). All methods except overnight digestion (6.1D) released a large population of CD45+ cells that could clearly be separated into T and B cells based on CD3 and CD19 expression. Inclusion of liberase enzyme (thermolysin low) permitted a higher proportion of lymphocytes to be released in comparison to gentleMACs dissociation alone (6.1A&C vs. 6.1B). GentleMACs compared to manual dissociation by pipetting had no detectable effect on extraction efficiency. Therefore, for extraction of lymphocytes from prostate tissue, a short digestion sufficed.

6.3.2. Cell yield and surface antigen (CD45, CD19 and CD3) expression is not effected by liberase blend or concentration

Liberase enzymes are available in a number of blends, varying ratios of thermolysin and dispase. To determine whether some combinations of enzymes improved recovery of lymphocytes, or whether cleavage of surface molecules could occur, 5 blends were investigated in conjunction with gentleMACs dissociation (Figure 6.2). MFI of CD45, CD3 and CD19 was analysed and no enzyme blend was found to consistently impact on fluorescence of the molecules tested (data not shown). Cell yield was not impacted as determined by trypan blue exclusion prior to antibody labelling.

Further to this, concentration of enzyme (thermolysin low, as in 6.2A) was titrated before analysing cell yield and MFI. In this case, the highest concentration of enzyme reduced yield of cells but not MFI (Figure 6.3C). As a result, a concentration of 0.2mg/ml of thermolysin low was used for the digestion of prostate tissue.

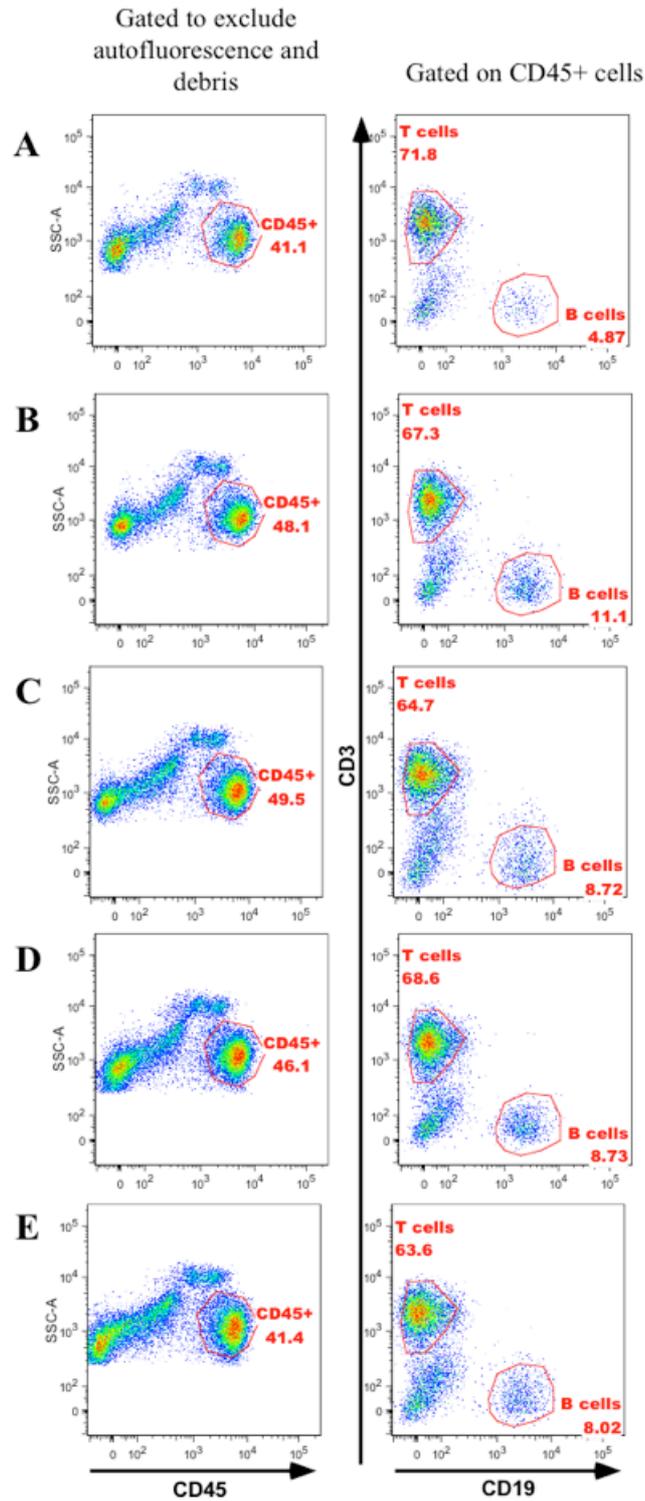


Figure 6.2: Improving extraction of prostate infiltrating immune cells for analysis by flow cytometry; blend of liberase

Prostate tissue collected from a patient undergoing a transurethral resection of the prostate was divided into 5 groups for digestion by different liberase blends before the released cells were labelled and analysed by flow cytometry. Tissue was digested for 15 minutes with Liberase (A) thermolysin low, (B) thermolysin medium, (C) thermolysin high, (D) dispase low and (E) dispase high, combined with dissociation by gentleMACs. Cells were labelled with antibodies targeting immune cell surface molecules; CD45, CD19 and CD3 before analysing on a flow cytometer. Data is a representative example of 2 separate patient tissue samples.

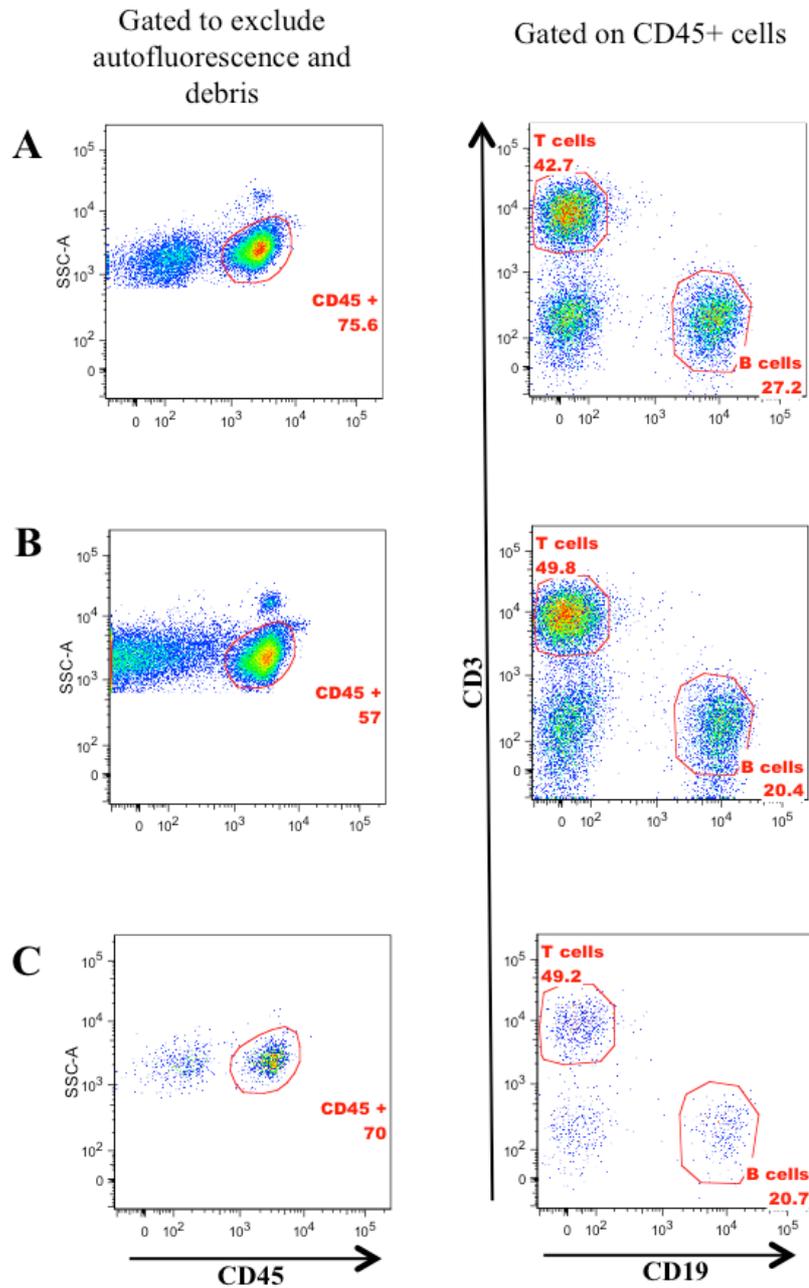


Figure 6.3: Improving extraction of prostate infiltrating immune cells for analysis by flow cytometry; concentration of liberase

Prostate tissue collected from a patient undergoing a transurethral resection of the prostate was divided into 3 groups for digestion by thermolysin low liberase at increasing concentrations before the released cells were labelled and analysed by flow cytometry. Tissue was digested for 15 minutes with liberase thermolysin low at (A) 0.2 mg/ml, (B) 0.35 mg/ml and (C) 0.5 mg/ml combined with dissociation by gentleMACs. Cells were labelled with antibodies targeting immune cell surface molecules CD45, CD19 and CD3 before analysing on a flow cytometer. Data is a representative example of 3 separate patient tissue samples.

6.3.3. Analysis of human prostate infiltrating lymphocytes demonstrates the importance of the PD-1/PD-L1 axis in the prostate

Human prostate tissue collected during TURP procedures was digested and immune cells extracted. In each case, patients were diagnosed with BPH; due to logistics with the tissue collection system, only four samples could be analysed before a disruption in sample retrieval occurred. With two of these samples patient matched peripheral blood lymphocytes were analysed as an internal.

MNCs derived from tonsil tissue were aliquoted and stored in liquid nitrogen for use as a control between experiments in the analysis prostate infiltrating immune cells. Isotype controls on tonsil MNCs were performed to assess unspecific binding of antibodies and used to set gates. Isotype controls and unstained controls are demonstrated in a representative example in Figure 6.4. The unstained control data from the prostate tissue demonstrate a degree of auto-fluorescence remains in the PD-1 channel (BV421, 405nm laser; 450/50 filter). To control for this, the background fluorescence in each channel is removed in the normalised MFI calculations (as described in section 2.8.5 methods).

The analysis of prostate infiltrating immune cells revealed CD8⁺ T cells express a higher level of the PD-L1 receptor PD-1 on the cell surface than their peripheral blood counterparts. Furthermore, there are a higher proportion of PD-1 positive CD8⁺ and CD4⁺ T cells in the tissue. Tissue infiltrating lymphocytes are enriched for CD8⁺ compared to CD4⁺ T cells. This data is summarised in Figure 6.6, with a representative example in Figure 6.5.

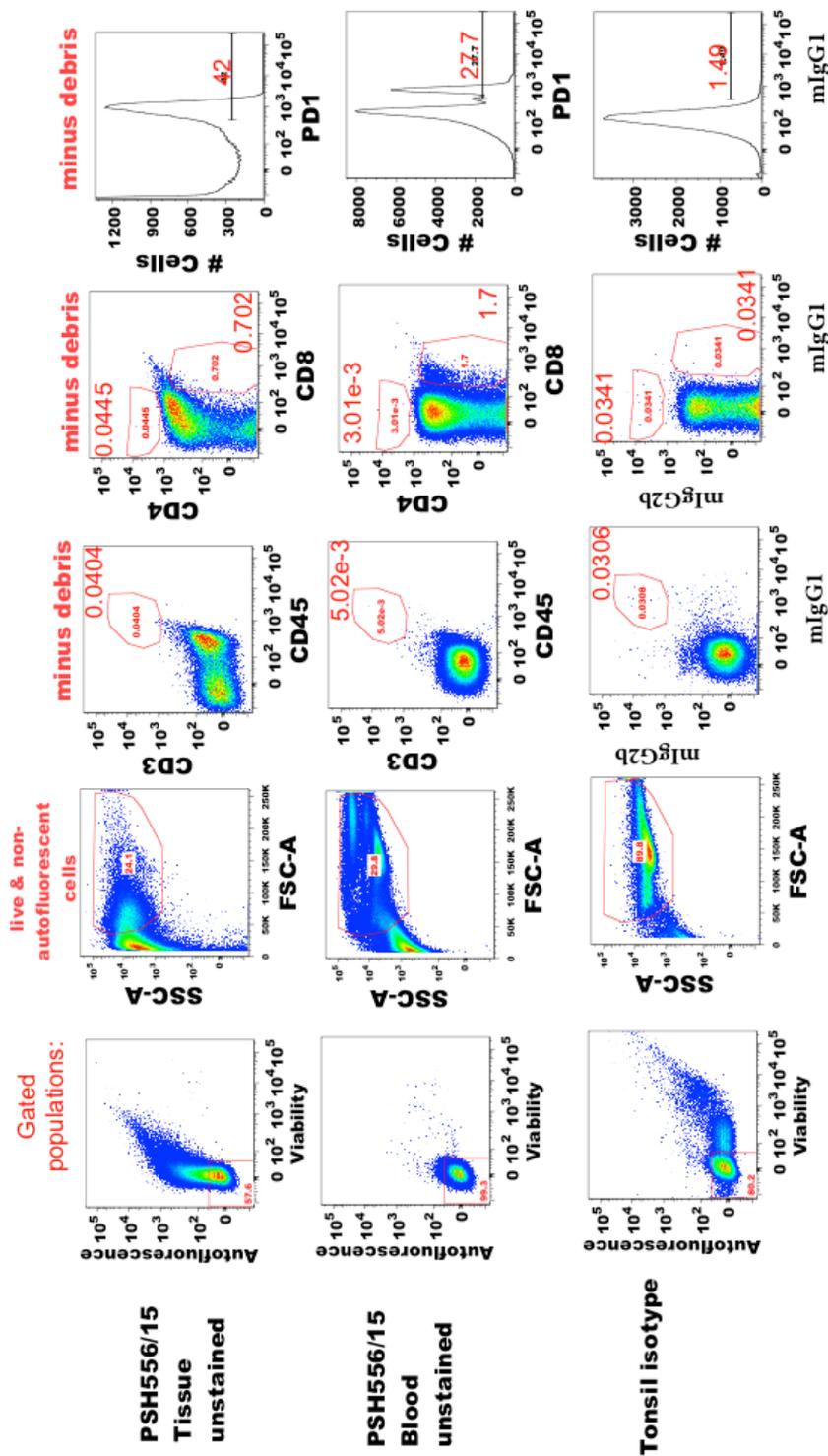


Figure 6.4: Characterising prostate infiltrating immune cells: controls

Prostate tissue collected from a patient undergoing a transurethral resection of the prostate was processed by thermolysin low liberase digestion followed by a ficoll gradient. Released cells were subsequently labelled with antibodies specific to CD45, CD3, CD4, CD8 and PD1. A fixable live/dead dye was included and the FITC channel was kept clear for the exclusion of dead and autofluorescent cells respectively. With every analysis a batch of mononuclear cells derived from the same tonsil was used as a consistency control. Where possible the peripheral blood of the patient corresponding to the prostate tissue was also analysed. Unstained cells from tissue and blood were run for autofluorescence consideration (top and middle rows). The tonsil MNCs were labelled with isotype control antibodies to consider non-specific binding (bottom row). Gated population are indicated in red above the plots

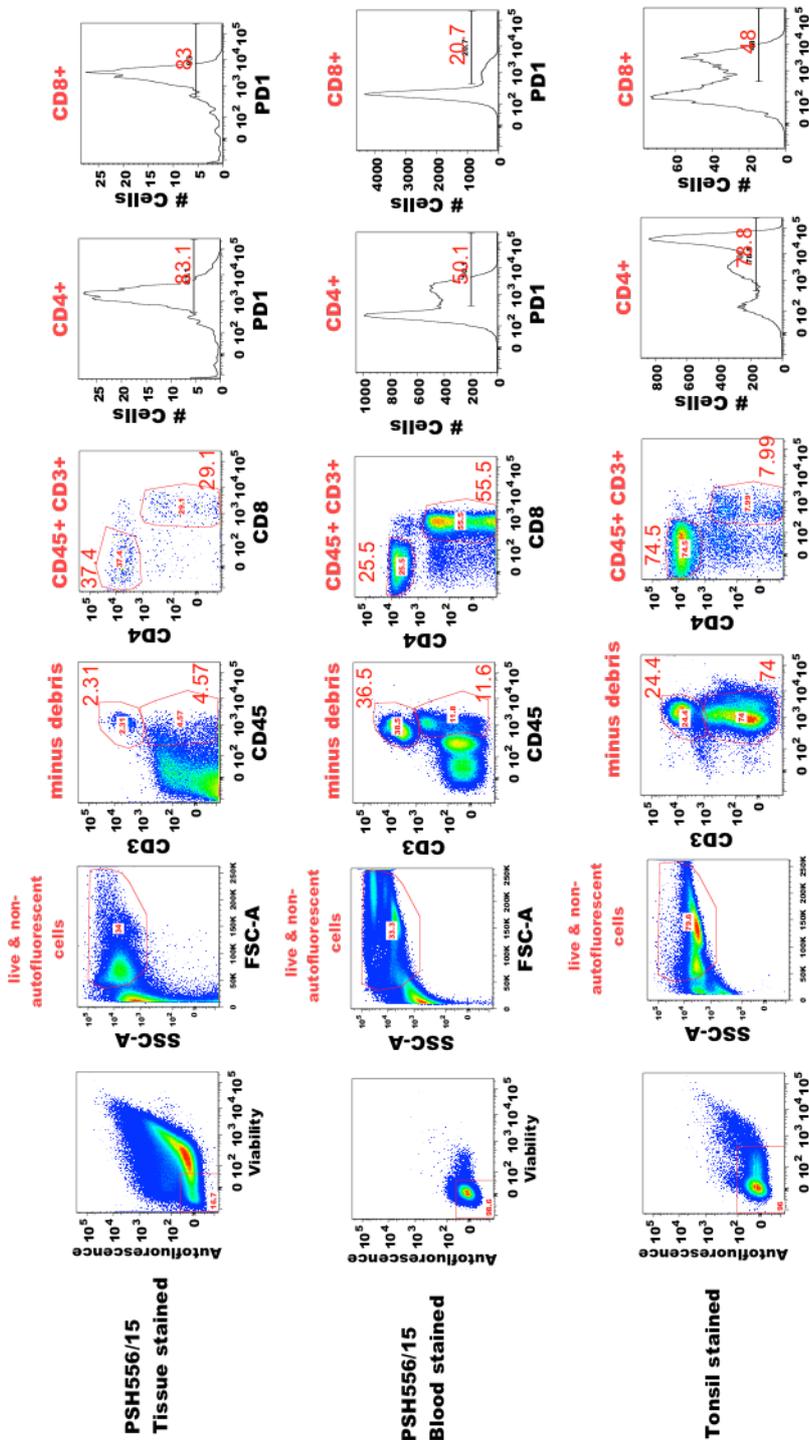


Figure 6.5: Characterising prostate infiltrating immune cells

Prostate tissue collected from a patient (PSH556/15) undergoing a transurethral resection of the prostate was processed by thermolysin low liberase digestion followed by a ficoll gradient. Released cells were subsequently labelled with antibodies specific to CD45, CD3, CD4, CD8 and PD1. A fixable live/dead dye was included and the FITC channel was kept clear for the exclusion of dead and autofluorescent cells respectively. With every analysis a batch of mononuclear cells derived from the same tonsil was used as a consistency control. Where possible the peripheral blood of the patient corresponding to the prostate tissue was also analysed. The gating strategy demonstrated allows the PD-1 status of CD4+ and CD8+ T cells infiltrating the prostate tissue (top) in comparison to peripheral blood T cells (middle) and the tonsil control (bottom). Data is a representative example of tissue from 4 patients. Peripheral blood of 2 of these patients was available for analysis. Gated population are indicated in red above the plots

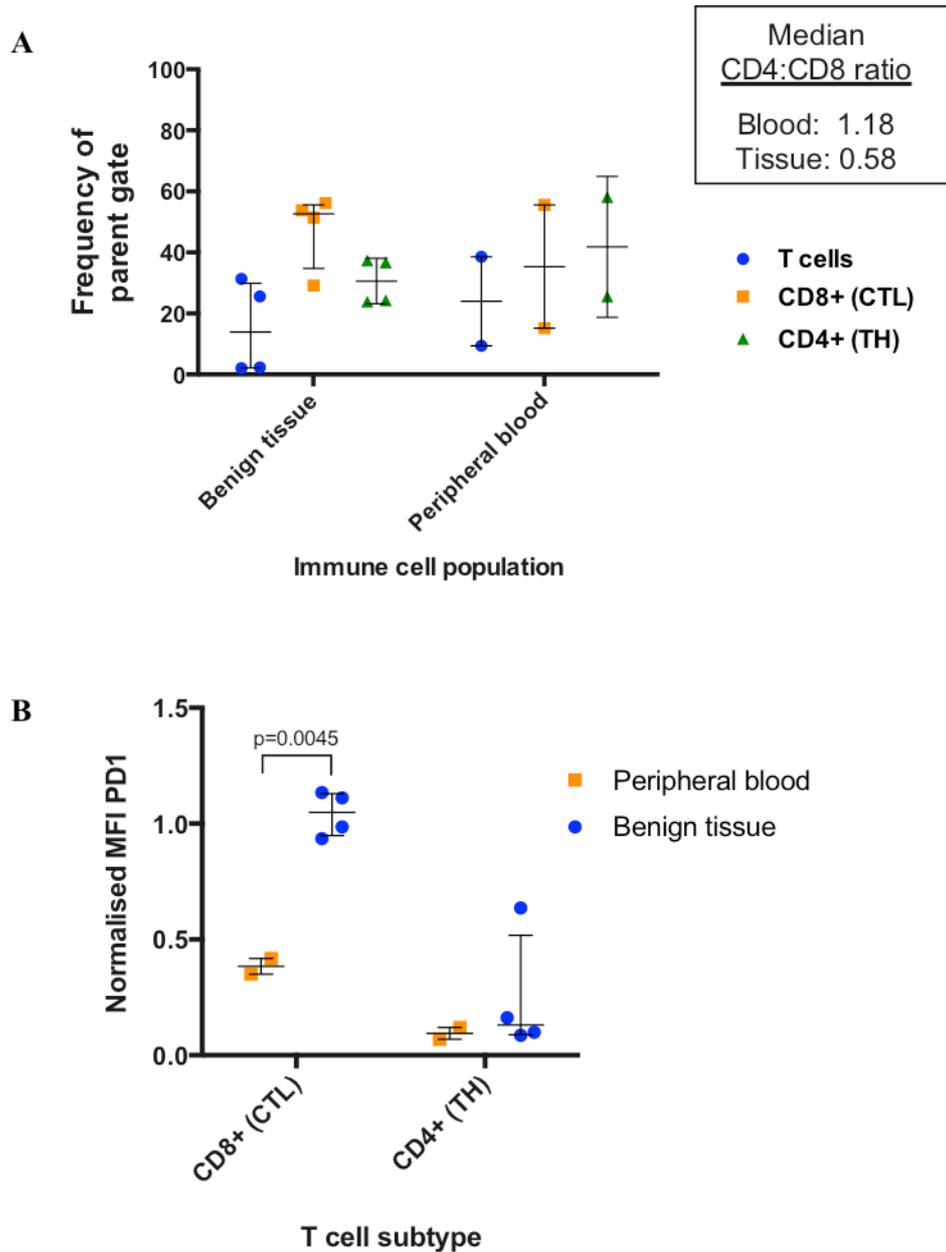


Figure 6.6: Characterising prostate infiltrating immune cells

Prostate tissue collected from patients undergoing a transurethral resection of the prostate was processed as described. Liberated cells were subsequently labelled with antibodies specific to CD45, CD3, CD4, CD8 and PD1, with inclusion of a live/dead dye. The MFI of tissue and blood immune cell populations was normalised to the MFI of the tonsil control of the given run, allowing comparability of separate experiments. The frequency of total T and CD8+/CD4+ T cells in parent gate (CD45+ cells for T cells and CD3+ cells for CD8+/CD4+ T cells) in tissue and blood is considered and the CD4:CD8 ratio stated (A). The normalised MFI of PD-1 on CD8+ and CD4+ is presented (B).

6.4. Summary of results

- A short digestion with liberase enzymes is required in order to analyse live TILs from prostate tissue by flow cytometry.
- Differences between liberase blends did not impact on the yield or level of detection of CD45, CD3 or CD19 surface markers
- The BPH tissue CD4:CD8 ratio of T cells is decreased in comparison to that of T cells in peripheral blood
- CD8+ CTL cells extracted from BPH tissue express a significantly higher proportion of PD-1 on their cell surface than circulating CD8+ CTLs

6.5. Discussion

The prostate is a dense tissue with high proportions of ECM components that would negatively impact on cell isolation. TURP procedures, outlined in Chapter 1, entail removal of prostate tissue that impedes on the urethra. This removal is facilitated by a heated element (resection loop) that separates chips of tissue, leaving a charred perimeter on the tissue. In addition to creating tissue auto-fluorescence detectable by fluorescent microscopy, this charred tissue likely affects the viability of cells within the tissue. Consistent with this theory, anecdotal evidence indicated a higher proportion of viable cells isolated from core biopsy tissue, despite a vast reduction in the weight of tissue that was processed (not shown). Dead and dying cells contain intracellular molecules that fluoresce, which makes multicolour flow cytometric analysis difficult due to ambiguous/apparent populations and false positive staining (Hulspas *et al.*, 2009; Monici, 2005). Historically our lab demonstrated an extensive digestion was required for the isolation of stromal and epithelial cells (Lang *et al.*, 1998). Importantly, cultured stromal and epithelial cells demonstrate comparable transcriptional profiles to that of *in situ* counterparts (Rane *et al.*, submitted). However, it was demonstrated here that this lengthy digestion reduced TIL viability, which resulted in extensive auto-fluorescence that made flow cytometry unachievable. To overcome this, a short digestion combined with mechanical disruption released enough cells for immuno-phenotyping. In the future, this protocol is expected to provide a means for cell selection and *ex vivo* analysis, in addition to expanding on the small subset of patients (with BPH) that were analysed for this work, including PCa tissue. The protocol developed here is somewhat similar to that of the Norstrom papers discussed earlier. The main difference between the two methods is our inclusion of an enzymatic digestion (Norstrom *et al.*, 2014). Interestingly, the authors also described a high degree of background auto-fluorescence, which was induced in their case by enzymatic digestion. The enzyme use or the time digested for was not disclosed though. The authors also described post-disruption H&E staining of tissue, which indicated that a significant proportion of tissue remained unprocessed. In supplementary information a representative example of H&E stained remainder tissue contained significant clusters of lymphocytes, which were therefore left unanalysed. Wu and colleagues reported Treg cells within hepatocellular carcinoma that had differential activity depending on the portion of tissue from which they originated (Wu *et al.*, 2013). This importantly reinforces the heterogeneity of TILs and demonstrates that to best understand their activity, as many TILs should be analysed as possible.

An important outcome of the data presented in this chapter is the high proportion of PD-1+ T cells, predominantly in the CD8+ populations infiltrating prostate tissue. Since PD-1 is upregulated upon activation of T cells, these data suggest a specific recruitment of active T cells to the prostate. Given these data are derived from BPH tissue and not PCa, it supports the potential for an immunological target in BPH, a disease for which the aetiology is relatively unknown. Although the equivalent analysis could not be achieved on PCa tissue within the time frame of this research project, it would be very interesting to continue further.

The small sample numbers in these data pose a significant limitation to the analysis. The use of tonsil MNCs for isotype controls, though necessary for this project due to number of cells available is not ideal. However, now that a protocol has been developed that permits the analysis of live PILs a greater sample set can be analysed. Particular areas of interest would be:

- Further immunophenotyping of TILs in both BPH and PCa tissue, including analysis of TH1 and TH2 subsets, Tregs and CTL cell
- Focus on the activity of the aforementioned subsets, including IFN γ , IL-4 secretion
- Characterisation of classical “exhaustion” markers; PD-1, LAG-3, CTLA-4
- Correlations of above with disease stage and disease progression

6.5.1. Concluding remarks

This chapter aimed to elucidate the proportions and activity of TILs in BPH ad PCa, comparing to circulating lymphocytes when possible. To do so, a protocol was developed that permits the isolation of viable TILs, which can be used for downstream analysis. In BPHs these TILs, particularly CTLs were PD-1 high, consistent with an exhausted phenotype (and susceptible to PD-L1 mediated inhibition), which is supported by similar data. However, tumour TILs could not be analysed due to the absence of fresh tissues. Despite this, the chapter presents interesting preliminary data worthy of further research in the future, and potentially incredibly valuable in the analysis of patient response to immunotherapies in the future.

Chapter 7

General Discussion

7.1. Summary of findings and significance

7.1.1. BPH and PCa stroma; counterparts in distinct diseases

It is important to appreciate that BPH is itself a hyperplastic disease with associated inflammation and therefore may not be considered an ideal control to investigate tumour-associated stroma. Examination of normal prostate stromal cultures may reveal that both BPH and G19 PCa stroma differ from normal. However, since similar transcriptional changes with cytokine treatments occur in HFF cells it is unlikely that normal prostate stroma differs in response to inflammation, thus making it easier to discriminate between general inflammatory signals and those driven by tumours. It would be interesting to understand whether morphological changes occur in normal prostate stroma with cytokine treatments and therefore whether the common inflammatory setting in BPH and PCa could account for the similar occurrence of myofibroblasts in BPH and PCa cultures but not HFFs. In this context, BPH may be a more relevant control for malignancy since both BPH and PCa have associated inflammation but only PCa stroma have grown in an environment containing malignant tumour cells.

There have been a number of other studies that find few variations between normal/BPH stroma and PCa stroma. Eiro and colleagues for example, examined the gene expression profile of cultured BPH and PCa stroma and found only 3 genes differentially expressed at the time of analysis; IL-17RB, CXCL14 and MMP2 (Eiro *et al.*, 2017). Intriguingly, the MMP2 finding is contradictory to the common perception of tumour stroma, as it was found overexpressed in BPH stroma compared to tumour stroma. It should be noted that in our system, neither IL-17RB nor CXCL14 were detected in prostate stroma by nanoString analysis (supplementary), suggesting discrepancies in the cells cultured between the two systems. This could be accounted for by the distinctions in the isolation and routine culture of stromal cells in the current study and the study by Eiro. For example, the authors did not disclose for how long stroma was cultured, but indicated that differential trypsinisation occurred over several passages in order to separate epithelial and stromal cell subsets. This is important as prolonged culture results in loss of physiologically relevant characteristics. Stromal cells additionally were cultured in DMEM-F12 10% FCS media compared to R10% in the current study. Moreover, MMP2 was previously shown in our lab to be slightly (2 fold) upregulated in G18 PCa stroma (Lakins, 2012). Here, it is shown that TGF- β 3, contrary to the literature, is downregulated at the mRNA level in tumour stroma (Figure 4.8 pg127). However, the increased TGF- β commonly found in the tumour stromal compartment may either derive from populations of infiltrating immune cells rather than the mesenchymal cells themselves, or could be due to increased active TGF- β protein. It should be an important consideration in all cancer research studies, to definitively show the cell of origin of differential signals (Rane *et al.*, 2015). Many studies, for example, by microdissection or whole tumour analysis show differential expression of key molecules without consideration of the infiltrating immune cells that potentially reside in variable ratios or activation states.

7.1.2. Potential sources for reactive stroma

Although the source of myofibroblasts in PCa has remained elusive, there are a number of potential possibilities, which may not be mutually exclusive. (i) Tissue resident fibroblasts may differentiate and become activated (as they are understood to during wound healing) to fulfil SMC roles (Mueller *et al.*, 2007), fitting with the likeness of cancer as the “never healing wound” (Dvorak, 1986). (ii) Dedifferentiation of SMCs may occur, which in the prostate at least, may explain loss of SMCs and accumulation of CAFs in PCa (Janssen *et al.*, 2000). (iii) Either resident or BM derived circulating MSCs may give rise to progeny to facilitate expansion or regrowth of the stromal compartment (Placencio *et al.*, 2010). (iv) EMT of prostatic tumour cells may also contribute to the myofibroblast pool (Ronnov-Jessen *et al.*, 1995).

The expression of immunosuppressive molecules detailed in Chapter 4 bare striking similarities to those well characterised in MSCs, so may support a MSCs source. Consistent with this, there are substantial reports to support either resident or BMSCs as a source of reactive stroma in PCa. A proportion of prostate stromal cells isolated from prostate biopsies fit the minimal criteria to appropriate MSCs and can support prostate repair (Brennen *et al.*, 2013; Lin *et al.*, 2007; Placencio *et al.*, 2010). It is possible that the data presented in this thesis are not representative of the *in vivo* environment and instead are an artefact of *in vitro* culture. Brennan and colleagues recently detail overgrowth of minor populations of MSCs in prostate stromal cultures (R10%), which dominate (80%) by passage three and show differentiation to the osteoblast and chondrocyte lineages when cultured in specific induction media (Brennen *et al.*, 2016). Here, it was found that patient cultures remained heterogenous, for example by basal PD-L1 expression (Figure 4.6, pg124) morphology (Figure 5.13, pg155) and proliferative capacity (Figure 5.17-18, pg160-161). Furthermore, while the lineage studies presented in Chapter 5 supported a potential for overgrowth of certain lineages, this had not yet occurred in the low passage cultures analysed in this thesis (Figure 5.17-18, pg160-161).

In response to inflammation during wound repair, BMSCs are recruited due to inflammatory signals (e.g. G-CSF) and impart immunomodulatory effect in the local environment (Kassis *et al.*, 2006). NanoString analysis highlighted that G-CSF is neither expressed by, nor induced in cultured prostate epithelial or stromal cells (supplementary), though infiltrating immune cells may be a potential source. The recruitment of BMSCs in response to inflammation (an occurrence in both BPH and PCa) potentially explains why, in this study, BPH and PCa stroma have similar properties in culture. However, since distinctions have been documented between BPH and PCa stroma it indicates that it is potentially a mixture a number of mechanisms that give rise to reactive stroma, in addition to the signals in the local environment.

7.1.3. Implications for the cancer immunity cycle

The data presented in this thesis suggests a highly immunomodulatory role for stromal cells that at least in PCa has been overlooked. Importantly, this data may account for the difficulties in treating PCa patients with immunotherapy. Prostate stroma creates an immunosuppressive environment that likely negatively affects many of the 7 stages in the cancer immunity cycle (Figure 7.1).

Impaired anti-tumour immunity can arise due to faults in *the immunisation stage* can occur by impeding DC maturation, triggering a tolerogenic response in cognate T cells and ultimately an inability to develop an active immune response. In the current study, a number of molecules expressed by prostate stroma may contribute to this, including IL-6, CSF1, VEGF, COX-2 (Figure 3.16, pg93, 3.23, pg103 and supplementary), as well as the low expression of TAA and HMGB1 by tumour cells (supplementary) (Gabrilovich *et al.*, 1998; Menetrier-Caux *et al.*, 1998; Sharma *et al.*, 2003). This block on immunisation is supported by responses in patients treated with Sipuleucel-T, which replaces endogenous APC activation in the prostate with artificial APC activation *in vitro* (Flanigan *et al.*, 2013; Fong *et al.*, 2014). However these patients benefit only moderately from Sipuleucel-T, suggesting further blockages occur in the cycle.

While *trafficking of T cells* to the prostate appears to be supported by stromal expression of chemokines like CXCL9 and CXCL10 (particularly in response to IFN γ release; supplementary) the efficacy of killing is greatly encumbered by stromal cells. Stroma express a plethora of the inhibitory molecules linked with blocking effective *killing of cancer cells* in the cancer-immunity cycle through preventing active immune responses within tissue. At the mRNA level TGF- β (Figure 3.17 pg94, 4.8, pg127), IDO, PD-L2, CD276 (Figure 3.23 pg103, Figure 4.8, pg127), non-classical MHCIIb molecules (Figure 3.19 pg99) and MICa/MICb (Figure 3.23 pg103) were expressed substantially either endogenously or is induced in response to local TH1/CTL cytokines. Given that CTLs were enriched in the BPH tissue analysed by flow cytometry compared to peripheral blood (Figure 6.6 pg177), it indicates a likelihood of local IFN γ production (if activated), though the CD4 $^{+}$ T cells were not subtyped into TH1 and TH2 cells. At the protein level MHCII (HLA-DR specifically) was upregulated on the stromal cell surface upon treatment with TH1/CTL cytokines (Figure 4.12 pg128). Correspondingly there was low level upregulation in CLIP expression on the cell surface and LAG-3 binding (Figure 4.12 pg128), indicating a capacity for stromal cells to inhibit TILs via the LAG-3 inhibitory receptor, but not present antigen as traditional APCs. Likewise, MHCIIb (HLA-E) (Figure 4.9 pg128) was expressed and upregulated on the cell surface, a molecule traditionally known as an NK cell inhibitor (or activator, depending on the receptor), so may therefore provide an additional method of preventing active immunity and tumour escape. The inhibitory receptor (CD94/NKG2A) has been shown at an elevated level on CTLs in cervical cancer and was associated with a worse prognosis when HLA-E was expressed (Gooden *et al.*, 2011; Sheu *et al.*, 2005). It should be noted though, that this

mechanism is complex as evidence is accumulating for a role in antigen presentation and activation of HLA-E restricted CTLs (Mazzarino *et al.*, 2005).

Most strikingly though, PD-L1 was expressed at significant levels on the stromal cell surface and further upregulated with TH1/CTL cytokines (Figure 4.6, pg124), suggesting both an endogenous capacity for PD-1 mediated inhibition and an elevated capacity in response to local inflammation. Flow cytometric analysis of PILs demonstrated that trafficking of T cells was not prevented, as immune cells were detected in the tissue. It further supported a physiological importance for stromal PD-L1 expression since PD-1 was correspondingly upregulated on PILs compared to peripheral blood (Figure 6.6 pg177). The expression of both PD-L1 and TGF- β in the prostate would make a favourable setting for Treg cells, since both of these molecules provide positive signals for Treg survival (Miller *et al.*, 2006). The failure of immunotherapies in PCa patients is potentially a trait of the redundancy of immunomodulatory mechanisms employed by stroma. Simply inhibiting PD-1/PD-L1 is not sufficient, as it is not the only inhibitor expressed by stroma. Further, the volatility of patient responses may be due to variability in the ratio of stroma to tumour cells. The data presented in this thesis together suggests that the quantity of TAA activated T cells needs to be greatly in excess so as to overwhelm the tumour and not be dissuaded by the stroma.

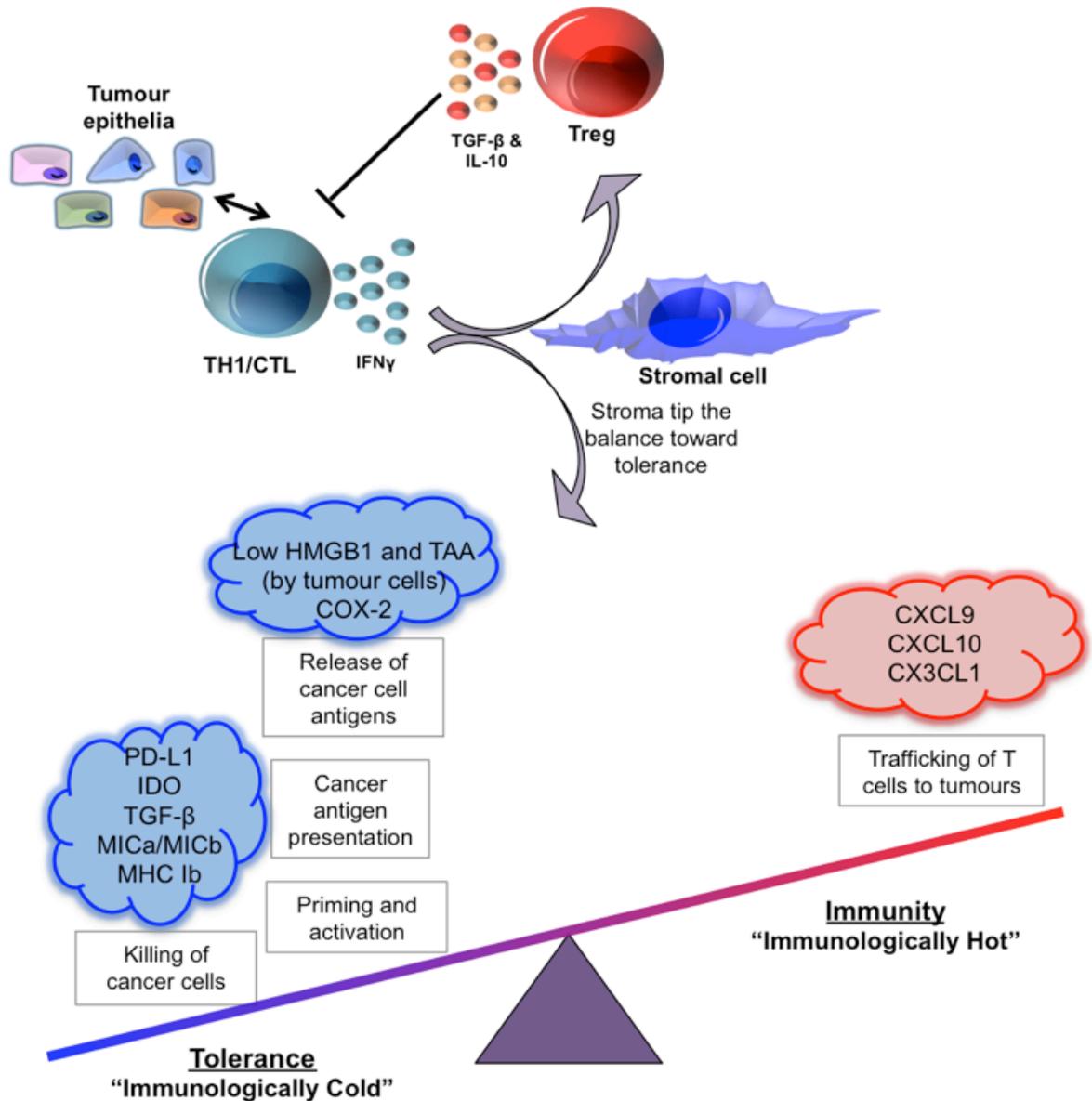


Figure 7.1 Role of stroma in modulating the cancer-immunity cycle.

Prostate stroma provides an immunosuppressive environment through expression molecules such as TGF- β and Cox-2. Activation of TH1/CTL cells stimulates local production of IFN γ , which induces a regulatory response by prostate stroma. Expression of chemokines contributes to increased trafficking of T cells to the prostate and providing an “immunologically hot” environment. Although, the “immunologically cold” aspects of prostate stroma far out-weighs this response. These molecules both directly inhibit T cells and provide a favourable environment for Treg cells.

7.2. Remaining questions and future work

7.2.1. Immunosuppression by prostate stroma

The data in this thesis have suggested a capacity of stromal cells to inhibit infiltrating immune cells via the expression of various molecules including PD-L1, MHCII and MHC1b, which was supported by analysis of PILs demonstrating expression of PD-1. However this should be tested further. PILs should be further analysed in more detail and an expanded cohort of tissue from both BPH and PCa. This could not be achieved in this current project due to complications with procuring further samples, although an experimental protocol for doing so was developed. Additionally, co-cultures of untreated and IFN γ &TNF α treated patient stroma with stimulated patient-matched peripheral blood lymphocytes could be utilised to determine whether stromal expression of immunomodulatory molecules is functionally capable of inducing T cell inhibition. Systematically blocking the molecules expressed and determining changes in T cell killing capacity can verify this. IHC analysis of PD-L1 expression in prostate tissue and correlation to local T cell infiltrates may determine *in vivo* relevance of this mechanism.

7.2.2. Is there a role for prostate stromal cells in biasing local T cell subsets?

In addition to outstanding questions directly associated to the work presented in this thesis, there are many lines of research that arose during nanoString analysis but could not be pursued. Prostate stromal cells exhibit an immunosuppressive phenotype through the expression of TGF- β and IL-6, which together have been shown to contribute to the differentiation of TH17 cells from naive T cells. These are a more recent T cell subset that are not fully understood as they are understudied (Weaver *et al.*, 2006). TH17 are most commonly associated with autoimmunity and organ specific chronic inflammation, but were also shown to be required for tumour development *in vivo*. They require IL-23 for sustenance and through production of IL-17 trigger further IL-6 expression. This implies that in the presence of APCs (a source for IL-23), prostate stroma may support local differentiation of naive T cells to a TH17 phenotype, perhaps in the context of cancer associated TLT formation. On the contrary, TGF- β production in the absence of IL-6 protein will instead skew T cells toward a Treg phenotype and propagate an immunosuppressive environment. Both of these scenarios have been documented in patient tissue, suggesting a patient dependent context that may rely on factors such as presence of infection, patient age and treatment history (Sfanos *et al.*, 2008).

7.3. Concluding remarks and schematic summary

While the local inflammatory setting will invoke similar adaptations by prostate stroma in BPH and PCa stroma, the setting these changes occur in may result in very different outcomes. For example, attempted homeostatic correction by BPH stroma due to inflammation and resultant inhibition of immune cells can attribute to expansion of the stromal compartment and unresolved inflammation, attributing to the chronic condition. In the context of malignancy, there is more at stake. Here, if inflammation is improperly inhibited, tumour cells are able to expand with moderate immune pressure, supporting tumour microevolution. This, and previous work from the Coles' and Maitland's labs have demonstrated that while it is beneficial to produce an anti-tumour response, the local cytokine response may paradoxically provide tumour supportive stroma. Previous data demonstrated a TH2 mediated induction of VCAM1 that supported stromal/tumour migration and metastasis (Lakins, 2012)(Figure 7.2 B). However, this thesis mainly focused on the stromal response to TH1/CTL cytokines revealing a key role for stroma in local immune inhibition, which in the context of malignancy will be detrimental for anti-tumour immunity. Treatment of PCa, specifically by immunotherapy, will prove a significant challenge in the future owing to this demonstrated role of stromal immune inhibition. Altogether, this is summarised in Figure 7.3.

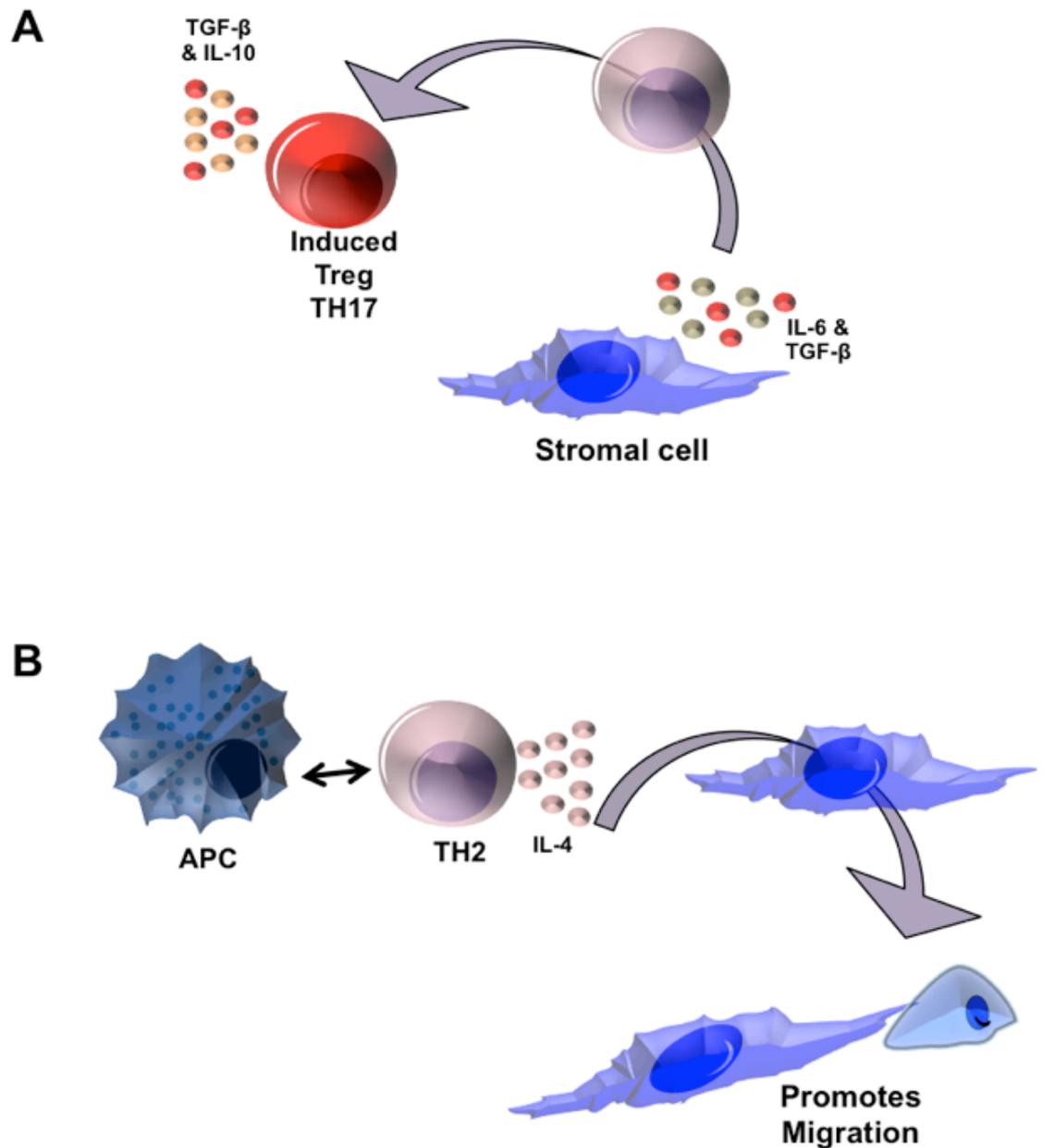


Figure 7.2: Interactions between stroma and immune cells

(A) A proposed model of stromal mediated skewing of local T cells. In the presence of both IL-6 and TGF- β , naive T cells differentiate to TH17 cells. In the absence of IL-6, Tregs are induced (iTregs). (B) Activation of TH2 cells (by APCs) induces local expression of IL-4, inducing stromal expression of VCAM1. Previously, this was shown to mediate crosstalk between prostate stromal and epithelia and stimulate increased migration. Epithelial cells expressing VLA-4, attach to VCAM-1 expressing stroma. This stimulates secretion of SPARC by proficient epithelia, which outcompetes VLA-4 for binding and provides a mechanism for detachment (Lakins, 2012).

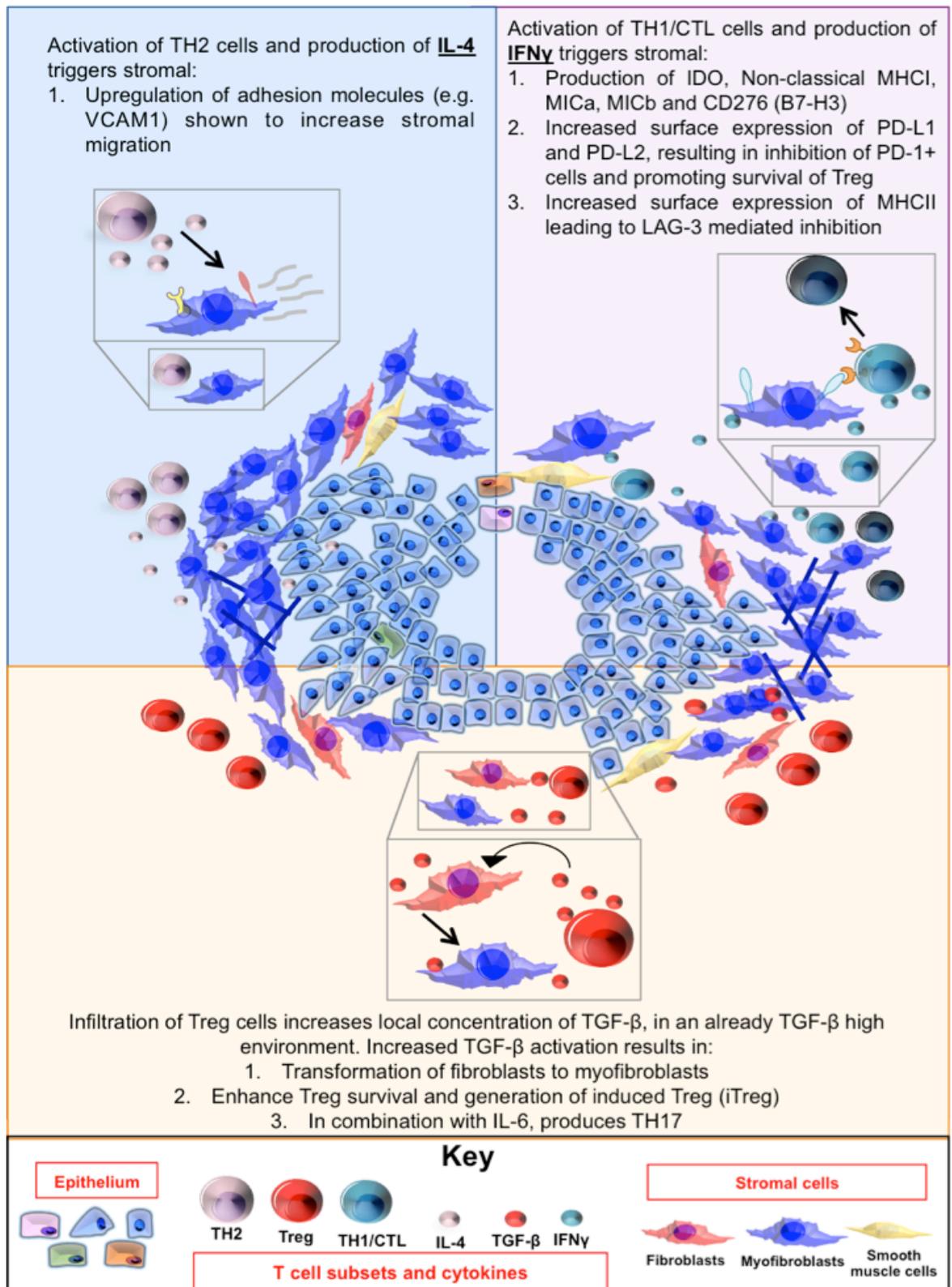


Figure 7.3: The changing faces of prostate stroma

Prostate stromal cells are highly responsive to local inflammation (top left and right). TH2 activation and subsequent IL-4 secretion stimulates stromal VCAM-1 expression, which mediates migration as previously described (Figure 7.2B). Activation of TH1/CTLs and production of IFN γ induces stromal expression of various molecules shown to inhibit T cell activity and therefore prevents tumour cell killing. Prostate stroma provides an immunosuppressive environment by the expression of IL-6 and TGF- β . Increased activation of TGF- β (e.g. Treg infiltration or MMP secretion) increases myofibroblast accumulation and skews local T cell subsets (Figure 7.2A).

Abbreviations

α SMA	α -smooth muscle actin
γ c	Common γ chain
μ g	Microgram
μ l	Microlitre
μ m	Micrometer
μ M	Micromolar
3D	3-dimensional
ADT	Androgen deprivation therapy
AE	Adverse events
AIPC	Androgen independence prostate cancer
AM	Adhesion molecules
APCs	Antigen presenting cells
AR	Androgen receptor
ATF4	Activating transcription factor 4
BCa	Breast cancer
BM	Bone marrow
BMSCs	Bone marrow mesenchymal stromal cells
BPH	Benign prostatic hyperplasia
BSA	Bovine serum albumin
CAFs	Cancer associated fibroblasts
cDNA	Complementary DNA
CEACAM	Carcinoembryonic antigen-related cell adhesion molecules
CO ₂	Carbon dioxide
CP/CPPS	Chronic prostatitis/chronic pelvic pain syndrome
CR	Complete responses
CRADs	Conditional replicating adenoviruses
CRC	Colorectal cancer
CRPC	Castrate resistant prostate cancer
CSCs	Cancer stem cells
Ct	Threshold cycles
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
CTLs	Cytotoxic T lymphocytes
CZ	Central zone
DAPI	4',6-Diamidino-2-Phenylindole, Dilactate
DC	Dendritic Cell
DHT	Dihydrotestosterone
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide

EC	Endogenous control
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
FACS	Fluorescence activated cell sorting
FAP	Fibroblast activation protein
FCS	Foetal calf serum
FDCs	Follicular dendritic cells
FGF	Fibroblast growth factor
FOV	Field of view
FRCs	Follicular reticular cells
FSC	Forward scatter
g	Grams
<i>g</i>	<i>g</i> -force
GAG	Glycosaminoglycan
gapdh	Glyceraldehyde 3-phosphate dehydrogenase
GAS	IFN γ activated site
GCN2	General control nonderepressible protein 2
GI	Gleason
GLM	Generalised linear model
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GOI	Genes of interest
H&E	Haematoxylin and eosin
HBSS	Hank's Balanced Salt Solution
HFF	Human foreskin fibroblasts
HGF	Hepatocyte growth factor
HLA	Human leukocyte antigen
HMGB1	High-mobility group box 1 protein
HNSCC	Head and neck squamous cell carcinoma
HS	Heparin sulfate
ICAM1	Intercellular Adhesion Molecule 1
IDO	Indoleamine 2,3-deoxygenase
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
ITIM	Immunoreceptor tyrosine-based inhibitory motif
ITSM	Immunoreceptor tyrosine-based switch motif
JAK	Janus Kinase

kD	Kilo Dalton
KIRs	Killer cell inhibitory receptors
LAG	Lymphocyte activation gene
LAP	Latency associated protein
LCM	Laser capture microdissection
LCMV	Lymphocytic Choriomeningitis Virus
LFA	Leukocyte function associated antigen-1 (LFA-1)
LLC	Large latent TGF-b complex
LN	Lymph node
LN ₂	Liquid nitrogen
LPS	Lipopolysaccharide
LT	Lymphotoxin
LTb	Lymphotoxin-b
LTBP	Latent TGF-b binding protein
Lti	Lymphoid tissue inducer
LTin	Lymphoid tissue initiator
LTo	LT organiser
LT α 1 β 2	Lymphotoxin α 1 β 2
LT β R	Lymphotoxin β Receptor
LUTS	Lower urinary tract symptoms
M	Molar
mCRPC	Metastatic CRPC
MFI	Median fluorescence intensity
MHCI	Major Histocompatibility Complex
min	Minutes
ml	Millilitre
mM	Millimolar
MMPs	Metallomatrix proteases
MNCs	Mononuclear cells
mOS	Median overall survival
MPL-A	Monophosphoryl Lipid-A
MRCs	Marginal reticular cells
MSCs	Mesenchymal stem/stromal cells
ng	Nanogram
NGF	Nerve growth factor
NHS	National health service
NK	Natural killer
NSAIDs	Non-steroidal anti-inflammatory drugs
NSCLC	Non-small cell lung cancer
OCT	Optimal cutting temperature

PAMPs	Pathogen associated molecular patterns
PAP	Prostatic acid phosphatase
PBS	Phosphate buffered saline
PCA	Principal components analysis
PCa	Prostate cancer
PD-1	Programmed death-1
PD-L1	Programmed death ligand 1
PFA	Paraformaldehyde
PFS	Progression free survival
pg	Picogram
PIA	Proliferative inflammatory atrophy
PILs	Prostate infiltrating lymphocytes
PIN	Prostatic intraepithelial neoplasia
PR	Partial responses
PSA	Prostate specific antigen
pSTAT1	Phosphorylated STAT1
PVDF	Polyvinylidene difluoride
PZ	Peripheral zone
qRT-PCR	Quantitative real time-PCR
RA	Rheumatoid arthritis
RCC	Renal cell carcinoma
RP	Radical prostatectomy
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SD	Standard deviation of the mean
SLC	Small latent complex
SLE	Systemic lupus erythematosus
SLO	Secondary lymphoid organs
SMCs	Smooth muscle cells
SSC	Side scatter
STAT	Signal transducer and activator of transcription
STING	Stimulator of IFN genes
TAAAs	Tumour-associated antigens
TCR	T cell receptors
TGF- β	Transforming growth factor- β
TH2	T helper 2
THBS-1	Thrombospondin-1
TICs	Tumour initiating cells
TIL	Tumour infiltrated T cell
TL	Thermolysin Low

TLRs	Toll like receptors
TLS	Tertiary lymphoid structures
TLT	Tertiary lymphoid tissue
TME	Tumour microenvironments
TNF	Tumour necrosis factor
TRANCE	TNF related activation-induced cytokine
Treg	T regulatory cells
TSAs	Tumour-specific antigens
TURP	Transurethral resection of the prostate
TZ	Transitional zone
UGE	Urogenital epithelia
UGM	Urogenital mesenchyme
UGS	Urogenital sinus
UTI	Urinary tract infections
V	Volt
VCAM1	Vascular cell adhesion protein 1
VLA	Very late antigen-4 (VLA-4)

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