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A review on the interactions between the tumour microenvironment and androgen receptor signaling in prostate cancer

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Abbreviations: ADT (androgen deprivation therapy), Akt (protein kinase B), AR (androgen receptor), Bcl-2 (B-cell lymphoma 2), BPH (benign prostatic hyperplasia), CaP (prostate cancer), CCL (C-C motif chemokine ligand), CD (cluster of differentiation), CTLA (cytotoxic T-lymphocyte-associated protein), CXCL (C-X-C motif chemokine), DC (dendritic cells), DRE (digital rectal examination), FcγR (Fc receptors for IgG), FGF (fibroblast growth factor), FOXP3 (forkhead box P3), GM-CSF (granulocyte-macrophage colony stimulating factor), GPI (glycosylphosphatidylinositol), HER2 (human epidermal growth factor receptor 2), HSP (heatshock protein), ICOS (inducible T-cell costimulatory), IFN-γ (interferon gamma), IL (interleukin), IP-10 (interferon gamma-induced protein 10 or CXCL10), JAK/STAT (janus kinase/

signal transducers and activators of transcription), LAG-3 (lymphocyte-activation gene 3), MAPK (mitogen-activated protein kinase), mCRCP (metastatic castrate resistant prostate cancer), M-CSF (macrophage colony stimulating-factor), MDSC (myeloid-derived suppressor cells), MHC-II (major histocompatibility complex), MMP9 (matrix metalloproteinase-9), MSI (microsatellite instability), NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells), NK (natural killer), NLR (neutrophil lymphocyte ratio), PD-1 (programmed cell death 1), PD-L (programmed death-ligand), PI3K (phosphatidylinositol 3-kinase), PIA (proliferative inflammatory atrophy), PKC (protein kinase c), PSA (prostate specific antigen), PSCA (prostate stem cell antigen), PSMA (prostate specific membrane antigen), PTEN (phosphatase and tensin homolog), PTPN1 (protein tyrosine phosphatase non-receptor type 1), PTTG1 (pituitary tumour-transforming 1), SMRT (silencing mediator for retinoic acid and thyroid hormone receptor), SRC-1 (steroid receptor coactivator-1), TAM (tumour associated macrophages), TCR (T-cell receptor), TGF- β (transforming growth factor beta), Th1 (T-helper 1 cell), TLR (toll like receptor), TME (tumour microenvironment), TNF- α (tumour necrosis factor alpha), TNM (tumour, node, metastasis), Tregs (regulatory T-cells), TREM1 (triggering receptor expressed on myeloid cells 1), TRUS (transrectal ultrasound), VCAM (vascular cell adhesion protein),

Abstract (250 words)

Prostate cancer growth is controlled by androgen receptor signaling via both androgen-dependent and androgen-independent pathways. Furthermore, the prostate is an immune competent organ with inflammatory changes both within the systemic and local environment contributing to the reprogramming of the prostatic epithelium with consistently elevated lymphocyte infiltration and pro-inflammatory cytokines being found in prostate cancer. The crosstalk between the tumour microenvironment and androgen receptor signaling is complex with both pro-tumorigenic and anti-tumorigenic roles observed. However, despite an increase in immune checkpoint inhibitors and inflammatory signaling blockades available for a range of cancer types, we are yet to see substantial progress in the treatment of prostate cancer. Therefore, this review aims to summarize the tumour microenvironment and its impact on androgen receptor signaling in prostate cancer.

Introduction

Prostate cancer (CaP) is the most common non-cutaneous cancer amongst men in Europe (1). Incidence rates have been increasing rapidly over the past 20 years due to the introduction of prostate specific antigen (PSA) testing and the increased diagnosis of asymptomatic disease (2). Initial investigations for CaP include serum PSA measurements and digital rectal examinations (DRE). Histopathological analysis is further performed via Trans Rectal Ultra Sound (TRUS) guided biopsies which are currently the gold standard for diagnosing and staging CaP. Prognosis and treatment decisions are based on the tumour grade using the Gleason sum, the clinical stage using the TNM (Tumour, Node, Metastasis) system, and a patient's serum PSA level. Active surveillance, radical prostatectomy, brachytherapy, and external beam radiotherapy are currently the most common treatments for localized prostate cancer (3). Conversely, following the development of locally advanced or metastatic CaP hormonal therapies and/ or chemotherapies are administered (4). However, despite these well-used clinical pathological predictive and prognostic factors, drastically variable outcomes are observed between patients with similar stages and disease grades. Therefore, this has highlighted the importance of identifying novel biomarkers within the tumour and its microenvironment.

Androgens acting upon the androgen receptor (AR) control the development, growth, and progression of CaP by inducing transcription of AR regulated genes that increase cellular proliferation and the cells ability to evade apoptosis (5-7). In addition, as AR is present in almost all primary and metastatic prostate tumours independent of stage or grade, AR is the primary target of multiple therapies through androgen deprivation (8). However, AR and downstream pathway is subject to alteration by numerous factors including posttranslational modifications such as methylation and phosphorylation, and crosstalk with other signaling pathways such as the PI3K/Akt pathway (9, 10). In spite of AR being the main target for CaP and its expression being significantly associated with reduced survival and a reduced time to biochemical relapse (11, 12), AR as a prognostic marker has

not translated into routine clinical practice due to the lack of reproducible methods and defined thresholds.

The association of the host immune response and the development and progression of cancer has long been recognized, with an estimated 20% of adult cancers attributable to chronic inflammation (13). Both systemic inflammation and local immune infiltrate in the tumour microenvironment (TME) may associate with the upregulation of various hallmarks of cancer, where a state of immune tolerance is established through regulatory immune cells and immune inhibitory cytokines (14, 15). This hallmark of cancer is widespread throughout the majority of cancers, so much so that the past 20 years has been spent developing inhibitors that target molecular pathways which control the activation and effector functions of immune cells. A vast amount of data has been published highlighting the importance of modulating the immune response to provoke antitumour behaviour specifically in melanoma, colorectal cancer, and renal cell carcinoma (16-18).

Moreover, the prostate gland is an immune-competent organ containing both stromal and infiltrating T and B cells mainly within the fibromuscular stroma and peri-glandular tissue (19, 20). The composition of both tumour cells and stromal cells forming the tumour stroma make up the TME along with multiple cell types including bone marrow-derived mesenchymal stem cells, cancer-associated fibroblasts, pericytes, and multiple inflammatory cells. This is found to have a significant effect on the development of CaP (21). Inflammatory changes both within the systemic and local environment may contribute to the reprogramming of the prostatic epithelium with consistently elevated lymphocyte infiltration and pro-inflammatory cytokines being found in prostate cancer. Furthermore, epigenetic alterations due to inflammatory stress may promote this fatal transformation (22). Therefore, due to the increased elevation of immune cells within the prostate and inflammation driven oxidative stress and increased reactive oxygen species following the transition from normal prostate epithelium to prostate adenocarcinoma, it is evident the association between the host inflammatory response and CaP needs to be fully evaluated (22, 23)

In this review, we aim discuss the roles of the AR both within CaP epithelial cells and within the cells of the TME and how AR interacts with local inflammation.

Inflammation and Cancer

The last two decades have shown a drastic increase into the research surrounding the host immune response and cancer using markers of systemic inflammation and local immune infiltrate, with promising evidence suggesting immunotherapies could provide control of disease progression. It has long been understood that the capacity to avoid immune destruction is one of the 'hallmarks of cancer' such as activated immune cells directly killing tumour cells and secreting cytokines to control malignant cell growth (24). Immunoediting, comprising of elimination (immune system eradicates malignant cells), equilibrium (immune system controls malignant cells), and escape (malignant cells evade destruction by immune system), has identified the roles between the immune system and malignant cells (25). A combined heterogeneous population of immune cells, mesenchymal cells, and extracellular matrix has shown to influence malignant progression and prognosis (26). However, more recently a vast amount of literature has supported the idea that infectious agents, chronic non-infectious inflammatory conditions, dietary factors, hormonal variations/ exposures, and autoimmune responses are the most probable causes of chronic inflammation. Chronic inflammation is the source of a variety of enabling characteristics leading to multiple protumourigenic effects such as stimulating angiogenesis and inducing DNA damage in an estimated 20% of all adult cancers and gaining interest in almost all solid tumours (13). These contradictory findings highlight that the immune system can control and repress malignant cell progress progression, but also promote disease advancement.

The cells within the immune system can be divided into adaptive and innate immune cells along with immune-suppressive cells, with each group further subdivided into individual cell types. Adaptive immunity, also known as the acquired immune system, is comprised of highly specialized B and T lymphocytes that contain memory responses following initial exposure to a specific antigen providing long-lasting protection. In contrast, innate immunity provides a first line defense mechanism against many organisms as well as stimulating the adaptive immune response, and comprises of

dendritic cells, macrophages, natural killer (NK) cells and granulocytes (25). In addition, natural killer T cells and $\gamma\delta$ T-cell receptor expressing T cells are also part of the immune system, with links seen between both responses (27). Furthermore, regulatory T cells, regulatory B cells and myeloid-derived suppressor cells are just some of the cells with immune-suppressive functions found within the immune system (28).

Targeting the immune system has therefore been the forefront of cancer research due to its applicability across a range of cancer types. Both innate and adaptive immune cell functions are held in check through immune checkpoints that suppress the activation and functionality of these cells to maintain self-tolerance and prevent autoimmunity. Cytotoxic T lymphocyte antigen 4 (CTLA-4) is a pivotal immune checkpoint receptor responsible for the suppression of T-cells by binding CD28's ligand B7 along with depleting CD80 and CD86, reducing T-cell receptor signaling (29). However, anti-CTLA-4 therapies have shown to be associated with increased risk of inflammatory side effects and upregulation of circulating T-cells (30-32). Furthermore, programmed cell death protein 1 (PD-1) is highly expressed on a variety of immune cells following T cell receptor (TCR) engagement including B cells, NK cells, T cells and regulatory T cells, with its primary function being to enable tumour cells to evade the host's immune response through inhibition of downstream TCR signaling and CD3 phosphorylation and subsequently T-cell activation (33, 34). In contrast to CTLA-4, PD-1 expression occurs 6-12 hours following TCR engagement predominantly within the TME rather than primarily within the lymphoid organs (35). Immune checkpoint inhibitors including anti-PD-1 antibodies such as nivolumab and pembrolizumab have proved to regulate immune responses through altering T-cell activity in a variety of cancers (16, 36). Perhaps more important are the PD-1 ligands (PD-L1 and PD-L2) that suppress T-cell activation expressed extensively on both tumour and stromal cells, with limited expression in healthy tissues (37).

Inflammation and Prostate Cancer

Like most organs, prostatic tissue is scattered with a variety of immune cells including B and T lymphocytes, macrophages, and dendritic cells. Chronic inflammation is highly prevalent with high

levels of lymphocytes and macrophages and low levels of eosinophils and plasma cells found within the prostate. For example, total overall leukocyte expression (CD45+ leukocyte) is markedly increased following the formation of benign prostatic hyperplasia (BPH) compared to normal prostatic tissue with CD3+ T-lymphocytes and CD19+ or CD20+ B lymphocytes comprising of 70-80% and 10-15% respectively (38). Prostatitis is a heterogeneous condition divided into acute bacterial prostatitis, chronic bacterial prostatitis, chronic prostatitis (chronic pelvic pain syndrome), and asymptomatic inflammatory prostatitis. Links between chronic inflammation and CaP have been demonstrated and may serve as precursor lesions to CaP due to increased proliferative epithelial cells and inflammatory infiltrate in prostatic focal atrophic lesions, termed proliferative inflammatory atrophy (PIA) particularly within the peripheral zone of the prostate (39). Both negative and positive results have been reported on the correlation between prostatitis and CaP risk (40, 41). A history of long term symptomatic prostatitis has been linked to an increased risk of CaP (relative risk=1.3; 95% confidence interval=1.10-1.54) (42).

It has previously been identified that inflammation is capable of driving CaP progression via multiple mechanisms including: promoting angiogenesis and tissue repair along with providing highly proliferative conditions within the tumour microenvironment (43). Furthermore, persistent inflammation has been hypothesized to drive the malignant progression from prostatic intraepithelial neoplasia to CaP due to dedifferentiation of the prostate epithelium (44). This hypothesis is supported by similar mechanisms identified in other cancers, with hepatitis B or C viruses increasing the risk of hepatic carcinoma and *Bacteroides* species increasing the risk of colorectal cancer (45, 46). In a prospective randomized controlled trial looking at males with normal DRE and PSA levels between 2.5-10ng/ ml, over 45% of 328 males had leucocytes within their prostatic secretions (47). Furthermore, chronic inflammation in ≥ 1 biopsy cores of benign prostate tissue is significantly associated with an increased risk of developing high grade CaP (Gleason sum 7-10) than those with no inflammation (Overall risk=2.24, 95% CI 1.06-4.71) (48). However, intratumoural CD3+ T cells and stromal CD4+ T cells have previously been reported to positively correlate with increased survival in epithelial ovarian

cancer and non-small cell lung cancer respectively (49, 50). Additionally, concurrent expression of both CD4+ and CD8+ T cell infiltrate in squamous cell esophageal cancer has been associated with improved survival (51). This has highlighted the paradoxical role the host immune response plays in cancer.

The Androgen Receptor and Prostate Cancer Tumour Microenvironment

Recent efforts have been made to understand both cellular and non-cellular components surrounding a tumour termed the TME. This intratumoural niche contains pro-inflammatory cytokines, both adaptive and innate immune cells, and fibroblasts all of which contribute to an inflamed TME shown to promote and enhance prostate cancer progression. The immune response in CaP is predominantly via the adaptive immune system, particularly CD8+ T-cells, with extensive infiltration observed following the transition from normal prostatic tissue to CaP. The adaptive immune response generates a range of CD4+ T cell clones that express unique T-cell receptors that recognize antigen presenting major histocompatibility complex class II (MHC-II) (52). The majority of findings previously published highlight a pro-tumourigenic effect of specific immune cells on CaP. Inflammation, be it acute or chronic, enhances the immune cell infiltrate surrounding the prostate, in particular T-lymphocytes and macrophages. Surrounding the prostate gland are high levels of CD8+ cytotoxic T cells with B-lymphocytes and CD4+ T-cells residing within the stroma. Interestingly, lymphocytes, stromal cells, and epithelial cells all alter the local immune response due to cell surface cytokine receptors (43, 53). However, the innate immune response, primarily through tumour associated macrophages also plays a significant role in CaP, for example through enhancing angiogenesis and constructing a metastatic niche. However, dendritic cells, killer lymphocytes, leukocytes, NK cells, granulocytes, and mast cells all contribute to the innate immune response within prostate cancer.

There has been recent interest into how stromal AR signaling correlates with CaP and a loss of expression has been observed during CaP progression (54). A decrease in stromal AR expression has been significantly associated with reduced time to biochemical relapse as well as reduced cancer specific survival, suggesting stromal AR has a protective role over CaP progression (55, 56). The

mechanism by which stromal AR depletes during CaP progression remains undefined but one hypothesis proposes that androgen uptake is greater by CaP cells and therefore outcompetes stromal AR and leading to a reduction in stromal AR expression (57). Therefore, it is evident that the exact mechanisms and characterizations of AR expression in the TME and how it associates with CaP progression needs to be fully determined.

Pro-inflammatory Cytokines and Prostate Cancer

Growth factors and pro-inflammatory cytokines have been linked to uncontrolled cellular proliferation and prostate cancer progression in both patient tumours and experimental models. Following insult or an inflammatory trigger, prostate epithelial, stromal, and inflammatory cells secrete multiple pro-inflammatory cytokines such as CXCL-2, CXCL12, and TNF- α and interleukins (ILs) such as IL-6, IL-8, IL-11 and IL-33 which stimulate various inflammatory pathways generating an inflammatory TME (58). These infiltrating immune cell secreted cytokines are known to activate a variety of pathways including JAK-STAT3, NF- κ B, RAS-RAF-MAPK, and PI3K-AKT, all of which are shown to promote AR activation following androgen deprivation (Figure 1) (59).

Interleukin-6 and Prostate Cancer

IL-6, expressed in both prostate tumours and its TME, is the most investigated cytokine, with extensive evidence suggesting its role in CaP progression. This multifunctional inflammatory cytokine is upregulated in response to both nuclear factor kappa B (NF κ B) and transforming growth factor-beta (TGF- β) with high expressions observed in AR-negative DU-145 and PC3 human prostate cancer cell lines and several studies suggesting its role in androgen-independent CaP (60-62). Recently it has been identified that a glycosylphosphatidylinositol (GPI)-anchored cell surface protein, prostate stem cell antigen (PSCA), can positively regulate the p38/NF κ B/IL-6 pathway and result in increased proliferation, migration, and invasion in CaP cells thus reducing biochemical recurrence-free survival (63). NF κ B suppression by androgenic hormones inhibits IL-6 expression and may result in the development of CRCP via increased activation of AR (60). Furthermore, IL-6 increases pituitary tumour transforming gene 1 (PTTG1) expression, whose expression can increase the tumorigenicity of LNCaP

cells following ADT, via activating signal transducer and activator of transcription 3 (STAT3) and providing possible resistance to ADT in CRPC patients (64). Contradictory to this, despite the anti-IL6 antibody siltuximab demonstrating a reduction in prostate tumour growth *in vitro* and *in vivo*, in a phase II study this monotherapy was not successful due to its anti-apoptotic effects and role in the development of enzalutamide resistance (65). Until recently, little evidence was available to suggest whether IL-6 induced AR activation or *visa versa*. However, IL-6 has now been shown in LNCaP cells to induce cellular proliferation by enhancing AR-steroid receptor coactivator-1 (SRC-1) interactions and decreasing AR-silencing mediator for retinoid and thyroid hormone receptors (SMRT) interactions (66). In LNCaP cell lines, IL-6/STAT3 activation has shown to enhance the secretion of multiple neurohormones following the increase in neuroendocrine differentiation, resulting in paracrine stimulated CaP growth and poorer prognosis (67). However, divergent responses have been identified over the role of IL-6 in CaP. Both pro- and anti-proliferative roles of IL-6 have been identified, with strong links between HER2 and IL-6 activity. Overexpression of this growth receptor has been associated with prostate tumourigenesis, along with IL-6 inducing HER2 tyrosine phosphorylation and forming complexes with the IL-6 receptor at the gp130 subunit. Importantly, HER2 has shown to induce the ability of IL-6 to stimulate the mitogen-activated protein kinase (MAPK) pathway and ultimately the proliferative ability of CaP cells (68). In another study, IL-6 has shown to be secreted in an autocrine fashion at high levels by PC3 cells via the phosphatidylinositol-3-kinase (PI3K) pathway (69). Both PI3K/Akt and MAPK/Erk signaling have been implicated in the development and progression of androgen-independent disease as a result of IL-6 activity. However, when taken into mouse models, loss of IL-6 or STAT3 lead to widespread metastases and reduced life span suggesting a tumour suppressive role of the IL-6/STAT3 pathway (70). Contradictory to this study, in PTEN-deficient tumours, STAT3 inactivation reduced tumour size by 70% and reduced the invasive ability of CaP (71). These conflicting results again highlight the need to fully elucidate the role of IL-6 in CaP.

CXC-chemokines and Prostate Cancer

Chemokines are the largest subfamily of cytokines with a major role in mediating immune responses with immune cell tumour trafficking of various lymphocytes into the TME being a key role of CXC-chemokines. In the TME, both tumour cells and immune cells express these chemokines. Natural killer cells, CD8+ T cells and TH1 cells all express C-X-C receptor 3 (CXCR3), the receptor for C-X-C ligand 9 (CXCL9) and 10, that enables them to move into the TME via a chemogradient. High levels of CXCL9 and CXCL10 are associated with increased CD8+ T cell infiltration and improved patient survival in ovarian and colon cancers (72-74).

Stromal cell-derived factor 1 or CXCL12 is one of the most well studied chemokines for its diverse cellular functions from immune surveillance and promoting inflammatory responses to inducing tumour growth and metastasis. Acting as a chemoattractant, the CXCL12/CXCR4 axis induces metastasis of prostate cancer cells to the bone as prostate cancer cells express abundant levels of CXCR4, the receptor for CXCL12 (75). It has been found that the TMPRSSP-EGF fusion gene regulates CXCR4 expression, with androgen induced ERG expression regulating CXCR4 expression in prostate cancer and contributing to prostate cancer bone metastasis (76, 77). However, inhibiting CXCR4 in vivo only partially relieves CaP metastasis.

Furthermore, activation of the CXCL12/CXCR4 axis has been found to promote; AR-regulated PSA secretion; PI3K-dependent AR phosphorylation; PI3K-dependent PSA expression in the absence of androgens; nuclear accumulation of the AR; and AR-dependent proliferative responses (78). This study was able to therefore demonstrate that the CXCL12/CXCL4 axis may stimulate AR phosphorylation in an androgen-independent manner via the PI3K/AKT pathway. Another possible mechanism by which the CXCL12/CXCR4 axis could potentially stimulate AR activity is via MAPK-mediated phosphorylation of SRC-1 on threonine residues. Inhibition of Src family kinases and PKC, upstream signals to both PI3K and MAPK signaling, reduces PSA secretions in LNCaP cells therefore suggesting the CXCL12/CXCR4 axis may act via this manner (78).

Moreover, CXCL8 (IL-8) expression is undetectable in hormone-sensitive prostate cancer cell lines (LNCaP and LAPC-4) but is drastically overexpressed in hormone-resistant PC-3 cells (79). It has

previously been reported that IL-8 expression can be suppressed following the presence of androgens, with levels increasing following androgen deprivation (80). However, in both LNCaP and 22Rv1 androgen-dependent cell lines, CXCL8 stimulation increases both mRNA and protein expression of AR and increases its transcriptional ability (81). Interestingly however, IL-8 levels increase following the development of castrate resistance, but it remains unclear whether IL-8 promotes androgen-independence or is a consequence of this transition to castrate disease (82). Taken together, these reports suggest that chemokines such as CXCL8 and CXCL12 may promote androgen-dependent and independent AR-mediated transcriptional activity.

TNF- α and Prostate Cancer

Tumour necrosis factor alpha (TNF- α) is a pro-inflammatory cytokine, produced primarily by macrophages but also CD4+ lymphocytes and NK cells, with vital roles in inflammation, proliferation, and cell death. In addition to infiltrating immune cells secreting TNF- α into the TME, TNF- α can also be produced by CaP cells, with high TNF- α in CaP associating with reduced overall survival (83). This pro-inflammatory cytokine is also known to rapidly activate NF κ B, a pathway often constitutively active in CaP and is seen to promote ADT resistance, increase cellular proliferation, and induce anti-apoptotic signaling (84-86). *In vitro* studies have identified high constitutively active levels of NF κ B in androgen-independent cell lines such as PC-3 and DU-145, whereas androgen-dependent cell lines such as LNCaPs express only low constitutive NF κ B activation (87). Additionally, in androgen-dependent CaP cells NF κ B activation is shown to inhibit proliferation whereas in androgen-independent cells no proliferative inhibition is observed (88).

Once activated, NF κ B complexes promote anti-apoptotic signaling and ultimately increased cell survival in CaP cells, with its activation also observed in inflammatory cells. Upregulation of this pathway has been observed in androgen-independent cell lines including PC3 and DU-145 and reduced expression seen in the androgen-dependent cell line LNCaP (87). This association of NF κ B DNA binding with CaP has shown positive associations between high Gleason Grade, biochemical relapse, and ultimately reduced cancer-specific survival (89, 90). A mutually exclusive role between

NFκB and AR has been suggested; however, contradictory results have been seen. For examples, AR expression in PC3 cells decreases NFκB expression with the aid of dihydrotestosterone, whilst in the androgen-dependent cell line LNCaP NFκB is shown to reduce AR transcriptional activity (91, 92). These changes suggest that following the transition from androgen-dependent CaP to CRPC, AR may lose its ability to repress NFκB expression along with its androgen-dependent ability. Furthermore, it has been postulated that NFκB could induce AR reactivation following the development of castrate resistant disease.

Adaptive Inflammatory Infiltrate and CaP

Regulatory T Cells

Reducing host tolerance to inducing infiltrating T-cells is of great importance in tumour immunotherapies. Predominantly CD4+ T-cell infiltration, with comparatively fewer CD8+ T-cells, has been observed in CaP following 7-28 days after androgen deprivation therapy (14). Increased CD4+ regulatory T (Tregs) cells associating with reduced cancer specific survival and biochemical relapse (14, 93, 94). A near twofold increase in the risk of dying from prostate cancer has been found in the high quartile compared to the lowest quartile of CD4+ Treg cells (odds ratio: 1.98; 95% confidence interval: 1.15-3.40). Interestingly, it has been found that for every additional CD4+ Treg cell there is a 12% increase in odds of prostate cancer death (odds ratio: 1.12; 95% confidence interval: 1.02-1.23) (95). However, the prevalence of regulatory T (T_{regs}) cells and their suppressive nature within prostate cancer is still not fully defined with some studies suggesting that prostate cancer derived TILs differ from those from other cancer types. For example, in a prostate dysplasia transgenic mouse model, during the progression of the tumour, increased levels of CD4+CD25+ T_{regs} were found along with elevated inhibitory cytokine productions which correlated with reduced T cell function. This study was able to decrease, but not fully diminish, tumour growth following anti-cd25 antibody treatment (96).

CD4+ T_{regs} act to suppress the autoreactive behavior of T cells and can be identified through the coexpression of CD4+ CD25^{high} surface markers, however the fork head family transcription factor FOXP3 is currently used to define this immune cell subset. In a study on 52 TIL cell lines from human

prostate cancers, 72% of cell lines expressed elevated CD4+CD25+ T_{reg} cells and showed a potent function in suppressing naïve T-cell proliferation (97). Their ability to suppress both the activation and effector function of immune cells is seen widespread throughout the immune system in particularly in CD4+ and CD8+ T cells, macrophages, dendritic cells, NK cells, and B cells (98). Interestingly, T_{regs} expression is now being investigated for its role as an antitumour immune response suppressor and increased tumourigenic activity (99). Elevated T_{regs} infiltration is markedly increased in CaP when compared to its corresponding normal prostatic epithelium and reduces patient outcome (100, 101). Furthermore, when T_{regs} are present in the epithelium of normal prostatic tissue, a fourfold increased risk of developing CaP is observed (102).

This immune suppressive behavior seen within prostate cancer has been hypothesized to occur through both secretion of pro-inflammatory cytokines including IL10 and TGF- β as well as cell-cell contact (103). Several possible mechanisms are hypothesized to increase Treg infiltration. For example, in ovarian cancer, tumour cells and/ or macrophages within the tumour secrete the chemokine CCL22 that binds with great affinity to the receptor CCR4 on Treg cells (104). Along with affecting typical ADT, low expression of FOXP3+ T_{regs} has correlated with prolonged progression free survival and overall survival in those who received salvage radiotherapy. IL10 secretion acts as an anti-inflammatory cytokine of the immune system with pleiotropic actions, in particular on T lymphocytes, dendritic cells, and macrophages. IL10 expression has therefore been linked with inhibition of angiogenesis, the downregulation of the macrophage pro-inflammatory cytokine IL6, and the regulation of immunoglobulin class switching (105-107). Overexpression of this cytokine has been observed in CaP tissue, as well as also being associated with decreased stemness *in vitro* and positively correlating with serum PSA levels (108). It has previously been revealed that serum IL6 and IL10 expression correlates with reduced survival and an overall worse prognosis for CRPC patients. AR-mediated gene expression has been highlighted in a paracrine fashion by IL6 in LNCaP cells (androgen-sensitive) but an autocrine fashion by IL6 in PC3 cells (androgen-independent). This androgen-independent mechanism has been linked to HSP-90 which holds the AR within the cytoplasm when

unbound to androgens and maintains the AR in a high affinity androgen-binding conformation with positive correlations observed between HSP90 and IL6 in both stroma and tumour epithelium, suggesting importance associations with CaP progression (109). More recently, lymphocyte activation gene 3 (LAG-3) has been identified as a marker of T_{regs} and has the capacity to reduce anti-tumour activity. This subpopulation of LAG-3 expressing T_{regs} display a terminal-effector phenotype with expression identified in peripheral blood of CaP patients (110). When present within the TME, T_{regs} exert varying functions than those present within the periphery. Overexpression of cell surface molecules such as LAG3, T-cell immunoreceptor with Ig and ITIM domain (TIGIT), CTLA4, and inducible T-cell costimulatory (ICOS) have been seen in a range of primary and metastatic tumours (111).

TGF- β expression in both normal and CaP cells shows an autocrine growth inhibitory action that increases the proliferation and survival of transformed cells as well being highly prevalent in metastatic prostate cancer (112, 113). Secretions from both the stroma and the TME suppress cytotoxic T lymphocyte function as well as induces FOXP3 expression. Once bound to either the TGF- β type 1 or TGF- β type 2 receptors, an upregulation of phosphorylation and activation of the transcription factors Smad2 and Smad3 occurs (114). Multiple survival signals are transcriptionally altered and result in pathological epithelial-mesenchymal transition (EMT) in tumour cells. Interactions between TGF- β and AR have been demonstrated with TGF- β signaling inducing an AR-mediated transcription of two androgen-responsive promoters, probasin and PSA. Furthermore, it has been discovered that DHT enhances TGF- β -mediated apoptosis in androgen-dependent LNCaP- TGF β II cells via an interaction between AR and Smad4 (115). Decreases in tumourgenicity are observed through the decrease in the expression of the anti-apoptotic protein bcl-2, the increase in the cell cycle regulator p21, and the increase in the expression of the apoptotic executioner procaspase-1 expression (116). However, in the androgen-independent cell line PC3, TGF- β is unable to promote apoptosis despite the overexpression of AR within the cell line (115). Furthermore, a reduction in FOXA1 has been shown to enhance enzalutamide resistance via increased activation of the TGF- β signaling pathway and ultimately IL-8 expression (117). In conjunction with this, a recent clinical trial

has aimed to combined the TGF- β receptor inhibitor, Galunisertib (Y2157299), with enzalutamide to aid in the treatment response for metastatic castrate resistant prostate cancer patients. Preliminary results within mice models discovered a significant reduction in cellular proliferation following combined Galsunisertib and enzalutamide when compared to the inhibitor as a monotherapy (118). Interestingly, over the past decade, the development of genetically engineered chimeric antigen receptor (CAR) T-cell immunotherapies has increased with great success observed in hematological malignancies (119). However, little success has been demonstrated in solid tumours due to the immunosuppressive nature of the TME caused by cytokines such as TGF- β and IL-6. Therefore, efforts are underway to inhibit TGF- β signaling either by CRISP gene editing knockdown of TGR- β receptor II or overexpressing a dominant negative TGF- β receptor II in CAR T-cells (120). These findings suggest that TGF- β -mediated apoptosis is enhanced by androgens via mechanisms involved in both controlling the cell cycle as well as regulating apoptosis, highlighting the importance for maximizing apoptotic induction during ADT. However, due to the recurrence of CRPC there is a need for further therapeutic interventions.

To further understand how infiltrating T cells can alter AR signaling in CaP, downstream metastasis genes were investigated following the co-culture of CaP cell lines with T-cells. MMP9 expression was elevated and its suppression notably reduced infiltrating T cell-enhanced CaP cell invasion (121). Furthermore, this study investigated the expression FGF11 in the CaP C4-2 cell line and observed that an increased expression of FGF11 was associated co-cultures of C4-2 with T-cells. Knockdown of FGF11 partially reduced T cells-enhanced CaP cell invasion along with reducing AR and MMP9 expression, possibly by the inhibiting protein translation and/ or degradation of mRNAs activity of micro-RNA-541. Therefore, this study postulates the theory that infiltrating T cells could secrete more FGF11 and thus provides a positive feedback mechanism by which down-regulation of the AR could in turn increase the recruitment of infiltrating T cells and consequently enhance the invasive nature of CaP. This has therefore highlighted a potential flaw in the suppression of AR signals via anti-androgens such as Enzalutamide, increasing T-cell infiltration and CaP invasion (121).

T Helper cells

CD4+ T helper cell activation is largely controlled by the most important antigen presenting cells, dendritic cells, which convert naïve T cells to their activated counterparts. From here, it is the CD4+ T helper cells, along with CD8+ cytotoxic T cells, which mainly carry out an immune response through the activation of the Fas/FasL and the perforin pathways (28). CD4+ T cell expression was markedly reduced in CaP tissue than BPH and PIN tissue, with suppressed CD4+ T cell expression observed with increasing Gleason Grade and PSA levels. These findings suggest that dendritic cell activation and function is inhibited following CaP progression. A potential mechanism for this is the positive correlation between increasing VEGF expression and PSA expression along with induction of PDL-1 in CaP and the negative association of VEGF with dendritic cell maturation and function (122).

Despite many studies suggesting the inhibitory role testosterone plays on inflammation, few molecular mechanisms have been defined. Androgen deprivation *in vivo* increased RNA expression patterns involved in interferon (IFN) signaling as well as in T-cell differentiation. Testosterone is shown to inhibit IL12 induced Stat4 phosphorylation therefore regulating T helper 1 cells. In mouse models, it is found that the AR inhibits the IL12 signaling through directly binding to the phosphate Ptpn1 in CD4+ T cells. An AR binding site has been discovered between exon 3 and 4 of the Ptpn1 gene. One possible mechanism for this interaction is through chromatin modifications by AR associated factors. Once upregulated, the Ptpn1 enzyme dephosphorylates both Tyk2 and Jak2 with Tyk2 phosphorylation being partially restored following Ptpn1 inhibition suggesting T helper cell differentiation could be upregulated following androgen inhibition. These findings were mirrored in patients undergoing ADT for CaP, with androgens inhibiting CD4+ T cell differentiation to T-helper cells, suggesting that androgens should be targeted to upregulate CD4- mediated immunity and together these findings support the possible use of ADT as an adjuvant for immunotherapy (123, 124).

ADT increases levels of circulating naïve T cells with increased CCL25 expression on thymic epithelial cells observed, suggesting a function in reversing thymic (125). Furthermore, androgens are also known to influence T helper cell bias and an upregulation of IFN- γ in T cells of castrate mice was

observed following restimulation ex vivo with a vaccine encoding a prostate antigen. This suggests that androgens may shift the T helper cell bias from a T helper 1 type population (126). 3 days following castration in mice, a significant increase in IFN- γ expression CD4⁺ T cells was observed and by day 30 significant expression of TNF α and IL17A expressing T cells was observed. This suggests the acute infiltrating T cell response seen in CaP following castration is predominantly T helper 1 cells and chronically T helper 17 cells (127). However, in this study this T helper 1 cell population was seen to diminish by 90 days castration, despite T helper 1 cell biased genes associating with better prognosis, similar to that seen in colon and lung cancer (128). This temporary infiltration of T Helper 1 cells was observed within androgen-deprived prostates with increased prostate epithelial gene expression of the chemoattractant IP-10/ CXCL10, highlighting a possible mechanism for this increased T-cell attraction (129). Therefore, it is hypothesized that increasing the duration of T helper 1 cell responses following castration may provide a great therapeutic response for CaP patients.

Correlations between T helper 17 cells and both pro- and anti-tumourigenic effects in CaP have been reported. Within the periphery, T helper 17 cells have been associated with a reduced time to metastatic progression; however, others have reported that a higher T helper 17 expression within the prostate tumour is associated with a lower Gleason Grade (130, 131). However, these studies were looking at the different localization of these T helper 17 cells, suggesting the systemic versus local expression may effect mechanism of action. Furthermore, T helper 17 cell development is known to be increased following STAT3 activation, which is significantly increased following castration. Contradictory to this, inhibition of STAT3 significantly reduces CaP growth in castrated mice (85).

Cytotoxic T cells

High Cytotoxic CD8⁺ T cells expression within the TME is proven to be a favourable prognostic feature for multiple cancers including colorectal (132). Once activated, effector CD8⁺ T cells are characterized by CCR-cd62L-CD45RO+CD95+IL-2b+, along with PD-1 expression and high levels of IFN γ and TNF α secretions.

Immunotherapies currently used for the treatment of metastatic CRPC (mCRPC) have shown little efficacy, with mCRPC reducing CD8+ T-cell anti-tumoural behavior. Chimeric antigen receptor retroviral constructs have been developed to increase CD8+ T-cell reactivity to prostate-specific membrane antigen (PSMA) and desensitize them to the immunosuppressive transforming growth factor- β (TGF- β). Increased tumour apoptosis and CD8+ T-cell infiltration in immuno-deficient RAG-1^{-/-} mice with PC3-PSMA tumours was observed, potentially providing a method to overcome the immunosuppressive effects of the mCRPC TME in patients who fail androgen deprivation therapy (133). Anti-Cytotoxic-T-Lymphocyte-associated protein 4 (CTLA4) or anti-programmed cell death/programmed cell death 1 (PD-1) ligand immune checkpoint blockade antibodies have provided significant therapeutic effects across a broad range of cancers. PD-1 inhibitors have shown little efficacy in men with metastatic prostate cancer so was generally left unexamined using this class of immunotherapies. However, in an ongoing phase II trial published in 2016, three out ten patients with evidence of disease progression on enzalutamide showed a rapid decrease in PSA to ≤ 0.2 ng/ml following 200mg IV of the PD-1 inhibitor pembrolizumab every 3 weeks for 4 doses. Furthermore, two out of the three responders showed the presence of CD3+, CD8+, and CD163+ leukocyte infiltration, PD-L1 expression, and markers of microsatellite instability (MSI) within a baseline tumour biopsy (134). A possibility for these conflicting results could be due down to the MSI status of these patients, with similar efficacies observed in colorectal cancer and other types with similar mismatch repair defects (17).

A more recent study, however, found immune checkpoint blockade resistance to be highly prevalent in mCRPC with increased myeloid-derived suppressor cells (CD11b+Gr1+) inducing tumour progression in mouse models and correlating with prostate-specific antigen levels and metastasis in CaP patients. Targeting either CTLA4/ PD-1 or MDSCs alone showed only modest efficacy and limited anti-tumoural activity. However, when anti-CTLA4 and anti-PD1 antibodies (upregulation of interleukin-1 receptor antagonists) were combined with multi-kinase inhibitors such as cabozantinib and BEZ235 which neutralizes MDSCs through the suppression of MDSC-promoting cytokines secreted

from prostate cancer cells, robust synergistic effects were observed both in primary and metastatic CRCP tumours (135).

Interestingly, in a recent *in silico* analysis on two prostate cancer cohort, overexpression of the androgen receptor was significantly associated with a decrease of CD8+ T cell infiltration ($p < 0.0001$) along with a significant reduction in PD-1 and CTLA-4 expression ($p < 0.0001$ and $p = 0.009$ respectively). In the same study however, they found that loss of PTEN was associated with an increase in PD-1 expression and an increase in CD8+ T cell infiltration within the tumour micro environment ($p < 0.0001$ and $p < 0.0001$ respectively). Furthermore, when patients experienced both a loss of PTEN and an increase in CD8+ T cell infiltration, they had a significant reduction in time to recurrence from diagnosis ($p = 0.029$). This study showed conflicting results as AR overexpression and loss of PTEN are both poor prognostic factors within prostate cancer but resulted in opposing inflammatory outcomes, therefore highlighting that a further in-depth immune cell profiling within the tumour microenvironment is required to allow novel immunotherapies to promote a better response in prostate cancer (136).

Innate Inflammatory Infiltrate and CaP

Androgens and the effects they have on the innate immune system remains largely unexplored. However, multiple mechanisms have been proposed to determine how androgens drive AR signaling in innate immune cells such as macrophages, neutrophils, dendritic cells (DC), and myeloid-derived suppressor cells. Despite macrophages and neutrophils being quite heavily investigated, little evidence is available determining the role of AR in dendritic and myeloid-derived suppressor cells. However, few studies have found that AR expression within DC reduces pro-inflammatory cytokine secretions such as IL-6 and increases anti-inflammatory secretions such as IL-4 and IL-10 (137, 138).

Tumour Associated Macrophages

Tumour-associated macrophages (TAM) originating from circulating blood monocytes are recruited to tumour sites via chemokine and cytokine signaling, with strong evidence suggesting both

tumouricidal activity through TNF- α and IL-12 production and tumourigenesis activity (139-141). During the development of CaP, AR expressing macrophages including both inflammatory-associated M1 and cancer promoting M2 CD68+ macrophages are recruited to the TME. The function of the AR in these cells currently remains unknown, however AR nuclear translocation has been observed following testosterone stimulation. Furthermore, following AR-ChIP sequencing, Macrophage Triggering Receptor 1 (TREM1) signaling was identified as being regulated by AR and several cytokines involved in TREM1 signaling and a pro-tumour phenotype of macrophages such as CCL2, CXCL8, and IL-1 β were significantly upregulated following testosterone stimulation (142).

In a study on 71 CaP patients following hormonal therapy, a high TAM infiltration was significantly associated with a reduced recurrence-free survival ($p < 0.001$) and associated with higher serum PSA level, stage, and Gleason score (143). Likewise, increased M2-macrophage infiltration has been associated with extracapsular extension and reduced biochemical recurrence free survival following radical prostatectomy (144). It has been reported that CaP cells secrete chemo-attractants such as GM-CSF, which may contribute to tumour infiltrating macrophages (145).

Furthermore, persistent co-culturing of RWPE-1 or BPH-1 cells (immortalized prostate epithelial cells) with THP-1 macrophage cells induces CCL4-STAT3 activation, epithelial-to-mesenchymal transition, down regulation of p53/PTEN, and ultimately prostate tumourigenesis. It has previously been confirmed that CCL4 is a crucial gene involved in tumourigenesis and is responsive to AR signaling (146). Interestingly, by neutralizing CCL4 activity, they were able to block STAT3 activation, THP-1 cell migration, and macrophage-associated cytokine expressions. However, following direct CCL4 stimulation they were unable to provoke any downstream signaling in RWPE-1 cells. The study further demonstrated an *in vivo* role between macrophage AR and CaP in macrophage-AR knockout PTEN^{+/-} mice, showing a decrease CCL4 expression and consequently a reduction in the development of prostatic intraepithelial neoplasia (147).

Interestingly, following androgen stimulation, macrophage expression of the receptors for the Fc region of IgG (FcγR) has been shown to be significantly downregulated, a process critical for inflammation and phagocytosis (148).

The interaction between CaP cells and macrophages is mediated by VCAM-1 adhesion and subsequently leads to macrophage activation and IL-1β secretion, a possible mechanism of resistant towards selective androgen receptor modulators and the development of castrate resistant disease (149). It has previously been identified that AR function in macrophages is significantly associated with wound healing-associated inflammation in mice, with AR enhancing inflammatory responses through increased TNF-α expression (150). This data suggests that following ADT and a deficit in AR expression, an immunosuppressive microenvironment may be created that favours wound healing, a process with similar gene signatures to those seen in aggressive breast cancers (151). Additionally, significant roles between CCL2 directed macrophage infiltration and advanced prostate tumour growth/ metastasis *in vivo* has been identified (152). One study has established a role between AR and downregulation of CCL2 expression. siRNA targeted AR in CaP cells resulted in the upregulation of CCL2 and subsequent increase in macrophage recruitment in a STAT3 dependent manner, suggesting that ADT may induce CCL2 activity and help establish an immunosuppressive TME. The study went on to simultaneously target AR with siRNA and the CCL2/CCR2-STAT3 axis and reported a reduction in CaP and metastasis in mice, potentially identifying a novel therapeutic strategy in advance CaP (153).

The same research team did follow on experiments and treated CaP cells and macrophage cell co-cultures with anti-androgens such as enzalutamide or bicalutamide. However, they discovered enhanced CaP cell invasion and macrophage migration towards CaP cells. These common anti-androgens were shown to reduced AR-mediated PIAS3 expression and induce pSTAT3-CCL2 signaling. However, when co-cultures were treated with the AR degradation enhancer ASC-J9, suppression of both macrophage and CaP cell migration was reported, suggesting ASC-J9 could potentially inhibit AR dependent signaling via inhibiting PIAS3 expression and AR independent signaling via inhibiting STAT3 signaling simultaneously (154).

Additionally, down regulation of toll-like receptor 4 (TLR4) on murine macrophages following AR stimulation has been found to decrease the expression of multiple pro-inflammatory molecules via MyD88-dependent and MyD88-independent signaling (155). For example, a decrease in TNF- α , CXCL10, IL-6, and IL1- β has all been observed (156). These results in combination or alone have distinguished the affects AR signaling plays on macrophage function and possibly identified that higher TAM infiltration can increase the aggressiveness of CaP cells.

Tumour Associated Neutrophils

Neutrophil-to-lymphocyte ratio (NLR) in the peripheral blood of CaP patients is highly prognostic in castrate resistant patients, with high NLR reducing 2-year overall survival to just 3% as well as reducing abiraterone and docetaxel responses (157). Additionally, in mCRPC patients treated with Abiraterone, an NLR-change to <5 after eight weeks of Abiraterone was associated with a reduction in overall survival as well as possibly marking early treatment response (158). Much like that seen with macrophages, neutrophils within the TME can be classified as either tumouricidal N1-like neutrophils or pro-tumourigenic N2-like neutrophils and are shown to exert their functions via phagocytosis or oxygen-free radical damage (159). AR expression within human neutrophils is yet to be defined, however some studies have shown high overexpression of AR within mouse neutrophils and knockdown of AR associating with reduced neutrophil proliferation (160, 161). As a result of AR knockdown, multiple inflammatory molecules such as TNF- α and IL-6 were also significantly reduced in granulocytes suggesting AR expression within neutrophils reduced neutrophil expression and supports their immunosuppressive abilities (161). Furthermore, high neutrophil-lymphocyte ratio in CaP is significantly associated with reduced time to biochemical relapse and poorer survival, suggesting ADT may result in a reduction in neutrophil populations and aim in better patient survival (162, 163). Additionally, carozantinib, a promiscuous receptor tyrosine kinase inhibitor, showed eradication of prostate adenocarcinomas 48 hours following administration into PTEN/p53 deficient CRPC model mice along with enhanced neutrophil infiltration and the release of neutrophil chemotactic factors such as CXCL12 and HMGB1. Importantly, when neutrophil chemotaxis was

blocked via CXCR4 inhibitors or HMGB1 neutralization, the tumour clearance demonstrated by carozantinib was reversed (164). Therefore, this suggests an anti-tumour response elicited by neutrophil infiltration.

Cancer Associated Fibroblasts

Amongst the stromal cells surrounding a tumour, large populations of cancer associated fibroblasts (CAFs) are found and are seen to have crucial roles in modulating tumourigenesis and immune responses via the production of various soluble molecules such as cytokines and chemokines (165). The precise mechanism by which normal-associated fibroblasts become activated CAFs remains unknown. Secreted pro-inflammatory cytokines from CAFs stimulate a massive immune cell infiltration into the TMA including macrophages, neutrophils, and lymphocytes along a chemotactic gradient. Co-cultures of AR knockout CAFs with PC3 prostate cancer cell showed decreased invasion and epithelial cell growth mediated through the secretion of various factors including FGF10, TGF β 2, and IGF1 (166). Furthermore, AR positive human fibroblasts co-cultured with LNCaP prostate cancer cells following DHT stimulation significantly enhanced LNCaP cellular proliferation (167). Contradictory to these findings, it has recently been found that AR negative CAFs overexpress IFN- γ and M-CSF resulting in increased stem cell markers and CaP cell growth (168).

Conclusion

Despite immunotherapies being widely used across a range of cancer types including non-small cell lung cancer and colorectal cancer, little efficacy has been demonstrated in CaP and an overwhelming *de novo* resistance to immune checkpoint blockade (ICB) has been observed in mCRPC (169). A possible explanation for the limited efficacy observed in CaP cells following immunotherapies is the immunosuppressive TME that surrounds untreated CaP with limited PD1+ expressing T cells as well as increased CD25+ and FoxP3+ T_{regs} (170). Increased TME infiltrated fibroblasts may also present as a possible resistant mechanism through their release of CCL2 and IL-6 which promotes DC infiltration and differentiation into tumour-associated DCs and therefore reduces antigen presentation to CD8+ T cells (171). Furthermore, ADT has been reported to reduce PD-L1 expression

and hence a reduction in the efficacy of anti-PD-L1 therapy (172). Additionally, due to the impact of pro-inflammatory cytokines on AR signalling, the use of receptor tyrosine kinase inhibitors or Src inhibitors for example could be combined with traditional ADT therapies for late stage prostate cancer. However, further understanding of how the TME and tumour cell signalling is required to understand how immunotherapy can be appropriately applied for prostate cancer treatment as it is possible this might only be suitable for a small subset of patients.

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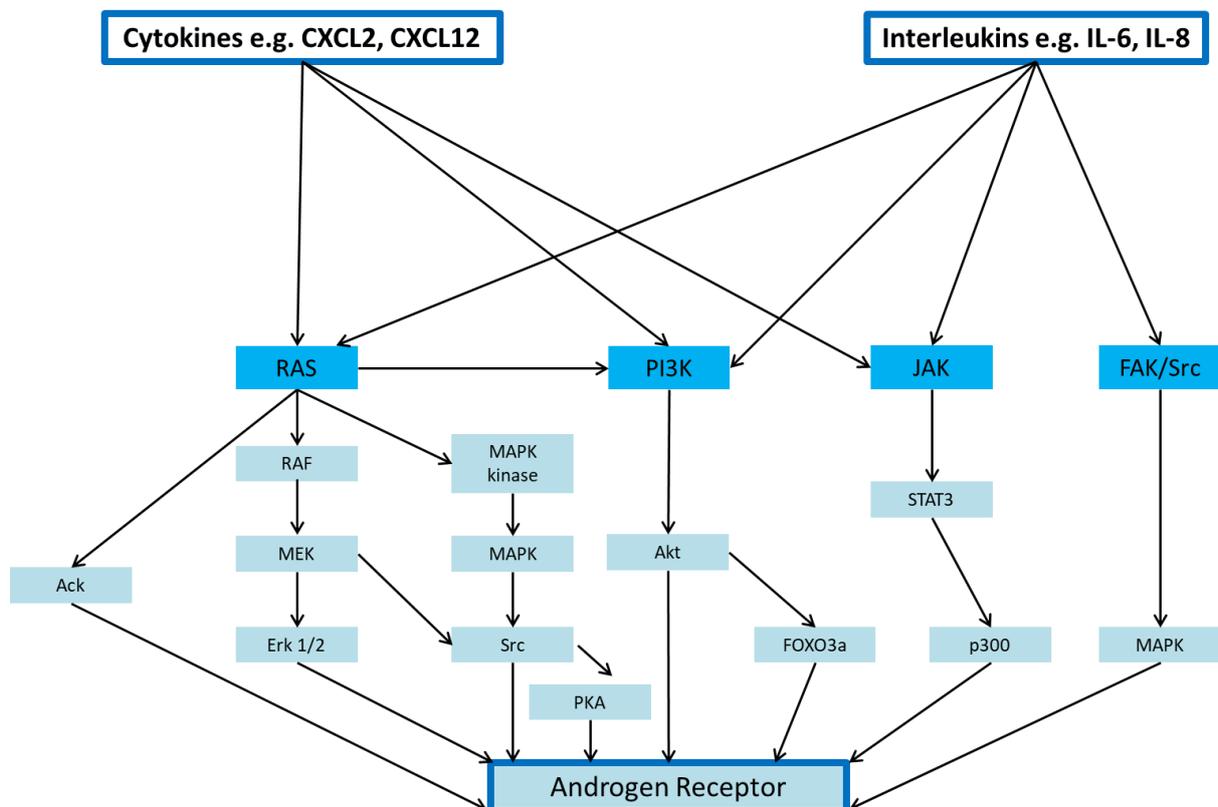


Figure 1: Pro-inflammatory chemokines and cytokines activate multiple pro-inflammatory pathways which activate androgen receptor signaling

