Prostate Cancer Cells differentially express anti-inflammatory and pro-inflammatory cytokines and chemokines: implications for prostate cancer immunotherapy.

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ABSTRACT

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PROSTATE CANCER CELLS DIFFERENTIALLY EXPRESS ANTI-INFLAMMATORY AND PRO-INFLAMMATORY CYTOKINES AND CHEMOKINES: IMPLICATIONS FOR PROSTATE CANCER IMMUNOTHERAPY

Advisor: Dr Godwin A. Ananaba
Thesis dated December 2007

Anti-inflammatory specific cytokines and chemokines are elevated in many advanced tumors and correlate with poor prognosis. However, the differential expression of anti-inflammatory cytokines and chemokines in prostate cancer is not known. We investigated the hypotheses that androgen unresponsive DU145 and PC3 prostate cancer cells and androgen responsive LNCaP prostate cancer cells, differentially expressed selected anti-inflammatory and pro-inflammatory cytokines and chemokines and that, dendritic cells pulsed with prostate tumor antigens will induce mainly pro-inflammatory cytokines and chemokines in T cells using mouse models. Our results indicated that anti-inflammatory specific cytokines IL-10, IL-4, and anti-inflammatory specific chemokine CCL-17 (TARC) and cognate receptor CCR4 are expressed in prostate cancer cell lines. Quantitative real-time PCR (qRT-PCR) revealed an almost five-fold increase in chemokine CCL17 and its cognate receptor CCR4 mRNA in androgen unresponsive DU145 and PC3 prostate cancer cell
lines compared to androgen responsive prostate tumor LNCaP. Protein analysis indicated significantly increased secretion of anti-inflammatory cytokine IL-10 by DU145 and PC3 compared to LNCaP. Furthermore, pro-inflammatory cytokine IFN-\(\gamma\) and pro-inflammatory chemokine IP-10 secretion were significantly less in these prostate cancer cells, when compared to immortalized normal prostate epithelial cells. Our \textit{in-vivo} analysis revealed that T cells were activated by pulsed dendritic cells shown in the increase mRNA expression of pro-inflammatory cytokine IFN-\(\gamma\) and pro-inflammatory chemokine IP-10, and cognate receptor CXCR3. However, a predominant pro-inflammatory response was not observed as anti-inflammatory cytokines and chemokines were also seen. The production of anti-inflammatory cytokines and chemokines suggests a possible mechanism for prostate cancer to evade host immune responses by negatively modulating immune responses that are necessary for destroying cancers cells. Cytokine and chemokine profiles could be used as potential prognostic markers for disease progression. Additionally, an efficacious vaccine will depend on its ability to inhibit the recruitment of known distinct functional anti-inflammatory effector molecules, implicated in prostate cancer progression.
PROSTATE CANCER CELLS DIFFERENTIALLY EXPRESS ANTI-INFLAMMATORY AND PRO-INFLAMMATORY CYTOKINES AND CHEMOKINES: IMPLICATIONS FOR PROSTATE CANCER IMMUNOTHERAPY

A THESIS
SUBMITTED TO THE FACULTY OF CLARK ATLANTA UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE

BY
KEREEEN S. BIRD-GORDON

DEPARTMENT OF BIOLOGICAL SCIENCES

ATLANTA, GEORGIA
DECEMBER 2007
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ABBREVIATIONS

APCs- Antigen Presenting Cells
Bcl-xl- B Cell Lymphoma Extra Long
EDTA- Ethylenediamine
ELISA - Enzyme Linked Immunosorbent Assay
FBS - Fetal Bovine Serum
GAPDH - Glyceraldehyde-3- Phosphate Dehydrogenase
Gro-α - Growth Regulated Oncogene Alpha
GMCSF - Granulocyte Macrophage Colony Stimulating Factor
IP-10 - Interferon Inducible Protein 10
IFN-γ - Interferon Gamma
I-PrEC - Immortalized normal prostate epithelial cell
kDA - Kilo Dalton
MEM - Minimum Essential Medium
MHC - Major Histocompatibility Complex
MIG- Monocyte Induced by Interferon Gamma
mRNA- messenger Ribonucleic Acid
PCR - Polymerase Chain Reaction
PMA - Phorbol 12- myrisate 13- acetate
RPMI 1640 - Roswell Park Memorial Institute (1640) Medium
TAAs - Tumor associated antigens

TARC - Thymus and activation regulated chemokine

TNF-α - Tumor necrosis factor alpha

VEGF - Vascular Endothelial Growth Factor
CHAPTER 1

INTRODUCTION

Prostate cancer is the second leading cause of cancer-related death in men in the United States of America. The American Cancer Society, 2007 reported that more than 65% of all prostate cancer cases occur in older men and it is most likely due to the accumulation of genetic changes over time (Giovannucci and Platz, 2002). There is also evidence to suggest that prostate cancer is becoming more prevalent in younger men (Elkord, 2007). The chances of survival are increased if early detection by means of serum testing for elevated levels of prostate specific antigen (PSA), surgical intervention and radiation (Abate-Shen et al., 2000) are done. However, Tjoa et al., 1999 reported that almost 20% of men succumb to the disease after these conventional treatments. This agrees with recent findings (Elkord, 2007) that 35% - 61% of patients receiving treatments had residual disease. Even though androgen ablation is considered a very effective therapy for advanced prostate cancer progression of the disease can continue to the point of becoming androgen unresponsive (Elkord, 2007).

With this in mind, immunotherapeutic approaches that are based upon the premise that the immune system plays a critical role in suppressing tumors (Tjoa et al., 1998) are becoming increasingly popular in cancer treatments. There is mounting experimental evidence to support the notion that elevated levels of T cell mediated immune responses, particularly Th1-driven anti-prostate cancer antigen specific or pro-
inflammatory responses, are necessary to stymie the effects of prostate cancer by eliciting protective immunity against it (Shibuya et al., 2004; Mullins et al., 2004). Immune cells involved in T cell mediated immunity are CD4+ T helper type-1 cells and CD8+ T cytotoxic cells. Anti-tumor cell mediated immunity are comprised of pro-inflammatory cytokines such as IFN-γ, IL-2, IL-12 and chemotactic cytokines or chemokines, such as IFN-γ inducible protein 10 (IP-10) and monokine induced by γ interferon (MIG) (Tannenbaum et al., 1998). Anti-inflammatory cytokines IL-4 and IL-10 and anti-inflammatory chemokines IL-8; GRO-α, CCL17 (TARC) are known to contribute to an immunosuppressive environment (Conticello et al. 2004; Mullins et al., 2003; Ishida et al., 2006 and Maccron et al., 2002). Conticello et al., 2004 reported that in-vivo production of IL-4 supported tumor growth by up regulating anti-apoptotic genes such as cFLIP/Fas-associated death domain-like anti-apoptotic molecule (FLAME) 1 and Bcl-xL in breast, bladder, and prostate tumors. McCarron et al., 2002 reported that elevated serum IL-10 levels were detected in patients with various solid tumors. Elkord, 2007 reported that cancers such as melanoma, ovarian carcinoma and B-cell lymphoma produced IL-10. Additionally, IL-10 secretion by tumors can convert immature dendritic cells (potent antigen presenting cells) into TGF-β and IL-10 secreting T suppressor cells (Ghiringhelli et al., 2005). Furthermore, IL-10 may help tumors evade the immune system by interfering with IFN-γ production and suppressing the expression of chemokines (IP-10) and MIG (Gasperini et al., 1999).

IP-10 and MIG are ELR-CXC chemokines that play an important role in inhibiting angiogenesis (the formation of new blood vessels from pre-existing ones),
in a tumor model system (Strieter, 2001). IP-10 has also been demonstrated to exert anti-proliferative effects in LNCaP prostate tumor cells (Nagpal et al., 2006). Frederick et al., 2001 demonstrated that along with MIG, IP-10 was shown to reduce the growth of new blood vessels stimulated by either vascular endothelial growth factor (VEGF) or angiogenic CXC chemokines such as IL-8 in a rat corneal micropocket assay.

Angiogenesis is important for tumor survival, growth and invasion as well as metastasis (Strieter, 2001). Chemokines and their cognate receptors in addition to being chemotactic are also implicated in the induction of proteases such as metalloproteinases (MMPs) (Payne et al., 2002). Chemokines have the ability to direct the trafficking of T lymphocytes and dendritic cells among other immune cells to the tumor microenvironment. TARC is known to be produced by tumor cells and preferentially attract T suppressor cells and other cells that bear its cognate receptor CCR4, to the tumor microenvironment, thus creating a suitable environment for survival and growth (Ishida et al., 2006).

The skewing of Th1-driven pro-inflammatory cytokine and chemokine responses to a predominant Th2 driven anti-inflammatory cytokine and chemokine response, is therefore necessary for tumor progression. Hence, cytokines, chemokines and their cognate receptors may be potential targets for prostate cancer immunotherapy. Furthermore, cytokine and chemokine expression profiles can be useful as biomarkers for potential disease progression and could be used to identify patients who may benefit from immune modulation treatments (Quatan et al., 2006). Additionally, identifying and targeting cellular and molecular factors that mediate the
induction of Th1 inducing pro-inflammatory and cytolytic anti-tumor cellular responses are of prime importance in the design of effective therapies against prostate cancer. Therefore, the intent of this study was to examine the expression profiles of anti-inflammatory cytokines; IL-4, IL-10 and chemokine, TARC and cognate receptor CCR4 and, pro-inflammatory cytokine; IFN-γ and chemokines, IP-10 and MIG and chemokine cognate receptor CXCR3 in prostate cancer cells; DU145, PC3 and LNCaP. These selected cytokines and chemokines, have biological importance in immunity and cancer progression. In addition, influence of prostate tumor associated antigen pulsed dendritic cells on cytokine and chemokine profiles in T lymphocytes was investigated.

We hypothesized that anti-inflammatory and pro-inflammatory cytokines and chemokines are differentially expressed in androgen unresponsive DU145 and PC3 prostate cancer cell lines and androgen responsive LNCaP prostate cancer cell line.

We also hypothesized that, anti-inflammatory and pro-inflammatory cytokines and chemokines expressed by prostate cancer cells will affect the profile of cytokines, chemokines and their receptors expressed by T helper and dendritic cells, which would influence the presentation of prostate cancer antigens to anti-tumor pro-inflammation specific T cells and potentiate a more robust pro-inflammatory response. To test the above hypotheses, the following aims were investigated.

1. To profile anti-inflammatory and pro-inflammatory chemokines and cytokines in two androgen unresponsive prostate cancer cell lines, DU145 and PC3, and androgen responsive LNCaP prostate cancer cell line compared to PZ-HPV-7 immortalized normal prostate epithelial cell line
(I-PrEC).

2. To profile cytokines and chemokines expressed by T cells when stimulated with dendritic cells pulsed with prostate tumor associated antigens from DU145, PC3, and LNCaP prostate cancer cell lysates.

By fulfilling these aims, a better understanding and knowledge of the functions of cytokines and chemokines in prostate cancer will be gained.
CHAPTER 2

REVIEW OF THE LITERATURE

T Cell Mediated Immunity

T lymphocytes or T cells are described as groups of small leukocytes that are the hallmark of cell-mediated immune responses (Jiang and Chess 2006). The regulation of the immune response by T lymphocytes relies on the nature and mode of action of these cells. They are responsive to an infinite number of antigens, mainly due to the extremely large repertoire of T-cell clones and epitopes (Jiang and Chess, 2006).

Immature T cells unlike B cells, which mature within the bone marrow, migrate from the bone marrow to the thymus where they become mature by expressing receptors for antigens (Jiang and Chess 2006). There are several distinct subsets of T lymphocytes. However, significant to this study are the T helper cells, T cytotoxic cells and T regulatory cells also known as T suppressor cells. T helper cells are also characterized based on their unique glycoprotein cell surface cluster of differentiation 4 or CD4 (Pitcher et al., 1999). The three primary phenotypes of T helper cells are Th0, Th1, and Th2. Th0 or naïve CD4+ expressing T cells recognize only antigens that are presented by major histocompatibility complex (MHC) class II molecules presented by antigen presenting cells (APCs) (Ribas et al., 2000). Upon stimulation by antigen, naïve CD4+ T cells or Th0 cells, differentiate into Th1 specific or Th2
by antigen, naïve CD4+ T cells or Th0 cells, differentiate into Th1 specific or Th2 specific T cells hence, T helper cell polarization depends on the cytokine milieu and antigen presenting cells (Benjamini et al., 2002).

T cytotoxic or CD8+ T cells, are distinct from T helper cells primarily on the basis of the expression of the glycoprotein cell surface marker known as cluster of differentiation-8 or CD8 (Wong et al., 2003). They are known as the primary effectors of the immune system and become activated by antigens presented only by MHC class I molecules and cytokines produced by proliferating T helper cells (Ribas et al., 2000). CD8+ T cells mediate their effector function by releasing IFN-γ and tumor necrosis α (TNF-α) or, through a direct cytotoxic mechanism which involves the release of the cytolytic protein perforin, which creates pores on the membrane of the target cell and the release of Granzyme B, an exogenous serine protease, which enters the target cells through the pores created. Granzyme B triggers the activation of apoptotic signaling cascade and induces cell death (Ribas et al., 2000).

A distinct type of lymphocytes called T suppressor or T regulatory lymphocytes constitute 1% - 3% of T lymphocyte cell population in humans and 5% - 10% in rodents (Ghiringhelli et al., 2005). They are known to suppress antibody mediated and cell mediated responses.
**Cytokines: the chemical messengers**

Cytokines are a group of polypeptide molecules and are produced by dendritic cells, and are responsible for activating T lymphocytes among other cells of the immune system. Cytokines play a crucial role in regulating both humoral and cell-mediated immune responses (McCarron et al., 2002). Their mechanism of action can be autocrine (exerting effect on cells that produce them) or paracrine (targeting neighboring cells), thereby eliciting a signaling cascade by binding to their cognate glycoprotein receptors, consequently altering gene transcription and translation (Simons et al., 2002). The production of cytokines is one of the signature functions of activated CD⁴⁺ T helper cells. CD⁴⁺ T cells are important regulators of CD⁸⁺ T cells, B-lymphocytes, and macrophages.

Cytokine secretion is also important in causing T helper cells to differentiate into type 1 and type 2 phenotypes depending on the cytokine milieu and antigen presenting cells. The proliferation and differentiation of B-lymphocytes into antibody producing cells are affected by type 2 T helper CD⁴⁺ cells (Simons et al., 2002). Cytokine cascades determine what systems are activated, which are suppressed and the duration of activation or suppression (which are usually ephemeral or short-lived) (Simons et al., 2002). In other words, they may have synergistic effects; for example, IL-12 can up-regulate IL-2 receptors. IL-2 is secreted by type one T helper cells (Th1) and act in an autocrine manner to secrete IL-2. Others act antagonistically, suppressing the biological functions of certain cytokines; for instance, high levels of IL-12 can inhibit the action of anti-Th1 cytokines such as IL-10 and vice versa. Furthermore, high levels of IL-12 are important in the augmentation and maintenance
of Th1 cellular responses by secreting pro-inflammatory cytokines such as IFN-γ, IL-2, GMCSF, and TNF-α. Cytokine IL-4 also mediates the differentiation of Th0 into Th1 and Th2 cells (Benjamini et al., 2000). High levels of IL-4 contributes to the Th2 type of immune response and modulates the production of Th2 cytokines IL-5, IL-6, IL-8, IL-10, and IL-13, and will inhibit Th1 development because IL-4 down regulates IL-2, an important mediator in Th1 mediated proliferation and development (Ghiasi et al., 2001), (Fig. 1). IL-4 and IL-10 as mentioned in the previous chapter have important implications in cancer progression. IL-10 is a pluripotent Th2 cytokine with potent effects on numerous cell populations particularly, circulating and resident immune cells, as well as epithelial and some other parenchymal cells (Benjamini et al., 2002). The biological effects of IL-10 are mediated through cell surface receptors. The functional receptor complex of IL-10 consists of at least two subunits, IL-10R1 and IL-10R2 (Asadullah et al., 2003). Two signaling pathways, the Janus kinase-1 (JAK1) and the Tyrosine Kinase-2 (TYK2) pathways are said to be associated with IL-10 R1 and IL-10R2 respectively (Dang et al., 2006). It is suggested that by the modulation of cell surface IL-10R1, IL-10 may have immunostimulatory or immunosuppressive effects (Ding et al., 2001). Cytokine IL-10 is also known to suppress the maturation of dendritic cells (Beckebaum et al., 2004). This effect is mainly due to the down-regulation of MHC class I, MHC class II, and B7 molecules (Matsuda et al., 1994; Salazar-Onfray et al., 1999). T suppressor cells, once mature, secrete high concentrations of IL-10 and TGF-β proteins (Ghiringhelli et al., 2005).

The implications of IL-10 is also demonstrated in a one study to show that IL-10 knockout APCs or antisense oligonucleotide treated antigen presenting cells
(APCs), were more potent activators of the Th1 response from naïve or immune cells and were effective cellular vaccines against genital chlamydia infection (Igiestme et al., 2000).

The proper balance between anti-inflammatory and pro-inflammatory immune response is therefore necessary for averting autoimmunity by and maintaining protective immunity. The immunological consequences of deregulation of anti-inflammatory and pro-inflammatory responses are therefore important in the context of prostate cancer immunotherapy.

Figure 1. Schematic representation of cytokine production. Antigen presenting cells are necessary for the activation of T helper CD4+ cells, natural killer cells (NK), T killer cells (TK) which secrete, among other cytokines, IL-12 and IL-4. Tilting the balance between Th1 type cytokines and Th2 type cytokines, will determine the type of T helper cell phenotype that dominates. (Adapted by Scientific Research Enterprise, 2007).
Chemokines: potent attractors

Chemokines are a family of small (8-14kDa) soluble chemo-attractant cytokines (Tanaka et al., 2005). To date, four subfamilies of chemokines have been characterized (CXC, CC, C and CX3C) based primarily on their cystein motifs (Allavena et al., 2005). Binding to their cognate receptors triggers signaling events through heterotrimeric seven transmembrane G-protein-coupled receptors (GPCRs) (Fredrick et al., 2001). Such signaling cascades may promote cytoskeletal rearrangement, the adhesion of tumor cells or leukocytes to endothelial cells as well as the directional trafficking and migration of cancer cells (Singh et al., 2004). There is reason to believe that chemokines regulate tumor growth in humans and other vertebrate species by modulating angiogenesis, activating tumor specific immune responses and autocrine growth stimulation (Frederick and Clayman, 2001). In humans, there are approximately 50 known chemokines (Frederick et al., 2001) and 18 known receptors (Tanaka et al., 2005). Chemokine receptors are differentially expressed by immune and non-immune cell populations (Fredrick et al., 2001). Chemokine receptor expression and association with Th1 and Th2 phenotypes are affected by other cytokines present during polarization (Sallusto et al., 1998). Th1 and Th2 lymphocytes differentially express chemokine receptors and migrate in response to the different chemokines (Bonecchi et al., 1998).

As mentioned in the previous chapter, Interferon inducible protein-10 (IP-10) or CXCL10 is known as a potent angiostatic CXC chemokine that is induced by pro-inflammatory stimuli in many cell types (Arenberg et al., 1996). It is 10kDa in size and lacks a Glu-Leu-Arg amino acid sequence or (ELR motif). In contrast, CXC
angiogenic chemokines such as IL-8 and Gro-α (Moore et al., 1999), contain the ELR motif (Arenberg et al., 1996). IP-10 is a pleiotropic molecule capable of eliciting many biological effects, including stimulation of monocytes, natural killer cells, T cell migration, bone marrow progenitor maturation, and the modulation of adhesion molecule expression (Neville et al., 1997). IP-10 is also known to induce the expression of transcription factor and tumor suppressor p53, which is responsible for cell cycle arrest and the induction of apoptosis in cancers (Zhang et al., 2005).

Thymus and activation-regulated chemokine (TARC) or CCL17, is a CC chemokine that is expressed constitutively in the thymus (Imai et al., 1997). It selectively attracts or directs the trafficking of Th2 lymphocytes and other cells bearing its cognate receptor CCR4 especially CD4⁺ CD25⁺ T regulatory cells (Ghiringhelli et al., 2005) and mature dendritic cells (Charo et al., 2006). The roles of TARC, the endogenous ligand for CCR4, is produced by tumors and the tumor microenvironment and as mentioned previously, is responsible for recruiting CCR4 expressing T suppressor cells to the tumor microenvironment and support its tumor growth potential (Ishida et al., 2006).

**Role of Dendritic Cells**

Dendritic cells (DC) are potent antigen presenting cells (Tjoa et al., 1999) and induce antigen specific T cell response. They have been used in clinical pilot trials to elicit tumor specific immunity as well as clinical response in selected patients with success (Nestle, et al., 2000). Dendritic cells (DCs) are known to provide an elaborate mechanism for activation of CD4⁺ type one T helper lymphocytes (Igiestme et al.,
Immature DCs actively acquire antigens through macropinocytosis (Sallusto et al., 1995). When dendritic cells mature, co-stimulatory proteins CD40, CD 80 (B7.1), CD86 (B7.2), MHC class II, CD54, and intercellular adhesion molecule (ICAM) become up regulated which is necessary for binding to T cell receptors and activation of naïve T cells (Sattaporn et al., 2001).

Activation of T cells further augment the biological effect of DCs by secreting cytokines and chemokines that modulate their activity (Simons et al., 2002). The Th 1 cytokine IFN-γ stimulate dendritic cells to produce IP-10 and MIG, which enhance them to attract CD8+ cells that preferentially express cognate receptor CXCR3 (Barnes, 2004). However, because most tumors express a small amount of tumor-associated antigen that can be cryptic, T cells may not easily recognize them (Benjamini et al., 2002). Furthermore, tumor cells tend to lack co-stimulatory molecules necessary for proliferation of T cells, cytokines, and activation of tumor specific CD8+ T lymphocytes (Schott et al., 2006). The effectiveness of tumor immunity therefore relies on the presentation of tumor associated antigens by potent dendritic cells, the activation of specific T cells, and the homing of tumor associated antigens (TAAgs) specific T cells to the tumor site for effective elimination of malignant cells (Fig. 2) (Schott, 2006).
Figure 2. Illustration of role of dendritic cells and their effect in anti-tumor immunity (Adapted: Schott, Matthias-Endocrine-Related Cancer, 2006. (A) Immature dendritic cells will present tumor associated antigens (TAAs) to T lymphocytes without the presence of co-stimulatory molecules; this can lead to T cell anergy. (B) Mature DCs will induce immunity. Co-stimulatory molecules are up regulated and TAAs are displayed by MHC class II to T lymphocytes the paracrine interaction of DCs and T lymphocytes leads to further activation and stimulation of cytotoxic T lymphocytes, which are important in anti-tumor immunity.
CHAPTER 3
MATERIALS AND METHODS

Prostate Cell Lines and Immortalized Cell Line Procurement: Androgen responsive prostate cancer cell LNCaP and androgen unresponsive prostate cancer cell lines DU-145 and PC3 are human prostate adenocarcinoma cell lines taken from patients inflicted with prostate cancer. PC3 was derived prostatic adenocarcinoma metastatic to the bone, DU145 was derived from prostatic adenocarcinoma metastatic to the brain, and LNCaP was derived from a needle biopsy of the supraclavical lymph node of a patient with metastatic prostate adenocarcinoma (Moore, et al., 1999). These prostate cancer cell lines were kindly provided by Dr. Jaideep Chaudary, Graduate Student Advisor and Dr. Shafiq Khan, Director of the Center for Research and Therapeutic Development, Clark Atlanta University. Dr. Nathan Jideama, Clark Atlanta University, kindly provided PZ-HPV-7 immortalized normal prostate epithelial cell line.

Cell Culturing: DU145 and PC3 were maintained in MEM supplemented with 100U/ml penicillin (Atlanta Biological; Atlanta, GA), 100ug/ml streptomycin and 10% FBS in humidified 5% CO₂ incubator at 37° C. LNCaP and immortalized normal prostate cells were cultured in RPMI 1640 medium (Atlanta Biological, Atlanta, GA) supplemented with 100U/ml penicillin, 100ug/ml streptomycin (Atlanta Biological, Atlanta, GA) and 10% Fetal Bovine Serum (Atlanta Biological, Atlanta, GA) in humidified 5% CO₂. Cells grown to confluency were trypsinized with 0.25% Trypsin-EDTA and counted using Coulter Instrument (Beckman, USA). Culture medium containing 1.5 x 10⁴ cells /ml
supplemented with 10 % FBS were seeded on 6-well plates and monitored over a 2-day period.

**Figure 3: Morphology of Cell lines.** Confocal microscopy to reveal the morphology of cell lines used in this study. (A) PZ HPV-7 immortalized normal prostate epithelial cells; (B) LNCaP, (C) DU145; and (D) PC3. Magnification X10

**RNA Isolation:** Cells grown to confluency were harvested and total RNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA) following manufacture’s instructions. Total RNA was eluded from columns into a final volume of 30 μl in RNase free water and stored at -80° C or immediately used for cDNA synthesis. RNA concentration was measured with a Gene Quant Pro DNA/RNA/ protein calculator, Amersham Biociences (Pittsburgh, PA).

**Complementary DNA synthesis (cDNA):** Approximately 0.25 μg of total RNA was reverse transcribed using Enhanced Avian HS RT-PCR Kit, Sigma (St Louis, MO). Briefly, total RNA was heated at 70° C for 10 minutes with 1μl oligo (dT) primers at a
concentration of 3.5 μM. Samples containing a final volume of 10 μl were cooled on ice for two minutes. Ten micro liters of RT-PCR mixture that comprised of PCR water, 2 μl 10x RT Buffer, 1μl of RNase inhibitor and 1 μl Enhanced Avian reverse transcriptase, were added to each sample to make a final volume of 20 μl. Complementary DNA synthesis was performed on samples incubated in a 50° C water bath for 50 minutes. Synthesized cDNA was stored at -20° C until needed.

**Qualitative Polymerase Chain Reaction:** Polymerase chain reactions (PCR) were performed using Enhanced Avian HS RT-PCR Kit, Sigma (St Louis, MO). Each tube contained a total volume of 50 μl: 2 μl of cDNA from RT reaction; 1μl of dNTP mixture (100 μM); 5 μl of 10x Accutaq buffer; 1 μl of Jumpstart Taq polymerase (3.5 μg/ml); and 1μl each of (human forward and reverse primers). Primer pairs were designed by Integrated DNA technologies SciTools Primer Quest software, Coralville, IA (Tables 1 and 2). The conditions for DNA amplifications were set as follows: heating at 94° C for 5 mins, followed by 35 cycles of DNA denaturing at 94° C for 2 mins. The annealing temperature was set at 65° C for 1 min; with an extension at 72° C for 2 mins, with a final extension step at 72° C for 10 min. PCR products were analyzed in 2.5% agarose gel containing ethidium bromide under ultraviolet (UV) light.
Table 1. Primer pairs for selected human cytokine and chemokine genes of interest and their product size.

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<tbody>
<tr>
<td>IFN-γ</td>
<td>Sense- CATCAGGGTACCTGACACATTCA</td>
<td>184</td>
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<td></td>
<td>Anti-sense- ACTAGGCAGCCAACCTAAGCAAGA</td>
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<td>IL-4</td>
<td>Sense- TAATGAATGCAGTCCGCAATCGGGC</td>
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<td>Anti-sense- TCGGAAACAGGCCACTTGGGAGAT</td>
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<td>IL-10</td>
<td>Sense- TAAATGACTGCTCGCTGGCTTTTGCG</td>
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<tr>
<td></td>
<td>Anti-sense- TGTGGGTTCCAGCTAGATTCGAGAT</td>
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<tr>
<td>MIG</td>
<td>Sense- TCCTAAGCCTGTAGCATGCTGGT</td>
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<tr>
<td></td>
<td>Anti-sense- TGTGGGTTCCAGCTAGATTCGAGAT</td>
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<tr>
<td>TARC</td>
<td>Sense- ATCTCCCTCAGTGCTTCTGCTTT</td>
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<tr>
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<td>Anti-sense- TAATGAATGCAGTCCCAACTCGG</td>
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<td>CXCR3</td>
<td>Sense- TTCTGTGGTGGTTCTGGTCCTGT</td>
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<td></td>
<td>Anti-sense- AAGCCCAACAAAGTACATCCAGGAA</td>
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<tr>
<td>CCR4</td>
<td>Sense- TTCTGTGGTGGTTCTGGTCCTGT</td>
<td>192</td>
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<tr>
<td></td>
<td>Anti-sense- AGCCCAACAAAGTACATCCAGGAA</td>
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Table 2. Primer pairs for selected mouse cytokine and chemokine genes of interest and their product size

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<th>Genes of Interest</th>
<th>Primer Pairs</th>
<th>Product size bp</th>
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<td></td>
<td>Anti-sense-GGTCACGTGCTCTGAAT</td>
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<td>IL-4</td>
<td>Sense-CCAAGGTGCTTCGATATT</td>
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<td></td>
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<td>IL-10</td>
<td>Sense-TCCTTGAAAAACCTGTTG</td>
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<td></td>
<td>Anti-sense-TCCCAATGGAACAGCTTA</td>
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<td>TARC</td>
<td>Sense-CAAGCTCATCTGTGAGACC</td>
<td>153</td>
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<td></td>
<td>Anti-sense-CTGGTCACAGGCGTTTAT</td>
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<tr>
<td>CCR4</td>
<td>Sense-CCTCCCTACACAACCTGGAA</td>
<td>161</td>
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<td></td>
<td>Anti-sense-GAGGGGAATGGAGATGG</td>
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<td>IP-10</td>
<td>Sense-TCCTTGTCCTCCCTAGCTCA</td>
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<td>CXCR3</td>
<td>Sense-ACCTGGTGAGCTAGTG</td>
<td>154</td>
</tr>
<tr>
<td></td>
<td>Anti-sense-CTCCCAAAGGCAATAGAGC</td>
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Quantitative Real Time Polymerase Chain Reaction (qRT-PCR): Quantification of differences in mRNA expression of IL-10, IL-4; IFN-γ, IP-10; MIG and cognate receptor CXCR3, and CCL17 and cognate receptor CCR4 mRNA expression levels in normal and cancer cell lines were determined by real-time-PCR using iCycler Detection System, Bio-
Rad Laboratory (Hercules, CA). Fluorescent DNA-binding dye iQSYBR Green Super mix from Bio-Rad Laboratory was added to standard PCR mixture. Optimum conditions, annealing temperature, primer, and template concentrations were pre-determined. Briefly, the reaction conditions for real time PCR were the following: 30 cycles consisting of 15 s incubation at 95° C and 1 min incubation at 60° C on the I-Cycler IQ, Bio-Rad, (Hercules, CA). Quantitative gene expression results were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or Beta Actin before the fold change in gene expression was calculated. All standard and qRT-PCR were done in triplicates.

*Multiplex Enzyme Linked-Immunosorbent Assay (ELISA):* Supernatants from cell lysates were analyzed by multiplex ELISA from Quansys Biosciences (Logan, UT), according to manufacturer's instructions.

*Statistical Analysis:* Two tailed t test were performed using Graphpad to evaluate differences between prostate cancer cells and I-PrEC. Analysis of variance (ANOVA) was performed using Sigma Stat S statistical software to evaluate differences among multiple experimental groups. All statistical analysis was judged at the P<0.05 level of significance. Samples were analyzed in triplicates, and reported as the mean standard error.
Protein Determination: LNCaP, PC3, DU-145 and immortalized normal prostate epithelial cells were grown to confluency in 150 cm² flasks and lysed by freeze thawing. Lysates were centrifuged at 3000 rpm at 4°C for 1 hour and supernatant collected and stored at -80°C until needed. The protein concentration was determined by Bradford assay method according to manufacture’s instruction Bio-Rad (Hercules, CA). Each data point calculated to represent the mean ± SEM of triplicate samples. Calculation of protein concentration from Bradford Assay was used to determine the amount of tumor lysates to be added to DCs at a cell ratio that range from 1 to 100 DCs for each 500 μg of tumor lysate protein (Weiss et al., 2004).

Animal Procurement, Care and Maintenance of Animal Subjects: Twenty 10-12 weeks old C57BL/6J male mice were purchased from The Jackson Laboratory (Bar Harbor, MA). The animals were kept in a pathogenic free condition in Laminar flow racks at 12 hours light and 12 hours darkness and fed with food and water ad libitum, at the Atlanta University Center Animal Care Facility, Morehouse School of Medicine Atlanta, GA.

Dendritic Cell Isolation: Dendritic cells were harvested from the bone marrow of five C57BL/6 male mice. The femoral plugs were forced into cell culture medium (through the lumen of the femur) by using a 5 cc syringe with a 23-gauge needle. This procedure was repeated until the femur is clear. The cells were refluxed several times to remove cell aggregates. The numbers of cells were counted re-suspended in medium containing 10 ng/ml of GM-CSF and 5 ng/ml of IL-4 at a concentration of 3.2 X 10⁶ cells/ml. Ten milliliters of suspension were cultured in a flask and incubated for 3 days at 37°C using a shaker. Non-adherent cells were collected and discarded. The medium was replaced with fresh medium containing GM-CSF and IL-4. After five days, non-adherent cells were
transferred to culture dishes and incubated for 2 hrs at 37° C. Cells were re-extracted to remove possible contaminants, including macrophages. Dendritic cells were pulsed with androgen responsive and androgen unresponsive cells at the calculated optimal cell and lysate protein concentration of (500 µg /ml) overnight.

**Immunization of C57BL/ 6J Mice:** Five experimental groups each containing three mice of wild type C57BL/6J background were immunized subcutaneously with TAA pulsed dendritic cells for one week. Group one was pulsed with PC3 tumor lysate. Group two were pulsed with DU145 tumor lysate. Group three were pulsed with LNCaP tumor lysate. Group four were pulsed with lysates from normal prostate epithelial. Group five served as positive control.

**T Lymphocyte Isolation:** Briefly, the spleen from wild-type mice previously immunized intramuscularly with tumor lysates were harvested. T cells were isolated and purified using the nylon wool column method. Purified T cells were then co-cultured with UV-irradiated splenocytes (used as feeder cells), normal prostate epithelial, PC3 and DU145 and LNCaP prostate tumor cell lysate at rate of 2x 10^5 cells/ml for 5 days at 37° C in a 5% CO₂ incubator. Supernatants and pellets were collected by centrifugation and stored at -80° C until needed.
CHAPTER 4
RESULTS

Pro-inflammatory responses are down regulated prostate cancer cells.

Qualitative PCR analysis of Th1 pro-inflammation specific chemokine IP-10 (CXCL10) was observed to be significantly down regulated in prostate cancer cells DU145, PC3 and LNCaP (Fig. 5). Furthermore, ELISA measurements for IFN-γ and IP-10 secretion revealed an almost 10 fold and 5-fold decrease respectively in these prostate cancer cells when compared immortalized normal epithelial cells (Fig 6 and 7). Interestingly, the mitogen PMA caused a significant increase in the chemokine IP-10 that is known to have anti-proliferative effects (Nagpal et al., 2006) (Fig. 8). This highlights the role of IP-10 in mediating anti-proliferative and angiostatic events in cancer cells. Additionally, qualitative PCR analysis showed that there were no significant differences in mRNA expression of pro-inflammatory chemokine and angiostatic MIG in prostate cancer cell lines DU145, PC3, and LNCaP compared to I-PrEC (Fig. 8). MIG may be less important as an antagonist to angiogenic events in prostate cancer cells.

IP-10 down regulation suggests that prostate cancer cells are less likely to recruit cytotoxic T cells expressing cognate receptor CXCR3. Moreover, the down regulation of IFN-γ may enhance the survival and growth in these cancer cells.
Figure 4. Qualitative analysis of IP-10 mRNA Expression. Shown is qualitative analysis of PCR products run on 2.5% agarose gel and visualized using ethidium bromide under UV light. Lane 1; PZ-HPV-7 Immortalized prostate epithelial cells (I-PrEC): Lane 2; DU145, Lane 3: PC3 and Lane 4: LNCaP prostate cancer cell lines.

Figure 5. IP-10 secretion in prostate cancer cells. Shown are ELISA measurements of IP-10 in prostate cancer cell lines: DU145, PC3 and LNCaP compared to I-PrEC (P <0.0001). Data analyzed by Graphpad Software, San Diego, CA. Standard error bars represent ± SEM of triplicate samples.
Figure 6. IFN-\(\gamma\) secretion in prostate cancer cells. Shown are ELISA measurements of IFN-\(\gamma\) in prostate cancer cell lines: DU145, PC3 and LNCaP compared to I-PrEC (\(P <0.0001\)). Data analyzed by Graphpad Software, San Diego, CA. Standard error bars represent \(\pm\) SEM of triplicate samples.
Figure 7. Mitogen regulation of chemokine IP-10 in LNCaP prostate cancer cells. The level of IP-10 induction was significantly higher in PMA treated LNCaP cells. Two tailed t test revealed ($P < 0.0001$) which was considered statistically significant. Data were analyzed using Graphpad Software, San Diego, CA.
Figure 8. Qualitative analysis MIG mRNA expression in prostate cancer cells. Shown are PCR products run on 2.5 % agarose gel and visualized with ethidium staining under UV light. Lane 1: PZ-HPV-7 Immortalized prostate epithelial cells, Lane 2: DU145, Lane 3: PC3 and Lane 4: LNCaP.
Anti-inflammatory responses in prostate cancer cell lines

Qualitative PCR was performed to evaluate the level mRNA expression of Th2 chemokine TARC and its cognate receptor CCR4 in prostate tumor cell lines DU145, PC3, and LNCaP. Results indicated CCL17 and its receptor CCR4 were up regulated in DU145 and PC3 as compared to LNCaP prostate cancer cells and I-PrEC (Fig. 9). Qualitative PCR analysis of mRNA expression also revealed that anti-inflammatory cytokines IL-4 and IL-10 mRNA were also expressed in these prostate cancer cells (Fig. 10).

![Figure 9. Qualitative analysis of chemokine TARC and cognate receptor CCR4 mRNA. Shown is the qualitative analysis of TARC and CCR4 mRNA in prostate cancer cells. PCR products were resolved on 2.5% agarose gel stained with ethidium bromide and visualized under UV light. Lane 1: PZ-HPV-7 immortalized prostate epithelial cells, Lane 2: DU145, Lane 3: PC3 and Lane 4: LNCaP prostate cancer cells. GAPDH used as an internal control.](image)
Figure 10. Qualitative analysis of cytokines IL-4 and IL-10 mRNA expression in prostate cancer cells. Shown is the qualitative PCR analysis of cytokines IL-4 and IL-10 mRNA. PCR products were resolved on 2.5% agarose gel stained with ethidium bromide and visualized under UV light. Lane 1: PZ-HPV-7 immortalized prostate epithelial cells, Lane 2: DU145, Lane 3: PC3 and Lane 4: LNCaP prostate tumor cells. GAPDH used as an internal control.
Differential expression of anti-inflammatory cytokine and chemokine mRNA in prostate cancer cells

Quantitative real time PCR was used to determine differential mRNA expression in prostate cancer cells DU145, PC3 and LNCaP. Results indicated that anti-inflammatory chemokine CCL17 and its receptor CCR4, as well as anti-inflammatory cytokines IL-4 and IL-10 were up regulated in expressed in the prostate cancer cell lines (Fig. 11). The over-expression of anti-inflammatory cytokines and chemokines in androgen unresponsive prostate cancer cell lines DU145 and PC3, suggest degree of aggressiveness and a possible mechanism to evade host immune responses.
Figure 11. Quantitative analysis of anti-inflammatory cytokine and chemokine mRNA in prostate cancer cells. Shown are the differential expressions of increase fold change mRNA in cytokines IL-4, IL-10 and; chemokine CCL17 (TARC), and cognate receptor CCR4, in prostate cancer cells, DU145, PC3, and LNCaP compared to PZ-HPV-7 immortalized normal prostate epithelial cells (I-PrEC).
DU145 and PC3 Prostate Cancer Cells Secrete Higher levels of IL-10

Multiplex ELISA performed to quantitate the levels of IL-4, IL-10 and TARC secreted, revealed that there was a statistically significant increase of IL-10 secretion in DU145 and PC3, (P < 0.0001) when compared to LNCaP prostate cancer cells and I-PrEC (Fig. 12). However, IL-4 and TARC secretion in prostate cancer cells were not significant when compared to I-PrEC. High levels of IL-10 in DU145 and PC3 may also indicate degree of aggressiveness.

Figure 12. IL-10 secretion in prostate cancer cells. IL-10 secretion in DU145 and PC3 prostate tumor cells was statistically significant (P <0.001) when compared LNCaP prostate cancer cell and I-PrEC cells. Data was analyzed by (Graphpad Software, San Diego, CA). Standard error bars represent ± SEM of triplicate samples.
T cell Chemokine Expression upon Stimulation by Pulsed Dendritic Cells (DCs)

Bone marrow derived dendritic cells cultured ex-vivo and pulsed with tumor antigens were used to stimulate T cells. Results from qualitative and quantitative mRNA of Th1 pro-inflammatory chemokine, IP-10 and its receptor CXCR3 (Fig. 13) showed that IP-10 and its receptor are expressed in T cells. Figs. 14 and 15 are results from quantitative PCR analysis to show the differential expression of chemokine IP-10 mRNA and cognate receptor CXCR3 respectively, among the five experimental groups shown in columns 2 – 6, compared to naïve or non-stimulated T cells. Qualitative PCR analysis revealed that anti-inflammatory chemokine TARC mRNA and its cognate receptor CCR4 (Fig. 16) were also expressed in T cells.

The induction of a robust and pre-dominant pro-inflammatory response was not observed in T cells stimulated with DCs pulsed with prostate tumor associated antigens. Differences among the five experimental groups, as indicated by quantitative mRNA expression of chemokine IP-10 (Fig. 14) and TARC (Fig. 17), were not statistically significant. A similar observation was seen in the quantitative mRNA expression of chemokine receptors CXCR3 (Fig. 15) and CCR4 (Fig. 18). Nevertheless, similar chemokine expression profiles seen in prostate cancer cells and T cells, suggest that they may interact in-vivo in a paracrine manner.
Figure 13. Chemokine IP-10 mRNA and cognate receptor CXCR3 mRNA expression in mice T cells. Qualitative analysis shows PCR products that were resolved on 2.5% agarose gel containing ethidium bromide and visualized under UV light. Lane 1: DCs only, Lane 2: DCs w/ PC3 TAAs, Lane 3: DC w/ DU145 TAAs; Lane 4 DCs w/ LNCaP TAAs, and Lane 5: DCs w/ I-PrEC As. Beta Actin used as an internal control.
Figure 14. Differential expression of chemokine IP-10 mRNA in mice T cells. Shown is the quantitative analysis of fold change increase in chemokine IP-10 mRNA in mice T cells stimulated with dendritic cells (DCs). Column 1 represents non-stimulated T cells. Columns 2 - 6 represent T cells stimulated with dendritic cells. *- Represent T cells stimulated with DCs pulsed with tumor associated antigens (TAAs).
Figure 15. Differential expression of chemokine receptor CXCR3 mRNA in mice T cells. Shown is quantitative analysis of chemokine CXCR3 mRNA in T cells stimulated with dendritic cells (DCs). Column 1 represents non-stimulated T cells. Columns 2 - 6 represent T cells stimulated with dendritic cells. * represents T cells stimulated with DCs pulsed with tumor associated antigens (TAAs).
Figure 16. Qualitative analysis of chemokine TARC and cognate receptor CCR4 mRNA in mice T cells. PCR products were resolved on 2.5% agarose gel containing ethidium bromide and visualized under UV light. Lane 1: DCs only; Lane 2: DCs w/PC3 TAAs, Lane 3: DC w/DU145 TAAs, Lane 4: DCs w/LNCaP TAAs, and Lane 5: DCs w/I-PrEC TAAs.
Figure 17. Differential expression of chemokine TARC mRNA. Shown is the quantitative analysis fold change increase in chemokine TARC in T cells stimulated with pulse dendritic cells. Column 1, represents non-stimulated T cells. Columns 2 – 6, represent T cells stimulated with dendritic cells (DCs). *- represents T cells stimulated with DCs pulsed with tumor associated antigens (TAAs).
Figure 18. Differential expression of chemokine receptor CCR4 mRNA in mice T cells. Shown quantitative analysis of fold change increase in chemokine receptor CCR4 mRNA in T cells stimulated with pulse dendritic cells (DCs). Column 1 represents non-stimulated T cells. Columns 2 - 6 represent T cells stimulated with dendritic cells. *-represents T cells stimulated with DCs pulsed with tumor associated antigens (TAAs).
T lymphocyte Cytokines Expression upon Stimulation by Pulsed Dendritic Cell (DCs)

Results from qualitative and quantitative PCR analysis showed that pro-inflammatory cytokine IFN-γ mRNA (Figs. 19 and 20). Anti-inflammatory cytokine IL-10 mRNA expression was also observed (Figs. 21 and 22). In line with the preceding observations of chemokine profiles in T cells, a more robust pro-inflammatory Th1-driven response was not potentiated by TAA-pulsed dendritic cells. Nevertheless, quantitative PCR showed that IFN-γ mRNA expression was significantly up regulated (P< 0.05) in T cells stimulated with dendritic cells compared to naïve T cells (Fig. 20). This highlights the important role of antigen presenting cells in initiating T cell responses.
Figure 19. Qualitative analysis of IFN-γ cytokine mRNA expression in mice T cells. Shown are the PCR products of cytokine IFN-γ mRNA in mice T cells stimulated with dendritic cells. PCR products were resolved on 2.5% agarose gel and visualized with ethidium bromide under UV light. Lane 1: DC only; Lane 2: DC w/ PC3 TAA; Lane 3: DC w/ DU145 TAA; and Lane 5: I-PrEC.
Figure 20. Differential expression cytokine IFN-γ mRNA in mice T cells. Shown is quantitative analysis of fold change increase in cytokine IFN-γ in T cells stimulated with dendritic cells. Column 1 represents non-stimulated T cells. Columns 2 - 6 represent T cells stimulated with dendritic cells. *- represents T cells stimulated with DCs pulsed with tumor-associated antigens (TAAs).
Figure 21. Cytokine IL-10 mRNA expression in mice T cells. Shown is Qualitative analysis of PCR of cytokine IL-10 mRNA in mice T cells stimulated with dendritic cells. PCR products were resolved on 2.5% agarose gel and visualized with ethidium bromide under UV light. Lane 1: DC only; Lane 2: DC w/ PC3 TAAAs, Lane 3: DC w/ DU145 TAAAs, and Lane 5: I-PrEC
Figure 22. Differential expression of cytokine IL-10 mRNA in mice T cells. Shown is the quantitative analysis of fold change increase in cytokine IL-10 mRNA in T cells stimulated with pulse dendritic cells. Column 1 represents non-stimulated T cells. Columns 2 - 6 represent T cells stimulated with dendritic cells. *- represent T cells stimulated with DCs pulsed with tumor-associated antigens (TAAs).
CHAPTER 5

DISCUSSION

Cytokines, chemokines, and chemokine receptors are essential immune effector molecules that play important regulatory roles in immune responses (Grutz, 2005). Additionally, chemokine and chemokine receptor interactions mediate the trafficking and migration of immune cells and non-immune cells including cancers (Tanaka et al., 2005). The hypothesis that prostate cancer cell lines differentially expressed anti-inflammatory and pro-inflammatory cytokines and chemokines was supported by our findings. We demonstrated that the androgen unresponsive prostate cancer cell lines DU145 and PC3 expressed anti-inflammatory cytokines IL-4 and IL-10, the anti-inflammatory chemokine TARC and chemokine cognate receptor CCR4 compared to androgen responsive LNCaP prostate cancer cells. Comparative protein analysis revealed that pro-inflammatory cytokine IFN-γ and the pro-inflammatory chemokine IP-10 were significantly down regulated in prostate cancer cells DU145, PC3, and LNCaP compared to the immortalized normal prostate epithelial cell line, PZ-HPV-7 used in this study.

Implications of Th2 driven anti-inflammatory cytokine and chemokine production in prostate cancer cells

The roles of anti-inflammatory cytokines and chemokines in immunity and cancer progression have been well documented (Sharma, et al., 1999; Grutz, 2005;
Moore et al., 1999). The up regulation of the Th2-driven anti-inflammatory cytokine IL-10 suggests a possible mechanism for prostate cancer to evade host immune responses. This suggestion is plausible, because high levels of IL-10 have been reported in patients with melanoma, ovarian and colon cancers (Elkord, 2007).

Moreover, increased serum levels of IL-10 have been observed in patients with breast cancer compared to healthy women (Chavey et al., 2007). Sharma et al., 1999 reported that IL-10 had the ability to inhibit a wide array of pro-inflammatory effector molecules and also, that elevated levels may negatively modulate antigen presentation thus enhancing the evasiveness of tumors. Indeed, Sharma et al., 1999 demonstrated that readily induced and augmented levels of IL-10 were produced by T lymphocytes in IL-10 transgenic mice. The group also demonstrated that Lewis lung carcinoma cells were able to potently induced T lymphocyte IL-10 production in vitro and had a more aggressive growth potential in IL-10 transgenic mice compared to control littermates. In vivo IL-10 is also known to play a vital role in the induction of CD4⁺CD25⁺CCR4⁺T regulatory cells (Ghiringhelli et al., 2005). T regulatory cells are important immune regulators because of the suppressive effects on the immune system. They exert their suppressive effects by secreting in an autocrine manner of IL-10 and TGF-β (Miller et al., 2006). Miller at al., 2006 also reported that significant levels of T regulatory cells were detected human cancer specimens. Ghiringhelli et al., 2005 also reported similar observations in rodent models of cancer. The importance of immunosuppressive effects of IL-10 is further exemplified by the impact that they have on antigen presentation. Antigen presenting cells such as dendritic cells (DCs) are potent antigen presenting cells (Igietseme et al., 2000) and
are impacted negatively by IL-10 (Mcbride et al., 2002). In one study, IL-10 treated dendritic cells were capable of inducing anergy in melanoma specific CD8\(^+\) T cells (Gerlini et al., 2004). Sharma et al., 1999 reported that APCs from IL-10 transgenic mice had significantly suppressed capacity to up regulate MHC, cytolytic responses and IL-12 production that are necessary for activating T cells. Miller et al., 2006 further demonstrated by using 5-bromo-2'-deoxyuridine (BrdU) incorporation assay, they were able to show that T cell function can be compromised by prostate cancer tissue supernatants because they significantly inhibited T cells proliferation. The up regulation of immunosuppressive cytokine such as IL-10 therefore may have important implications in cancer immunotherapy.

Although high levels of the anti-inflammatory cytokine IL-4 have been reported in many advanced tumors including prostate cancer tissues (Concetta et al., 2004), we did not detect high levels of IL-4 the prostate cancer cell lines used in this study.

We also demonstrated that unlike anti-inflammatory responses, pro-inflammatory responses such as cytokine IFN-\(\gamma\) and chemokine IP-10 were significantly down regulated. These effector molecules are important in mediating anti-tumor responses. The role of IFN-\(\gamma\) as an anti-tumor mediator is supported by a study done by Frederick et al., 2001 to show that IFN-\(\gamma\) inhibited the growth of PC3 and DU145 prostate cancer cell lines in a dose dependent manner. Additionally, IFN-\(\gamma\) has been shown to have direct cytotoxic effects on squamous cell carcinoma of head and neck cancers in-vitro and can enhance cell-mediated responses to head and neck cancers (Quinn, 1998). It has also been demonstrated that mice that lack IFN-\(\gamma\) or
IFN-γ receptors developed tumors more rapidly upon exposure to carcinogens (Elkord, 2007).

Pro-inflammatory chemokine IP-10 down regulation in prostate cancer cells suggests that prostate cancer cells are likely to promote angiogenesis, by negatively modulating IP-10 production. This observation is in line with Sato et al., 2007 who reported that the levels of IP-10 decreased with the advancement of uterine cervical cancers where as, the levels of angiogenic chemokine VEGF were high and the prognosis of the cancer with low IP-10 expression was poor. Moreover, they would be less likely to recruit T cytotoxic cells bearing the cognate receptor CXCR3 which are the main effector cells that are involved in the targeted killing of cancer cells (Nouri-shirasi et al., 2000; Arenberg et al., 1996). This correlates with Mullins et al., 2004, who reported that the expression of CXCR3, the receptor for IP-10 by activated T cells, correlated with greater survival of patients with stage III melanoma cancer. A similar mechanism of IP-10 regulation presumably, will destroy prostate tumors and reduce their progression. *In-vivo*, IP-10 is known to preferentially target natural killer cells and will activate cells of the Th1 phenotype through the CXCR3 receptor (Nagaoka et al., 2002). Sato et al., 2007 concluded that IP-10 could be used as a prognostic biomarker for disease progression.

An interesting finding was that, Phorbol-12- Myristate-13 Acetate (PMA), a potent mitogen that activates the PKC signal transduction pathway (Gonzalez, G et al., 2005), caused significant up regulation of interferon inducible protein IP-10 in androgen responsive LNCaP prostate cancer cells. The over expression of IP-10 has been shown to have anti-proliferative effects and decreased PSA production in LNCaP
prostate cancer cells (Nagpal et al., 2006). Gonzalez, et al., 2005, showed that PMA mediated PKC pathway, specifically the PKC isozyme PKC δ, induced apoptosis in LNCaP prostate cancer cells. There is a strong possibility, that PMA mediated apoptosis in LNCaP is via induction of IP-10. IP-10 induction has also been reported to up regulate transcription factor and tumor suppressor gene p53, known to be important in cell cycle arrest and apoptosis (Zhang et al., 2005). The effects of PMA on the up regulation of cytokines IFN-γ, IL-4 and IL-10 as well as chemokine were not significant. From all indications, IP-10 could be used as a potential therapeutic to interfere with prostate cancer progression.

Through gene expression studies, we were also able to demonstrate that Th2 anti-inflammatory chemokine TARC and cognate receptor CCR4 were up regulated in androgen unresponsive prostate cancer cell lines DU145 and PC3. This autocrine regulation, presumably indicates degree of aggressiveness because the aggressiveness of cancers is often characterized by their resistance or unresponsiveness to androgen ablation therapy (Elkord, 2007). Miller et al., 2006 showed that ELISA analysis of culture supernatants of LNCaP cells, secreted low levels of chemokine CCL22 or MDC (0.12 ± 0.05 ng/ml) which share the same receptor CCR4 as TARC (Charo et al., 2006). Higher levels were detected in malignant prostate tissue.

**Dendritic Cells as Immunotherapeutic Adjuvants**

The *in-vivo* studies conducted to test the hypothesis that prostate tumor antigen pulsed dendritic cells would influence the profile of cytokines, chemokines and their receptors, revealed that dendritic cells were able to activate T cells, but we did not
observe a pre-dominant pro-inflammatory response indicative of the upregulation of
cytokine IFN-γ and chemokine IP-10, as well as the chemokine receptor CXCR3
which usually indicates presence of activated T killer cells (Tannenbaum et al., 1998).
Indeed our gene expression studies detected elevated levels of anti-inflammatory
cytokine IL-10 mRNA and anti-inflammatory chemokine TARC mRNA, as well as its
cognate receptor CCR4. These observations did not support the hypothesis that TAAs
pulsed dendritic cells would potentiate a more robust pro-inflammatory response.
Limitations to this particular study could be the antigenic dosage used, the efficiency
of antigenic uptake by dendritic cells and the presence of endogenous immune
suppressive factors. Indeed, the mixed Th1-pro-inflammatory and Th2- anti-
-inflammatory cytokine and chemokine profiles could be attributed to the recruitment
of the CCR4+ Treg subset of T cells. Since mature dendritic cells are known to express
the TARC (Charo et al., 2006), it is possible that they may have influenced the
recruitment of this distinct subset of T cells. Troy, A et al., 1998, demonstrated that
dendritic cells that have been identified in prostate tissue could induce the formation
of T regulatory cells. Nevertheless, the similar cytokine and chemokine profiles seen
in prostate cancer cells and T cells, suggests that they can interact in-vivo in a
paracrine manner.

Taken together, the immune response to cancer can often be compromised by
the immune suppressive effects of anti-inflammatory cytokines and chemokines.
Additionally, the inadequacy of dendritic cells due to the immune suppressive effects
of cancer has been well documented to be one of the main reasons for ineffective anti-
tumor responses (Sharma et al., 1999).
CHAPTER 6

CONCLUSION

Prostate cancer cells DU145, PC3 and LNCaP were shown to differentially express anti-inflammatory and pro-inflammatory cytokines and chemokines. The down regulation of key mediators involved in anti-tumor responses, IFN-γ and IP-10 in these prostate cancer cell lines and concomitant increase in IL-10 and TARC suggest that prostate cancer cells take advantage of the anti-inflammatory responses that are often immune suppressive, to negatively modulate functions that are necessary for the killing cancer cells. The similar cytokine and chemokine expression profiles in prostate cancer cells and T cells, gives us reason to believe that there are paracrine interactions involved in immunity against prostate cancer.

Ongoing studies demonstrate that dendritic cells are a potential vehicle to deliver specific target antigens to T cells, specifically those involved in anti-tumor responses. Dendritic cell biology is also important in the context of cancer immunology since its functions are known to be severely compromised by IL-10. The influence of paracrine and autocrine interactions between T cells, dendritic cells and prostate cancer cell lines and their effects on migration and survival of prostate cancers, should be further investigated. Cytokine and chemokine profiles could be used as prognostic markers for prostate cancer progression.
REFERENCES


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