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Immuno-oncology of human prostate cancer : phenotypical characterization and study of the tumor-derived, androgen-regulated immunosuppressive microenvironment

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RÉSUMÉ

Le cancer de la prostate est le cancer le plus fréquemment diagnostiqué chez les hommes canadiens et la troisième cause de décès relié au cancer. Lorsque diagnostiqué à un stade précoce de la maladie, le cancer de la prostate est traité de manière curative par chirurgie et radiothérapie. Par contre, les thérapies actuelles ne peuvent éradiquer la maladie lorsqu'elle progresse à des stades avancés. Ces thérapies, comme la chimiothérapie et l'hormonothérapie, demeurent donc palliatives. Il est primordial d'optimiser de nouvelles thérapies visant l'élimination des cellules cancéreuses chez les patients atteints des stades avancés de la maladie. Une de ces nouvelles options thérapeutiques est l'immunothérapie.

L'immunothérapie du cancer a fait des progrès considérables durant les dernières années. Cependant, les avancements encourageants obtenus lors d'essais précliniques ne se sont pas encore traduits en des résultats cliniques significatifs. En ce qui concerne le cancer de la prostate, les résultats négligeables suivants des interventions immunothérapeutiques peuvent être causés par le fait que la plupart des études sur le microenvironnement immunologique furent effectuées chez des modèles animaux. De plus la majorité des études sur l'immunologie tumorale humaine furent effectuées chez des patients atteints d'autres cancers, tels que le mélanome, et non chez les patients atteints du cancer de la prostate. Donc, le but central de cette thèse de doctorat est d'étudier le microenvironnement immunologique chez les patients atteints du cancer de la prostate afin de mieux définir les impacts de la tumeur sur le développement de la réponse immunitaire antitumorale. Pour réaliser ce projet, nous avons établi deux principaux objectifs de travail : (i) la caractérisation précise des populations des cellules immunitaires infiltrant la tumeur primaire et les ganglions métastatiques chez les patients atteints du cancer de la prostate; (ii) l'identification et l'étude des mécanismes immunosuppressifs exprimés par les cellules cancéreuses de la prostate. Les résultats présentés dans cette thèse démontrent que la progression du cancer de la prostate est associée au développement d'un microenvironnement immunosuppressif qui, en partie, est régulé par la présence des androgènes.

L'étude initiale avait comme but la caractérisation du microenvironnement immunologique des ganglions drainant la tumeur chez des patients du cancer de la prostate. Les résultats présentés dans le chapitre III nous a permis de démontrer que les ganglions métastatiques comportent des signes cellulaires et histopathologiques associés à une faible réactivité immunologique. Cette immunosuppression ganglionnaire semble dépendre de la présence des cellules métastatiques puisque des différences immunologiques notables existent entre les ganglions non-métastatiques et métastatiques chez un même patient. La progression du cancer de la prostate semble donc associée au développement d'une immunosuppression affectant les ganglions drainant la tumeur primaire.

Par la suite, nous nous sommes intéressés à l'impact de la thérapie par déplétion des androgènes (TDA) sur le microenvironnement immunologique de la tumeur primaire. La TDA est associée à une augmentation marquée de l'inflammation prostatique. De plus, les protocoles d'immunothérapies pour le cancer de la prostate actuellement évalués en phase clinique sont dirigés aux patients hormonoréfractaires ayant subi et échoué la thérapie. Cependant, peu d'information existe sur la nature de l'infiltrat de cellules immunes chez les patients castrés. Il est donc essentiel de connaître la nature de cet infiltrat afin de savoir si celui-ci peut répondre de manière favorable à une intervention immunothérapeutique. Dans le chapitre IV, je présente les résultats sur l'abondance des cellules immunes infiltrant la tumeur primaire suivant la TDA. Chez les patients castrés, les densités de lymphocytes T $CD3^+$ et $CD8^+$ ainsi que des macrophages $CD68^+$ sont plus importantes que chez les patients contrôles. Nous avons également observé une corrélation entre la densité de cellules NK et une diminution du risque de progression de la maladie (rechute biochimique). Inversement, une forte infiltration de macrophages est associée à un plus haut risque de progression. Conjointement, durant cette étude, nous avons développé une nouvelle approche informatisée permettant la standardisation de la quantification de l'infiltrat de cellules immunes dans les échantillons pathologiques. Cette approche facilitera la comparaison d'études indépendantes sur la densité de l'infiltrat immun. Ces résultats nous ont donc permis de confirmer que les effets pro-inflammatoires de la TDA chez les patients du cancer de la prostate ciblaient spécifiquement les lymphocytes T et les macrophages.

L'hypothèse intéressante découlant de cette étude est que les androgènes pourraient réguler l'expression de mécanismes immunosuppressifs dans la tumeur primaire.

Dans le chapitre V, nous avons donc étudié l'expression de mécanismes immunosuppressifs par les cellules cancéreuses du cancer de la prostate ainsi que leur régulation par les androgènes. Notre analyse démontre que les androgènes augmentent l'expression de molécules à propriétés immunosuppressives telles que l'arginase I et l'arginase II. Cette surexpression dépend de l'activité du récepteur aux androgènes. Chez les patients castrés, l'expression de l'arginase II était diminuée suggérant une régulation androgénique *in vivo*. Nous avons observé que l'arginase I et l'arginase II participent à la prolifération des cellules du cancer de la prostate ainsi qu'à leur potentiel immunosuppressif. Finalement, nous avons découvert que l'expression de l'interleukin-8 était aussi régulée par les androgènes. De plus, l'interleukin-8, indépendamment des androgènes, augmente l'expression de l'arginase II. Ces résultats confirment que les androgènes participent au développement d'une microenvironnement immunosuppressif dans le cancer de la prostate en régulant l'expression de l'arginase I, l'arginase II et l'interleukin-8.

En conclusion, les résultats présentés dans cette thèse témoignent du caractère unique du microenvironnement immunologique chez les patients atteints du cancer de la prostate. Nos travaux ont également permis d'établir de nouvelles techniques basées sur des logiciels d'analyse d'image afin de mieux comprendre le dialogue entre la tumeur et le système immunitaire chez les patients. Approfondir les connaissances sur les mécanismes de régulation du microenvironnement immunologique chez les patients atteint du cancer de la prostate permettra d'optimiser des immunothérapies mieux adaptées à éradiquer cette maladie.

MOTS CLÉS

Cancer de la prostate; Immunologie; Immunosuppression; Androgène; Arginase; Immunohistochimie

SUMMARY

Prostate cancer is the most frequently diagnosed cancer in Canadian men and the third cause of cancer related death. When diagnosed at an early stage, prostate cancer can be effectively cured by surgery and radiotherapy. However, current therapies do not eradicate the advanced stages of the disease. Treatment of prostate cancer via chemotherapy or hormonotherapy remains palliative. It is thus essential to optimize novel therapies whose goal is to eliminate tumor cells in patients with advanced prostate cancer. One such approach is immunotherapy.

Cancer immunotherapy has made important strides in recent years. The encouraging progress observed in pre-clinical trials has nonetheless not translated to significant results in the clinical setting. Concerning prostate cancer, the limited clinical efficacy of current immunotherapeutic protocols could be explained by the lack of studies directly evaluating the immunological microenvironment in prostate cancer patients and not in animal models or in patients afflicted by other malignancies, such as melanoma. Thus, the fundamental goal of this Ph.D. thesis is to study the immunological microenvironment in prostate cancer patients in order to better understand the impact of the tumor on the development of the anti-tumoral immune response. To realize this project, we established two main working objectives: (i) to precisely characterize the immune cell populations in tumor draining lymph nodes (LNs) and in the primary tumor of prostate cancer patients; (ii) to identify and to study the immunosuppressive pathways expressed by prostate cancer cells. The results detailed in this thesis demonstrate that prostate cancer progression is associated with the development of an immunosuppressive microenvironment, which is regulated, in part, by the presence of androgens.

The initial study was based on the characterization of the immunological microenvironment of tumor draining LNs of prostate cancer patients. The results presented in chapter III allowed us to demonstrate that metastatic lymph nodes displayed cellular and histopathological evidence associated with a reduced immunological reactivity. This LN immunosuppression seemed to be dependant on

the presence of metastatic cells as we noted significant immunological differences between non-metastatic and metastatic lymph nodes of the same patient. Prostate cancer progression was thus associated with the development of an immunosuppressive state, which affected tumor-draining lymph nodes.

Next, we studied the impact of androgen deprivation therapy (ADT) on the immunological microenvironment of the primary tumor. Following ADT, there is a marked augmentation in intra-prostatic inflammation. Immunotherapeutic protocols currently evaluated in clinical trials are targeted at hormone refractory patients, which have received and failed ADT. However, very little information is available regarding the nature of the post-ADT immune infiltrate. Thus, it becomes essential to understand whether this post-ADT infiltrate could positively react to immunotherapy. In chapter IV, I present the results of the quantification of the immune cell abundance within the primary tumor. In patients who have received ADT prior to surgery, there was an elevated density of $CD3^+$ and $CD8^+$ T lymphocytes as well as $CD68^+$ macrophages compared to control patients. We also observed an inverse correlation between the NK cell density and the risk of disease progression (biochemical recurrence). Conversely, an elevated macrophage infiltration was associated with a higher risk of progression. Furthermore, for this study, we developed a novel computerized approach allowing for the standardization of the quantification of immune cell infiltrate. This approach could facilitate the interpretation of results from independent studies on the density of immune cells within pathological specimens. This study confirmed that the pro-inflammatory impact of androgen deprivation therapy in prostate cancer patients target specifically the T lymphocyte and macrophage populations. The interesting hypothesis arising from this study was that androgens could positively regulate the expression of immunosuppressive pathways within the primary tumor.

In chapter V, we evaluated the immunosuppressive mechanisms expressed by prostate cancer cells and regulated by androgens. Our analysis demonstrate that androgens increase the expression of molecules with immunosuppressive properties, such as arginase I and arginase II in an androgen receptor dependent manner. This androgen regulated expression of arginase II was also observed in prostate cancer

patients treated by ATD. We observed that arginase I and arginase II participate in prostate cancer cell proliferation as well as in their immunosuppressive potential. Finally, we discovered that interleukin-8 expression was also regulated by androgens. Moreover, interleukin-8, independently of androgens, increased the expression of arginase II. Altogether, these results confirmed that androgens participate in the development of an immunosuppressive microenvironment in prostate cancer by regulating the expression of arginase I, arginase II and interlukin-8.

In conclusion, the results presented in this thesis attest to the unique character of the immunological microenvironment in prostate cancer patients. Our work has also allowed to establish novel software-based analysis methods in order to better understand the dialogue between the tumor and the immune system. Further understanding of the regulatory pathways involved in the immunological microenvironment will allow for the optimization of immunotherapies better suited to eradicate prostate cancer.

KEY WORDS

Prostate cancer; Immunology; Immunosuppression; Androgen; Arginase; Immunohistochemistry

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LIST OF ABBREVIATIONS

ACN	Acetonitrile
ADC	Arginine Decarboxylase
ADCC	Antibody-Dependent Cell Cytotoxicity
ADT	Androgen Deprivation Therapy
AGAT	Arginine:Glycine Amidinotransferase
APC	Antigen-Presenting Cell
apc	adenomatosis polyposis coli
AR	Androgen Receptor
ARE	Androgen Receptor Response Element
ARG1	Arginase I
ARG2	Arginase II
ASL	Argininosuccinate Lyase
ASS	Argininosuccinate Synthase
BCR	B Cell Receptor
BPH	Benign Prostatic Hyperplasia
BRCA2	Breast Cancer Type 2 Susceptibility Protein
BrdU	Bromodeoxyuridine
cAMP	Cyclic Adenosine Monophosphate
CAPB	Cancer Prostate and Brain
CAT	Cationic Amino Acid Transporters
CD40L	CD40 ligand
CDK4	Cyclin-Dependent Kinase 4
cGMP	Cyclic Guanosine Monophosphate
CMV	Cytomegalovirus
COX-2	Cyclooxygenase-2
cPGES	cytosolic Prostaglandin E_2 Synthase
CTL	Cytotoxic T Lymphocyte
CTLA-4	Cytotoxic T Lymphocyte-Associated Antigen 4

DAB	3,3'-Diaminobenzidine
DC	Dendritic Cell
DHT	Dihydrotestosterone
EAE	Experimental Autoimmune Encephalomyelitis
eIF2	Eukaryotic Initiation Factor 2
ELAC2	Elac Homolog 2 (Escherichia Coli)
ELISA	Enzyme-Linked Immunosorbent Assay
eNOS	Endothelial Nitric Oxide Synthase
ER	Estrogen Receptor
ERE	Estrogen Receptor Responsive Elements
FasL	Fas Ligand
FFPE	Formalin-Fixed Paraffin-Embedded
Foxp3	Forkhead Box P3
GCN2	General Control Non-Derepressible-2
GITR	Glucocorticoid-Induced Tumor Necrosis Factor Receptor Family Related Protein
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GnRH	Gonadotropin-Releasing Hormone
GPCR	G-Protein-Coupled Receptor
HA	Hemagglutinin
H&E	Hematoxylin and Eosin
H_2O_2	Hydrogen Peroxide
HHV8	Human Herpes Virus Type 8
HPC-1	Hereditary Prostate Cancer Locus On Chromosome-1
HPC-2	Hereditary Prostate Cancer Locus On Chromosome-2
HPC-X	Hereditary Prostate Cancer Locus On Chromosome-X
HPV	Human Papillomavirus
HRP	Horseradish Peroxidase
HRPC	Hormone Refractory Prostate Cancer
HSV2	Human Herpes Simplex Virus Type 2
HTLV-1	Human T-Cell Leukemia/Lymphoma Virus-1

iAR	Intracytoplasmic Androgen Receptor
IDO	Indoleamine 2,3-Dioxygenase
IFN-γ	Interferon-γ
IL	Interleukin
IMPACT trial	Immunotherapy For Prostate Adenocarcinoma Treatment trial
iNOS	Inducible Nitric Oxide Synthase
IRF3	Interferon Regulatory Factor 3
ITIM	Intracytoplasmic Inhibitory Motif
KAR	Killer-Activating Receptor
KIR	Killer-Inhibitory Receptor
LHRH	Luteinizing Hormone Releasing Hormone
LN	Lymph Node
L-NMMA	N^G-Monomethyl-L-Arginine
LPS	Lipopolysaccharide
MAPK	Mitogen-Activated Protein Kinases
mAR	Membrane Androgen Receptor
MCA	20-Methylchol-Anthrene
MCP-1	Monocyte Chemotactic Protein-1
mDC	Myeloid Dendritic Cell
MDSC	Myeloid-Derived Suppressor Cell
mER	Membrane Estrogen Receptor
MHC-I	Major Histocompatibility Complex Class I
MHC-II	Major Histocompatibility Complex Class II
MIC1	Macrophage Inhibitory Cytokine 1
MICA/B	MHC Class I-Related Chain Molecules A/B Molecule
mPGES-1	membrane PGE_2 Synthase-1
MS	Multiple Sclerosis
MSR1	Macrophage Scavenger Receptor 1
MyD88	Myeloid Differentiation Primary-Response Gene 88
NDA	Naphthalene-2,3-Dicarboxaldehyde

NK cell	Natural Killer cell
NKT cell	Natural Killer T cell
nNOS	Neuronal Nitric Oxide Syntase
NO	Nitric Oxide
NOHA	N-Hydroxy-Nor-L-Arginine
NOS	Nitric Oxide Syntase
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
NSCLC	Non-Small Cell Lung Cancer
OAT	Ornithine Aminotransferase
ODC	Ornithine Decarboxylase
PABPN1	Poly(A) Binding Nuclear Protein 1
PAP	Prostatic Acid Phosphatase
PBMC	Peripheral Blood Mononuclear Cell
PCa	Prostate Cancer
PCAP	Predisposing for Cancer of the Prostate
pDC	Plasmacytoid Dendritic Cell
PGG_2	Prostaglandin G_2
PHA	Phytohemagglutinin
PhIP	2-Amino-1-Methyl-6-Phenylimidazol[4,5-B]Pyridine
PI3K	Phosphatidyl-Inositol 3-Kinase
PIN	Prostatic Intraepithelial Neoplasia
PKA	Protein Kinase A
PKC	Protein Kinase C
PLC	Phospholipase-C
PSA	Prostate-Specific Antigen
PSCA	Prostate Stem Cell Antigen
PSMA	Prostate Specific Membrane Antigen
qPCR	Quantitative Polymerase Chain Reaction
RA	Rheumatoid Arthritis
Rag-2	Recombinase Activating Gene-2
RECIST	Response Evaluation Criteria in Solid Tumors

rf-PSA	Recombinant Fowlpox Virus Expressing PSA
RNASEL	Ribonuclease L
ROS	Reactive Oxygen Species
RRM	RNA-Recognition Motif
rv-PSA	Recombinant Vaccinia Virus Expressing PSA
SCID	Severe Combined Immunodeficiency
SHBG	Sex Hormone Binding Globulin
SLE	Systemic Lupus Erythematosus
STAT1	Signal Transducer and Activator of Transcription-1
TAA	Tumor-Associated Antigen
TAMs	Tumor-Associated Macrophages
TCR	T Cell Receptor
TEAA	Triethylammonium Acetate
TGF-β	Transforming Growth Factor-β
T_H	T Helper lymphocyte
TIL	Tumor-Infiltrating Lymphocyte
TLR	Toll-Like Receptor
TMA	Tissue Microarray
TNF-α	Tumour Necrosis Factor-α
TRAIL	TNF-Related Apoptosis-Inducing Ligand
TRAMP	Transgenic Adenocarcinoma Mouse Prostate
T_{REG}	Regulatory T Cell
TURP	Transurethral Resection of the Prostate
VEGF	Vascular Endothelial Growth Factor

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CHAPTER I

INTRODUCTION

The introductory chapter of this Ph.D. thesis contains five sections. Following a summary of important aspects of prostate cancer, I will describe basic concepts of the immune system and of the current theories regarding the anti-tumoral immune response. Finally, I will review the literature on arginase and on the immunoregulatory properties of sexual hormones.

1.1 PROSTATE CANCER

1.1.1 CANCER STATISTICS

Cancer affected 166,400 Canadians and claimed the lives of 73,800 patients in 2008 (1). According to the current cancer incidence rate, 40% of Canadian women and 45% of Canadian men will develop cancer in their lifetime. In Canada, cancer is the second most common cause of mortality (30.2% of deaths) after circulatory diseases and fourth cause of hospitalization (7.4%) (2).

As for prostate cancer, in 2008, 24,700 Canadian men were diagnosed with the disease and 4,300 patients died from prostate cancer related complications making it the most diagnosed cancer (28.4% of newly diagnosed cancer in men) and third cause of cancer related deaths (11.1% of all male cancer related deaths) (1). Improvements in early detection protocols through the widespread use of prostate-specific antigen (PSA) screening accounts for the continuing increasing numbers of patients diagnosed with prostate cancer, which are now being diagnosed at a younger age and with less aggressive tumors (3). Over 95 % of prostate cancer patients have a relative survival rate exceeding five years and prostate cancer mortality has decreased by 2.9% annually between 1995 and 2004. This decreased in prostate cancer associated mortality is attributed to PSA screening, surgery, higher doses of radiotherapy and earlier onset of androgen deprivation therapy (ADT) (1).

1.1.2 THE PROSTATE GLAND

The prostate is an exocrine gland part of the male reproductive system. The prostate surrounds the urethra below the bladder. Its organogenesis begins at the onset of puberty and is under the control of androgens. In healthy men, the prostate is roughly the size of a walnut. Through the contraction of prostatic muscles during ejaculation, the prostate secretes a milky alkaline fluid that constitutes 25-30% of the semen along with spermatozoa and seminal vesicle fluid. This fluid is primarily composed of sugars and electrolytes (citrate, zinc) (4) with less than 1% being proteins such as proteolytic enzymes, prostatic acid phosphatase (PAP) and PSA. The prostatic fluid protects the genomic material of spermatozoa and promotes their motility and survival by providing the necessary nutrients as well as by regulating the pH of the environment (5).

The prostate contains five distinct glandular zones: central zone, fibromuscular zone, transitional zone, peripheral zone and periurethral zone. There is a distinctive prevalence of prostatic pathologies between the different zones (Figure 1 on page 3). Benign prostatic hyperplasia (BPH) is mostly present within the transition zone. Adenocarcinomas are most often located in the peripheral zone, with few in the transition zone and with rare occurrence in the central zone. The peripheral zone is also more susceptible to inflammation, high-grade prostatic intraepithelial neoplasia (PIN) and more aggressive adenocarcinomas. Adenocarcinomas within the transition zone are generally less aggressive (6).

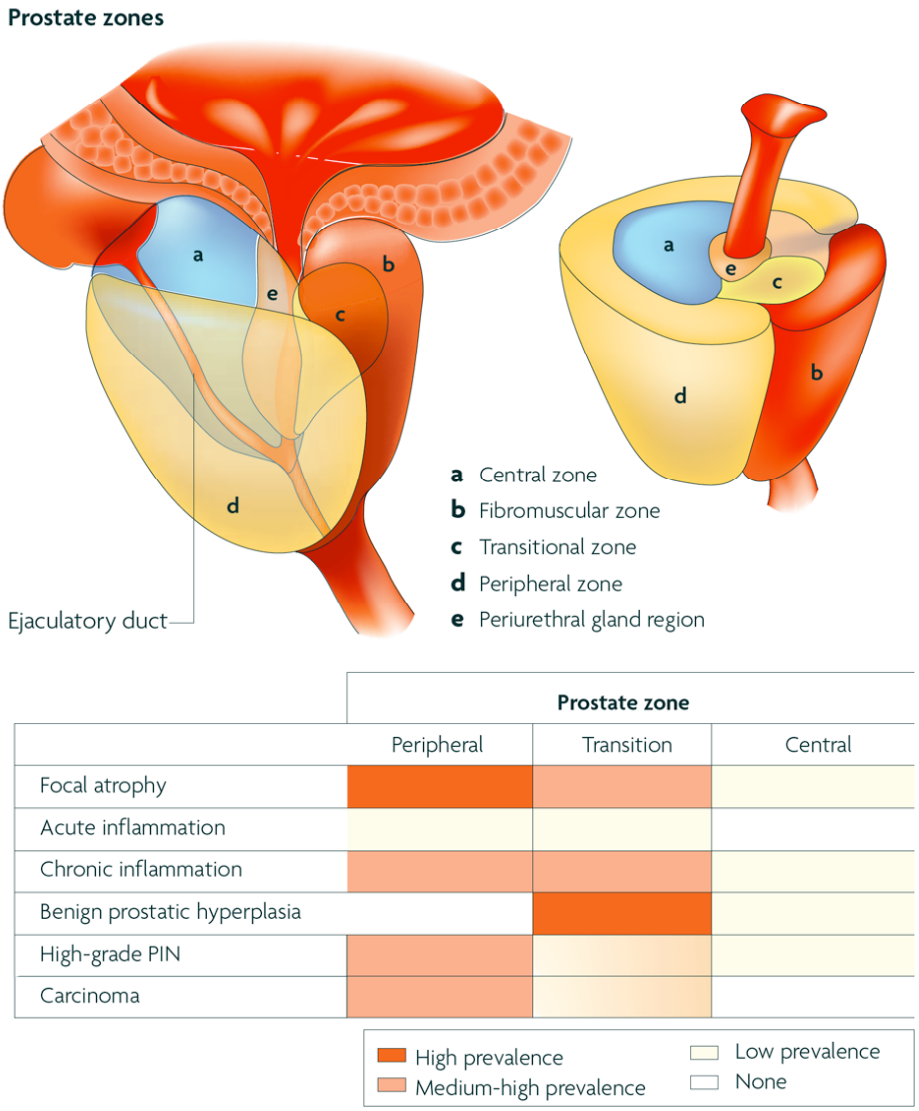

Figure 1. Zonal predisposition of prostate disease.

Most cancers develop in the peripheral zone, with few in the transition zone and with rare occurrence in the central zone. Focal atrophy, chronic inflammation and high-grade PIN are also more prevalent in the peripheral zone. BPH lesions develop preferentially in the transition zone without affecting the peripheral zone.

Adapted from (6).

1.1.3 PATHOLOGIES OF THE PROSTATE

1.1.3.1 Prostatitis

Prostatitis is the inflammation of the prostate gland. Symptomatic prostatitis affects 3 % to 16 % of men (7). The American National Institute of Health (NIH) recognizes four categories of prostatitis (8). Acute (class I) and chronic (class II) symptomatic bacterial prostatitis are caused by *Escherichia coli* or other Gram-negative bacteria. However, 90% of patients with prostatitis have symptomatic chronic non-bacterial prostatitis of unknown etiology (class III) (9). The prevalence of asymptomatic prostatitis (class IV) is unknown. More details on prostatic inflammation and its implication in prostate cancer can be found in section 1.1.6 on page 8.

1.1.3.2 Focal Atrophy

Histologically, areas of focal epithelial atrophy are frequently associated with acute or chronic immune cell infiltration. These atrophic lesions contain elevated numbers of proliferative epithelial cells, which fail to differentiate as columnar secretory cells. This pathology is defined as proliferative inflammatory atrophy (10). Proliferative inflammatory atrophy lesions occur in the peripheral zone and are associated with the development of high-grade PIN and prostate cancer (6).

1.1.3.3 Prostatic Intraepithelial Neoplasia

PIN is the benign proliferation of prostate epithelium cells within the glandular lumen in the absence of basal membrane invasion. PIN can remain unchanged or regress with time. It is classified as either low-grade or high-grade PIN. High-grade PIN is associated with an increased risk of developing prostate cancer.

1.1.3.4 Benign Prostatic Hyperplasia

BPH refers to the non-malignant proliferation of the prostate's stromal and epithelial cells frequently diagnosed in older men. Over 70% of 60 year old men and > 90% of 70 year old men have histological evidence of BPH (11). BPH is also caused by the proliferation and increased muscle tone of the prostate's stromal smooth muscle cells, which lead to the formation of nodules and the enlargement of the transition zone. The enlarged prostate constricts the prostatic urethra thereby causing urinary difficulties, a common symptom of BPH and prostate cancer. BPH can be treated by medication such as α-adrenoreceptor blockers (tamsulosine, alfuzosin), by 5-α reductase inhibitors (finasteride, dutasteride) or by surgery. Surgery for BPH is usually through transurethral resection of the prostate (TURP).

1.1.4 PROSTATE CANCER

Prostate cancer is a slow progression cancer, which can remain asymptomatic for a relatively long period. Approximately 70% of men in their 60s have asymptomatic prostate cancer (12). Prostate cancer is an adenocarcinoma caused by the uncontrolled proliferation of prostate epithelial cells. Prostate cancer statistics are detailed in section 1.1.1 on page 1.

Prostate cancer diagnosis is based on digital rectal examination and the pathological evaluation of prostate biopsies. Serum PSA level is used in diagnosis and in disease monitoring. Nomograms are used for risk assessment and prostate cancer prognosis. Current nomograms are composed of clinico-pathological features such as Gleason score, pTNM stage, pre-operative serum PSA levels and seminal vesicle invasion (13). The Gleason scoring system categorizes the degree of tissue differentiation, with "1" representing well-differentiated and "5" undifferentiated tissues. The sum of the two most prevalent histologies is used as the Gleason score. Current nomograms however lack the desired precision for identification of patients at higher risk of prostate cancer progression. An important domain of prostate cancer research focuses on the optimization of nomograms using molecular and/or cellular markers. Such markers could be evaluated on biopsy samples prior to surgery and

complement current clinico-pathological markers in the early prognosis of prostate cancer patients.

1.1.5 RISK FACTORS FOR PROSTATE CANCER

1.1.5.1 Age

Age is the primary risk factor for prostate cancer, with an average age at diagnosis of 70. Older age is associated with an increased risk of prostate injury and infection resulting in chronic prostate inflammation, as well as with hormonal changes and decreasing immunological functions. All of these factors participate in the development of prostate cancer.

1.1.5.2 Environmental causes

Similar to other cancers, prostate cancer has a multi-factorial etiology. Several environmental factors increase the risk of developing the disease. The importance of environmental factors in the development of prostate cancer is apparent in the population of Southeast Asian men who immigrate to a westernized country. These men, who naturally have a low incidence of prostate cancer, develop an increased rate of prostate cancer often within one generation following immigration. This rise is attributed to diet, pattern of sexual behavior, alcohol consumption, exposure to ultraviolet radiation and occupational exposure. A diet rich in red meat and animal fat escalate the risk of prostate cancer. Conversely, an Asian diet rich in soy as well as a Nordic diet with high content of rye lowers the risk of developing prostate cancer. Epidemiological data suggests that consumption of dietary anti-oxidants and micronutrients, such as lycopene, selenium, vitamin D and vitamin E, may also be protective (14), but remains unproven in prospective studies to date.

1.1.5.3 Hereditary causes

Studies comparing the occurrence of prostate cancer in monozygotic and dizygotic twins reveal that prostate cancer has the strongest hereditary component of

any cancer (15). Prostate cancer is hereditary in 5-10% of cases and 10-20% of prostate cancer patients have a family history of the disease (16). While canonical cancer mutations have been identified in other cancers (*k-ras* in pancreatic cancer and *adenomatosis polyposis coli* (*apc*) in colon cancer), few specific genetic risk factors have been identified in prostate cancer. Linkage analyses have however identified several gene locus associated with an increased risk of developing the disease. These locus include the hereditary prostate cancer locus on chromosome-1 (HPC1), HPC-2, HPC-X, predisposing for cancer of the prostate (PCAP) locus, cancer prostate and brain (CAPB) locus, which together contain a hereditary mutations in genes such as *elaC homolog 2 (Escherichia coli)* (*elac2*), *ribonuclease l* (*rnasel*) and *macrophage scavenger receptor 1* (*msr1*) [reviewed in (17)]. Mutations in *breast cancer type 2 susceptibility protein* (*brca2*) also augment the risk of prostate cancer and could be attributed to 5% of cases in patients younger than 55 years (13).

1.1.5.4 Race

Southeast and East Asian men have the lowest incidence of prostate cancer (18). Conversely, compared to Caucasian men, African American men have a 34% higher incidence of prostate cancer, less favorable stages at diagnosis and a two-fold higher risk of prostate cancer associated mortality (19). PSA levels are also higher and more variable in African American men without prostate cancer (20) or with localized prostate cancer (21).

1.1.5.5 Androgens

Although there is no direct association between androgen serum levels and the development of prostate cancer (22), androgens do play an important role in the disease. The initial stages of prostate cancer are termed "hormone sensitive" and medical castration causes prostate atrophy and a temporary elimination of symptoms associated with prostate cancer metastasis (discussed in section 1.1.7.3 on page 15) Testosterone is the most abundant sex hormone in men and is converted to dihydrotestosterone (DHT) by 5α-reductase. Compared to testosterone, DHT has a

greater affinity (8-fold) for the androgen receptor (AR) (23). Finasteride, an inhibitor of 5α-reductase, is the only agent to date proven to reduce the risk of developing prostate cancer (24, 25). Testosterone can also be converted into estrogen by the enzymatic activity of a cytochrome p450 aromatase. Exposure to environmental or developmental estrogen is also associated with the development of prostate cancer (26, 27).

1.1.5.6 Inflammation

Prostatic inflammation is associated with an increased risk of prostate cancer (28) and will be discussed in details in the following section.

1.1.6 PROSTATIC INFLAMMATION

From an immunological standpoint, the prostate is a rather complex organ. The prostate was long thought to be an immunologically privileged organ similar to the eye (29, 30). Such is no longer the case. Immune cells secreting a wide-array of cytokines infiltrate the prostate and there is evidence of immune responses directed against prostate specific antigens.

The prostate may nonetheless contain a unique immunological microenvironment. For instance, during puberty the androgen-dependent organogenesis of the prostate causes the expression of novel prostate specific antigens. Remarkably, there is no immune response targeting these novel prostate antigens. The prostate also has a low density of lymphatic vessels. Furthermore, androgens, which are present at their highest tissue concentration within the prostate, have documented immunosuppressive functions (discussed in section 1.5.4 on page 74) and could regulate a state of immunological tolerance (31, 32). Such observations suggest that the prostate may possess a strong immunoregulatory potential that accompanies its organogenesis.

1.1.6.1 Inflammation in the normal prostate

B and T lymphocytes, macrophages and mast cells infiltrate the normal prostate. Within the normal prostate tissue, most T lymphocytes are $CD8^+$, whereas $CD4^+$ T lymphocytes predominate in prostatitis lesions. Compared to non-activated T lymphocytes in normal tissues, T lymphocytes in inflamed tissues express major histocompatibility complex of class II (MHC-II) and CD45RO suggesting that they are activated and antigen experienced (33).

1.1.6.2 Causes of prostatic inflammation

As discussed earlier, 90 % of men diagnosed with prostatic inflammation have prostatitis of unknown etiology. Various factors have been described to have pro-inflammatory effects within the prostate such as: infection, urine reflux, dietary factor, age, and hormonal imbalance [reviewed in (6)] (see Figure 2 on page 10 and Table 1 on page 11).

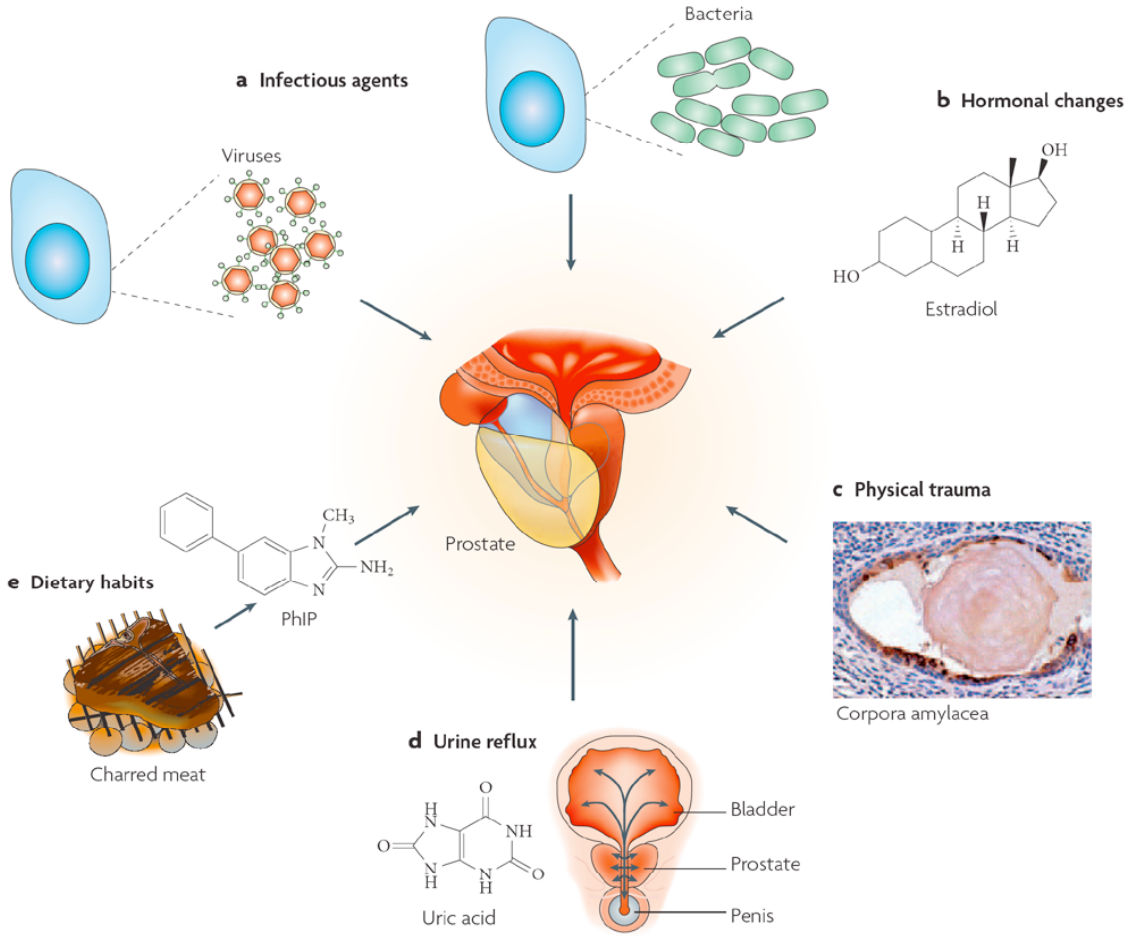

Figure 2. Causes of prostatic inflammation.

Details on the sources of prostatic inflammation are found in Table 1.

Adapted from (6).

Sources of prostatic inflammation. Adapted from (6)	
Bacteria	Acute and chronic bacterial prostatitis: Gram-negative bacteria (Escherichia coli) (34). Bacteria acquired from sexual transmitted diseases : Neisseria Gonorrhoeae, Chlamydia trachomatis, Trichomonas vaginalis and Treponema pallidum (Shyphilis) (28). Bacteria acquired from non-sexually transmitted disease : Propionibacterium acnes
Viruses	Recently, a study demonstrated a strong association with the presence of Xenotropic murine leukemia virus–related virus (XMRV) and prostate cancer (35). Human papillomavirus (HPV), human herpes simplex virus type 2 (HSV2), cytomegalovirus (CMV) and human herpes virus type 8 (HHV8) are also associated with prostate cancer.
Urine reflux	Chemical irritation of the prostate due to the presence of uric acid, which can directly activate immune cells.
Corpus amylacea	Amorphous small nodules or concretions located in the lumen of benign prostate acini and ducts that accumulate with age and which can lead to the erosion of the epithelium and promote the expression of cyclooxygenase-2 (COX-2). Corpus amylacea contribute to prostate inflammation, increase the risks of persistent infection and carcinogenesis.
Dietary factors	Charred meats contain heterocyclic aromatic amines and polycyclic aromatic hydrocarbon carcinogens such as 2-amino-1-methyL-6-phenylimidazol[4,5-b]pyridine (PhIP). PhIP causes prostate inflammation and glandular atrophy.
Estrogens	Increases in prolactin production through the hypothalamic-pituitary-gonadal axis leads to prostate inflammation (36). Direct effects on the stroma (ER-α) and epithelial cells (ER-β) by estrogens at specific developmental stages causes tissue architectural defects, which lead to inflammation.
Break of immune tolerance	Breaks of immune tolerance following prostate injury are associated with proliferative inflammatory atrophy.
Allelic variants of inflammatory genes.	Increased risk of prostate cancer with: - Inactivating mutation in RNASEL diminishes the anti-viral response of innate cells. - Inactivating mutation in MSR1 promotes bacterial infection due to reduced macrophage functions. - Mutation in macrophage inhibitory cytokine 1 (MIC1), a member of the TGF-β family also diminishes macrophage activity.

Table 1. Source of intra-prostatic inflammation.

1.1.6.3 Association between inflammation and prostate cancer

Chronic inflammation is linked to an increased risk of developing prostate cancer (37). Prostatitis increases the risk of prostate cancer and this preferentially in younger men (38). Several studies demonstrate that the infiltration of various immune cell populations correlates with disease progression or with various clinico-pathological parameters of prostate cancer patients. Increased immune cell infiltrate correlates with an increased rate of tumor recurrence (39), whereas elevated density of $CD4^+$ T lymphocytes (40) and mast cells (41) are associated with poor survival and higher Gleason score, respectively. Strong tumor-infiltrating lymphocytes (TILs) density also correlates with perineural and capsular invasion and a shortened time to PSA recurrence (42). Conversely, one study finds that a high TILs density was protective against disease progression (43). Results from these studies are however difficult to analysis due to different staining and analysis methods (discussed in Chapter IV on page 116)

Furthermore, it is only recently that the activation status through detailed phenotypical analysis has been documented. $CD3^+$ and $CD4^+$ T lymphocytes do infiltrate the tumor tissue but do not express perforin and interferon-γ (IFN-γ) suggesting that they are functionally inactive (44). Several studies have also evaluated the presence of $Foxp3^+$ regulatory T cells (T_{REGs}) in prostate cancer. T_{REGs} are important for the maintenance of immune tolerance and the inhibition of the anti-tumoral immune response (more details in section 1.2.5.3 on page 43). In a transgenic adenocarcinoma mouse prostate (TRAMP) mouse model expressing the influenza hemagglutinin (HA) antigen under a prostate specific promoter, adoptive transfer of HA-specific $CD4^+$ T lymphocytes resulted in a skewing into a T_{REG} phenotype both at the transcriptional and functional level attesting to the tolerogenic power of the prostate microenvironment (45). In prostate cancer patients, a recent study demonstrates an increased infiltration of T_{REGs}, PD-1^+ and B7-$H1^+$ immune cells within the prostate primary tumors (46). $Foxp3^+$ T lymphocytes were also more present in tumor tissue than in BPH tissue or normal prostate samples from healthy men (46). The T_{REG} skewing was apparent even at the earlier stages of the disease and

elevated levels of T_{REGs} with increased suppressive potential (47) are also present in the blood and primary tumors of prostate cancer patients (48). Although present at a higher density in malignant tissues compared to benign tissues, T_{REG} density does not correlate with disease progression (49). Another study illustrated that $CD4^+$ T lymphocyte population was skewed towards a T_H17 and a T_{REG} phenotypes (50). High abundance of T_H17 $CD4^+$ T cells did correlate with lower Gleason scores (50). The roles of T_H17 cells in prostate cancer remain undefined. Altogether, as the primary tumor is able to convert antigen-specific T lymphocytes into immunosuppressive immune cells thereby promoting immune evasion, these results argue that the simple assessment of immune cell numbers is inaccurate as it is their activation status that is relevant.

Finally, there are correlations between cytokines levels and the risk of prostate cancer progression. For example, elevated serum levels of IL-6, IL-8, transforming growth factor-β (TGF-β) and tumor necrosis factor-α (TNF-α) are associated with higher Gleason score, development of metastatic disease and poor prognosis (51, 52). The cytokines are produced by infiltrating immune cells and by prostate cancer cells through the activation of NF-κB signaling (7). The prolonged presence of infiltrating lymphocytes and the production of cytokines and other immunological mediators can inhibit the anti-tumoral immune response and provide pro-angiogenic and tumor growth factors.

Altogether, these data suggest that prostatic inflammation correlates with a more aggressive disease and a more rapid disease progression. Interestingly, T_{REG} removal in murine model of prostate cancer decreased prostatic inflammation and reduced the risk of developing prostate cancer (53). Nonetheless, from these studies, it is difficult to establish the roles of prostatic inflammation in actual prostate cancer development. Due to a lack of appropriate experimental model suited for the evaluation of prostatitis prior to prostate carcinogenesis, it remains unclear whether inflammation is a causative agent of prostate cancer or whether inflammation is induced following the development of prostate cancer.

1.1.7 THERAPIES FOR PROSTATE CANCER

Localized prostate cancer is highly curable through radical prostatectomy or radiotherapy. Treatment of advanced prostate cancer is however palliative (54). Advanced prostate cancer is characterized by: (i) high-risk locally advanced disease or metastatic disease, (ii) PSA recurrence after local therapy or (iii) increasing PSA level despite treatment with ADT, which is also termed hormone-refractory prostate cancer (HRPC) (55). The median survival of patients with HRPC ranges from 24 to 40 months for patients diagnosed with skeletal metastasis and averages 68 months for patients without skeletal metastasis (56).

1.1.7.1 Active Surveillance and Radical Prostatectomy

Prostate cancer is well-suited for active surveillance as the cancer grows relatively slowly in most patients. Approximately 70% of men in their 60s have asymptomatic prostate cancer (12). A study evaluating the influence of radical prostatectomy versus active surveillance demonstrates that the benefits of surgery on cancer-related mortality, risks of metastasis and local progression were mainly apparent at 10 years post-surgery (57). As such, it is recommended that men with a life expectancy of less than 10 years and who are diagnosed with early-stage prostate cancer be actively monitored without undergoing aggressive therapy in the absence of progression (13). Side effects also remain a major problem following radical prostatectomy, which include erectile dysfunction (frequent) and urinary incontinence (less frequent).

1.1.7.2 Radiotherapy

Radiotherapy, by brachytherapy with the insertion of radioactive sources within the prostate, or by external beam radiotherapy, is a curative therapy for early-stage prostate cancer. Patients with locally advanced prostate cancer (positive surgical margins, seminal vesicle invasion) can also be cured by radiotherapy through dose escalation and by combination with ADT.

1.1.7.3 Androgen Deprivation Therapy

ADT is the primary treatment option for patients with advanced prostate cancer. Huggins and Hodges first reported in 1941 that ADT causes prostate cancer regression and alleviation of pains associated with metastatic prostate cancer (58). Dr Charles B. Huggins, born in Halifax, Nova Scotia, won the Nobel Prize in Medicine in 1966 for his discovery. Their work demonstrated the androgen dependency of normal and neoplastic prostate cells. ADT blocks cellular proliferation and causes the involution of the prostate gland through the apoptosis of hormone sensitive epithelial cells (59). Successful in 70-80% of patients, ADT was the only treatment clinically proven to prolong patient survival, until 2005 when two docetaxel (Taxotere) regiments proved to have survival benefits (60-63). Unfortunately, ADT remains a palliative treatment option with a response window limited 18 to 24 months (64).

ADT targets circulating androgens and/or the AR. Testicular androgens are eliminated through surgical castration or via agonists and antagonists of the gonadotropin-releasing hormone (GnRH) receptors and luteinizing hormone releasing hormone (LHRH). AR activity is blocked with cyproterone (Androcur), cyproterone acetate (Androcur) and non-steroidal anti-androgens such as flutamide (Euflex), bicalutamide (Casodex) and nilutamide (Anandron).

1.1.7.4 Chemotherapy

Recent reports suggest that docetaxel increases the survival of metastatic HRPC patients by 2.9 months. Docetaxel is an anti-mitotic agent that promotes microtubule assembly and stability. Combination of docetaxel with prednisone (Deltasone) decreases PSA levels and increases survival of HRPC patients (65-67). Prednisone is a synthetic corticosteroid, which is converted to prednisolone in the liver, and acts mainly as an anti-inflammatory agent. Docetaxel-based therapy is now the standard of care for chemotherapy against HRPC as recommend by American and European guidelines.

1.1.8 CANCER IMMUNOTHERAPY

The goal of immunotherapy is the induction of a cytotoxic immune response targeting the tumor cell. The therapeutically induced anti-tumoral immune response must achieve three criteria: (i) *in vivo* generation of sufficient numbers of tumor-specific immune cells; (ii) trafficking and infiltration of these tumor-specific immune cells within the tumor: (iii) activation of the immune cell's effector functions within the tumor (68). Although a humoral immune response could be beneficial, it is the cell-mediated immune response that is essential for tumor rejection. In mouse models, transfer of T lymphocytes, and not antibodies, was protective against tumor challenges. Elimination of $CD8^+$ T lymphocytes also abrogated both the protective and therapeutic actions of the anti-tumoral immune response. Finally, in the context of a potent immunotherapy promoting the induction of a cytotoxic $CD8^+$ T lymphocytes response, both dendritic cells (DCs) and $CD4^+$ T lymphocytes need to be involved.

Cancer immunotherapy is classified as either active or passive. Active immunotherapy involves the *in vivo* stimulation of the immune system either specifically or non-specifically through administration of cytokines and interleukins. Passive immunotherapy involves the *ex vivo* activation of immune cells, which are transferred back into the patient. The inherent specificity of immunotherapy should theoretically decrease the normal tissue toxicity observed with chemotherapy. However, results from clinical trials demonstrate that patients that do develop clinically manageable autoimmunity associated with the development of the anti-tumoral immune response, such as vitiligo in the case of immunotherapy against melanoma, have a more favorable clinical response to therapy.

In theory, prostate cancer is ideally suited for immunotherapy. Innate and adaptive immune cells infiltrate the prostate and the therapeutic window is relatively long (69). Moreover, for patients diagnosed at the earlier stages of the disease, immunotherapy could be administered during the period of active surveillance or prior to ADT (see section 1.1.8.2.5 on page 19). The prostate also expresses several specific antigens (see section 1.1.8.1 on page 17), which are recognized by T

lymphocytes. Finally, prostate cancer patients are generally diagnosed at an age when they no longer have children. As such, the prostate becomes a non-essential organ and there is no need to discriminate between normal and neoplastic prostatic epithelium as the entire prostate can be targeted by immunotherapy. In practice however, the prostate's immunosuppressive mechanism may be the cause of the lack of success in the clinical setting.

1.1.8.1 Prostate Tumor-Associated Antigens

Several prostate tumor-associated antigens (TAAs) have been described as potential immunotherapeutic targets (70, 71). PSA is an active serine protease that participates in the liquefaction of the seminal fluid (72). PSA transcription is regulated by androgens and is specifically expressed by the prostate epithelium. Prostate cancer causes PSA levels to increase up to 10,000 fold. Serum PSA levels are also influenced by BPH, prostatitis, age, body-mass index and race (73). Increases in serum PSA levels are not caused by elevated PSA expression, which actually decreases during cancer, but by a higher release of PSA in blood caused by a disruption of prostate architecture (74). Circulating $CD8^+$ T lymphocytes are present in patients with prostatitis (75) or prostate cancer (76). Other prostate tumor-associated antigens include prostate specific membrane antigen (PSMA), a transmembrane glycoprotein overexpressed in primary tumors and metastases, prostate stem cell antigen (PSCA) (77) and PAP (78), a glycoprotein whose expression is more specific to the prostate than that of PSA or PSMA (79).

1.1.8.2 Clinical trials in immunotherapy of prostate cancer

Several immunotherapy strategies for prostate cancer using cell-based approaches, viral vectors or antibodies are currently in clinical trials. The following sections will describe the immunotherapies that have had the most promising results.

1.1.8.2.1 Sipuleucel-T (Provenge)

Sipuleucel-T (Provenge) is a DC-based vaccine. DCs are loaded with PAP peptides and granulocyte macrophage-colony stimulating factor (GM-CSF). PAP was chosen becase of its localization on the cytoplasmic membrane and its success in pre-clinical models, where it could elicit prostate-specific immune responses and autoimmune prostatitis (80). GM-CSF promotes DC differentiation into potent T_H1 activator (81). Activated DCs promote the activation of cytotoxic $CD8^+$ T lymphocytes targeted against PAP epitopes. In two phase III trials, Sipuleucel-T offered a survival advantage of 4.5 months for HRPC patients (82, 83). Following recent positive results from the IMPACT trial (Immunotherapy for Prostate AdenoCarcinoma Treatment) the American Food and Drug Administration has approved Sipuleucel-T for treatment of HRPC. It is the first immunotherapy to be approved for cancer treatment.

1.1.8.2.2 GVAX

GVAX is tumor cell-based vaccine in which LNCaP and PC3 cell lines are transfected with GM-CSF, irradiated and injected in patients. The premise of the GVAX vaccine is that the prostate cancer cells will be phagocytosed by the patient's DCs, which will then present several prostate TAAs to cytototoxic $CD8^+$ T lymphocytes. In a Phase II trial, GVAX increased median survival of HRPC patients by 8.2 months (84). A phase III clinical trial is currently underway in North America and Europe evaluating GVAX as a single agent (VITAL-1) in comparison to docetaxel plus prednisone in HRPC patients. A VITAL-2 phase III trial evaluating the combination of GVAX with docetaxel was terminated due to increased mortality in the GVAX docetaxel arm.

1.1.8.2.3 PROSTVAC-VF

PROSTVAC-VF is a recombinant vaccinia virus expressing PSA (rV-PSA). The premise is that the anti-viral response will promote the activation of PSA-specific $CD8^+$ T lymphocytes. Initial trials showed that pre-existing immunity to the virus and immunodominance of viral proteins limited the efficacy of the therapy (85). The protocol was modified to include a fowlpox virus (rF-PSA) as a boost to improve

effectiveness. A phase II trial showed that rV-PSA + rF-PSA increased time to progression from 9.2 months to 18.2 months compared to rV-PSA or rF-PSA individual injection and an increase survival of 8.5 months (86). A phase III trial (PARADIGM, Therion/NCI/ECOG) is currently underway.

1.1.8.2.4 Anti-CTLA-4 therapy

Cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) signaling inhibits activated T lymphocytes. Blocking of CTLA-4 causes the activation of $CD8^+$ T lymphocytes. In two phase I trials with HRPC patients, anti-CTLA4 therapy (Ipilimumab) was shown to be safe and cause a decrease in PSA levels (87, 88).

1.1.8.2.5 Combination of immunotherapy with conventional therapy

Combination of immunotherapy with radiotherapy has synergistic effects in prostate cancer (89) and in other cancers (90). In squamous cell carcinoma, the beneficial effects of combining radiotherapy and chemotherapy are associated with the elimination of T_{REGs} and an increase in homeostatic proliferation (91-93). In a TRAMP mouse model, cyclophosphamide temporarily decreases T_{REG} numbers and could potentially improve immunotherapy (94). In prostate cancer, chemotherapy with docetaxel however inhibits the anti-tumoral effect of immunotherapy (95).

The most beneficial combinatory effects have been obtained from co-therapy with ADT. ADT improves the survival benefits of immunotherapy (96) when vaccination occurs early in the treatment regiment (90) and prior to ADT (97, 98). The favorable effects of ADT are related to the improvements of DC maturation, costimulation marker expression and cytokine secretion. However, it is noteworthy that, in the current clinical settings for immunotherapy, patients undergoing immunotherapy have already failed other therapies including ADT. It is thus important to understand the immunological consequences of ADT, and other therapies, on the prostate's immunological environment.

1.1.9 SUMMARY

Prostate cancer remains untreatable for patients with advance form of the disease. Novel immunotherapeutic regiments offer to tackle HRPC. However, until the recent encouraging announcement of Sipuleucel-T's FDA approval for the treatment of HPRC, immunotherapy in prostate cancer had minimal success in clinical trials. The prostate must be regarded as an organ with a unique immunological microenvironment. This uniqueness, which remains to be fully characterized, must be taken into consideration during the optimization of immunotherapies against prostate cancer. To achieve this, three main aspects need to be addressed.

1.1.9.1 Studying the prostate's immunological environment in order to develop prostate specific immunotherapies.

The prostate's immuno-oncologic microenvironment remains understudied. It is important to recognize that each organ possesses unique mechanisms to maintain its distinct state of tissue homeostasis. This translates to different immune cell populations infiltrating different tissues thereby generating diverse immune environments (99). As such, the immunoregulatory pathways in one cancer, such as melanoma, are not representative of those in prostate cancer. This is especially important considering the prostate's unique immunological microenvironment (see section 1.1.6 on page 8). To date, most immunological studies on prostate cancer present correlative data between immune cell infiltration and disease progression. However, further studies are needed to understand the mechanisms responsible for regulating prostatic inflammation in order to identify key targets for immunotherapy.

1.1.9.2 Understanding the prostate's immunological environment in human

Prostate cancer immunology needs to be studied in human. Without detailing the immunological differences between mice and men [reviewed in (100)], key immunoregulatory pathways, which could affect the efficacy of the anti-tumoral immune response, are different between the two species. However, it is important to highlight that the study of TILs functions in human prostate cancer faces many

challenges. First, surgical samples are often only available from patients with early-stage prostate cancer or with a favorable prognosis for surgery. These patients may not necessarily represent patients that would be treated with immunotherapy. Second, isolation of true TILs is difficult due to the infiltrative and heterogeneous nature of prostatic adenocarcinoma and to the low numbers of TILs found in the tumor tissue. Finally, the generation of autologous prostate cancer cell lines allowing for direct measurement of anti-tumoral cytotoxicity is difficult. Resolving these challenges could offer essential knowledge on human prostate immunobiology and significantly improve current immunotherapeutic protocols.

1.1.9.3 Immunosuppression in human prostate cancer

Finally, the majority of immunotherapeutic protocols for prostate cancer have focused solely on the activation of tumor-specific cellular effectors. Similar to stepping on the accelerator while keeping one foot on the brake, not eliminating the tumor's endogenous immunosuppressive pathways prevents the full activation of tumor-specific cellular effectors. The prostate is the source of several immunosuppressive pathways (see sections 1.2.4 on page 34, 1.2.5 on page 42 and 1.3.4 on page 55), whose regulatory pathways responsible to their expression remain undefined. As part of this Ph.D. thesis, we chose to characterize the immunological microenvironment in prostate cancer and to study on the regulation of immunosuppression in human prostate cancer.

1.2 BASIC CONCEPTS OF AN IMMUNE RESPONSE

1.2.1 THE INNATE AND THE ADAPTIVE ARM OF THE IMMUNE SYSTEM

The immune system is responsible for the elimination of pathogens, virally infected cells and malignant cells. It is composed of two main arms participating in innate and adaptive immune responses. The innate immune response is the primary responder to pathogen invasion and tissue damage. The adaptive immune response takes a longer time to develop due to its antigen-specific activation, but results in the

establishment of immunological memory. Immunological memory is responsible for the rapid and robust immune response following a secondary exposure to a pathogen. Innate cellular effectors are phagocytes (neutrophils, DCs, monocytes and macrophages), cells that release inflammatory mediators (basophils, eosinophils and mast cells), and natural killer (NK) cells. Innate immune cells are an important source of cytokines, which regulate both the innate and adaptive immune responses. Adaptive effectors are the B and T lymphocytes, which proliferate and mature in an antigen-specific manner in secondary lymphoid organs through the recognition of their cognate antigen on the surface of antigen-presenting cells (APCs). The elimination of invading pathogens engages both innate and adaptive arms of the immune system.

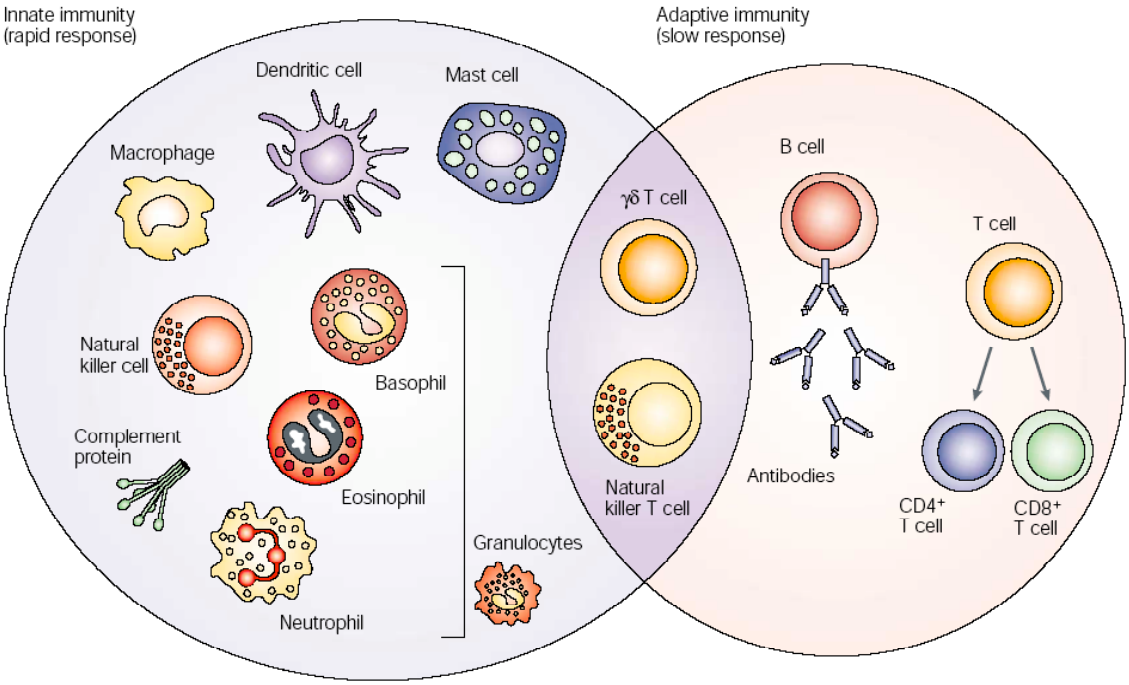

Figure 3. Immune and adaptive immune cells.

Innate immune cells (macrophages, DCs, mast cells, NK cells, granulocytes) initially recognize pathogens and tissue injury and initiate the inflammatory response. Adaptive immune cells (B lymphocytes, $CD4^+$ and $CD8^+$ T lymphocytes) take a longer time to develop in mature effector. They are however able to target the pathogens in an antigen-specific manner and are responsible for the development of immunological memory. Natural Killer T (NKT) cells and γδ T cells are hybrid innate / adaptive immune cells that recognize non-peptidic antigens such as lipids and carbohydrates.

Adapted from (101).

1.2.2 SECONDARY LYMPHOID ORGAN: THE LYMPH NODE

The adaptive immune response is activated within secondary lymphoid organs, such as LNs, the spleen, tonsils and Payer's patches. During infection, the LN acts as a mechanical sieve filtering the lymph draining from the infected tissue. In cancer, LNs are often the primary site of tumor cell metastasis. A layer of connective tissue encapsulates the LN with collagen fibers (trabeculae) extending from the capsule to the parenchyma of the LN (see Figure 4 on page 25). Immune cells continuously enter and exit LNs. From the blood, lymphocytes enter LNs through high endothelial venules and, from the lymphatic system, through afferent lymphatic vessels. The lymph containing immune cells and antigens flows through the LNs via medullary sinuses composed of macrophages known as histiocytes. B and T lymphocytes are segregated in specific zones within the LN, which promote antigen recognition and proper activation due to the close proximity of APCs (DCs and macrophages) and $CD4^+$ Helper T lymphocytes. The B lymphocyte zone located in the cortex contains follicules and germinal centers where memory B lymphocytes develop and somatic hypermutation and antibody class-switching occurs. The T lymphocyte zone surrounds the B lymphocyte follicle and extend towards the center of the LN. Antigen recognition within the T lymphocyte zone will either lead to the development of a MHC-II restricted, $CD4^+$ T lymphocyte-mediated humoral immune response or a MHC-I restricted, $CD8^+$ T lymphocyte-mediated cytotoxic immune response depending on the pathogen and on the cytokines present during antigen recognition.

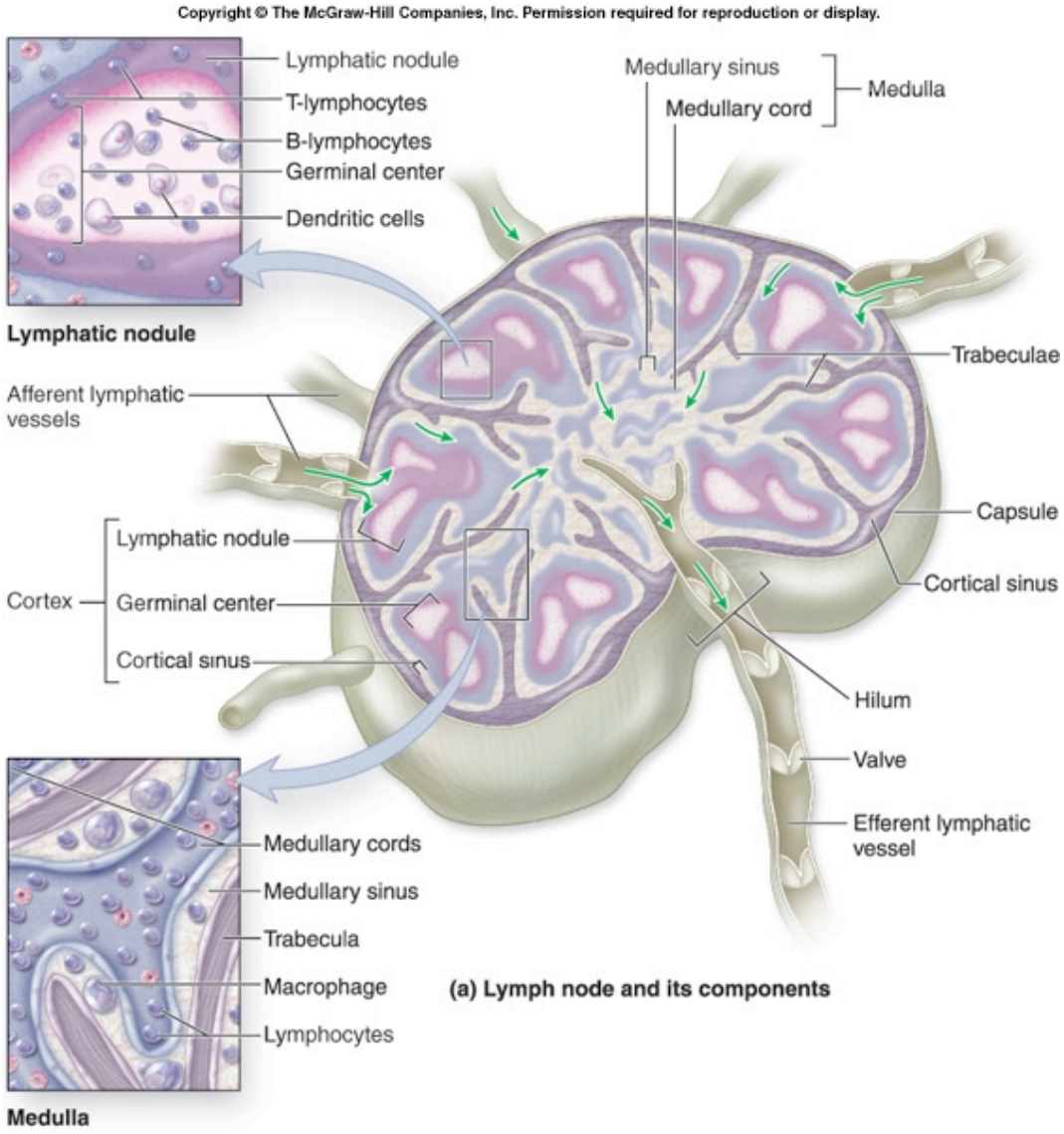

Figure 4. Histology of a lymph node.

Although LNs lack connective tissue, they nonetheless have a highly defined tissue architecture well-suited for the optimal recognition of cognate antigenic peptides and activation of naïve lymphocytes in distinct B and T lymphocytes zone.

Adapted from

http://academic.kellogg.cc.mi.us/herbrandsonc/bio201_McKinley/Lymphatic%20System.htm

1.2.3 IMPORTANT IMMUNOLOGICAL CONCEPTS

The following section will detail important immunological concepts pertaining to the anti-tumoral immune response.

1.2.3.1 Pathogen and Malignant Cell Recognition

Pathogen recognition differs between the innate and adaptive immune responses. Innate immune cell effectors directly recognize pathogens through pathogen-recognition receptors. For example, Gram-negative bacteria expressing lipopolysaccharides (LPS) are recognized by the Toll-like receptor-4 (TLR-4) of macrophages. Neutrophils and macrophages also express antibody-specific receptors (Fc receptors), which allow for the recognition and phagocytosis of antibody-coated pathogens. Activation of NK cells is discussed in section 1.2.4.2 on page 35.

For adaptive immune cells, the pathogen recognition process takes a longer time and accounts for the different response rate between the innate and adaptive immune responses. Naïve adaptive immune cells encounter their cognate peptides presented by APCs in secondary lymphoid organs. During the initial recognition of their cognate antigen, naïve B or T lymphocytes form an immunological synapse with the APC. This immunological synapse procures the first two of three signals necessary for lymphocyte activation: signaling through the T cell receptor (TCR) or B cell receptor (BCR) and signaling through co-stimulatory receptors such as CD80 and CD86. The third signal is provided by cytokines locally produced by APCs or helper $CD4^+$ T lymphocytes. This antigen specific activation causes the proliferation, maturation and expression of the cytotoxic machinery or production of antibodies as well as the development of immunological memory. Once activated in secondary lymphoid organs, lymphocytes migrate to the injured tissue and eliminate the pathogens, the infected cells or malignant cells.

In cancer, tumor cell recognition is similar to the recognition of virally infected cells. NK cells recognize and eliminate malignant cells that have lost MHC-I expression (section 1.2.4.2 on page 35). APCs also phagocytose malignant cells and

present TAAs to $CD8^+$ T lymphocytes in tumor-draining lymph nodes (LNs). However, once the tumor reaches a clinically detectable size, the activation of the anti-tumoral immune response is impaired (section 1.3.3 on page 52). For example, APCs lose their ability to properly activate TAA-specific lymphocytes. Furthermore, the development of an immunosuppressive tumor microenvironment prevents the activation of TAA-specific lymphocyte within the tumor.

1.2.3.2 Antigen Presentation

APCs present in the injured tissue will phagocytose pathogens and present enzymatically digested antigenic peptides through MHC-I or MHC-II. Peptides from endogenous proteins and intracellular pathogens, such as viruses, are presented by MHC-I to $CD8^+$ T lymphocytes. Conversely, peptides from extracellular pathogens, such as bacteria, are presented by MHC-II to B and $CD4^+$ T lymphocytes.

In cancer, $CD8^+$ T lymphocytes need to be activated by APCs (macrophages or DCs) in a TAA-specific manner in order to recognize and eliminate malignant cells. Since TAAs are extracellular antigens, the activation of $CD8^+$ T lymphocytes by APCs is achieved through cross-presentation [reviewed in (102)]. In this process, the APCs will phagocytose tumor cells, degrade its proteins into antigenic peptides and, instead of targeting them to MHC-II presentation, will present the TAAs onto MHC-I complexes. The machinery involved in cross-presentation remains to be clearly defined. Phagocytosis is the principal pathway for the internalization of extracellular antigens, but antigens acquired through macropinocytosis are also cross-presented. Once inside the phagosome, antigens can either be transferred to the cytosol where they are transfered to the classical MHC-I peptide processing machinery in the endoplasmic reticulum. On the other hand, specialized phagosomes degrade antigens via cathepsin S and directly load the resulting peptides on MHC-I that have trafficked to the phagosome. Activation of $CD8^+$ T lymphocytes by cross-presentation further necessitates the concomitant activation of $CD4^+$ T helper lymphocytes. In a tumor mouse model, in which TAAs were strictly presented by MHC-I and not by MHC-II, the $CD8^+$ T lymphocyte were rendered anergic due to lack of $CD4^+$ help (103).

Furthermore, considering that tumor-associated DCs promote T lymphocyte anergy, it is possible that they represent a distinct CD8α^+ DC population (104, 105), which poorly expresses MHC-II restricted peptides (106) and thus promote $CD8^+$ T lymphocyte anergy.

1.2.3.3 Diversity of the antigen repertoire

The strength of the immune system lies in its ability to generate T and B lymphocytes specific for a large diversity of non-self peptides. The vast repertoire of antigen-specific receptors, estimated at 10^{15} different variable regions, originates from genomic rearrangement during lymphocyte development of about 400 genes. DNA recombination of V (variable), D (diversity), J (joining) and C (constant) gene loci occurs during TCR and BCR rearrangements in primary lymphoid organs. T lymphocytes undergo a unique TCR rearrangement during thymic development. B lymphocytes undergo a first BCR rearrangement in the bone marrow and a second rearrangement during somatic hypermutation in germinal centers.

Following BCR and TCR rearrangement and expression at the cell surface, B and T lymphocytes must undergo negative and positive selection. This process ensures that the antigen-specific receptors are not specific for self-antigens while maintaining adequate affinity for self-MHC (for T lymphocyte only). T lymphocytes expressing a TCR specific for self-peptide die by apoptosis (negative selection) whereas a lack of MHC recognition deprives the lymphocyte of survival signal (positive selection). More than 95% of T lymphocytes will be eliminated during negative selection, which is the principal mechanism to maintain immunological tolerance to self.

In cancer, TAAs are self-peptides, which are either overexpressed or mutated by the tumor cells. Consequently, T and B lymphocytes with high-avidity for these TAAs are not present in cancer patients as they are eliminated during lymphocyte development through negative selection. Cancer patients do produce TAA-specific antibodies (107) and TAA-specific T lymphocytes (108) that recognize both MHC-I

and MHC-II restricted peptides (109). However, these TAAs-specific lymphocytes express low avidity TCR (110).

1.2.3.4 Termination of the immune response

An acute immune response will resorb itself prior to causing pathological tissue damages. The termination of an immune response involves the removal of the initial danger signals, of pro-inflammatory mediators, the apoptosis of cellular effectors and the promotion of tissue repair (111). The immune system has developed intrinsic mechanisms to inhibit its own effectors in order to prevent the exacerbation of the inflammatory response. These mechanisms include the secretion of anti-inflammatory lipoxins synthesized from arachidonic acids (112), prostaglandins and cytokines, the expression of inhibitory or pro-apoptotic receptors by lymphocytes immediately following their activation (CTLA-4, Fas) and the recruitment of immunoregulatory immune cells with immunosuppressive properties, such as T_{REGs} and macrophages.

In cancer, molecular and cellular effectors implicated in the termination of the immune response participate in the development of tumor immunosuppression. From an immunological point of view, a tumor that reaches clinically detectable size is similar to a wound that does not heal or to an immune response that did not end (113). The disruption of tissue homeostasis that accompanies tumor growth favors the sustained and excessive activation of innate immune cells and the recruitment of adaptive immune cells. At a certain stage in tumor development, the immune system is coerced to accept the tumor as self and initiates the termination of the anti-tumoral immune response thereby preventing more tissue damage. More details on anti-inflammatory processes present in prostate cancer are found in section 1.3.4 on page 55.

1.2.3.5 Inflammation-induced carcinogenesis

In 1863, Rudolf Virchow proposed that tumors originate from chronically inflamed tissues (114). Chronic inflammation is directly implicated in the development of liver, stomach, large intestine, biliary tree and bladder cancer and

increases the risk of developing of esophagus, lung, pancreatic and prostate cancer. For many of these cancers, chronic inflammation results from exposure to infectious agents or other pro-inflammatory environmental agents. Chronic inflammation leads to increased sensitivity to chemical carcinogens and can induce cellular transformation in the absence of pathogen-encoded oncogenes (115).

Inflammation-induced carcinogenesis involves a complex interaction between adaptive and innate immune cells. Generally, inflammation caused by innate immune cell activation promotes carcinogenesis whereas a lymphocyte-mediated immune response eliminates tumor cells. Activated innate immune cells release reactive oxygen species (ROS) (superoxide, hydrogen peroxide, singlet oxygen and nitric oxide) that initiate a free-radical reaction with phospholipids, which increases genomic instability. Chronic inflammation also participates in several physiological processes implicated in carcinogenesis: cell survival, tissue remodeling, angiogenesis and suppression of the anti-tumoral adaptive immune response (116).

Regulation of Cancer Development by Immune Cells	
Innate immune cells contribute to cancer. Tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSC), granulocytes.	Direct mechanisms: - Induction of DNA damages by the generation of free radicals - Paracrine regulation of intracellular pathways (NF-κB) Indirect mechanisms: - Promotion of angiogenesis and tissue remodeling via the secretion of growth factors, cytokines, chemokines, matrix metalloproteinases - Upregulation of COX-2 - Immunosuppression of the adaptive anti-tumoral immune response
Adaptive immune cells modulate cancer.	Direct mechanisms: - Inhibition of tumor growth by cytotoxic T lymphocytes - Inhibition of tumor growth by cytokine mediated tumor cell lysis (IFN-γ, TNF-β) Indirect mechanisms: - Promotion of tumor growth by T_{Regs} - Promotion of tumor growth via a humoral immune response that causes chronic inflammation

Table 2. Regulation of cancer development by immune cells.

Summary of the various pathways by which innate and adaptive immune cells can regulate tumor growth.

Adapted from (110).

Summary of the source and function of important cytokines		
Cytokines	**Source(s)**	**Function(s)**
Interleukin-1 β	Monocytes, macrophages, DCs, T and B lymphocytes. Non-immune cells such as fibroblasts and others.	T_H1 cytokine promoting inflammation by activating T lymphocytes and macrophages. IL-1 also induces fever, acute phase response and neutrophil activation. In cancer, IL-1 can promote the growth and progression of solid tumors (117).
Interleukin-2	Activated T_H1 T lymphocytes.	T_H1 cytokine, activation of T lymphocytes, NK cells and B lymphocytes. Necessary for the proliferation of T lymphocytes during activation.
Interleukin-4	T_H2 T lymphocytes, mast cells, basophils and eosinophils.	Activation of T_H2 T lymphocytes, B lymphocytes and monocytes Involved in immunoglobulin class switching to IgE
Interleukin-6	T_H2 T lymphocytes, B lymphocytes, monocytes and macrophages.	Stimulates T_H2 T lymphocytes and B lymphocyte differentiation Macrophage and NK cell activation In cancer, IL-6 favor the proliferation of hormone refractory prostate cancer cell lines (118), inhibit tumor cell apoptosis and promote angiogenesis s(119).
Interleukin-8	T lymphocytes, macrophages, granulocytes. Non-immune cells such as fibroblasts and others.	Chemokine Chemotaxis of neutrophils, basophils and T lymphocytes. In cancer, IL-8 promotes angiogenesis and metastasis of prostate cancer cells through the induction of MMP-9 expression (120).
Interleukin-10	Activated T_H2 $CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes and DCs.	T_H2 cytokine that inhibits a T_H1 immune response. Stimualtion of B lymphocyte proliferation and IgA secretion. Inhibits the production of pro-inflammatory cytokines (IL-1β, TNF-α) by T lymphocytes, NK cells, monocytes and macrophages. Decreases the expression of MHC-II on monocytes (121, 122).
Interleukin-12	Monocytes, Macrophages B lymphocytes and DCs.	T_H1 cytokine. Stimulate the production of IFN-γ by T_H1 $CD4^+$ T lymphocytes. Stimulates the cytotoxic properties of $CD8^+$ T lymphocytes, NK cells and macrophages.
Interleukin-17	T_H17 activated $CD4^+$ T lymphocytes	Pro-inflammatory cytokine. Induces macrophages to secrete IL-1β and TNFα. Promotes *in vivo* growth and angiogenesis of tumors.
Interferon-γ (IFN-γ)	T_H1 $CD4^+$ and $CD8^+$ T lymphocytes as well as NK cells.	Polarization of T_H1 $CD4^+$ T lymphocytes through STAT-1 and inhibition of T_H2 $CD4^+$ T lymphocytes. Activation of macrophages and NK cells. IFN-γ in pivotal in the anti-tumoral immune response. IFN-γ can enhance the immunogenicity of tumor cells by increasing the expression of MHC-I and MHC-I antigen processing components.
Transforming growth factor-β (TGF-β)	T lymphocytes, macrophages, B lymphocytes, mast cells as well as non-immune cells and platelets	T_H2 cytokine. Immunosuppressive cytokine that inhibits antigen presentation, T lymphocyte proliferation, NK cytotoxicity and activates Tregs. In cancer, TGF-β inhibits the growth of non-transformed and hematopoietic cells, while promoting the survival and proliferation of transformed cells, including prostate cancer cells.
Tumor necrosis factor-α (TNF-α)	Monocytes, macrophages, NK cells, mast cells, T and B lymphocytes	T_H1 cytokine. Pro-inflammatory cytokine causing T lymphocyte activation.

Table 3. Source and functions of important cytokines.

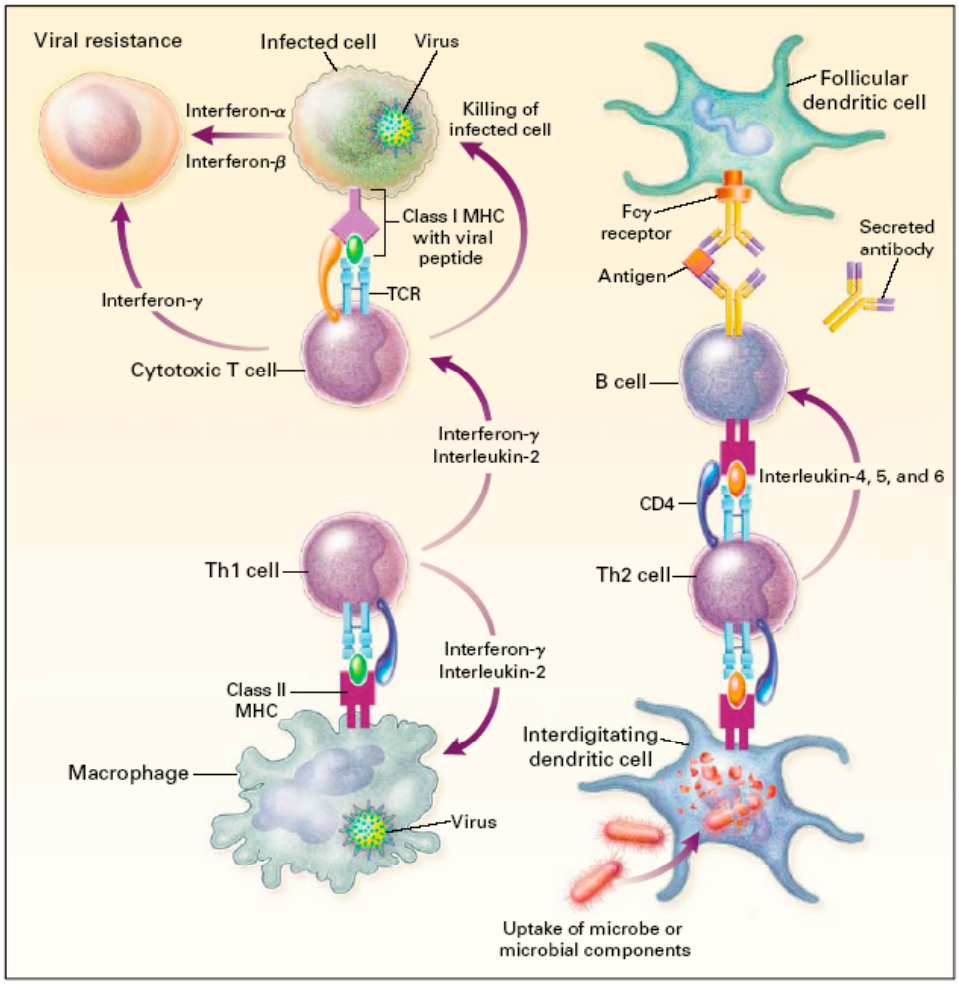

Figure 5. Lymphocyte immune response activation.

Left panel: Cell-mediated immune response with the activation of T_H1 helper $CD4^+$ and cytotoxic $CD8^+$ T lymphocyte in the context of a viral infection. Recognition of viral peptide presented by MHC-I on the APC and on the virally-infected cell as well as the production of T_H1 cytokines (IL-2, IFN-γ). Right panel: Humoral immune response with the activation of T_H2 helper $CD4^+$ and antibody-producing B lymphocyte in the context of a bacterial infection. Presentation of bacterial peptides following phagocytosis by the APC on MHC-II and production of T_H2 cytokines (IL-4, IL-5, IL-6). Adapted from (123).

1.2.4 CELLS OF THE INNATE IMMUNE SYSTEM

1.2.4.1 Granulocytes

Granulocytes constitute about 65% of all peripheral blood mononuclear cells (PBMCs) and regroup basophils (0.5% to 1%), eosinophils (3% to 5%) and neutrophils (90% to 95%). Neutrophils are the first cells to extra-vasate from the blood to the site of injury. Neutrophils are terminally differentiated, are incapable of cellular division, have a very short lifespan and produce very low *de novo* RNA and protein, which makes them exceptionally hard to study *in vitro*. Neutrophils phagocytose and kill pathogens by producing free radicals. GM-CSF, often utilize in cancer immunotherapy, potentiates the neutrophil functions, such as increases in migration and transmigration, phagocytosis, oxidative metabolism, lysozyme release and complement recruitment (124, 125). Eosinophils are not phagocytotic and kill pathogens through the release of reactive oxygen species (ROS), cytokines and prostaglandins. Basophils have FcεR specific for IgE. During allergic reaction, the binding of FcεR by IgE will trigger the release of histamine, protaglandins and leukotrienes.

The role of granulocytes in tumor promotion and tumor elimination remains poorly understood. Eosinophils expressing indoleamine 2,3-dioxygenase (IDO), a tryptophan-metabolizing enzyme with immunosuppressive properties, are found in lesions of non-small cell lung cancer (126). On the other hand, neutrophils expressing Fc receptors could help eliminate antibody-coated tumor cells (127). Through the use of an anti-Gr1 antibodies targeting neutrophils and eosinophils (128, 129) and of an attenuated measle virus transfected with GM-CSF (125), neutrophils were shown to be implicated in the rejection of human tumors in SCID mice. Neutrophils are also thought to possess a more important role in tumor rejection than eosinophils (130, 131) and to directly eliminate tumor cells via the release of hydrogen peroxide (132, 133), the nitric oxide (NO) pathway (130) and neutrophils-mediated inhibition of glutamine uptake by the tumor cell (134).

1.2.4.2 Natural killer cells

NK cells are effector lymphocytes of the innate immune system [reviewed in (135)]. NK cells comprise 2 to 18% of PBMCs with a turnover rate of about two weeks. In humans, NK cells can be divided into 2 subsets: $CD56^{dim}$ (90% of blood and spleen NK cells) and $CD56^{bright}$ (mostly present in LNs). NK cells do not recognize antigens presented by MHC. They express killer-activating (KARs) and killer-inhibitory receptors (KIRs), which allows for the recognition and elimination of virally-infected or malignant cells. KARs, such as NKG2D, detect the presence of stress-induced ligands on the surface of the target cells. NK cells also express CD16, a low-affinity Fc receptor allowing the detection of antibody-coated cells and the activation of antibody-dependent cell cytotoxicity (ADCC). KIRs contain intracytoplasmic inhibitory motifs (ITIMs) and inhibit NK cell activation. KIRs recognize MHC-I expressed on all nucleated cells. MHC-I expression is often lost during viral infection causing the absence of presentation of viral to cytotoxic $CD8^+$ T lymphocytes. The loss of MHC-I expression results in an absence of NK cell inhibitory signaling, which induces NK cell activation and the destruction of the MHC-I deficient target cell.

Malignant cells also lose MHC-I expression, which renders them invisible to $CD8^+$ T lymphocytes but sensitive to NK cell recognition. NK cells kill tumor cells via TNF-related apoptosis-inducing ligand (TRAIL) or perforin-dependent pathways. In mouse models of spontaneous and induced tumors, depletion of NK cells augments the risk of tumor development (136, 137). To prevent NK cell killing, prostate cancer cells secrete soluble NKG2D ligand, such as MHC class I-related chain molecules A/B molecules (MICA/B), which causes NK cell activation away from the tumor cells.

1.2.4.3 Natural killer T cells

Natural killer T (NKT) cells are a subset of $CD4^+$ and $CD8^+$ T lymphocytes expressing a αβ-TCR and NK cell markers. Similar to γδ T lymphocytes, NKT cells recognize lipids and carbohydrates presented on the cell surface by non-classical

MHC such as CD1d. NKT cells produce both T_H1 (IL-2, GM-CSF, IFN-γ, TNF-α) and T_H2 cytokines (IL-4). The role of NKT cells in tumor development remains ambiguous.

1.2.4.4 Monocytes and Macrophages

Monocytes compose 5% to 10% of PBMCs. They have a short lifetime, spending an average of 24 hours in the circulation. Monocytes kill phagocytosed pathogens and produce a wide array of pro-inflammatory cytokines. In human, there are two main monocyte subtypes: classical $CD14^{hi}CD16^{-}$ monocytes (90% of total monocytes) and non-classical $CD14^{+}CD16^{+}$ monocytes. When monocytes extra-vasate from the blood, they differentiate into macrophages. Macrophages can further differentiate in various long-live tissue macrophages, such as osteoclasts (bones), microglial cells (alveoli, central nervous system), histiocytes (LNs) and Kupffer cells (liver) (138).

Macrophages play important roles in homeostasis and in immune responses [reviewed in (139)]. With regards to homeostasis, macrophages phagocytose 2×10^{11} erythrocytes each day and recycle iron and hemoglobin. Macrophages also remove cellular debris generated during tissue remodeling and apoptotic bodies from dying cells. Receptors involved in macrophage's homeostatic clearing functions are scavenger receptors, phophatidyl serine receptors, thrombospondin receptor and integrins. These receptors do not activate intracellular signaling linked to cytokine gene expression and development of an inflammatory response. Conversely, clearance of cellular debris from necrotic cells causes an inflammatory response due to the activation of TLRs, intracellular pathogen recognition receptors, IL-1R, and myeloid differentiation primary-response gene 88 (MyD88) signaling.

During an immune response, macrophages participate in pathogen phagocytosis, antigen presentation, cytokine production as well as innate and adaptive immune cells activation. Macrophages also play essential roles in the termination of the immune response and tissue repair. Innate immune cells cause transient

macrophage activation, whereas adaptive immune cells cause a prolonged activation, which can lead to the chronic inflammation.

Macrophage classification has been the focus of extensive reviews in recent years. Based on murine studies, macrophages are classified as M1 (classically activated, pro-inflammatory) and various subtypes of M2 macrophages (alternatively activated, anti-inflammatory, tissue remodeling) (140). However, studies have revealed that human macrophages cannot be categorized according to murine macrophage classification. Key molecular markers for M1 and M2 murine macrophages, such as inducible nitric oxide synthase (iNOS) and ARG1 respectively, are not expressed by human macrophages. The mannose receptor (CD206) is the only accepted marker for human M2 macrophages (141). Hence, compared to murine macrophages, population markers and overall classification of human macrophages remains rather blurred. In 2008, Mosser *et al.* proposed a classification based on the fundamental functions of macrophages: host defense, wound healing and immune regulation (139).

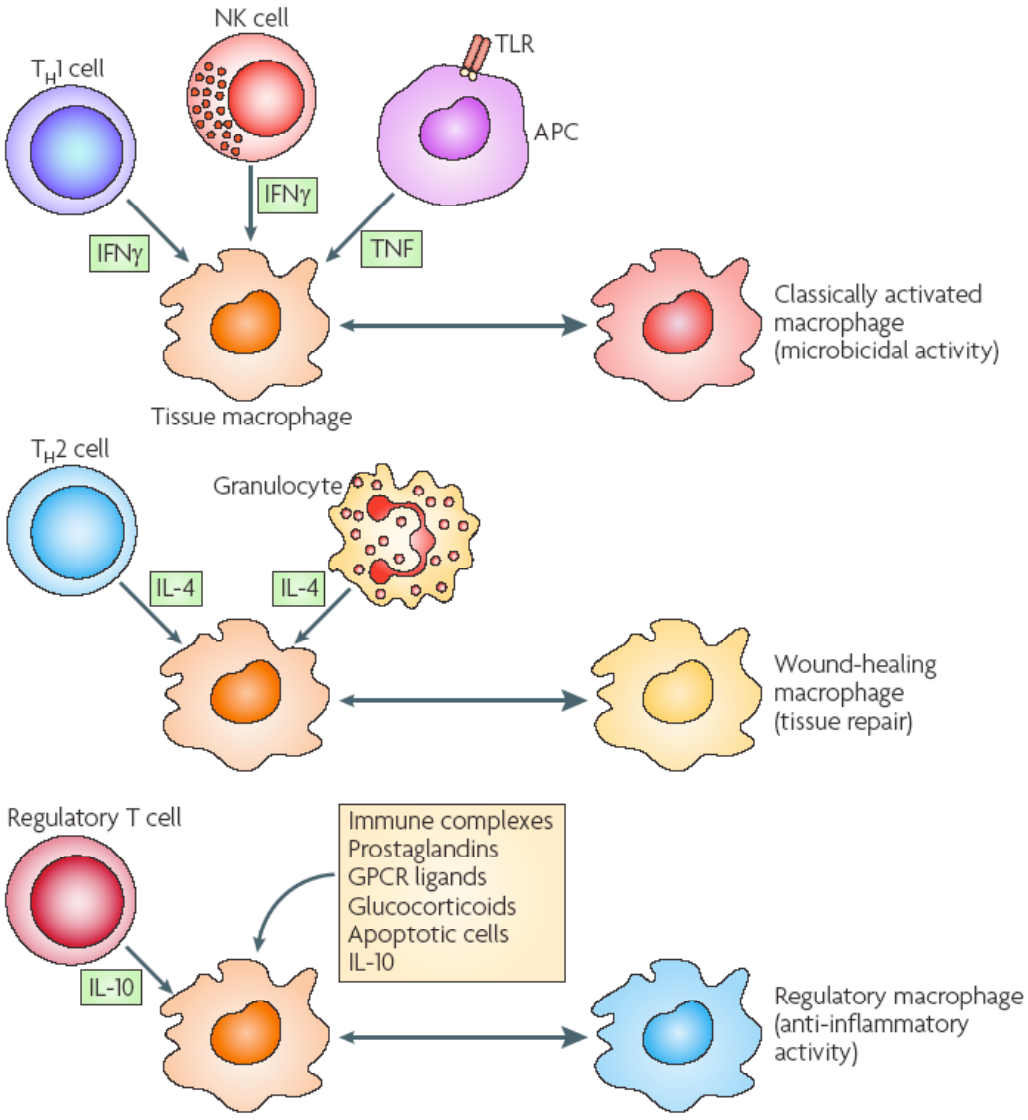

Figure 6. Human macrophage phenotypes.

Human macrophage classification according to functions: microbicidal, tissue repair and anti-inflammatory functions.

Adapted from (139).

Host defense or classically activated macrophages or M1 macrophages are effector macrophages produced during a cell-mediated immune response in the presence of IFN-γ and TNF-α. IFN-γ is initially produced by innate cells (NK cells) and later by adaptive cells (T_H1 $CD4^+$ T lymphocytes) allowing for the prolonged macrophage activation. Other pro-inflammatory mediators, such as TLR-ligands signaling through MyD88, IFN-regulatory factor 3 (IRF3) and IFN-β also activate this macrophage population. Classically activated macrophages have enhanced microbicidal and tumoricidal capacity and secrete high amounts of pro-inflammatory cytokines. They will produce high amounts of ROS enabling them to kill phagocytosed intracellular pathogens.

Wound-healing macrophages or M2 macrophages are activated primarily through IL-4, IL-13 and M-CSF produced by innate and adaptive immune cells. Basophils, mast cells, neutrophils and other granulocytes are responsible for the initial release of IL-4 following tissue injury (142, 143). Compared to classically activated macrophages, wound-healing macrophages secrete minimal amount of pro-inflammatory cytokines, do not present antigen to T lymphocytes and are less efficient at killing intracellular pathogens due to lower ROS production. Wound-healing macrophages have immunosuppressive effects on the immune response.

Regulatory macrophages are also part of the M2 macrophage murine nomenclature. This macrophage population is implicated in the termination of the immune response. Regulatory macrophages produce IL-10, an anti-inflammatory cytokine, in response to glucocorticoids, immune-complexes, FcγR activation, prostaglandins, apoptotic cells, ligands of G-protein-coupled receptors (GPCRs), and IL-10 itself. Glucocorticoids released by adrenal glands in response to stress can promote the expansion of regulatory macrophage by inhibiting the transcription and decreasing mRNA stability of pro-inflammatory genes (144). Moreover, glucocorticoid-treated macrophages skew the T lymphocyte response to a T_H2 phenotype and induce the development of T_{REGs} (145). Interestingly, regulatory

macrophages are potent APCs and have a high expression of co-stimulatory molecules (CD80 and CD86) (146).

In cancer, macrophages activated by IFN-γ initially eliminate tumor cells through cell-contact dependant and independent mechanisms via the production of ROS (147, 148). However, this production of free radicals promotes DNA damages and increases genomic instability within the tumor microenvironement. Furthermore, tumor cells take advantage of the high level of plasticity between the various macrophage phenotypes. Tumor-associated macrophages (TAMs) acquire a hybrid wound healing/regulatory phenotype that inhibits the anti-tumoral immune response and restrain neighboring cytotoxic macrophages. TAMs also promote tissue remodeling and angiogenesis (149) through the expression of matrix degrading enzyme and pro-angiogenic factors (114, 139, 150-153).

In prostate cancer, the impact of macrophage infiltration within the prostate remains uncertain with only a few studies offering diverging conclusions on the association between macrophage density and disease progression. High TAMs density is associated with a worse prognosis and decreased survival (154). In a severe combined immunodeficiency (SCID) mouse model, inhibition of monocyte chemotactic protein-1 (MCP-1) causes a reduced macrophage infiltration resulting in a reduction of angiogenesis and tumor growth (155). In a rat prostate cancer model, only extra-tumoral macrophages were associated with tumor size and vasculature proliferation (156). However, high TAMs density correlated with a better five-year survival rate (157). Altogether, a better assessment of the macrophage phenotype is essential in understanding their function in prostate cancer.

1.2.4.5 Dendritic cells

DCs were first described by Paul Langerhans in the late 19th century. In 1973, the group of Dr. Ralph Steinman classified these cells as DCs (158). Myeloid DCs (mDCs) express TLR-4 and TLR-6 and activate T_H1 lymphocytes through IL-12 secretion. Plasmacytoid DCs (pDCs) express TRL-7 and TLR-9 and produce high levels of IFN-α. DCs are professional APCs that have the ability to present antigens

to both $CD4^+$ and $CD8^+$ T lymphocytes. DCs express MHC-I and MHC-II as well as several co-stimulatory molecules (CD40, CD80, CD86) leading to potent lymphocyte activation. Immature DC have high phagocytic activity and minimal lymphocyte activation potential. Following pathogen recognition through pathogen recognition receptors and their subsequent phagocytosis, DCs express CCR7, the chemokine receptor promoting LN homing. During LN migration, DCs begin their maturation process, where they lose their phagocytotic abilities and upregulate the expression of co-stimulatory molecules and MHC-peptide complexes (159, 160).

In cancer, dysfunctions in DC maturation, differentiation and migration promote the tumor's tolerogenic state [reviewed in (161)]. Instead of priming TAA-specific T lymphocytes, immature DCs secrete IL-10 and vascular endothelial growth factor (VEGF) causing T lymphocyte anergy. Cancer cells also alter bone marrow hematopoiesis and promote the generation of large numbers of immature DCs. Moreover, mature tolerogenic DC promote T_H2 polarization and the expansion of T_{REGs}. Furthermore, as previously mentioned, tumor-associated DC have defects in cross-presentation of TAAs. Monocytes from prostate cancer patients also differentitate less efficiently in myeloid DCs as in healthy controls (162, 163). Fortunately, circulating DC from prostate cancer patients can be activated into mature, fully functional DC suitable for immunotherapy (164, 165) (see section 1.1.8.2.1 on page 17).

1.2.4.6 Mast cells

Mast cells are tissue resident innate immune cells that contain cytoplasmic granules enriched in histamine and heparin. Mast cell degranulation occurs following tissue injury, IgE cross-linking of FcεRI or complement activation during allergic reaction and anaphylaxis shock.

In cancer, mast cells were one of the first immune cells identified in the tumor microenvironment (166). During acute inflammation, mast cells secrete several cytokines and inflammatory effectors with pro-angiogenic properties (heparin, heparanase, histamine), tissue-remodeling serine- and metallo-proteinases and various

growth factors (basic fibroblast growth factor and VEGF) (167). Thus, mast cell can promote extracellular matrix remodeling and tumor neo-angiogenesis (168).

1.2.4.7 Myeloid-derived suppressor cells

Myeloid-derived suppressor cells (MDSCs) are a phenotypically diverse population of immature myeloid precursors found in tumors. MDSC can inhibit the production of IFN-γ by $CD8^+$ T lymphocyte by secreting TGF-β or by L-arginine metabolism. In mice, MDSCs express high levels of ARG1 concomitantly with iNOS, which deplete the tumor microenvironment in L-arginine and potently inhibit T lymphocyte activity through peroxynitration of tyrosine residues (169, 170) (see section 1.4.2.3 on page 66). In human, MDSCs are undefined immunoregulatory innate immune cells with an immature differentiation phenotype (171). Human MDSC express CD11b, CD13, CD15, CD33 and CD34 and are devoid of CD14, and HLA-DR (163, 172). Human MDSC can express ARG1.

1.2.5 CELLS OF THE ADAPTIVE IMMUNE SYSTEM

1.2.5.1 T lymphocytes

Lymphocytes comprise about 30 % of PBMCs, with 85 % to 90 % being T lymphocytes. There are two main families of T lymphocytes based on their TCR: the αβ-T cells and γδ-T cells. αβ-T cells represent 95 % of all T lymphocytes and recognize antigenic peptides presented by MHCs on APCs. Within circulating αβ-T cells, the ratio between helper $CD4^+$ T lymphocytes and cytotoxic $CD8^+$ T lymphocytes is about 2 to 1. γδ-T cells represent 5 % of the total T lymphocyte population and participate in mucosal immunity. γδ-T cells do not recognize antigens through MHC-peptide complexes, but rather through non-classical MHCs, such as CD1, which present lipids and glycolipids. During TCR-MHC engagement, signaling through the associated CD3 molecules is essential for T lymphocyte activation. The CD3 complex is comprised of CD3γ, CD3δ, CD3ε and CD3ζ chains. The cytoplasmic tails of the CD3 chains are phosphorylated following TCR cross-linking

and initiate signal transduction. In the absence of co-stimulatory molecules (second signal), CD3 is improperly phosphorylated, which causes lymphocyte anergy or apoptosis.

In murine models of prostate cancer, infiltrating T lymphocytes have decreased expression of TCR-β, CD3ε and CD3ζ chains (173). L-arginine depletion in the prostate tumor microenvironment decreases the expression of CD3ζ chains and prevents T lymphocyte activation and proliferation (see section 1.4.2.3 on page 66).

1.2.5.2 $CD4^+$ T lymphocytes

$CD4^+$ T lymphocytes can be broadly defined as cytokine-secreting helper T lymphocytes. Depending on the pathogen and on the cytokine environment during the initial recognition of their cognate antigen, naïve T_H0 T lymphocytes will differentiate into various T_H phenotypes (T_H1, T_H2, T_H17). Each T_H phenotype is characterized by its cytokine profile and by the immune cell effector it activates. T_H1 helper $CD4^+$ T lymphocytes secrete T_H1 cytokines (IFN-γ, IL-2, IL-12), which promote the activation of cytotoxic $CD8^+$ T lymphocytes and classically-activated macrophages. T_H2 helper $CD4^+$ T lymphocytes secrete T_H2 cytokines (IL-4, IL-5, IL-6, IL-10, IL-13), which promote the activation of B lymphocytes and antibody production, granulocytes as well as wound healing and regulatory macrophages. T_H1 cytokines such as IFN-γ inhibits the activity of T_H2 cells and, reciprocally, IL-10 inhibits T_H1 cells (174). T_H17 $CD4^+$ T lymphocytes develop in response to IL-23 and secrete IL-17 [reviewed in (175)]. T_H17 T lymphocytes participate in the inflammation associated with arthritis and encephalitis. In prostate cancer, activated T lymphocytes were shown to secrete high amounts of IL-17 (176). However, the exact function of T_H17 T lymphocytes in prostate cancer remains undefined.

1.2.5.3 Regulatory T cells

Regulatory T cells (T_{REGs}) represent 10-15% of the $CD4^+$ T lymphocytes. T_{REGs} constitutively express CD25, the α-chain of the IL-2 receptor and forkhead box P3 (Foxp3), a transcription factor, which, to date, is the most reliable T_{REG} marker

(177). Other T_{REG} markers include glucocorticoid-induced tumor necrosis factor receptor (GITR), CD44, CD103, latency-associated peptide, intracellular IL-10 and CTLA-4. Two main subtypes of T_{REGs} have been identified, naturally-occurring thymic T_{REGs} and peripherally-induced T_{REGs}, whose development from naive T lymphocyte is regulated by TGF-β, IL-2 and retinoic acid. T_{REGs} are essential for the maintenance of immunological tolerance and immune cell homeostasis during the immune response (178, 179). T_{REGs} are activated in an antigen-specific manner in secondary lymphoid organs, but suppress effector cells irrespectively of their antigen specificity. T_{REGs} have multiple immunosuppressive mechanisms through cell-cell contact dependent pathways or secretion of immunosuppressive IL-10 and TGF-β. Naturally-occurring T_{REGs} suppress the proliferation and the differentiation of effector functions of naïve T lymphocytes. T_{REGs} also inhibit the effector activities of $CD4^+$, $CD8^+$ T lymphocytes, NK cells, NKT cells, B lymphocytes, macrophages, osteoclasts and DCs. Recently, the group of Dr. Sakaguchi observed that T_{REGs} promoted the down-regulation of the co-stimulatory molecules CD80 and CD86 on the surface of DCs, without affecting the expression of CD40 or MHC-II, thereby preventing naïve T lymphocyte activation via CD28 (180).

Pathologically, T_{REGs} limit the aberrant activation of immune cell effectors in autoimmune disorders and in allergy. In cancer, the presence of T_{REGs} within the primary tumor can inhibit the anti-tumoral immune response and can hinder the clinical efficacy of immunotherapy (181). In murine models, T_{REGs} accumulate in the primary tumor and blocking of CD25 with anti-CD25 antibody reduces tumor growth (182). T_{REGs} also accumulate in primary tumors of prostate cancer patients (48, 50) as well as a less described $CD8^+Foxp3^+$ T_{REGs} (183). Similar to ovarian cancer (184), the chemokine CCL22 could be implicated in the recruitment of T_{REGs} in the prostate tumor. Remarkably, in a murine mouse model, the presence of T_{REGs} correlated with a lower risk of prostate cancer as it inhibited the development of chronic inflammation (53).

1.2.5.4 $CD8^+$ T lymphocytes

Cytotoxic $CD8^+$ T lymphocytes recognize antigens presented by MHC-I. Virally-infected cells present viral peptides on their surface through MHC-I, which causes their recognition and elimination by $CD8^+$ T lymphocytes through the secretion of perforin and granzymes (185). $CD8^+$ T lymphocytes also express Fas ligand (FasL) and bind Fas receptor on the surface of the target cell, which leads to caspase activation and apoptosis. Cytotoxic $CD8^+$ T lymphocytes are central cellular effectors that need to be activated in the context of cancer immunotherapy.

As a method of immune evasion, prostate cancer cells lose MHC-I expression, which renders them invisible to cytotoxic $CD8^+$ T lymphocytes. Furthermore, in a recent study, $CD8^+$ T lymphocytes were shown to have undergone clonal expansion within the prostate (186). However, these $CD8^+$ T lymphocytes also express high level of PD-1, a cell surface receptor associated with an "exhausted" $CD8^+$ T lymphocyte phenotype (46, 186). Finally, following trafficking through the prostate, murine prostate-specific CD8+ T lymphocytes can inhibit the proliferation of naïve T lymphocytes in a TGF-β-dependent manner (187).

1.2.5.5 B lymphocytes

B lymphocytes are antibody producing cells representing 5% to 15% of circulating lymphocytes. B lymphocytes first develop in the bone marrow. They proliferate and mature in germinal centers through close interactions with follicular dendritic cells, macrophages and $CD4^+$ helper T lymphocytes. Ligation of CD40 on B lymphocytes by CD40 ligand (CD40L) expressed by $CD4^+$ helper T lymphocytes is necessary for their activation and leads to somatic hypermutation and class switching recombination causing the production of high-affinity antibodies. Conventional B lymphocytes (B2 cells) can present phagocytosed antigen to T lymphocytes and, reciprocally, get activated and develop into antibody-producing plasma cells (188-190). The smaller population of B1 cells can produce antibody independently of T lymphocytes and are suggested to be the source of auto-antibodies (191).

In cancer, B lymphocytes and antibody production participate in the maintenance of the tumor's chronic inflammatory state.

1.2.6 SUMMARY

An effective immune response necessitates the activation of both innate and adaptive immune cellular effectors. In cancer, the cell-mediated anti-tumoral T_H1 $CD8^+$ T lymphocyte immune response is rendered ineffective due to DC dysfunction, the recruitment of immunoregulatory cells, such as T_{REGs} and regulatory macrophages, and the development of an immunosuppressive microenvironment within the tumor bed. In the case of prostate cancer, it is essential to better understand the activation status of immune cells within secondary lymphoid organs and the primary tumor. The sole evaluation of cell numbers contributes to confusing conclusions in the literature as to the implication of lymphocytic and myeloid cell infiltration in disease progression.

1.3 THE ANTI-TUMORAL IMMUNE RESPONSE

It was 100 years ago when Dr. Paul Ehrlich demonstrated that the immune system could recognize and eliminate tumor cells (192). In the late 1950s, Drs. Thomas and Burnet, coined the concept of immune surveillance based on the increased cancer incidence from viral origins in immunosuppressed patients (193, 194). The cancer immune surveillance concept states that lymphocytes are responsible for eliminating continuously arising malignant cells. Early on, this concept however failed to be accepted by oncologists since athymic nude mice develop chemically induced tumors at the same rate as control mice suggesting that the absence of T lymphocytes did not favor tumor development (195). At the time, it was not known that athymic nude mice do in fact produce low, but detectable numbers of $\alpha\beta$ T lymphocytes, have normal numbers of functionally active NK cells, have a fully functional innate immune system, and that they could thus mount an effective anti-tumoral immune response. Recently, with the use of modern immunodeficient mouse models, it has been demonstrated that the immune system does eliminate tumor cells.

Nonetheless, the continued interactions between immune and tumor cells causes a survival selection pressure that promotes the outgrowth of tumor cells better suited to fight off the anti-tumoral immune response. In light of these results, Dunn and Schreider proposed the concept of immune editing, which is comprised of three phases: elimination (immune surveillance), equilibrium and escape (196) [and reviewed in (197)].

1.3.1 IMMUNE ELIMINATION

During the immune elimination phase, the immune system acts as an extrinsic tumor suppressor continuously killing spontaneously arising tumor cells. Similar to the eradication of invading pathogens, the elimination of tumor cells requires the collaborative effort of both adaptive and innate immune cells and the production of IFN-γ. Innate cells are first to recognize malignant transformation. NK cells can recognize tumor cells by the absence of MHC-I expression. Innate cells can also recognize the local tissue disruption associated with unregulated stroma remodeling caused by tumor neo-angiogenesis and tissue invasion. This initial tumor cell recognition leads to the production of IFN-γ and IL-12 by NK cells and macrophages. Tumor cell death and phagocytosis favor the presentation of TAAs to naïve tumor-specific T_H1 $CD4^+$ T lymphocytes and promote the activation tumor-specific $CD8^+$ cytotoxic T lymphocytes. These activated T lymphocytes eliminate the remaining tumor cells in an antigen-specific manner. It is interesting to note that most clinically diagnosed cancers occur in the aging population where there is a concomitant decline in immune functions.

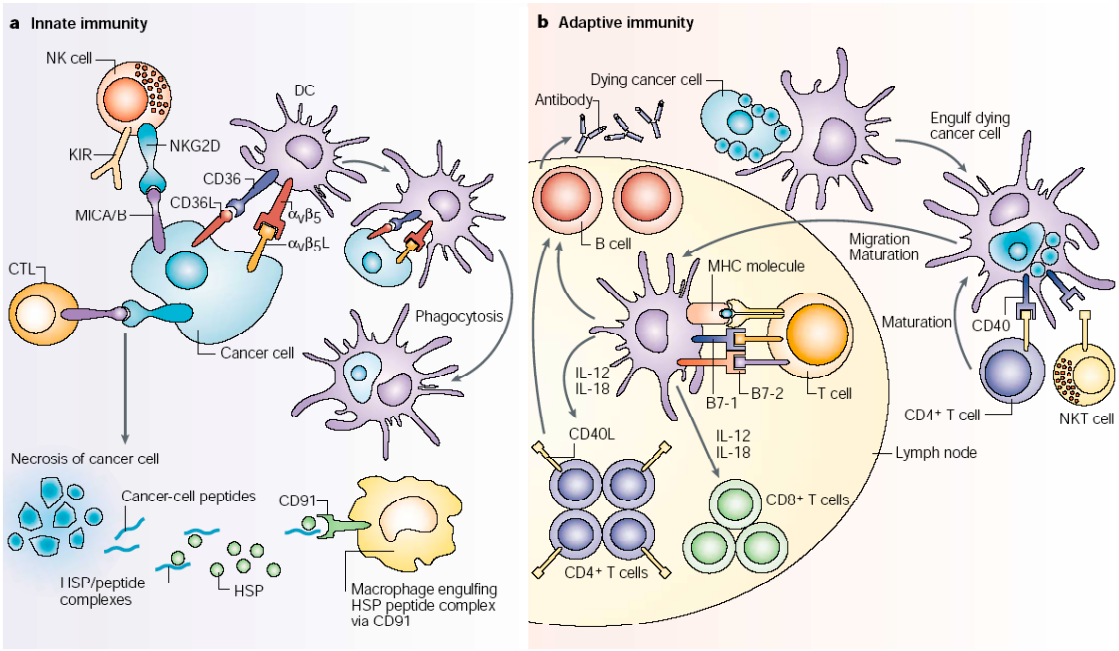

Figure 7. Direct and indirect pathways of tumor cell recognition by innate and adaptive immune cells.

Right panel: Tumor cell recognition by innate immune cells. NK cell recognizes the absence of MHC-I expression by the tumor cell and MICA/B expression by the tumor cell. Through CD36 and $\alpha_v\beta_5$ integrins, DC phagocytose the tumor cell, whose TAAs will be presented in the tumor draining LNs. Necrotic tumor cell will activate scavenger receptors (CD91) on tumoricidal macrophages. Left panel: DC present TAAs to $CD4^+$ T lymphocytes and $CD8^+$ T lymphocytes. B lymphocytes are also activated with the help of $CD4^+$ T lymphocytes. Reciprocally, $CD4^+$ T lymphocytes promote DC maturation, which also activate NKT cells.

Adapted from (101).

1.3.1.1 Evidence of immune elimination in mice

In the 1990s, two landmark studies renewed the interest for cancer immune surveillance. There was the demonstration that endogenous IFN-γ is central in the protection against transplanted tumors, chemically induced tumors and spontaneously arising lymphomas and lung adenocarcinomas (198). Furthermore, mice lacking perforin are more sensitive to chemically induced and spontaneous tumors (199). Definitive proof of cancer immune surveillance came through the use of recombinase activating gene-2 (RAG-2) knock-out mice, which lack B lymphocytes, T lymphocytes and NKT cells (200). Rag-2 -/- mice have a more rapid development of chemically-induced tumors as well as a higher incidence of spontaneous, non-virally induced tumors.

1.3.1.2 Evidence of immune elimination in humans

Individuals with congenital or acquired immunodeficiencies and patients undergoing immunosuppressive therapies have increased incidence of cancers of the anal and urogenital tracts and virally-induced cancer, such as Kaposi's sarcoma (Kaposi's sarcoma-associated herpes virus) and non-Hodgin's lymphoma (human T-cell leukemia/lymphoma virus and Epstein Barr virus) (201). Cancer incidence analysis of non-viral origins in immunocompromised individuals is problematic due to their higher sensitivity to infection. Nonetheless, there is evidence showing that immunosuppressed transplant patients have a higher incidence of non-viral cancers (197). Several studies demonstrated an increased incidence of colon, lung, bladder, kidney, pancreatic, ureter and endocrine tumors in transplanted patients (202, 203). In one study, the prevalence of lung cancer was 25-fold higher in transplanted patients. However, it remains unknown whether clinically induced immunosuppression increases the development of *de novo* tumors (related to the absence of immune elimination) or permits the proliferation of pre-existing cancers initially constrained by the immune system (related to the absence of immune equilibrium).

Dunn and Schreiber also suggest that immune surveillance is evidenced by the correlative data between the presence of tumor-infiltrating lymphocytes (TILs) and favorable prognostic indicator for patients with melanoma (204), ovarian (205), colorectal (206), esophageal squamous cell (207) and prostate cancer (43). NK cell infiltration of the primary tumor is also a favorable prognostic factor for patients with gastric carcinoma (208), squamous cell lung carcinoma (209) and colorectal cancer (210). Moreover, patients do develop adaptive and innate immune response to the tumors they bear. Some cancer patients develop paraneoplastic neurological disorder, a rare autoimmune neurological disease caused by the anti-tumoral immune response cross-reacting with the nervous system (211).

However, contrary to the murine studies, these correlative data based on immune cell infiltration and disease progression do not reflect a role for immune surveillance. Clinically detectable tumors have passed the stage of immune surveillance and thus the immune cell infiltrate could be drastically different from the immunological environment during the earlier stages of carcinogenesis. Nonetheless, cancer incidence in transplanted patients is insightful. If transplanted patients not only have a higher tumor incidence, but also have a more rapid onset of non-virally-induced tumors, then it could be suggested that these patients do lack anti-tumoral immune functions related to either immune elimination or equilibrium.

1.3.2 IMMUNE EQUILIBRIUM

Evidently, not all malignant cells are eliminated during the immune surveillance phase as patients do develop cancer. The carcinogenic process follows a darwinesque evolutionary route on which tumor cells that gain a survival advantage in the face of a selective pressure will be able to survive and to proliferate. The immune equilibrium stage of carcinogenesis is believed to be the longest stage in the life of the tumor during which it enters in a dynamic communication with the immune system. There are three eventual outcomes to this stage. The immune system can gain a definite advantage and fully eradicate the tumor mass. On the other hand, the immune system can be unable to eliminate the tumor cells, but nonetheless keep tumor cell numbers

below clinically detectable levels. Finally, tumor cells can overpower the immune system and grow into a clinically detectable mass. Through the release of ROS, the continuous presence of innate and adaptive immune cells effectors can increase genomic instability of malignant cells. It will thus become easier for these genetically unstable cells to mutate, reduce their immunogenicity and acquire weapons to fight off the immune system [reviewed in (212)]. By the time the tumor attains a clinically detectable size, it has developed sufficient immune invisibility and gained an effective arsenal to allow for its survival.

1.3.2.1 Evidence of immune equilibrium in mice

Studies in mouse models demonstrate that the immune system sculpts the immunogenicity of tumor cells. Cancer cells from wild type or Rag2 -/- mice both grow with similar kinetics in immunodeficient mice. However, Rag2 -/- tumors are rejected when transplanted in immunocompetent hosts, demonstrating that tumors arising from immunodeficient hosts are more immunogenic (197). In the prostate cancer TRAMP model, adoptive transfer of TAA-specific T lymphocytes offers a long-term protection against tumor outgrowth without completely eradicating the tumor bed (213). This state of immune-induced tumor dormancy is also observed in healthy mice receiving low doses of carcinogen 20-methylchol-anthrene (MCA). Without having evidence of growing tumors, these mice have dormant tumors kept in check by the immune system. Following specific immunosuppression (T lymphocyte depletion or anti-IL-12 or anti-IFN-γ antibodies), there is an outgrowth of the dormant tumor, which becomes fatal for the host (214).

1.3.2.2 Evidence of immune equilibrium in humans

Immune equilibrium is observed in cancer patients who are in remission for several years following therapy. A study shows that breast cancer patients have been in remission for more than 20 years (free of clinically detectable tumor mass) still had tumor cells circulating in their blood (215). The immune equilibrium phase of cancer progression is also observed in the transmission of cancer from transplant donors to

recipients. The transplanted organs from donors with no clinical history of cancer or in durable cancer remission (histologically normal and tumor-free at harvest) can give rise to cancer in the recipient host (216-219). Results from the literature suggest that the immunosuppressive regiment of transplanted patients can allow the outgrowth of occult tumor cells, which have been maintained in the equilibrium phase by the immunocompetent donor. The rapid onset of tumor growth in these patients argues against *de novo* carcinogenesis.

1.3.3 IMMUNE ESCAPE

The immune escape phase is characterized by the outgrowth of immunologically sculpted tumor cells into clinically detectable size in immunocompetent host. At this point of carcinogenesis, tumor cells have acquired sufficient "immunity" against immunological attacks from both innate and adaptive immune cell effectors. In a murine model activated TAA-specific T lymphocytes are functionally tolerogenized, i.e. they are unable to degranulate and secrete IFN-γ or granzyme B (220). In fact, it is the entire tumor microenvironment that has become hostile to the anti-tumoral immune response. Stromal cells, which are also modified by their neoplastic neighbors, can express IDO (221) and COX-2 thereby directly participating in the development of the tumor's immunosuppressive microenvironment. DC expressing IDO can accumulate in tumor draining LNs preventing the activation and promoting the apoptosis of TAA-specific lymphocyte (220). In prostate cancer patients, we showed that tumor-draining lymph nodes are also immunosuppressed (222).

Immune escape involves several mechanisms. The selective immunological pressure renders the tumor cell less immunogenic through the loss of MHC-I expression. Cancer cells also express immunoregulatory molecules (T_H2 cytokines, arginase, IDO, COX-2) preventing the activation of innate and adaptive immune cells. Cancer cells also shed decoy receptors (Fas) and decoy ligands (FasL, MICA/B) forcing the improper activation or apoptosis of cellular effectors away from the tumor bed. Finally, immunosuppressive immune cells (MDSCs, T_{REGs} and regulatory

macrophages) that participate in the termination of the immune response are recruited to the tumor, further preventing the activation of the anti-tumoral immune response.

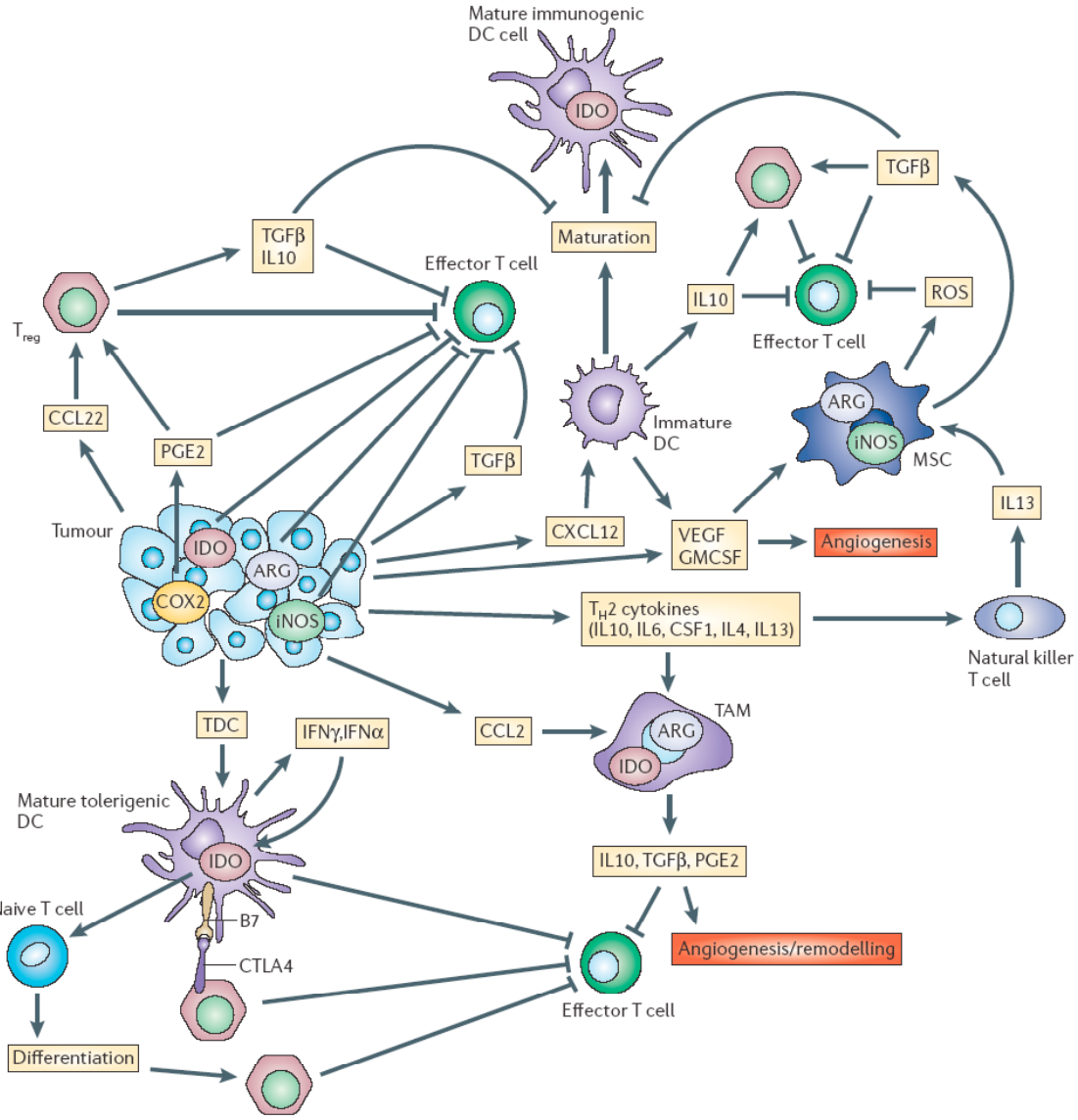

Figure 8. Immunosuppressive pathways in cancer.

Schematization of several immunosuppressive pathways possibly present in cancer. Note the large variety of immunoregulatory molecular mediators as well as the involvement of multiple innate and adaptive immune cells.

Adapted from (221).

1.3.4 IMMUNOSUPPRESSION IN PROSTATE CANCER

The following section will briefly describe the role of immunosuppressive molecules documented to be expressed in prostate cancer [reviewed in (223)]. Tumor-derived immunosuppression through cellular effectors such as T_{REGs}, immature DCs and regulatory macrophages were previously described in sections 1.2.5.3 on page 43, 1.2.4.5 on page 40 and 1.2.4.4 on page 36.

1.3.4.1 Defects in antigen presentation

Several studies have demonstrated a reduced or complete loss of MHC-I expression in primary tumors and LN metastases from prostate cancer patients compared to normal or BPH specimens (224-227). Loss of MHC-I expression prevents the presentation of TAAs on the surface of tumor cells and their recognition by $CD8^+$ T lymphocytes. Contrary to prostate cancer cell lines, defects in the antigen presentation machinery have not been detected in tissue samples from primary tumors.

1.3.4.2 Production of immunosuppressive cytokines

In the context of a T_H1 anti-tumoral immune response, the expression of T_H2 cytokines is considered immunosuppressive as it inhibits the expression of IFN-γ and the activation of cytotoxic $CD8^+$ T lymphocytes. The serum of prostate cancer patients contains elevated levels of T_H2 cytokines (IL-4, IL-6, IL-10, TGF-β) compared to men without cancer or to men with BPH (228-231). An increase in IL-6 levels is associated with a worst prognosis (232) and with direct growth promoting effects on prostate cancer cells (119). Mitogen-activated PBMCs of prostate cancer patients also produce less T_H1 cytokines (IL-2 and IFN-γ) than controls (233), which could explain the inefficiency of the CD8+ mediated anti-tumoral immune response.

1.3.4.3 Immunosuppression through amino acid depletion

Amino acid depletion is an in ancestral mechanism preventing the proliferation of invading cells or pathogens. From a tumor's point of view, the invading cells are activated tumor-specific $CD8^+$ T lymphocytes. As further detailed in section 1.4.2.3 on page 66, the activation status of $CD8^+$ T lymphocytes is sensitive to the concentration of specific amino acids (arginine, tryptophan) present in the tumor microenvironment. Overexpression of L-arginine metabolizing enzymes (arginase and NOS) and the consequent diminution of arginine extracellular concentration causes the inhibition of prostate-specific T lymphocytes [reviewed in (169)]. Compared to benign tissue, arginase activity is also increased in the tumor tissue of melanoma (234), breast (235) and colon cancer patients (236) and in the serum of colon cancer patients (237) where it is associated with disease progression (238).

In prostate cancer patients, arginase II (ARG2) is overexpressed in the peripheral and transition zones of the prostate. There is almost no ARG2 expression in the central zone of the prostate (239) (see section 1.1.2 on page 2). Two studies found an increased arginase activity (240, 241) as well as increased ARG1 and ARG2 expression (242) in prostate cancer patients compared to BPH patients, whereas one study demonstrated a lower arginase activity in tumor tissues (243). However, a high arginase activity also correlates with lower Gleason score (244) and with increased survival rate (245). This inverse correlation between arginase expression and tumor aggressivity is also observed in prostate cancer cell lines. Androgen-sensitive prostate cancer cell lines (LNCaP and 22RV1) express higher levels of ARG2 than androgen-independent cell lines (Du145 and PC3) (244). Moreover, LNCaP cells derived to an androgen-independent state lose their ARG2 expression, which is compensated by an increased ornithine ornithine aminotransferase (OAT) expression (244). LNCaP cells are also dependent on the generation of polyamines from the metabolism of L-arginine, which are essential for their growth (246). Expression of iNOS is also upregulated in prostate cancer (247-249) and correlates with poor survival (250). Altogether, these data suggest that arginase expression and arginase activity are

increased within tumor tissue. However, the importance of this elevated arginase expression seems to be restricted to the earlier stages of the disease.

IDO, which metabolizes tryptophan, is another enzyme that modulates $CD8^+$ T lymphocyte activation through amino acid metabolism. Tryptophan depletion leads to decreases in T lymphocyte proliferation, maturation of effector functions and survival [reviewed in (251)]. Increased IDO expression has been demonstrated in primary tumors of prostate cancer patients (252).

1.3.4.4 COX-2 and Prostaglandin E_2

Cyclooxygenase-2 (COX-2) converts arachidonic acid to prostaglandin G_2 (PGG_2), which is further converted to PGE_2 by PGE_2 synthase (PGES). COX-2 is overexpressed in several tumors (253). In prostate cancer, COX-2 expression correlates with higher Gleason score and neoangiogenesis (254). COX-2 expression is linked to prostatic inflammation with TILs promoting COX-2 expression by prostate cancer cells (255). Epidemiologically, individuals taking non-steroidal anti-inflammatory drugs (NSAIDs) have a 15% lower incidence of prostate cancer, suggesting a role for COX-2 in prostate cancer (256). Three different PGES have been identified: membrane PGES-1 (mPGES-1) and mPGES-2 and cytosolic PGES (cPGES). Of the three only mPGES-1 is inducible during inflammatory responses. None of the three PGES have been demonstrated to be expressed in prostate cancer.

PGE_2 regulates T lymphocyte proliferation, lymphocyte cytokine production as well as macrophage and NK cell cytotoxicity (257). The immunosuppressive action of PGE_2 is associated with the production of IL-10 (221) and with an increased T_{REG} suppressive activity (258, 259).

1.3.4.5 Induction of T lymphocyte death through Fas-FasL

FasL is a type II transmembrane TNF family protein that triggers apoptosis in Fas expressing cells. $CD8^+$ T lymphocytes induce tumor cell apoptosis through this Fas-FasL interaction. Conversely, there is an increased secretion of soluble Fas in serum of prostate cancer patients (260), which act as decoy receptors preventing the

elimination of Fas-bearing tumor cells. There is also an increased secretion of FasL by prostate cancer cell lines promoting the apoptosis of Fas-expressing lymphocytes (261).

1.3.5 SUMMARY

According to the immune editing theory of cancer development, at the time a tumor reaches clinically detectable size, it has to have gained an immunoregulatory status rendering it "immune" to the anti-tumoral immune response by diminishing its immunogenicity, by actively expressing immunosuppressive molecules and by recruiting immunosuppressive cellular effectors. In the context of an immunotherapy, these immunosuppressive mechanisms need to be identified and overcomed to allow for the activation of cytotoxic cellular immune effectors within the tumor bed. Concerning prostate cancer, the challenge regarding the elimination of immunosuppressive mechanism is two-fold. First, we must identify which immunosuppressive mechanisms are expressed at specific stages of prostate cancer progression. We must characterize the immunological environment in patients with advanced prostate cancer, as they are the patients targeted to undergo immunotherapy. Secondly, we must understand the regulation of the immunosuppressive pathways in prostate cancer patients. Studying the regulatory pathways will provide essential information on which factors allow prostate tumors to evade the immune system and possibly identify new therapeutic targets.

1.4 L-ARGININE AND ARGINASE

1.4.1 L-ARGININE HOMEOSTASIS

L-arginine and its metabolites participate in protein synthesis, cell division, wound healing, reproduction, ammonia removal, neurotransmission and immunity. In humans, L-arginine is a conditionally non-essential amino acid, which is only nutritionally essential in infants. Circulating L-arginine is derived from diet, endogenous synthesis and protein turnover. In diet, it is abundant in dairy products, meat, seafood, wheat germ and cereals, nuts, seeds, chick peas and soybeans (262).

Due to the high arginase activity of the small intestines about 40% of dietary L-arginine is degraded in first pass metabolism (263, 264). In adults, L-arginine is principally synthesized via the intestinal-renal axis. L-citrulline is released in the blood from the small intestine and metabolized to L-arginine in the proximal tubule of the kidney. L-citrulline is converted into L-arginine through the activity of argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL). There are also minor contributions of adipocytes, endothelial cells, macrophages, neurons and myocytes in L-arginine synthesis (265). The liver produces the highest amount of L-arginine, however, due to its high arginase activity, there is no net L-arginine synthesis (265). In healthy adults, a balance between uptake and catabolism maintains L-arginine homeostasis (266). Through various metabolic pathways, L-arginine is the source of NO, agmatine, proline, glutamine, glutamate, creatine and several polyamines.

1.4.1.1 Polyamines and tumor cell proliferation

Polyamines are important substrates in the regulation of cellular proliferation and differentiation [reviewed in (267)]. The prostate has the highest polyamine concentration of any tissue and it is one of the only organs that produce polyamines for export in the seminal fluid (267). The seminal fluid is rich in polyamines, such as putrescine, spermidine and spermine, which are essential for cell growth and differentiation during spermatogenesis.

Compared to normal cells, tumor cells require higher amounts of polyamines to sustain their proliferative rate (268). Malignant cells augment polyamine synthesis through the overexpression of ornithine decarboxylase (ODC), the first enzyme in polyamine biosynthesis from L-ornithine (269). Tumor cells also express high levels of arginase (270, 271). Hepatocellular carcinoma and melanoma cells are termed auxotrophic for L-arginine, which means that they are unable to synthesize the sufficient amount of L-arginine necessary for their growth (272, 273). Clinically, systemic release of endogenous arginase following transhepatic arterial embolisation

caused hepatocellular carcinoma remission due to diminished L-arginine bioavailability (274).

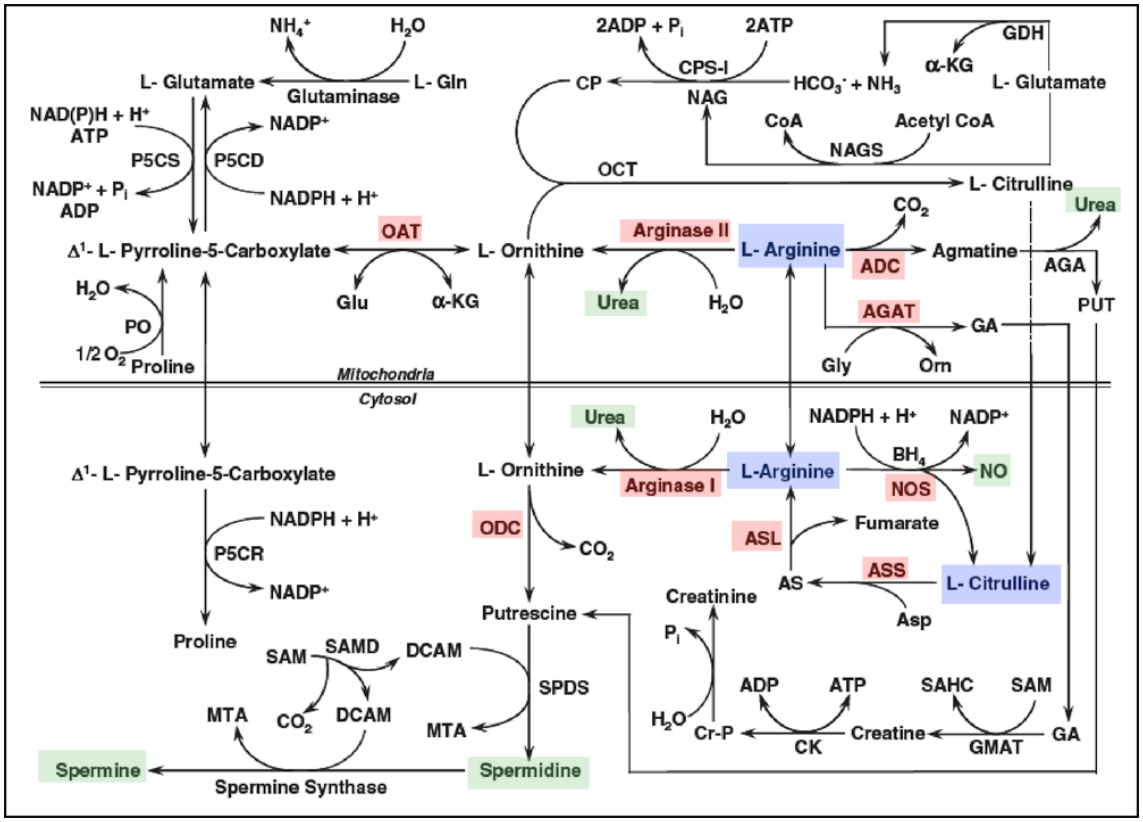

Figure 9. L-arginine metabolism.

Metabolic pathways of L-arginine and polyamine synthesis. Important enzymes are highlighted in red, L-arginine and L-citrulline in blue and important metabolic by-products in green.

ADC (Arginine Decarboxylase); AGAT (Arginine:Glycine Amidinotransferase), ASL (Argininosuccinate Lyase); ASS (Argininosuccinate Synthase); OAT (Ornithine Aminotransferase); ODC (Ornithine Decarboxylase).

Adapted from (262).

1.4.1.2 L-arginine intracellular transport

L-arginine is actively transported inside cells via high-affinity cationic amino acid transporters (CATs), which are either Na^+-independent (system y^+) or Na^+-dependent ($b^{0,+}$, $B^{0,+}$ and y^+L) (275, 276). In inflammatory cells, the system y^+ is an absolute requirement for L-arginine transport (277). The system y^+ is composed of five confirmed members: CAT-1, CAT-2A, CAT-2B, CAT-3 and CAT-4 (278). Whereas CAT-1 is constitutively expressed, CAT-2 expression is inducible. CAT-2A expression is limited to hepatocytes while CAT-2B is expressed by various cells including inflammatory cells, such as macrophages (279, 280). Inflammatory signaling will induce the expression of CAT-2B concomitantly with arginine-metabolizing enzymes such as NOS (281). The expression of CAT-1, CAT-2 and CAT-3 has been confirmed in humans.

1.4.1.3 Pathological disorders associated with L-arginine deficiency.

An imbalance in L-arginine availability leads to serious pathological conditions. In premature infants, L-arginine deficiencies cause life-threatening hyperammonemia as a result of a lack of urea necessary for ammonia detoxification (282). Patients with pulmonary tuberculosis have significantly lower plasma L-arginine levels, which correlates with impaired T lymphocyte activation (283) (see section 1.4.2.3 on page 66 for the immunosuppressive effects of L-arginine depletion). Conversely, high levels of L-arginine metabolites can also be detrimental. Renal cells are quite sensitive to high concentration of proline, polyamine and NO (262). Overproduction of proline, and the consequent elevated collagen formation, causes extracelllular matrix fibrosis. Elevated polyamine levels lead to excessive renal cell proliferation. Excessive NO production causes elevated levels of peroxynitrite anion, nitration of protein tyrosine and production of hydroxyl radical, all of which may worsen immune-mediated glomerulonephritis and post-ischemic renal failure.

1.4.1.4 Regulation of L-arginine metabolism

L-arginine is metabolized by five enzymes. ARG1 (cytoplasm) and ARG2 (mitochondria) hydrolyze L-arginine into L-ornithine. Cytoplasmic NOS oxidizes L-arginine into NO. Mitochondrial arginine decarboxylase (ADC) decarboxylates L-arginine in agmatine and arginine:glycine amidinotransferase (AGAT) catalyses the transfer of an amidino group on L-arginine to form glycine. Glucocorticoids augment arginase expression and L-arginine hydrolysis in hepatocytes and enterocytes (284) as well as reducing NOS expression and consequently NO production (285, 286). Cytokines (IL-4, IFN-γ) and other pro-inflammatory molecules, such as LPS and cyclic adenosine monophosphate (cAMP), are important regulators of L-arginine metabolism (287). Rats injected with LPS will have a 10- to 20-fold increase in NO within 24 hours (288). In murine macrophages, T_H1 cytokines favor the expression NOS, whereas T_H2 cytokines favor the expression of arginases. The polyamine synthesis pathways, which include ornithine decarboxylase (ODC) and spermidine synthase, are also regulated by androgens (267). More details on the regulation of arginase expression will be discussed in the following section.

1.4.1.4.1 NO production in cancer

At low concentration, NO has tumor-promoting effect by acting as a second messenger and by promoting tumor vascularization (289, 290). On the other hand, at high concentration, NO causes DNA and protein nitrosylation leading to cell-cycle arrest and apoptosis (291). Myeloid cells are the major source of iNOS within the tumor bed and their NO production, which can be induced by IFN-γ, has direct tumoricidal effects (292, 293). Interestingly, iNOS expression in tumor is often loss during tumor progression (294), possibly due to the phenotypic changes of M1 into arginase-expressing M2 macrophages (295) and through the production of TGF-β by myeloid suppressor cells (296).

1.4.2 ARGINASE I AND ARGINASE II

The two arginase isoforms, ARG1 and ARG2 are encoded by two separate genes and share ~60% protein homology. ARG1 and ARG2 have different biochemical and immunological functions (287). Cytoplasmic ARG1 is primarily expressed by hepatocytes and to a limited extent by enterocytes, endothelial cells, immune cells and red blood cells. ARG1 directs ornithine to polyamine synthesis due to its colocalization with ODC. Mitochondrial ARG2 is widely expressed at a low level in most mitochondria-containing extra-hepatic cells. ARG2 participates in the synthesis of proline and glutamine and colocalizes with OAT. ARG2, and not ARG1, seems to be responsible for the inhibition of iNOS expression through L-arginine bioavailability, whereas ARG1 is more important in polyamines synthesis necessary for tumor growth (297). ARG1 knock-out and ARG1/ARG2 double knock-out mice develop severe hyperammonemia and die within 10 to 14 days after birth (298). ARG2 knock-out display cardiovascular anomalies associate with hypertension (299) due to increased NO production.

Arginase is the principal pathway for L-arginine catabolism and the limiting step in polyamine synthesis. High arginase expression has been described in chronic inflammation, asthma (300), psoriasis (301), infection diseases (283, 302, 303) and cancer (304). Arginase can also be released in extracelllular fluids (plasma, wounds, intestinal lumen) where it hydrolyzes L-arginine into ornithine and urea. In inflammation and injury, high plasma arginase activity can results in L-arginine deficiency, reduced NO production and increased parasite and bacterial survival (305). Serum arginase activity is also elevated in association with type-2 diabetes mellitus (306), asthma (307), burn victims (308) and in sickle cell anemia (309).

1.4.2.1 Regulation of arginase expression in animal models

In mice, ARG1 expression is upregulated by T_H2 cytokines (IL-4, IL-6, IL-10, IL-13, TGF-β) (310), cyclic adenosine monophosphate (cAMP), GM-CSF (311), LPS (171), PGE_2 (312), catecholamines (313) and NF-κB signaling (314). In murine macrophages, ARG1 induction by T_H2-cytokine is dependent on signal transducer

and activator of transcription-6 (STAT-6) (315). Murine ARG2 is also regulated by LPS and cAMP (316). In murine macrophages, *Heliobacter pylori* activates NF-κB signaling causing ARG2 expression (314). Liver X receptors increase ARG2 expression whereas IRF-3 prevents this Liver X receptor-mediated ARG2 induction (317).

Contrary to T_H2 cytokines, T_H1 cytokines (TNF-α, IFN-α, IFN-β, IFN-γ) promote iNOS and not ARG1 expression (318). Moreover, arginase and NOS also directly inhibit each other through NO by-products and L-arginine bioavailability (319, 320). Through the production of the NO intermediate hydroxy-L-arginine, iNOS inhibits arginase expression. Conversely, depletion of L-arginine by ARG2 directly inhibits the translation of iNOS mRNA (286, 321, 322).

1.4.2.1.1 Androgenic regulation of arginase expression in animal models

In rats, castration decreases arginase activity by 50%, which was prevented by DHT administration (323). Testosterone injection also upregulates ARG2 and ODC, whereas it down-regulates OAT expression by murine female kidneys (324). Conversely, castration in male mice decreases the expression of ARG2 and ODC by kidney cells and upregulates OAT (324). The murine *ARG2* promoter does not contain putative androgen receptor response elements (AREs). It has been proposed that testosterone can bind sex hormone binding globulin (SHBG) and the SHBG receptors, which would increase cAMP in an AR-independent signaling (324). (See section 1.5.5.2.2 on page 76 for details on AR non-genomic signaling).

1.4.2.2 Regulation of arginase expression in humans

It remains unclear what factors regulate the expression of ARG1 and ARG2 in human cells. Contrary to murine regulatory macrophages and DCs, human macrophages and DCs do not express ARG1 (171). Human neutrophils, but not eosinophils (325), constitutively express ARG1, which however does not vary following exposure to T_H2 cytokines IL-4 and IL-13 (326). IRF-3 signaling increases ARG2 expression in Jurkat cells infected with Sendai-virus (327). Recent reports suggest that COX-2 and PGE_2 may also regulate arginase expression in human tumor

cells (170). Finally, gene-chips studies evaluating LNCaP cells treated with DHT identified ARG2 as an androgen-regulated gene (328, 329). DHT also upregulates the expression of ODC in LNCaP cells (330).

1.4.2.3 Immunosuppressive effects of L-arginine depletion

L-arginine depletion following elevated L-arginine metabolism by the tumor causes the anergy of activated $CD8^+$ T lymphocytes (169, 304, 326, 331-335). Physiologically, L-arginine depletion mediated T lymphocyte hyporesponsiveness is present in pregnancy (336).

Activated T lymphocytes are quite sensitive to L-arginine levels. L-arginine depletion causes T lymphocyte anergy by blocking protein synthesis, proliferation and CD3ζ TCR signaling. Reduction of intracellular L-arginine concentration activates general control non-derepressible-2 (GCN2) kinase, a stress-induced kinase activated by elevated levels of uncharged tRNA. GCN2 activation leads to the inhibitory phosphorylation of eIF2, which inhibits protein expression by halting translation initiation (316, 337). L-arginine depletion also causes a decreased phosphorylation of Rb. Moreover, following activation in an L-arginine-depleted microenvironment, T lymphocyte will fail to upregulate the expression of cyclin D3 and cyclin-dependent kinase 4 (CDK4), which causes cell cycle arrest in G_0-G_1 (334, 335, 338). T lymphocytes are also unable to upregulate CD3ζ as a result of reduced protein synthesis and a shorter CD3ζ mRNA half-life in the absence of L-arginine (331). Increased production of polyamines can also inhibit the release of pro-inflammatory cytokines (339). Finally, within the tumor, a high concentration of NO, due to the expression of NOS, can directly induce T lymphocyte apoptosis and inhibit the expression of intracellular signaling proteins participating in lymphocyte activation such as JAK kinases (340-343).

NK cells are also sensitive to L-arginine depletion. In a recent study, constitutive arginase expression by granulocytes inhibits human NK cell proliferation as well as IL-12 and IFN-γ secretion (344). NK cell viability and granule exocytosis were not affected. However unlike T lymphocytes and NK cells, murine macrophages

are insensitive to L-arginine depletion and maintain expression of cytokines, chemokines and activation markers in the absence of L-arginine (345). Since macrophages do not proliferate following their activation, L-arginine depletion may have less repressive effects on macrophages than on the actively proliferating T lymphocytes. Moreover, macrophages have a functional urea cycle and can thus synthesize their own L-arginine, which allows for their activation in an L-arginine depleted environment (346).

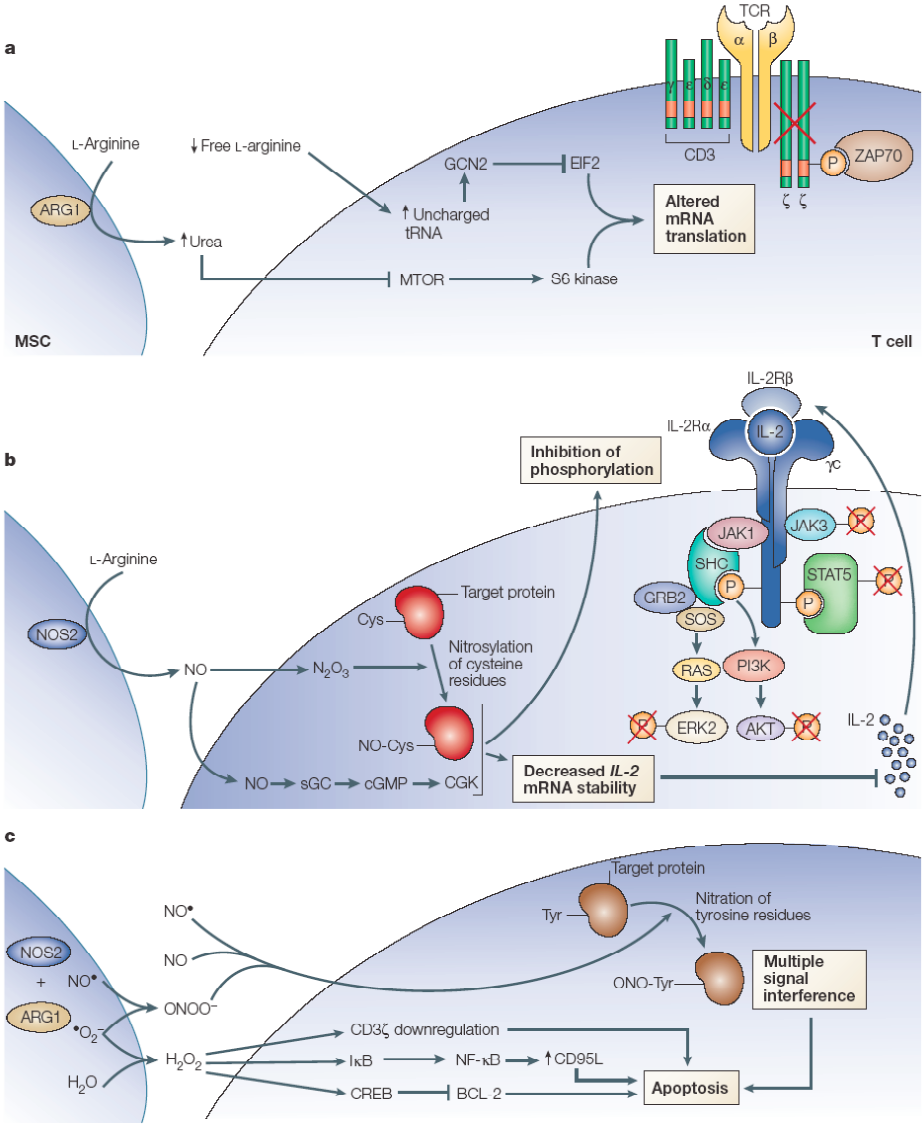

Figure 10. Potential inhibitory pathways of L-arginine depletion.

A) Blockade of CD3ζ expression through the activation of GNC2. B) NO production causes nitrosylation of cysteine residues and activation of cyclic guanosine monophosphate (cGMP), which affect IL-2R/CD25 signaling and IL-2 mRNA stability. C) Expression of both ARG1 and iNOS causes the production of ROS and reactive nitrogen-oxide species, which can induce lymphocyte apoptosis.

Adapted from (169).

1.4.3 L-ARGININE SUPPLEMENTATION

The involvement of L-arginine depletion in several pathologies and tumor-derived immunosuppression suggests that L-arginine supplementation could have therapeutically effects. Several studies demonstrated that L-arginine supplementation is beneficial for patients with cardiovascular disorders (347), obesity (262, 348), sickle-cell anemia (349), cystic fibrosis (350) as well as to increase wound healing (351) and muscular endurance (352) in healthy individuals. However, regarding L-arginine supplementation in cancer patients, the answer is not so simple.

1.4.3.1 L-Arginine Supplementation in Cancer

In cancer models, L-arginine supplementation reverses the immunosuppressive effects of increased L-arginine metabolism (262). In tumor-bearing rats, L-arginine supplementation increases thymic weight and cellularity, T lymphocyte proliferation, IL-2 production and IL-2R expression as well as lymphocyte, macrophage and NK cell cytotoxicity (353). Mice given low doses of oral L-arginine for one year had decreased tumor incidence and increased survival due to the activation of NO-dependent tumor cytotoxicity mediated by macrophages and lymphocytes (354). However, the concomitant polyamine synthesis associated with increased L-arginine bioavailability has tumor growth promoting effects, which depends on tumor stage. L-arginine supplementation to patients with colorectal adenoma (benign) or during the earlier stage of colorectal carcinogenesis increases ODC activity, reduces cellular proliferation decreases tumor mass, whereas during later stages, L-arginine promotes tumor growth and increases the tumor expression of NOS and NO serum levels, two factors that favor colorectal cancer progression (355-357). Furthermore, administration of L-arginine, which increases NO production, would be detrimental to patients with severe infections, inflammatory or autoimmune disorders and pathological angiogenesis. As such, L-arginine supplementation does not apply to every pathological disorder and should be carefully evaluated in specific stages of cancer progression.

1.4.3.2 Polyamine inhibition to minimize tumor growth

Conversely to L-arginine supplementation, the dependency of tumor cells for L-arginine and arginine can be targeted to eliminate tumors (272, 273). A recombinant human ARG1 linked to a polyethylene glycol linker (rhArg-PEG), which depletes the extracellular L-arginine, causes the inhibition of tumor cell proliferation *in vitro* and reduces the growth of xenografts *in vivo* (358). Another group has generated a pegylated L-arginine deiminase (ADI-PEG), which, like the rhArg-PEG, causes L-arginine depletion and tumor growth inhibition (359). However, tumor cells have been shown to be resistant to these novel drugs by overexpressing enzymes of the urea cycle, such as ASS and OTC, and replenish their store of L-arginine (272). Furthermore, these studies did not evaluate the consequence of L-arginine depletion on the inhibition of the anti-tumoral immune response.

In prostate cancer, reduction of intracellular polyamines through the inhibition of ODC caused a decreased proliferation of prostate cancer cell lines *in vitro* and PC3 xenograft on nude mice (360). Such an approach is interesting as it inhibits the growth promoting effects of polyamine, while preventing the depletion of extracellular L-arginine.

1.4.3.3 Arginase inhibitors

Treatment with *N*-hydroxy-nor-L-Arginine (NOHA) impaired tumor formation of Lewis lung carcinoma cells in syngenic animals but not in SCID mice suggesting of an immune-mediated tumor rejection (333, 361). N^G-monomethyl-L-arginine (L-NMMA) is a NOS inhibitor. Addition of both NOHA and L-NMMA can restore T lymphocyte cytotoxic functions in prostate cancer patients *ex vivo* models (362). Nitroaspirin (NCX-4016) also restores T lymphocyte proliferation in the presence of MDSC by inhibiting arginase and NOS (363).

1.4.4 SUMMARY

Arginase activity leads to increased synthesis of polyamines, which are necessary for the prostate's physiological roles in reproduction. However, during

carcinogenesis, aberrant arginase expression leads to increased polyamines synthesis, which sustains the growth of prostate cancer cells. Furthermore, the consequent L-arginine depletion associated with elevated arginase activity causes local immunosuppression within the tumor microenvironment. Inhibition of arginase or NOS reverses T lymphocyte anergy and promotes a tumor-specific anti-tumoral immune response. However, current data suggests that human arginases are not regulated by similar factors as murine arginases. The regulatory factors leading to arginase expression in human immune and tumor cells remain unknown. Data suggest that androgens could regulate the expression of ARG2 and other enzymes involved in polyamine synthesis.

1.5 REGULATION OF IMMUNE RESPONSES BY SEXUAL HORMONES

There is accumulating evidence that sexual hormones play determining roles in the regulation of immune responses, which results in considerable differences between women and men. Epidemiological data reveal a gender-based difference in the predisposition to autoimmune diseases and other pathologies. Studies with experimental animal models demonstrate that sexual hormones are directly implicated in this dichotomy and that they can modulate immune cell numbers, cytokine production and activation of the lymphocyte cytotoxic machinery.

The literature on the immunoregulatory impact of sexual hormones largely describes the actions of estrogens with relatively scarce information on the impact of testosterone on immune cells. In prostate cancer, estrogens do participate in the disease progression as the intra-prostatic estrogen concentration may regulate the local immunological microenvironment. In the normal prostate, prostate stromal cells express the cytochrome p450 aromatase. The aromatization of testosterone and DHT by the p450 aromatase results in an elevated concentration of estrogens within the prostate (364). During carcinogenesis, malignant prostate epithelial cells also acquire aromatase expression (365). Furthermore, stromal cells express both ER-α and ER-β (366), with ER-α playing a prominent role in the pro-inflammatory actions of

estrogens and ER-β having an anti-inflammatory role (367). As such, the immunoregulatory functions of estrogens could play important role in the carcinogenesis of the prostate. In the following section, the immunoregulatory properties of both androgens and estrogens will be discussed. A larger focus for this introductory section will be devoted to estrogens due to aforementioned predominance in the current literature.

1.5.1 HIGHER INCIDENCE OF AUTOIMMUNE DISEASES IN WOMEN

There are contrasting differences in the incidence of autoimmune disorders between women and men. The women to men ratio for the incidence of systemic lupus erythematosus (SLE), Grave's disease, Hashimoto's thyroiditis and Sjögren's syndrome is 7-10 : 1 and 2-3 : 1 for the incidence of multiple sclerosis (MS), rheumatoid arthritis (RA) and scleroderma. In contrast, ankylosing spondylitis, Goodpasture syndrome, Reiter syndrome and vasculitis are more present in men (368, 369). In animal models of experimental autoimmune encephalomyelitis (EAE), transfer of male T lymphocytes leads to less severe EAE symptoms than female T lymphocytes (370). Estrogens increase the expression of CCR5 and CCR1 by $CD4^+$ T lymphocytes, which participate in T lymphocyte homing during infection and autoimmune disease (371). Gender-based immunological differences also exist with regards to infection. With the exception of sexually transmitted infections (HIV and herpes simplex virus-2), men have higher incidence and increased degree of severity for viral, bacterial, fungal and parasitic infection (372, 373). Overall, these epidemiological data suggest that women have stronger cell-mediated and humoral immune responses to antigenic challenges than men and may also explain the lower incidence of cancer in women (374, 375).

1.5.2 SEXUAL HORMONES IN THYMIC DEVELOPMENT

With age comes a profound thymic atrophy (almost 90% loss of function). This thymic atrophy begins at the onset of puberty under the action of sexual hormones (376, 377). Thymic atrophy is characterized by a degeneration of the stromal thymic

network, which sustains the survival and differentiation of developing T lymphocytes, and by a diminution in the homing of thymocyte progenitors (378).

Estrogens (i) decrease the number of precursors that migrate from the bone marrow to the thymus, (ii) deplete early thymic progenitors within the thymus, (iii) reduce the ability of double-negative (CD4-CD8-) progenitors to proliferate in response to pre-TCR signaling, and (iv) cause the apoptosis of double-positive ($CD4^+CD8^+$) thymocytes (379), through upregulation of FasL (380). Finally, estrogens increase $CD4^+CD8^-$ and inhibit the production of IL-7, an important regulator of T lymphopoiesis (381). Together, these data suggest that estrogens prevent the proper development of T lymphocytes within the thymus. Nevertheless, estrogens do favor the development of the $CD4^+$ T lymphocyte compartment over $CD8^+$ T lymphocytes.

Conversely, if given early in thymic development, androgens contribute to thymic hypercellularity and favor the development of mature single positive $CD4^+$ and $CD8^+$ T lymphocytes and naturally occurring T_{REGs} (381, 382). Contrary to estrogen, androgens favor the immigration and development of CD4-$CD8^+$ single-positive thymocytes through the overexpression of Thy-1 (383, 384). Castration does however reverse the age-related thymic atrophy suggesting that androgens also have a detrimental role in thymic physiology.

1.5.3 ESTROGENS PROMOTE A T_H2 SKEWING OF THE IMMUNE RESPONSE

Estrogen favor the development of a greater number of $CD4^+$ T lymphocytes in women compared to men. Estrogen also reduce the production of pro-inflammatory T_H1 cytokines (IL-1β, IL-2, IL-12 and TNF-α) by monocytes (385) and lymphocytes (386, 387). Women PBMCs also secrete less T_H1 cytokines (IL-2, IFN-γ) and more T_H2 cytokines (IL-4, IL-10) following mitogen activation (387, 388). Estrogens favor the secretion of T_H2 cytokines by regulating the expression of T-bet (regulator of T_H1 differentiation) and IRF1 (389). Estrogens promote the development of B lymphocytes and the production of auto-reactive antibodies (390,

391) through the expression of activation-induced deaminase in B lymphocytes, which participates in somatic hypermutation and class switching recombination (392). Conversely, testosterone prevents the production of auto-reactive antibodies (393, 394). Moreover, estrogens reduce NK cell numbers and cytotoxicity (395-398). On murine macrophages, estrogens promote the expression of TLR-4 and a stronger anti-bacterial immune response (399). Physiologically, high estrogen concentration during the luteal phase of the menstrual cycle or during pregnancy causes an elevated production of T_H2 cytokines (IL-4, IL-6) and reduced production of T_H1 cytokines (IL-2) (400-403). For women with autoimmune disorders, these variations in estrogenic concentration exacerbate T_H2 autoimmune disease (SLE) and improve T_H1 diseases (asthma, MS and RA) (369, 389, 404, 405). These variations may also be caused by T_{REGs} (406, 407), whose numbers are diminished during the luteal phase (high estrogen) (408). Altogether, these data suggest that estrogen promotes a T_H2 skewing of the immune response.

1.5.4 ANDROGENS ACT AS NON-SPECIFIC IMMUNOSUPPRESSANT

Contrary to the T_H2-promoting action of estrogens, androgens broadly suppress the immune system by inhibiting B and T lymphocyte proliferation (409-412) and by causing T lymphocyte apoptosis (413). $CD4^+$ T lymphocytes produce more IL-10 following testosterone stimulation preventing a T_H1 cell-mediated immune response (414). On monocytes and macrophages, testosterone decreases the expression of TLR4, the receptor for LPS responsible for the activation of the innate immune system in response to Gram-negative bacterial infection (415). Testosterone does not however affect the production of IL-2, IFN-γ by lymphocytes and TNF-α by monocytes (416), nor does it changes NK cells count (417). In a recent study, androgens increase telemorase expression in human PBMCs from healthy donor following aromatization and signaling through ERα (418). Altogether, androgens act as immunosuppressants preventing the activation of cell-mediated and innate immune response, which accounts for the higher rate of infection in men.

1.5.5 EXPRESSION OF ESTROGEN AND ANDROGEN RECEPTOR BY IMMUNE CELLS

Until recently, it remained unknown whether immune cells expressed steroid hormone receptors and whether sexual hormones could directly modulate their activity. Evidence now suggest that immune cells express classic cytoplasmic steroid receptors and non-classical steroid receptors located on the cytoplasmic membrane.

1.5.5.1 Estrogen receptor

Estrogens bind to two cytoplasmic steroid receptors: estrogen receptor α (ERα or ERS1) and estrogen receptor β (ERβ or ERS2). Upon ligation, there is a conformational change, dimerization and nuclear translocation of the ER. The ER regulates the expression of genes containing ER responsive elements (ERE) in their promoter region and through the recruitment of co-regulatory proteins on ERE-negative promoters (419). ERα and ERβ bind to identical ERE, but differ in their trans-activating subunits.

The transcription role of estrogens varies depending on which ER is expressed. ERα and ERβ are expressed by B and T lymphocytes, DCs, macrophages, neutrophils and NK cells (369). T lymphocytes express higher levels of ERα, whereas B lymphocytes express more ERβ (389). Estrogens induce Fas/FasL-mediated apoptosis of monocyte expressing ERβ, but not of macrophage expressing ERα (420). Similarly, through ERβ, estrogens cause the age related thymic involution that begins at puberty (421). Moreover, ERα activation has protective anti-inflammatory effects in EAE symptoms whereas specific ERβ activation has no effect (422). Finally, a membrane ER (mER) is present in T lymphocytes, monocytes and granulocytes (389, 423). mER signals through non-genomic pathways, which can increase intracellular calcium concentration (423), SRC kinase as well as downstream MAPK and AKT activation (389).

1.5.5.2 Androgen Receptor

The classical cytoplamsic AR (iAR) is a member of the steroid hormone superfamily of ligand-activated transcription factors. It is composed of four main domains: (i) a N-terminal regulatory domain involved in interaction with co-regulators; (ii) a DNA-binding domain composed of two zinc fingers that recognize AREs; (iii) a hinge region that includes the nuclear translocation signal; and (iv) a ligand-binding domain.

1.5.5.2.1 iAR genomic signaling

The binding of androgens (testosterone or DHT) to the iAR in the cytoplasm induces conformational changes, liberation from heat-shock proteins, homodimerization and nuclear translocation. Nuclear androgen receptor binds to ARE and activate transcription by inducing conformational changes in the chromatin, by promoting RNA polymerase activity and by recruiting the transcription machinery (25). This pathway is defined as iAR genomic signaling.

1.5.5.2.2 iAR non-genomic signaling

Non-genomic signaling needs to be further studied, as its impact remains largely undefined. What is known is that iAR non-genomic signaling involves the rapid induction (seconds and minutes) of second messengers such as intracellular Ca^{2+} independently of the iAR transcriptional activity. This rapid action implicates protein-protein interactions, which are insensitive to transcription or translation inhibitors (424). Depending on the cell type, non-genomic signaling causes the activation of protein kinase A (PKA), protein kinase C (PKC), phosphatidyl-inositol 3-kinase (PI3K) and mitogen-activated protein kinases (MAPK) (425) [reviewed in (426)]. DHT is reported to induce non-genomic signaling in LNCaP causing an accumulation of intracellular Ca^{2+} in a pathway dependent on an unidentified GPCR (427). The iAR can also bind to and activate the non-receptor tyrosine kinase Src via its SH3 domain. Finally, non-genomic signaling can lead to transcription changes

causing an increased expression of c-fos protein and to an increase in iAR genomic signaling by phosphorylating iAR or its coactivators (426).

Murine studies revealed that T lymphocyte and macrophages do not express the classical iAR. Murine $CD4^+$ and $CD8^+$ T lymphocytes express a plasma membrane AR (mAR) whose ligation causes the rapid (< 5 seconds) rise of intracellular Ca^{2+} dependent on the influx of extracellular Ca^{2+} (428). In contrast, murine B lymphocytes solely express the iAR and testosterone stimulation of B lymphocytes does not induce a rise in intracellular Ca^{2+} (429). Murine macrophages express an undefined plasma membrane G-protein coupled receptor, which, following testosterone stimulation also cause a rapid increase in their intracellular Ca^{2+} concentration, but this time due to the release of intracellular Ca^{2+} stores (430). This Ca^{2+} mobilization is dependent on the activation of phospholipase-C (PLC), ERK1/2, JNK/SAPK and p38 (430, 431).

Testosterone can also bind to the SHBG receptor. Approximately 60% of serum testosterone and DHT is bound to SHBG and the remainder is bound to albumin. Ligation of testosterone-SHBG complexes to SHBG receptors, also a G-coupled receptor, activates cAMP and PKA independently of the AR (432). LNCaP cells express SHBG receptors (433, 434) and SHBG receptor activation in this prostate cancer cell line also causes an induction of cAMP and the subsequent activation of PKA (434). Unfortunately, the non-genomic signaling of iAR or mAR in human immune cells remains needs to be further studied.

1.5.6 IMMUNOREGULATORY PROPERTIES OF MEDICAL CASTRATION

By taking into account the immunosuppressive functions of androgens, it is not surprising that medical castration stimulates the pro-inflammatory functions of the immune system. Medical castration has the dual effect of eliminating circulating testosterone and reducing intracellular estrogens derived from testosterone and DHT aromatization. It is thus important to keep in mind that the immunoregulatory

properties of medical castration are dependent on the loss of both androgens and estrogens.

1.5.6.1 Castration in animal models

Castration of male mice augments T lymphocytes in peripheral lymphoid tissues due to an increase in bone marrow, spleen and thymus cellularity and B and T lymphopoiesis (32, 435). Castration reduces the serum concentration of T_H2 cytokines (IL-10 and IL-17) and increases T_H1 cytokines (IL-1a and IL-12p40) without affecting the concentration of IL-6, TNF-α and IFN-γ (98). T lymphocytes from castrated mice also proliferate more vigorously *in vitro* during anti-CD3 and anti-CD28 stimulation as well as during antigen-specific activation (32, 436). Castration also increases DC numbers in LNs, DC maturation and DC expression of co-stimulatory marker (CD80, CD83, CD86, CD40, OX-40L) (98). Antigen-experienced $CD62L^+CD4^+$ T lymphocytes in the presence DCs from castrated mice secrete more IL-2, IL-4, IL-12p70, GM-CSF, IFNγ and TNF-α (98) suggesting that DC from castrated mice have increased T lymphocyte priming functions. Conversely, in a myocarditis murine model, castration increases the population of "alternatively activated" or immunosuppressive macrophages (437). In a murine model of prostate cancer, 55% of castrated mouse develop an autoantibody response against poly(A) binding nuclear protein 1 (PABPN1) as well as a T cell response against PABPN1 (438). Paradoxically, mice that developed these autoantibody and T cell response have a shorter time to tumor recurrence. Finally, castration does improve tumor-recognition by T lymphocytes. In a mouse model expressing influenza antigens under a prostate-specific promoter, androgen-deprivation leads to recognition of the influenza antigens in tumor draining LNs (439). Nonetheless, T lymphocytes fail to fully mature into cytotoxic effectors and undergo abortive proliferation. This is possibly as a result of the tumor's tolerogenic state, which is not completely abolished following castration. Altogether, these results suggest that medical castration has pro-inflammatory effects by expanding B and T lymphocytes numbers, favoring DC stimulation and increasing T_H1 cytokine production.

1.5.6.2 ADT and prostatic inflammation

The effect of ADT on the prostate's immunological network is complex. ADT leads to an increase infiltration of macrophages, T lymphocytes and DCs (31, 59, 440, 441). In healthy individuals, medical castration leads to a decreased percentage of circulating $CD4^+CD25^+$ T lymphocytes (442). The immunoregulatory actions of ADT could be related to its impact on thymocyte development and the increases of naïve T lymphocyte pool (443). Moreover, the massive epithelial cell apoptosis could lead to increased infiltration of phagocytic APCs, which present TAAs to naïve lymphocyte in the draining LNs. Finally, as stated previously, androgens may directly participate in the development of the prostate's immunological tolerogenic microenvironment by either directly inhibiting the activation of immune cells or by promoting the expression of immunosuppressive molecules. ADT thus causes the reduced expression of androgen-dependent immunosuppressive pathways, and consequently the elevated immune cell infiltration.

1.5.7 SUMMARY

The immunoregulatory properties of estrogens and androgens are associated with distinctive predisposition to autoimmune disorders and infection between women and men. Estrogens promote thymic atrophy and a T_H2 skewing of the immune response whereas androgens have broad immunosuppressive effects. Medical castration has pleiotropic immunostimulatory effects on immune cells caused by the elimination of circulating androgens and a reduction of estrogens generated from androgen aromatization. Expression of sexual steroids receptors (iAR, mAR and SHBG receptors) was demonstrated in murine immune cells. However, their expression by human immune cells and their regulatory properties through genomic and non-genomic signaling remains to be understood. In prostate cancer, it remains to be demonstrated that medical castration eliminates an androgen-driven immunosuppressive microenvironment.

CHAPTER II

DOCTORAL THESIS OBJECTIVES

At the time when this doctoral project was initiated, the literature regarding the immuno-oncology of prostate cancer mainly consisted of correlative studies based on immune cell numbers and disease progression. A better understanding on the interactions between tumor and immune cells had to be gained. The all-encompassing goal of this doctoral thesis was thus to further the understanding of the immunological microenvironment in human prostate cancer. To achieve this we established two main objectives: (i) to precisely characterize the immune cell populations present in the tumor microenvironment; (ii) to identify and to study the immunosuppressive pathways expressed by human prostate cancer cells.

This project originated from a publication by our group evaluating the nuclear localization of NF-κB p65 in prostate cancer LN metastases (444). The authors demonstrated that a vast majority of lymphocytes neighboring metastatic cells had nuclear localized NF-κB p65. Conversely, lymphocytes in non-metastatic LNs had no or very low levels of nuclear NF-κB p65. This result suggested that only lymphocytes in the proximity of invading metastatic cells have activated NF-κB signaling. We thus set out to phenotypically characterize the immune cell populations within metastatic and non-metastatic LNs of prostate cancer patients by immunohistochemistry (222). The goal of this study was not to provide correlations between various immune cell markers and prostate cancer progression. Rather, we wanted to evaluate the immunological status of metastatic LNs of prostate cancer patients. For this study, we pioneered a novel software-assisted image analysis protocol to precisely quantify immune cell numbers within large tissue sections. Our results demonstrate that the presence of prostate cancer LN metastasis is associated with the development of an immunosuppressive microenvironment. Similar to the NF-κB p65 study, our data suggests that metastatic LNs have a unique immunological microenvironment. Furthermore, our data raise the possibility that metastatic cells may have direct

immunoregulatory properties within the LN thereby leading to a local immunosuppression.

Subsequently, we sought to further understand the immunosuppressive pathways expressed by prostate cancer cells. Specifically, we studied the contribution of androgen, an important player in the prostate's organogenesis and carcinogenesis, in the regulation of the immunosuppressive microenvironment in human prostate cancer. As stated in section 1.5.6.2 on page 79, a significant increase in intra-prostatic immune cell infiltration follows ADT. Our goal was to determine which immune cell population infiltrated the prostate following medical castration. We thus characterized the immune cell infiltrate in primary tumor specimens of a cohort of 35 ADT patients treated by ADT prior to radical prostatectomy and 40 Gleason-matched control patients treated by radical prostatectomy only (49). For this study, we optimized our software-based image analysis method, which was coupled to whole-slide image scanner. With this digital image-analysis approach, we were able to precisely quantify immune cell density on entire prostate specimens thereby removing significant analysis biases often present in similar studies. Our data enabled us to confirm that ADT promotes the infiltration of specific immune cell populations (T lymphocytes and macrophages) within the primary tumor. Furthermore, we validated a novel software-based approach, which may help standardize the quantification of immune cell populations within pathological samples.

Finally, we evaluated whether the pro-inflammatory state induced by ADT was caused by the removal of androgen-regulated immunosuppressive pathways. We thus setout to identify and to study which immunosuppressive molecules were expressed by prostate cancer cells and upregulated following androgen stimulation. Through bioinformatic analyses, we generated a list of several molecules reported to have immunosuppressive properties and to be expressed in prostate cancer. Following molecular biology experiments (qPCR and Western blot), we identified ARG1 and ARG2 as two immunosuppressive enzymes expressed by prostate cancer cells and upregulated following androgen stimulation (445). Importantly, we are the first group to demonstrate an expression of ARG1 by malignant cells. We also show that the androgen-regulated expression of ARG2 is also present *in vivo* using prostate samples

from our cohort of 35 ADT-treated patients and 40 control patients. Finally, we observed that interleukin-8 was also upregulated by androgens and could, on its own, promote the expression of ARG1 and ARG2. Together, our data clearly demonstrate that the immunosuppressive properties of androgens in prostate cancer implicate the expression of ARG1, ARG2 and IL-8.

In conclusion, our research has furthered the common understanding of the uniqueness of the prostate's immunological microenvironment in prostate cancer patients. We validated novel quantification methods allowing for a clearer understanding of the immune cells population that infiltrate the tumor bed. Furthermore, we demonstrate that androgen play have potent immunoregulatory functions in prostate cancer.

CHAPTER III

Presence of prostate cancer metastasis correlates with lower lymph node reactivity

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Author contributions:

For this paper, I did the majority of the experiments and wrote the manuscript in its entirety, although all co-authors played a role in the editing process. Mona Alam Fahmy and Louis Réal Bégin helped with the pathological evaluation. Audrey Djoukhadjian optimized immunohistochemical stainings. Abdelali Filali-Mouhim developed a computer algorithm for the image analyses.

Keywords:

Histopathology, Immunosuppression, Immunohistochemistry.

Abstract

Background: Several reports suggest that the dissemination of neoplastic cells and cancer progression are associated with the generation of an immunosuppressive environment.

Methods: In this report, we investigated immunological effects of prostate cancer by comparing metastastic and non-metastatic pelvic lymph nodes (LNs) from 25 patients with carcinomatous involvement of LNs to the non-metastatic LNs from 26 control patients with no metastatic involvement by immunohistochemistry and histological analyses.

Results: Our results showed a decreased abundance of $CD20^+$ B lymphocytes ($p=0.031$), $CD38^+$ activated lymphocytes ($p=0.038$) and $CD68^+$ macrophages ($p<0.001$) and less evidence of follicular hyperplasia ($p=0.014$), sinus hyperplasia ($p<0.001$) and fibrosis ($p=0.028$) in metastatic LNs comparatively to control LNs. Finally, we observed that metastatic LNs were significantly smaller than control LNs ($p=0.005$).

Conclusions: Our results suggest that the development of prostate cancer LN metastasis is accompanied with smaller LN size and decreased LN reactivity suggesting the development of an immununosuppressive microenvironment.

Introduction

One obstacle that metastatic cancer cells must circumvent in order to invade and proliferate in distant tissues is the immune system. Continual interactions between immune and cancer cells occur throughout the development of a tumor. The immunosurveillance properties of the immune system allow for the initial recognition and successful elimination of the early threat of growing neoplastic cells. However, this constant selective immunological pressure leads to the development of robust neoplastic cells, which are able to evade the immune system by mimicking immunosuppressive processes associated with the induction of tolerance and the prevention of auto-immune disorders (1, 2). Lymph nodes are essential in preventing the dissemination of tumor cells by acting as mechanical and biological filters (3-6). In spite of this, they are the primary metastatic sites for epithelial tumors. Regional LNs have an essential role in the development of a systemic anti-tumoral immune response. Professional antigen presenting cells (APCs), such as dendritic cells (DCs) and macrophages, migrate to tumor draining LNs carrying antigens from the tumor and participate in the activation and proliferation of tumor antigen-specific T and B lymphocytes. Activated lymphocytes subsequently migrate to the primary tumor and exert their newly acquired tumoricidal effector potential. It is now becoming evident that the tumor environment contributes to the suppression of the anti-tumor immune functions of tumor draining LNs thereby promoting the occurrence of cancer metastasis (7).

Prostate cancer is the second leading cause of cancer related death and the most frequently diagnosed cancer among North American men (8). Curative therapies, such as radical prostatectomy and radiotherapy, are effective only for patients with localized disease. Prostate cancer metastases are initially detected in the pelvic LNs prior to disseminating to the bones and lungs. Since the emergence and widespread use of serum prostate specific antigen (PSA) in prostate cancer screening, the clinical incidence of LNs metastasis has decreased by half (9-14). Although uncommon, even the presence of micrometastases in draining LNs has substantial clinical significance in the staging and prognosis of the disease (15-19). Given the

pivotal role of tumor-draining LN activity in cancer progression, merely focusing on the presence or absence of metastasis in this specialized secondary lymphoid compartment may not provide a comprehensive picture of the implication of the immune system in the eradication of cancer cells. Rather, the histological evaluation of LN reactivity and the thorough characterization of the immune cell population may potentially offer a more accurate assessment of the interaction between the immune system and prostate cancer cells.

The diverse histological patterns of reactivity observed in tumor draining LNs are indicative of the varied immune responses taking place (20). For example, a humoral immune response is associated with the proliferation of B lymphocytes in germinal centers and the development of follicular hyperplasia, whereas the proliferation and activation of T lymphocytes in a cell-mediated immune response is associated with paracortical hyperplasia (21). In tumor draining LNs, the presence of distinct histopathological patterns have also been linked to cancer progression. Sinus hyperplasia (histiocytosis), follicular hyperplasia, granulomatous inflammation, fibrosis (hyaline material) with or without secondary calcification have all been shown to correlate with cancer prognosis and may represent an immune response to the tumor or its secreted products (21-24). The presence of sinus hyperplasia in metastatic LNs of laryngeal squamous cell carcinoma patients correlated with survival (25). The presence of paracortical hyperplasia correlated with survival in squamous cell carcinoma of the oral cavity and thyroid carcinoma (23, 26). These morphological changes reflect past or present immunological responsiveness, and may even reflect tumor-specific reactivity.

Our recent studies on the immuno-environment of LNs draining prostate cancer suggest that the presence of metastatic cells promote a distinct immunological phenotype. Our report revealed an increased level of nuclear localization of NF-κB in lymphocytes surrounding metastatic prostate cancer cells in pelvic LNs suggesting a local activation of lymphocytes (27). We also documented, in a preliminary report based on a Ki67 immunohistochemical analysis, that lymphocytes in metastatic LNs have a higher proliferation index than lymphocytes in non-metastatic LNs (28). The

aim of the present study is to further characterize the immunological microenvironment of metastatic and non-metastatic LNs in prostate cancer based on the characterization of LN immune cells, LN histological patterns of immunological reactivity and LN size. To our knowledge, no other immunological and histopathological evaluations of prostate cancer draining LNs have been published describing the potential generation of an immunosuppressive microenvironment associated the presence of metastatic cells.

Materials and methods

Tissues:

Formalin-fixed paraffin-embedded (FFPE) archival tissue specimens were obtained from 51 patients who had undergone radical prostatectomy with pelvic lymphadenectomy at the CHUM Notre-Dame Hospital (Montréal, Québec, Canada) between 1990 and 2000. Clinical and pathological characteristics of metastatic and non-metastatic patients are summarized in Table 1. Specimens were obtained from patients who had not received hormone therapy prior to surgery since it was demonstrated that androgen depletion therapy intensifies the anti-tumoral immune response in prostate cancer (29-31). Of the 51 patients, 25 patients had carcinomatous involvement of LNs (metastatic LNs), whereas 26 patients had no evidence of metastasis or biochemical recurrence (PSA greater than 0.3) five years after surgery (control LNs). Furthermore, we studied the benign adjacent LNs from the 25 patients with a positive LN status (non-metastatic LNs, internal control group). In order to confirm their status, i.e. metastatic LN or non-metastatic LN, all LNs were subjected to immunohistochemical staining with anti-PSA antibodies (Figure 1L) and analysis by two pathologists to detect the presence of micro-metastases.

Immunohistochemistry:

Formalin-fixed paraffin-embedded specimens were immunostained as previously described (32). Briefly, 4μm thick tissue sections were de-paraffinized with toluene and rehydrated in an ethanol gradient. Microwave antigen retrieval was performed by heating tissue slides in 1 mM EDTA buffer (pH 8.0) or 10 mM sodium citrate buffer (pH 6.0) for 15 minutes. Non-specific antigen binding was blocked with a protein blocking serum-free reagent (DakoCytomation, California, United States) preceding the ninety-minute primary antibody incubation with: anti-CD4 (Ab-8) (LabVision, California, United States), anti-CD8 (M-7103), anti-CD20 (M-0755), anti-CD45RA (M-0754), anti-CD45RO (M-0742), anti-CD68 (M-0876), anti-PSA (A-0562) (all from

DakoCytomation, California, United States), anti-CD56 (Ab-5, Lab Vision NeoMarkers, California, United States), anti-Fascin (Ab-1, Lab Vision NeoMarkers, California, United States), anti-HLA-DR/DP/DQ (555557, BD Biosciences Pharmingen, California, United States) or anti-Ki-67 (SP-6, Lab Vision NeoMarkers, California, United States). Tissues were treated with 1% H_2O_2 in methanol or 3% H_2O_2 in distilled water to eliminate endogenous peroxidase activity. This was followed by consecutive incubations with the secondary biotinylated antibody and streptavidin-HRP (DakoCytomation, California, United States). Reaction products were developed using 3,3'-diaminobenzidine (DAB) substrate-chromogen system (DakoCytomation, California, United States). CD4 staining was performed with the Envision G|2 system and developed with permanent red (DakoCytomation, California, United States). Hematoxylin counterstaining was performed for ease of reading. Immunostaining with appropriate isotype control antibodies were used as negative controls.

Quantification:

Slides were examined under standard light microscopy in order to confirm an optimal quality of staining. Histological images of 30 randomly selected fields were captured using a 20x microscope objective (Arcturus Pix Cell® IIe system microscope, California, United States) through a video camera (Hitachi Digital KP-0590P CCD color video camera, Tokyo, Japan). Pictures were digitized in a 24 bits true color TIFF format. Positive signals were quantified using the Image-Pro Plus version 5.1 software (MediaCybernetics, Maryland, United States). The software was trained to discern the DAB or the permanent red immunostaining signal (brown or red coloration), the hematoxylin stain (blue) and areas devoid of tissue (white) using the color segmentation operation. Two filter ranges were also applied to eliminate background staining: an area range (50.0 to 10,000,000 pixels) and a mean density range (100 to 250 units). In order to obtain precise color recognition templates, the analysis was initially performed manually against a representative photograph of each tissue sample prior to the full analysis with an automated macro. The data was then exported to an Excel spreadsheet where the percentage of the immunostained area

was corrected for the area devoid of tissue *[% $area_{corr}$ = % area of positive signal / (1 - % area devoid of tissue)*]. For the histopathological evaluation of LN reactivity, morphological analyses were performed with light microscopy using hematoxylin & eosin (H&E) stained sections.

Statistics:

The % $area_{corr}$ was used in statistical analyses using non-parametric Mann-Whitney U-test or parametric Student T-Test. The Kolmolgorov-Smirnov test and a test of homogeneity of variances were used in the evaluation of the distribution and the variance of the data between the three groups of LNs studied. Correlations were performed with linear regression evaluations. A two-tailed $P \leq 0.05$ was considered statistically significant. All statistical tests were performed using Statistical Package for the Social Sciences (SPSS), version 11.0 (SPSS Inc., Illinois, United States).

Results

Immunological microenvironment of pelvic LNs

We characterized the lymphocyte populations within pelvic LNs of 51 prostate cancer patients consisting of 26 control LNs, 25 non-metastatic LNs and 21 metastatic LNs using immune cell population markers (CD4, CD8, CD20, CD56, CD68, Fascin, HLA-DR/DP/DQ), activation and maturation markers (CD38, CD45RA, CD45RO) and a proliferation marker (Ki67) (Figure 1A-L). In order to minimize the subjectivity of immunohistochemical analyses, we chose to quantify the area covered by positively stained cells with a digital image analysis software.

We first evaluated if the presence of prostate cancer metastasis affected different immune cell populations (T and B lymphocytes and NK cells) found within LNs. The abundance of T lymphocytes was evaluated by quantifying the expression of CD4 and CD8, co-receptors expressed respectively by helper T lymphocytes and cytotoxic T lymphocytes. We observed a significant increase in $CD8^+$ T lymphocytes in metastatic and non-metastatic LNs when compared to controls ($p = 0.047$ and $p = 0.05$, respectively, Mann-U test) (Figure 2a). No significant differences were detected in the $CD4^+$ helper T cell populations between metastatic and non-metastatic LNs when compared to controls ($p = 0.616$ and $p = 0.098$, respectively, Mann-U test) and between non-metastatic and metastatic LNs ($p = 0.062$, Mann-U test) (Figure 2a). We evaluated the abundance of B lymphocytes by quantifying the expression of CD20, a plasma membrane protein expressed by naïve and mature B lymphocytes. We found a significant decrease in CD20+ B lymphocytes in metastatic and non-metastatic LNs when compared to controls ($p = 0.031$ and $p = 0.009$, respectively, Mann-U test) (Figure 2a). Finally, we studied the presence of the NK cell population by quantifying the expression of the NK cell-specific marker CD56. Our results trend towards a reduction of $CD56^+$ NK cells in metastatic LNs when compared to control LNs, although not statistically significant ($p = 0.093$, Mann-U test) (Figure 2b).

We then determined if there were differences in the abundance of APCs in metastatic LNs by analyzing the abundance of DCs, macrophages and the expression

of HLA-DR/DP/DQ, the major histocompatibility complex of class II (MHC-II) expressed by APCs. We investigated the LN macrophage population by studying the expression of CD68, a lysosomal membrane protein strongly expressed in the cytoplasmic granules of macrophages. We observed that metastatic LNs had lower CD68 expression than control LNs ($p = 0.0003$, Mann-U test) (Figure 2b). The DC population was studied by evaluating the expression of the protein Fascin. Fascin is a 55kDa cytoskeletal actin bundling protein highly expressed by DCs (33-35). No significant differences were observed between metastatic and non-metastatic LNs when compared to the controls ($p = 0.732$ and $p = 0.547$, respectively, Mann-U test) (Figure 2a). Finally, we evaluated the expression of MHC-II primarily expressed by APCs, such as DCs, macrophages and B lymphocytes. Again, no significant differences were observed between metastatic and non-metastatic LNs when compared to the controls ($p = 0.668$ and $p = 0.835$, respectively, Mann-U test) (Figure 2a).

In order to evaluate whether lymphocyte maturation levels were affected by the presence of prostate cancer LN metastases, we assessed the expression of CD45, a transmembrane glycoprotein tyrosine kinase present on the surface of lymphocytes. Distinct CD45 isoforms are expressed at various stages of differentiation of hematopoietic cells: the CD45RA isoform is present on naïve cells whereas activated/memory cells express CD45RO. No significant differences in the expression of CD45RA and CD45RO were observed between the three groups (Figure 2a).

We further analyzed the activation status of the immune cells in metastatic LNs by quantifying the expression of the cell surface marker CD38, an ADP-ribosyl cyclase expressed by activated lymphocytes. We observed a significant reduction in the abundance of $CD38^+$ activated immune cells in metastatic LNs when compared to both control LNs and non-metastatic LNs ($p = 0.038$ and $p = 0.0003$, respectively, Mann-U test) (Figure 2b).

Finally, we examined the proliferation of lymphocytes in pelvic LNs by quantifying the expression of the proliferative marker Ki67. Only $Ki67^+$ lymphocytes were counted in this analysis. These cells were easily distinguishable from other $Ki67^+$

cells, such as macrophages or metastatic cells, in the computer-assisted analysis because of their small dense nuclei (Figure 1K). Metastatic LNs contained more lymphocytes expressing the proliferation marker Ki67 than non-metastatic LNs ($p = 0.042$, Mann-U test) and no statistically significant difference was observed between metastatic and control LNs ($p = 0.325$, Mann-U test) (Figure 2b). Together, these results suggest that the presence of prostate cancer LN metastasis alters the distribution of lymphocytic and macrophage populations, promotes lymphocytes proliferation and lowers the activation levels of lymphocytes without significantly altering their differentiation into memory cells.

Histological evaluation of pelvic LNs in prostate cancer

We evaluated if the presence of metastatic cells in pelvic LNs of prostate cancer patients was associated with histological changes related to LN reactivity. Without prior knowledge of the LN status, pathological reviews of the hematoxylin & eosin staining of the 72 LNs were analyzed for reactive LN changes, namely: follicular hyperplasia, sinus hyperplasia (histiocytosis), fibrosis (hyaline material), calcifications and granulomatous reactions. These parameters of LN reactivity are known to correlate with cancer prognosis (22, 23, 25).

Follicular hyperplasia is characterized by the proliferation of B lymphocytes in germinal centers alongside tingible body macrophages and DCs, which phagocytose apoptotic lymphocytes and participate in the antigen presentation necessary for B lymphocyte development. Follicular hyperplasia was detected in 9/26 (34.6%) of control LNs, 5/25 (20.0%) of non-metastatic LNs and only 1/21 (4.8%) of the metastatic LNs ($p = 0.014$ between control LNs and metastatic LNs, Mann-U test) (Table 2). Furthermore, in our study, the presence of follicular hyperplasia was associated with the lower expression of CD8 ($p = 0.007$, linear regression).

Sinus hyperplasia is characterized by distention and prominence of lymphatic sinusoids caused by the infiltration with histiocytes and marked hypertrophy of the lining endothelial cells (21). Sinus hyperplasia was detected in 88.5% (23/26) of control LNs and in 96.0% (24/25) of non-metastatic LNs, in contrast to only 4/21 (19.0%) of metastatic LNs ($p<0.001$ between control LNs and metastatic LNs,

Mann-U test) (Table 2). The occurrence of sinus hyperplasia also correlated with higher expression of CD38 ($p = 0.012$, linear regression), higher CD68 expression ($p = 0.029$, linear regression) and lower Ki67+ lymphocytes ($p = 0.006$, linear regression).

Fibrosis results from the formation of excessive collagen in a reactive process. LNs with fibrosis in the tissue parenchyma were observed in 24/26 (92.3%) of control LNs, 21/25 (84.0%) of non-metastatic LNs and 14/22 (66.7%) of the metastatic LNs ($p = 0.028$ between control LNs and metastatic LNs, Mann-U test) (Table 2). The occurrence of LN fibrosis was also associated with the higher expression of CD38 ($p = 0.05$, linear regression) and the incidence of sinus hyperplasia and calcification ($p = 0.025$, $p < 0.001$, linear regression, respectively).

Granulomatous inflammation is a distinctive pattern of chronic inflammation characterized by aggregates of activated macrophages activated by T lymphocytes often associated with infective agents such as tuberculosis (21). None of our study cases displayed evidence of granulomatous inflammation in draining LNs (Table 2). LN calcification is an abnormal deposition of calcium salt in the soft tissue in the absence of calcium metabolic derangements (21). There was no statistically significant difference in the presence of calcification between the three groups: 12/26 (46.2%) of control LNs, 18/25 (72.0%) of non-metastatic LNs and 10/21 (47.6%) of the metastatic LNs (Table 2).

LN size and presence of metastatic cells

We evaluated if the size of LNs was associated with the lower prevalence LN reactivity of metastatic LNs. We calculated the approximate surface area of the LNs by measuring the longest and shortest axes of the LN tissue section. A similar technique of assessment of LN size is performed in transesophageal echocardiography (36). We found that metastatic LNs and non-metastatic LNs were significantly smaller than control LNs from patients with localized prostate cancer ($p = 0.005$ and $p = 0.044$, Student T-Test, respectively) (Table 2). Furthermore, 71.4% of the metastatic LNs had a diameter < 10mm as compared to non-metastatic (52.0%) and controls (34.6%). In addition, the size of the LN correlated positively

with the presence of sinus hyperplasia, follicular hyperplasia and fibrosis ($p = 0.026$, $p = 0.019$ and $p = 0.008$, linear regression, respectively), thus confirming that the LN reactivity correlated with the size of the LN.

Discussion

Currently, LNs of cancer patients are subjected to a routine histological analysis focusing on the detection of metastatic tumor cells. However, considering that removal of metastatic LNs does not appear to provide significant improvement to the patient's overall survival rate, it is likely that the detrimental effects associated with the development of LN metastasis remain long after the removal of tumor-infiltrated LNs (15). The important roles of tumor draining LNs in the development of the anti-tumoral immune response might be compromised in advanced prostate cancer. As such, the evaluation of various cytoarchitectural characteristics of both metastatic and non-metastatic LNs might provide important clues regarding the activity of the immune system in events leading to the development of prostate cancer LN metastasis. Moreover, a detailed analysis of the immunological activity of tumor-infiltrated LNs would further our understanding of the mechanisms involved in immune system evasion and help in the development of clinically applicable immunotherapy for prostate cancer.

We observed an increased abundance of $CD8^+$ cytotoxic T lymphocytes in tumor-invaded LNs. Reports in the literature describe an augmentation of CD8+ T lymphocytes in metastatic LNs of breast cancer patients and lower abundance of $CD8^+$ T lymphocytes in metastatic LNs of patients with head and neck cancer, suggesting that variation in the $CD8^+$ T lymphocyte population could be cancer specific (37-39). An augmentation of $CD8^+$ T cells in metastatic LNs is significant since it implies that the microenvironment of tumor-invaded LNs favors the proliferation and/or the homing of cytotoxic T cells, which could participate in the elimination of the intruding cancer cells. Furthermore, the reduced level of $CD20^+$ B lymphocytes and follicular hyperplasia in metastatic LNs, which correlates with the augmented presence of $CD8^+$ T lymphocytes, may suggest that a cell-mediated immune response occurs more frequently than a humoral response. However, data suggest that $CD8^+$ lymphocytes in metastatic LN of melanoma patients displayed a precursor phenotype (pre-terminally differentiated state) and lack the expression of

key protein of the cytotoxic machinery (40). Further work would be required to specifically address the activation status of the $CD8^+$ lymphocyte populations in metastastic and non-metastatic LNs in order to fully understand the extent of immunosuppression associated with prostate cancer metastasis.

The increase abundance of $Ki67^+$ lymphocytes also implies that the microenvironment could favor some lymphocyte proliferation. However, the increased lymphocyte proliferation was very small and metastatic LNs were significantly smaller than non-metastatic LNs. Our results on lymphocyte proliferation require further investigation in order to determine whether this increased proliferation is counterbalanced by increased numbers of apoptotic lymphocytes in tumor-invaded LNs. It was shown that tumor cells could promote lymphocyte apoptosis through Fas-Fas ligand interaction and caspase activation (41). An augmentation in apoptotic lymphocytes in metastatic LNs could prevail over lymphocytic proliferation and further explain the smaller size of metastatic LNs in comparison to control LNs.

The microenvironment of metastatic LNs was shown to favor the activation of lymphocytes by increasing the immunogenicity of the invading neoplastic cells (42). However, our results on the expression of CD45RA and CD45RO suggest that metastatic LN lymphocytes did not attain levels of maturation higher than lymphocytes in non-metastatic or control LNs. Similar observations have been made in the study of lymphocytes in colon and stomach cancer (43). Nonetheless, contrary to expectation, the increased prevalence of sinus hyperplasia and/or follicular hyperplasia, two processes associated with the development of inflammatory responses, were not associated with elevated expression of CD45RO in tumor-free LNs. We observed, however, a lower abundance of $CD38^+$ activated lymphocytes in metastatic LNs, which suggest that the presence of neoplastic cells would prevent the activation of immune cells.

Furthermore, the expression of MHC-II was relatively similar between the three LN groups even though there was significant reduction in the abundance of B lymphocytes and macrophages, two APCs expressing MHC-II at their surface. There are three hypotheses that could explain why we did not observe a lower expression of

MHC-II in metastatic LNs. First, the lower abundance of macrophages in metastatic LNs compared to controls is significant but very low (3.28% ± 2.17% and 0.62% ± 0.52%) when compared to the expression of MHC-II (13.1% ± 1.98 and 9.88% ± 1.79%). The lower abundance of macrophage may not significantly account for lower expression of MHC-II. The second hypothesis is that the relative equal abundance of DCs between the three groups may be enough to counterbalance the lower abundance of B lymphocytes and, thus the lack of lower expression of MHC-II in metastatic lymph nodes. DCs in LNs are known to express higher levels of MHC-II than macrophages (44) and B lymphocytes (45-47). This higher expression of MHC-II by DC could prevent the reduction in MHC-II expression. Finally, immunohistochemical analysis is somewhat subjective. FACS analyses with double staining for MHC-II and DC, macrophages, T or B lymphocytes markers would be necessary to precisely address the question whether there is a lower expression of MHC-II within one immune cell population. Unfortunately, it is technically impossible to perform FACS analyses on archived tissues.

Our histopathological findings reinforces the idea that an immunosuppressive microenvironment may exist in tumor-invaded LNs. Our results indicate a diminished incidence of LN reactivity (sinus hyperplasia, follicular hyperplasia and fibrosis) in metastatic LNs. The absence of sinus hyperplasia in metastatic LNs was observed in an experimental rat model, which demonstrated a transient LN reactivity only in the early stages of the metastatic process (6). The presence of metastatic cells in LNs has also been documented to be associated with a decrease in the macrophage population, a result similar to our observation in metastatic LNs of prostate cancer patients (6). A lower incidence of follicular hyperplasia in metastatic LNs was also observed in squamous cell carcinoma of the oral cavity (26). The lower abundance of CD8+ lymphocytes correlated with decreased signs of sinus hyperplasia. An inverse relationship between paracortical hyperplasia (proliferation of T lymphocytes) and sinus hyperplasia has also been previously reported (23, 48). These results, combined with the correlation between follicular hyperplasia and $CD8^+$ T lymphocytes, indicate that the immunological response in metastatic LNs, if present, might predominantly be T lymphocyte mediated.

Finally, the development of an inflammatory reaction is generally associated with an augmentation of LN size. This reactive enlargement is caused by the generation of sinus, follicular and/or paracortical hyperplasia. In our study, the size of the LN showed a correlation with the presence of sinus hyperplasia, follicular hyperplasia, fibrosis and calcification, therefore suggesting that larger LNs are more immunologically reactive. In several cancers, it has been established that tumor-infiltrated LNs are larger than tumor-free LNs due to the development of an immune response against invading metastatic cells leading to reactive hyperplasia or to the growth of tumor cells (6, 26, 49). In contrast, our results indicate that metastatic LNs are significantly smaller than non-metastatic LNs. This has not previously been reported in prostate cancer. Interestingly, other investigators have reported similar observations, specifically in non-small cell lung cancer and endometrial cancer (36, 50). The finding that metastatic LNs are of smaller size than non-metastatic LNs would further indicate that the presence of prostate cancer LN metastasis is associated with the development of an immunosuppressive microenvironment.

Finally, both non-metastatic and metastatic LNs of patients with metastatic LNs were significantly smaller than LNs of the control groups. There are two reasons that may explain this result. First, the non-metastatic LNs could contain micro-metastases, which would affect LN size and LN reactivity. Although the presence of micro-metastases in non-metastatic LNs is possible, we feel it is unlikely since two pathologists analyzed all of the non-metastatic LNs and none were positive for the presence of micro-metastases. Furthermore, our results clearly show that the non-metastatic LN group is significantly distinct from the metastatic group based on cellular and histological characteristics. Secondly, the entire nodal basin could be subjected to the immunosuppressive action of a soluble factor, such as TGF-β. This is a plausible hypothesis since the expression of TGF-β, an immuno-inhibitory cytokine, is increased in advanced prostate cancer (51, 52). All tumor-draining LNs in metastatic patients could be less effective at mounting an immunological response than LNs in patients with localized prostate cancer. This phenomenon would nonetheless be further amplified in metastatic LNs.

Conclusions

Although this is consistent with published data, to our knowledge, this is the first report to characterize tumor-infiltrated pelvic LNs in prostate cancer both from an immunological and from a histopathological perspective. We observed that metastatic LNs are subjected to immunoregulatory interactions with the invading cancer cells, which tends to correlate with a smaller LN size and decreased LN reactivity. In other tumor models, it has also been previously reported that tumor-draining LNs and metastatic LNs show evidence of an immunological unresponsiveness (6, 48, 53-56). Several mechanisms have been postulated to account for lack of immunological reactivity in tumor-draining LNs, such as high expression of immunosuppressive cytokines, elevated activity of immunosuppressive cells (regulatory T cells and/or indoleamine 2,3-dioxygenase (IDO) expressing DCs) and modifications in the T lymphocytes and DCs zones (7, 54-57). Further research is now necessary in order to detail which immunosuppressive mechanisms are utilized by prostate cancer cells. Moreover, our results on LN size call into question the validity of assessing LN status (metastatic or non-metastatic) based on their macroscopic appearance. Our data reinforces the pressing need to develop new molecular and imaging tools in order to better stratify patients with metastatic disease and to help clinicians plan treatment modalities. This work may eventually lead to the development of immunotherapeutic modalities for prostate cancer by targeting the immunosuppressive properties of tumor cells, thereby enhancing the local tumoricidal immune response.

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References

1. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. Nat Immunol 2002;3(11):991-8.
2. Dunn GP, Old LJ, Schreiber RD. The immunobiology of cancer immunosurveillance and immunoediting. Immunity 2004;21(2):137-48.
3. Fisher B, Fisher ER. Studies concerning the regional lymph node in cancer. I. Initiation of immunity. Cancer 1971;27(5):1001-4.
4. Abe R, Taneichi N. Lymphatic metastasis in experimental cecal cancer. Effectiveness of lymph nodes as barriers to the spread of tumor cells. Arch Surg 1972;104(1):95-8.
5. Heys SD, Eremin O. The relevance of tumor draining lymph nodes in cancer. Surg Gynecol Obstet 1992;174(6):533-40.
6. Nagata H, Arai T, Soejima Y, Suzuki H, Ishii H, Hibi T. Limited capability of regional lymph nodes to eradicate metastatic cancer cells. Cancer Res 2004;64(22):8239-48.
7. Cochran AJ, Morton DL, Stern S, Lana AM, Essner R, Wen DR. Sentinel lymph nodes show profound downregulation of antigen-presenting cells of the paracortex: implications for tumor biology and treatment. Mod Pathol 2001;14(6):604-8.
8. Jemal A, Murray T, Ward E, Samuels A, Tiwari RC, Ghafoor A, et al. Cancer statistics, 2005. CA Cancer J Clin 2005;55(1):10-30.
9. Petros JA, Catalona WJ. Lower incidence of unsuspected lymph node metastases in 521 consecutive patients with clinically localized prostate cancer. J Urol 1992;147(6):1574-5.
10. Danella JF, deKernion JB, Smith RB, Steckel J. The contemporary incidence of lymph node metastases in prostate cancer: implications for laparoscopic lymph node dissection. J Urol 1993;149(6):1488-91.

11. Bundrick WS, Culkin DJ, Mata JA, Zitman RI, Venable DD. Evaluation of the current incidence of nodal metastasis from prostate cancer. J Surg Oncol 1993;52(4):269-71.
12. Mettlin CJ, Murphy GP, Ho R, Menck HR. The National Cancer Data Base report on longitudinal observations on prostate cancer. Cancer 1996;77(10):2162-6.
13. Krongrad A, Lai H, Lai S. Survival after radical prostatectomy. Jama 1997;278(1):44-6.
14. Fournier G, Valeri A, Mangin P, Cussenot O. [Prostate cancer: Diagnosis and staging]. Ann Urol (Paris) 2004;38(5):207-24.
15. Gervasi LA, Mata J, Easley JD, Wilbanks JH, Seale-Hawkins C, Carlton CE, Jr., et al. Prognostic significance of lymph nodal metastases in prostate cancer. J Urol 1989;142(2 Pt 1):332-6.
16. Ishida K, Katsuyama T, Sugiyama A, Kawasaki S. Immunohistochemical evaluation of lymph node micrometastases from gastric carcinomas. Cancer 1997;79(6):1069-76.
17. Cheng L, Bergstralh EJ, Cheville JC, Slezak J, Corica FA, Zincke H, et al. Cancer volume of lymph node metastasis predicts progression in prostate cancer. Am J Surg Pathol 1998;22(12):1491-500.
18. Cheng L, Zincke H, Blute ML, Bergstralh EJ, Scherer B, Bostwick DG. Risk of prostate carcinoma death in patients with lymph node metastasis. Cancer 2001;91(1):66-73.
19. Daneshmand S, Quek ML, Stein JP, Lieskovsky G, Cai J, Pinski J, et al. Prognosis of patients with lymph node positive prostate cancer following radical prostatectomy: long-term results. J Urol 2004;172(6 Pt 1):2252-5.
20. Tsakraklides V, Olson P, Kersey JH, Good RA. Prognostic significance of the regional lymph node histology in cancer of the breast. Cancer 1974;34(4):1259-67.
21. Kumar V, Cotran RS, Robbins SL. Robbins Basic Pathology. 7th ed. Philadelphia, Pennsylvania, USA; 2003.

22. Meyer EM, Grundmann E. Lymph node reactions to cancer. Klin Wochenschr 1982;60(21):1329-38.
23. Amar A, Rapoport A, Rosas MP. Evaluation of lymph node reactivity in differentiated thyroid carcinoma. Sao Paulo Med J 1999;117(3):125-8.
24. Black MM, Barclay TH, Hankey BF. Prognosis in breast cancer utilizing histologic characteristics of the primary tumor. Cancer 1975;36(6):2048-55.
25. Patt BS, Close LG, Vuitch F. Prognostic significance of sinus histiocytosis in metastatic laryngeal cancer. Laryngoscope 1993;103(5):498-502.
26. Okura M, Kagamiuchi H, Tominaga G, Iida S, Fukuda Y, Kogo M. Morphological changes of regional lymph node in squamous cell carcinoma of the oral cavity. J Oral Pathol Med 2005;34(4):214-9.
27. Ismail HA, Lessard L, Mes-Masson AM, Saad F. Expression of NF-kappaB in prostate cancer lymph node metastases. Prostate 2004;58(3):308-13.
28. Gannon PO, Lapointe R, Bellon-Gagnon P, Mes-Masson AM, Saad F. Comparison of Lymphocyte Populations in Lymph Node from Localized and Metastatic Prostate Cancer Patients. Immunology 2004 2004:397-402.
29. Armas OA, Aprikian AG, Melamed J, Cordon-Cardo C, Cohen DW, Erlandson R, et al. Clinical and pathobiological effects of neoadjuvant total androgen ablation therapy on clinically localized prostatic adenocarcinoma. Am J Surg Pathol 1994;18(10):979-91.
30. Montironi R, Schulman CC. Pathological changes in prostate lesions after androgen manipulation. J Clin Pathol 1998;51(1):5-12.
31. Mercader M, Bodner BK, Moser MT, Kwon PS, Park ES, Manecke RG, et al. T cell infiltration of the prostate induced by androgen withdrawal in patients with prostate cancer. Proc Natl Acad Sci U S A 2001;98(25):14565-70.
32. Lessard L, Mes-Masson AM, Lamarre L, Wall L, Lattouf JB, Saad F. NF-kappa B nuclear localization and its prognostic significance in prostate cancer. BJU Int 2003;91(4):417-20.
33. Pinkus GS, Pinkus JL, Langhoff E, Matsumura F, Yamashiro S, Mosialos G, et al. Fascin, a sensitive new marker for Reed-Sternberg cells of hodgkin's disease. Evidence for a dendritic or B cell derivation? Am J Pathol 1997;150(2):543-62.

34. Pinkus GS, Lones MA, Matsumura F, Yamashiro S, Said JW, Pinkus JL. Langerhans cell histiocytosis immunohistochemical expression of fascin, a dendritic cell marker. Am J Clin Pathol 2002;118(3):335-43.
35. Bros M, Ross XL, Pautz A, Reske-Kunz AB, Ross R. The human fascin gene promoter is highly active in mature dendritic cells due to a stage-specific enhancer. J Immunol 2003;171(4):1825-34.
36. Arita T, Matsumoto T, Kuramitsu T, Kawamura M, Matsunaga N, Sugi K, et al. Is it possible to differentiate malignant mediastinal nodes from benign nodes by size? Reevaluation by CT, transesophageal echocardiography, and nodal specimen. Chest 1996;110(4):1004-8.
37. Alam SM, Clark JS, George WD, Campbell AM. Altered lymphocyte populations in tumour invaded nodes of breast cancer patients. Immunol Lett 1993;35(3):229-34.
38. Verastegui E, Morales R, Barrera JL, Mueller A, Guzman B, Meneses A, et al. Immunological approach in the evaluation of regional lymph nodes of patients with squamous cell carcinoma of the head and neck. Clin Immunol 2002;102(1):37-47.
39. Kohrt HE, Nouri N, Nowels K, Johnson D, Holmes S, Lee PP. Profile of immune cells in axillary lymph nodes predicts disease-free survival in breast cancer. PLoS Med 2005;2(9):e284.
40. Mortarini R, Piris A, Maurichi A, Molla A, Bersani I, Bono A, et al. Lack of terminally differentiated tumor-specific CD8+ T cells at tumor site in spite of antitumor immunity to self-antigens in human metastatic melanoma. Cancer Res 2003;63(10):2535-45.
41. Gastman BR, Johnson DE, Whiteside TL, Rabinowich H. Tumor-induced apoptosis of T lymphocytes: elucidation of intracellular apoptotic events. Blood 2000;95(6):2015-23.
42. Santin AD. Lymph node metastases: the importance of the microenvironment. Cancer 2000;88(1):175-9.
43. Lores-Vazquez B, Pacheco-Carracedo M, Oliver-Morales J, Parada-Gonzalez P, Gambon-Deza F. Lymphocyte subpopulations of regional lymph nodes in

human colon and gastric adenocarcinomas. Cancer Immunol Immunother 1996;42(6):339-42.

44. Lutsiak ME, Robinson DR, Coester C, Kwon GS, Samuel J. Analysis of poly(D,L-lactic-co-glycolic acid) nanosphere uptake by human dendritic cells and macrophages in vitro. Pharm Res 2002;19(10):1480-7.
45. Masten BJ, Lipscomb MF. Comparison of lung dendritic cells and B cells in stimulating naive antigen-specific T cells. J Immunol 1999;162(3):1310-7.
46. Inaba K, Pack M, Inaba M, Sakuta H, Isdell F, Steinman RM. High levels of a major histocompatibility complex II-self peptide complex on dendritic cells from the T cell areas of lymph nodes. J Exp Med 1997;186(5):665-72.
47. Binder RJ, Anderson KM, Basu S, Srivastava PK. Cutting edge: heat shock protein gp96 induces maturation and migration of CD11c+ cells in vivo. J Immunol 2000;165(11):6029-35.
48. Hoon DS, Korn EL, Cochran AJ. Variations in functional immunocompetence of individual tumor-draining lymph nodes in humans. Cancer Res 1987;47(6):1740-4.
49. Eremin O, Roberts P, Plumb D, Stephens JP. Human regional tumour lymph nodes: alterations of micro-architecture and lymphocyte subpopulations. Br J Cancer 1980;41(1):62-72.
50. Ayhan A, Tuncer ZS, Tuncer R, Yuce K, Kucukali T. Tumor status of lymph nodes in early endometrial cancer in relation to lymph node size. Eur J Obstet Gynecol Reprod Biol 1995;60(1):61-3.
51. Danielpour D. Functions and regulation of transforming growth factor-beta (TGF-beta) in the prostate. Eur J Cancer 2005;41(6):846-57.
52. Li MO, Wan YY, Sanjabi S, Robertson AK, Flavell RA. Transforming growth factor-beta regulation of immune responses. Annu Rev Immunol 2006;24:99-146.
53. Wirth M, Schmitz-Drager BJ, Ackermann R. Functional properties of natural killer cells in carcinoma of the prostate. J Urol 1985;133(6):973-8.
54. Hoon DS, Bowker RJ, Cochran AJ. Suppressor cell activity in melanoma-draining lymph nodes. Cancer Res 1987;47(6):1529-33.

55. Cochran AJ, Pihl E, Wen DR, Hoon DS, Korn EL. Zoned immune suppression of lymph nodes draining malignant melanoma: histologic and immunohistologic studies. J Natl Cancer Inst 1987;78(3):399-405.

56. Lee JH, Torisu-Itakara H, Cochran AJ, Kadison A, Huynh Y, Morton DL, et al. Quantitative analysis of melanoma-induced cytokine-mediated immunosuppression in melanoma sentinel nodes. Clin Cancer Res 2005;11(1):107-12.

57. Battaglia A, Ferrandina G, Buzzonetti A, Malinconico P, Legge F, Salutari V, et al. Lymphocyte populations in human lymph nodes. Alterations in CD4+ CD25+ T regulatory cell phenotype and T-cell receptor Vbeta repertoire. Immunology 2003;110(3):304-12.

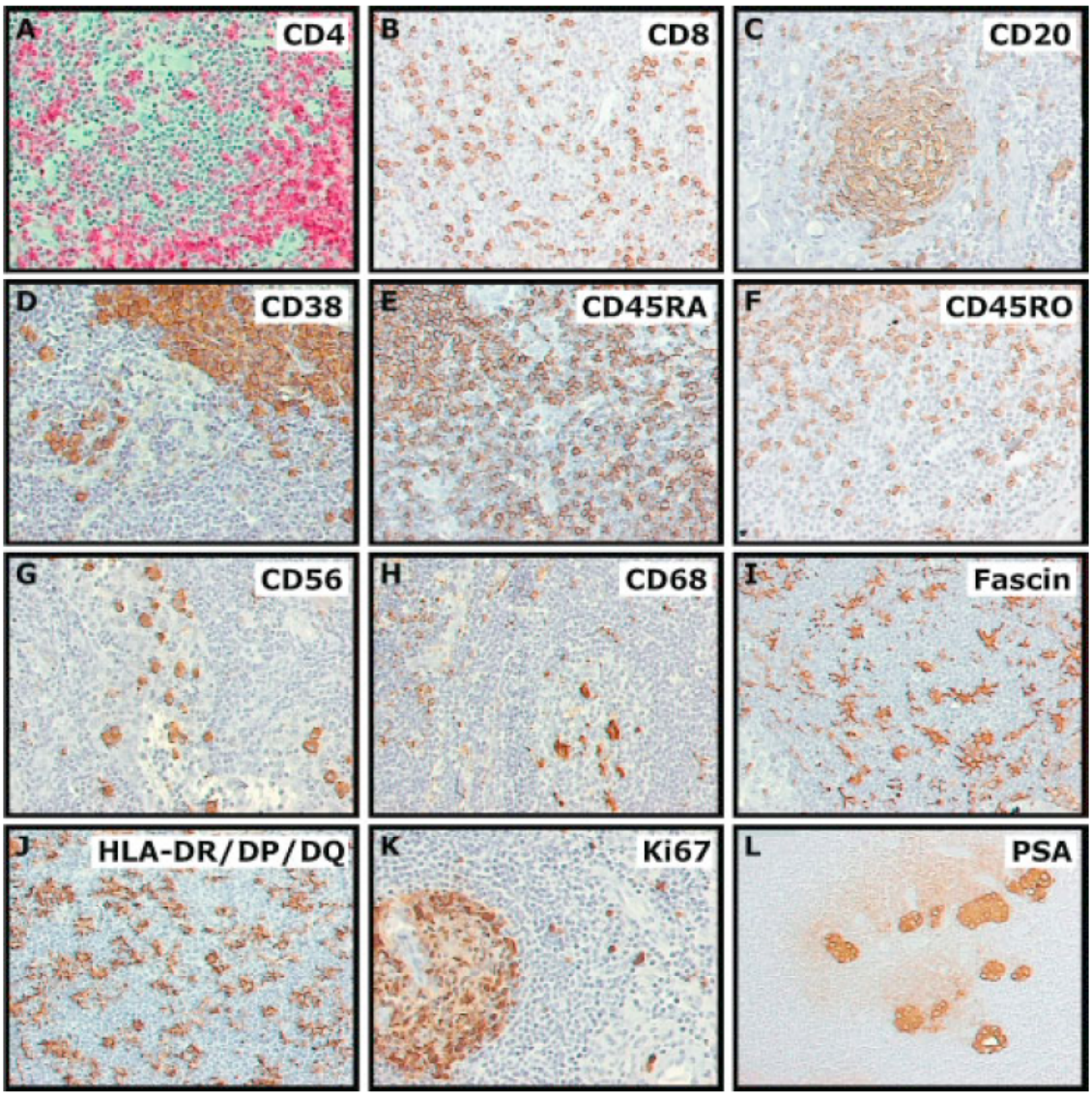

Figure 1. Representative images of immunohistochemical staining

Figure 1. Representative images (20x objective) of immunohistochemical staining of formalin-fixed paraffin embedded pelvic lymph nodes (LNs) tissue sections. (A) $CD4^+$ T lymphocytes marked with permanent Red; (B) $CD8^+$ T lymphocytes; (C) $CD20^+$ B lymphocytes; (D) $CD38^+$ activated lymphocytes; (E) $CD45RA^+$ naïve lymphocytes; (F) $CD45RO^+$ memory lymphocytes; (G) $CD56^+$ natural killer cells; (H) $CD68^+$ macrophages localized in a B lymphocyte follicle; (I) $Fascin^+$ dendritic cells in the paracortical area; (J) HLA-DR/DP/DQ^+ immune cells localized in the paracortical area (staining distribution similar to the Fascin staining); (K) $Ki67^+$ proliferating lymphocytes in a B lymphocyte follicle marked with DAB. $Ki67^+$ lymphocytes are easily distinguishable from other cell type based on their dense nuclei; (L) PSA^+ prostate cancer lymph node metastasis.

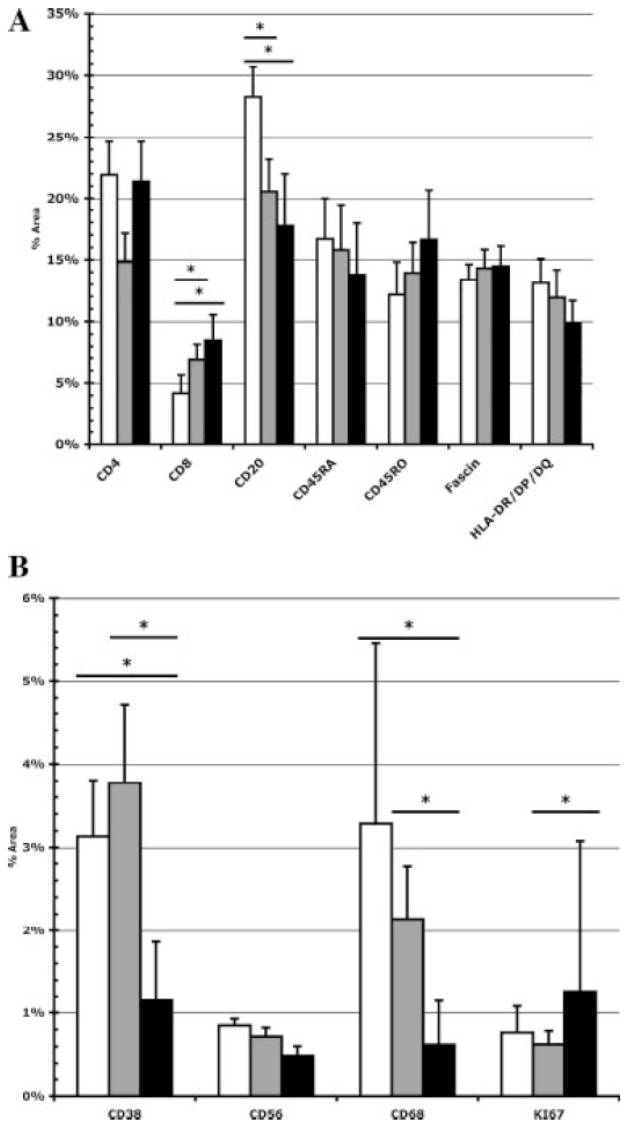

Figure 2. Percentage of area covered by positively stained cells

Figure 2. Percentage of area ($area_{corr}$) covered by positively stained cells analyzed by Image-Pro Plus v.5.1 in pelvic lymph nodes (LNs) (median ± s.e. median). (White bars) non-metastatic (control) LNs from patients with no PCa LNs metastasis, (Gray bars) non-metastatic LNs from patients with PCa LNs metastasis, (Black bars) metastatic LNs from patients with PCa LNs metastasis. (A) CD4, CD8, CD20, CD45RA, CD45RO, Fascin, HLA-DR/DP/DQ; (B) CD38, CD56, CD68, KI67.

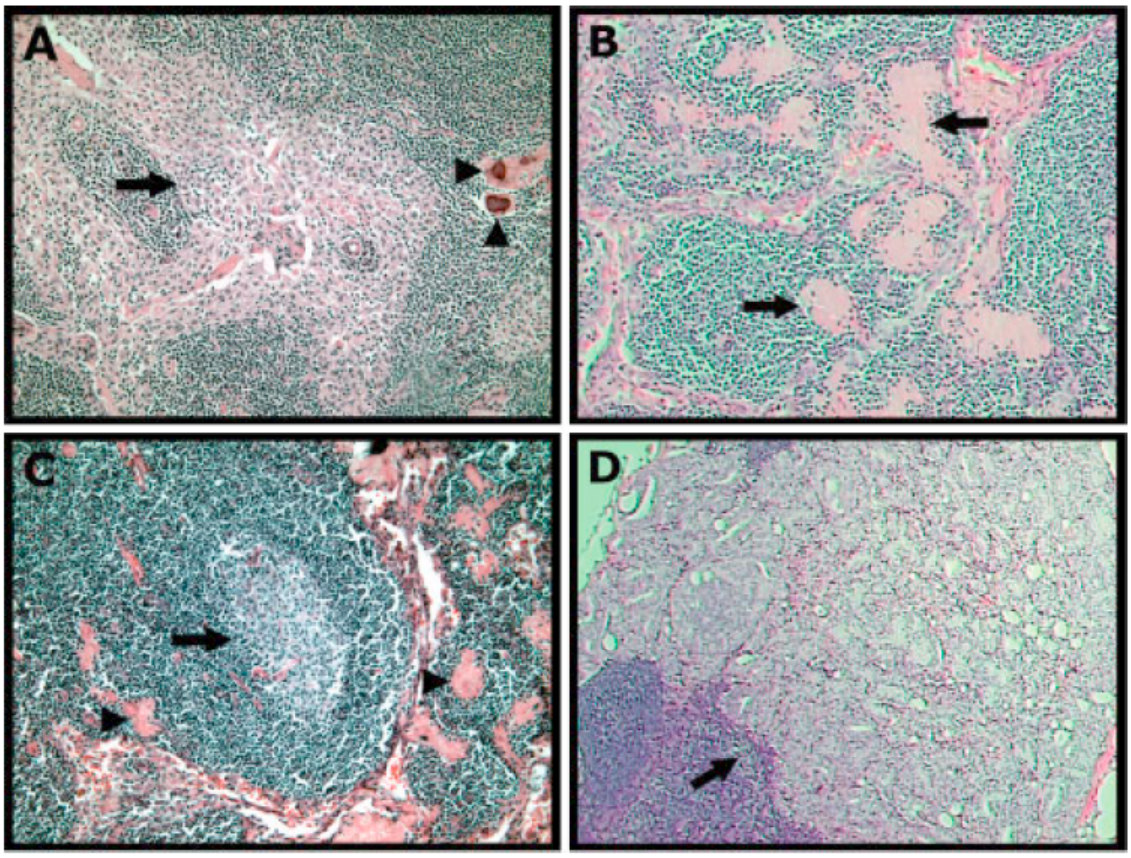

Figure 3. Histopathological features observed in hematoxylin and eosin (H&E) stained LN sections.

Figure 3. Histopathological features observed in hematoxylin and eosin (H&E) stained LN sections. (A) Sinus hyperplasia (arrow) and calcification (arrowheads) (10x objective); (B) Fibrosis (arrows) (10x objective); (C) Follicular hyperplasia (arrow) and fibrosis (arrowheads) (10x objective); (D) Prostate cancer lymph node metastasis (arrow) (4x objective).

TABLE I. Clinical Characteristics of Prostate Cancer Patients

	Control patients	Metastatic patients	*P*-values
Number of patients	26	25	
Age (mean ± SE)	62.58 ± 0.89	63.0 ± 1.00	0.650
PSA pre-op (mean ± SE)	8.64 ± 1.38	13.45 ± 1.92	0.018
Gleason score (mean ± SE)	5.33 ± 0.29	6.92 ± 0.21	<0.001
TNM stage			
PT2	11/26 (42.3%)	3/25 (12.0%)	0.016
PT2a	2/26 (7.70%)	1/25 (4.00%)	0.579
PT2b	6/26 (23.1%)	3/25 (12.0%)	0.304
PT3a	7/26 (26.9%)	7/25 (28.0%)	0.932
PT3b	0/26 (0.00%)	11/25 (44.0%)	<0.001
Biochemical recurrence at 5 years	0/26 (0.00%)	15/25 (60.0%)	<0.001

P-value determined by Mann–Whitney *U*-tests.

Table 1. Clinical Characteristics of Prostate Cancer Patients

TABLE 2. Pathological Analysis of LNs in Prostate Cancer Patients

Pathologies	Lymph nodes			*P*-values	
	Control	Non-metastatic	Metastatic	Control versus metastatic	Metastatic versus non-metastatic
Follicular hyperplasia	9/26 (34.6%)	5/25 (20.0%)	1/21 (4.8%)	0.014[a]	0.131[a]
Sinus hyperplasia	23/26 (88.5%)	24/25 (96.0%)	4/21 (19.0%)	<0.001[a]	<0.001[a]
Fibrosis	24/26 (92.3%)	21/25 (84.0%)	14/21 (66.7%)	0.028[a]	0.175[a]
Calcification	12/26 (46.2%)	18/25 (72.0%)	10/21 (47.6%)	0.921[a]	0.095[a]
Granuloma	0/26 (0.00%)	0/25 (0.00%)	0/21 (0.00%)	1.000[a]	1.000[a]
LN size (mm) (mean ± SE mean)	92.9 ± 12.5	63.7 ± 11.2	44.8 ± 6.2	0.005[b]	0.636[b]

[a]*P*-Values determines by Mann–Whitney *u*-test.
[b]*P*-Values determines by Student's *t*-test.

Table 2. Pathological Analysis of LNs in Prostate Cancer Patients

CHAPTER IV

CHARACTERIZATION OF THE INTRA-PROSTATIC IMMUNE CELL INFILTRATION IN ANDROGEN-DEPRIVED PROSTATE CANCER PATIENTS

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Author contributions:

For this paper, I did the majority of the experiments and wrote the manuscript in its entirety, although all co-authors played a role in the editing process. Alexis Poisson and Nathalie Delvoye optimized immunohistochemical stainings.

Keywords:

Prostate cancer; Androgen-deprivation therapy; Immunohistochemistry; Computer-assisted quantification; Tumor-infiltrating lymphocytes; NK cells; Macrophages.

Abstract

Introduction: Our goal was to study the hormonal regulation of immune cell infiltration in prostate cancer patients treated by androgen deprivation therapy (ADT) using an optimized computer-assistance quantification approach.

Methods: The relative density of immune cell subtypes ($CD3^+$, $CD8^+$, $CD20^+$, $CD56^+$, $CD68^+$ and $Foxp3^+$) was analyzed by immunohistochemistry in archived prostate specimens from control patients (radical prostatectomy only, n=40) and ADT-treated patients (ADT prior to radical prostatectomy, n=35) using an image analysis software and a whole-slide scanner.

Results : ADT-treated patients had significantly increased relative density of $CD3^+$ ($p<0.001$) and $CD8^+$ T lymphocytes ($p<0.001$) as well as $CD68^+$ macrophages ($p<0.001$). Elevated abundance of $CD56^+$ Natural Killer (NK) cells was associated with a lower risk of prostate cancer progression ($p=0.044$), while a high density of $CD68^+$ macrophages was related to an increased risk of biochemical recurrence ($p=0.011$).

Conclusions: Our results demonstrate that the infiltration of specific immune cell subtypes is modulated by ADT. Furthermore our data confirm that NK cells have a protective role against tumor progression while macrophages seem to favor the development of advanced prostate cancer.

Introduction

Prostate cancer remains the most frequently diagnosed cancer and third leading cause of cancer related deaths for North American men (Jemal et al., 2008). Androgens participate in the prostate's organogenesis and carcinogenesis (Grossmann et al., 2001). As such, the most common treatment for men with advanced stage or recurrent prostate cancer is androgen deprivation therapy (ADT). ADT promotes the apoptosis of the hormone sensitive prostate epithelial cells, which leads to the involution of the prostate (Montironi and Schulman, 1998; Ohlson et al., 2005). Unfortunately, generally within one to five years following ADT initiation, patients develop hormone refractory prostate cancer, the major contributor to prostate cancer related death, and a disease for which only palliative therapies are currently available (Oefelein et al., 2002; Tannock et al., 2004).

Novel therapeutic protocols are currently emerging with the goal of tackling hormone-refractory prostate cancer, including immune-based therapies. The common rationale between the various immunotherapeutic approaches is the activation of the anti-tumoral immu ne response within the tumor and/or metastases. To this day, immunotherapeutic protocols in clinical trial have yet to attain the optimistic results demonstrated in animal models. Recent data suggest that the prostate possesses a strong immunoregulatory potential, which may suppress the activation of the anti-tumoral immune response (Miller and Pisa, 2007). Therefore, the interactions between the immune system and prostate cancer cells within the patient's primary tumor needs to be better understood in order to develop clinically effective immunotherapies.

Several publications have demonstrated that different immune cell populations infiltrate the prostate and that, in some cases, the abundance of specific immune cells may correlate with cancer progression (Vesalainen et al., 1994; Irani et al., 1999; Shimura et al., 2000; McArdle et al., 2004; Karja et al., 2005). Nonetheless, in the context of an androgen dependant cancer such as prostate cancer, significant knowledge needs to be obtained on whether androgens can modulate the abundance and activity of the immune cell infiltrate within the primary tumor. It is known that

ADT fosters the development of a pro-inflammatory environment within the prostate (Civantos et al., 1996; Guinan et al., 1997; Mercader et al., 2007). Moreover, the immunosuppressive potential of the primary tumor can be dampened following ADT (Drake et al., 2005). With clinical trials combining immune-based therapies and ADT currently being evaluated, it is essential to gain insights on the various immunoregulatory changes present within the prostate following ADT.

Our goal was thus to characterize, using a computer-based approach, the immune cell infiltrate in patients treated by ADT. Using a cohort of 75 patients, we quantified the relative density of various adaptive and innate immune cell populations using a software-assisted protocol coupled to a whole-slide image scanner. The abundance of various immune cell populations was quantified using a freely available image-analysis software. With the idea that immune cell abundance could be used as a prognostic tool for prostate cancer progression, we optimized a system that would allow for the rapid and accurate quantification of various immunohistochemical markers on a large tissue sample thereby eliminating significant biases of analyses on smaller tissue sections. We believe that our method could heIp standardize the analysis of diverse immune cell populations within primary tumors and thus facilitate the interpretations of independent studies to better understand the biology of the immune system in prostate cancer.

Materials and methods

Patients

Paraffin-embedded formalin-fixed primary tumor specimens from 40 control patients (radical prostatectomy only) and 35 ADT patients (ADT prior to radical prostatectomy) who had undergone surgery between 1991 and 2001 were used in this study. The 35 patients in the ADT group had (i) histologic effects attributable to neoadujvant ADT (ii) histologically identifiable areas of tumor remaining within prostate samples. The control group was matched according Gleason score in order to eliminate immune infiltration variation due to the degree of differentiation of the tumors. All patients had a clinical follow-up of at least five years or until death (mean 99.8 months). Clinico-pathological characteristics of both control and ADT patients can be found in Supplementary Table 1. All ADT patients received either a luteinizing hormone-releasing hormone (LHRH) agonist (Cyproterone) or gonadotropin-releasing hormone (GnRH) antagonist (Leuprolide) in combination with an AR blocker (Flutamide). Time to biochemical recurrence was defined as the time elapsed between surgery and when prostate-specific antigen (PSA) level first rose from undetectable to > 0.3 ng/ml and increasing, as previously reported by our group (Fradet et al., 2004; Le Page et al., 2006; Koumakpayi et al., 2007; Diallo et al., 2008). The final pathological staging, grading and histopathological diagnosis was based on the pathology report. Specimens were obtained from consenting patients and the institutional ethics review committee approved this study.

Immunohistochemistry

Specimens were immunostained with anti-CD3 (NCL-L-CD3-PS1, Novocastra, Newcastle, UK), anti-CD8 (M-7103, Dako Diagnostics Inc. Carpinteria, CA, USA), anti-CD20 (M-0755, Dako), anti-CD56 (Ab-5, LabVision Neomarkers, Fermont, CA, USA), anti-CD68 (M-0876, Dako) and anti-Foxp3 (222510, Abcam, MA, USA). Staining was performed as previously described (Lessard et al., 2003; Gannon et al., 2006; Koumakpayi et al., 2006; Diallo et al., 2007). Briefly, specimens

were deparaffinized, rehydrated and antigen retrieval was performed with a Tris-EDTA buffer (10 mM Tris Base, 1 mM EDTA Solution, pH 9.0) using a commercial pressure cooker. The specimens were then blocked with a protein-blocking serum-free reagent (Dako) and incubated with the primary antibody for 90 min. Endogenous peroxidase activity was then blocked with 0.6% H2O2 in methanol for 30 min, which was followed by a 30 min incubation with a secondary goat anti-mouse IgG HRP-coupled antibody (sc-2005, Santa Cruz, CA, USA). Between each incubation, specimens were washed in PBS for 15 min. Positive signal was revealed using the LSAB 2 peroxidase system (Dako) and counterstained with Harris haematoxylin (Sigma-Aldrich, St-Louis, MO, USA). There were no non-specific stainings when IgG isotype controls were used in lieu of the primary antibody.

Image Analysis

Whole slide digital images were obtained for each specimen using a ScanScope XT automated high-throughput scanning system (Aperio Techonologies Inc, Vista, CA, USA). The resulting high-resolution digital images were analyzed using the Image ScopeTM software (Aperio) using the positive pixel count algorithm (version 9). The positive pixel-count algorithm generates four output values based on the pixel's intensity: haematoxylin or negative signal (blue in mark-up image), weak positive (yellow in mark-up image), positive (orange in mark-up image) and strong positive (brown in mark-up image) (Figure 1a, 1b). The Image Scope™ software contains several parameters that can be adjusted to precisely differentiate positive and background staining. Among all the parameters, we found that positively stained cells could be clearly differentiated from the non-specific and background staining with minor adjustments in the upper limit of intensity for weak-positive pixel or *Iwp(high)*, the lower limit of intensity for weak-positive pixel equals the upper limit of intensity for positive pixel or *Iwp(low)=Ip(high)* and the lower limit of intensity for positive pixel equals the upper limit of intensity for strong-positive pixel or *Ip(low)=Isp(high)*. In most case, the *Iwp(High)* value was set at 230, the *Iwp(Low)=Ip (High)* value between 80 and 140 and the *Ip(Low)=Isp(High)* between 20 and 40. Using these parameters, the strong non-specific (brown) as well as the weak positive (yellow) signals were

considered as debris background staining, whereas the positive value (orange) was considered as specific. To quantify the immune cell infiltrate, the number of positive pixels (orange) was divided by the total number of pixels of the specimens (blue + yellow + orange + brown) thus giving a relative abundance ratio (referred in the text as relative units).

Statistics

The nonparametric Mann-Whitney U test was used to assess statistical significance of differences in immune cell infiltration between control and ADT groups. Correlation coefficients were computed using Spearman's non-parametric test. Univariate analyses were completed using Cox regression with the Enter model. Multi-variate analyses were completed using the Forward Wald model. Statistical tests were performed using the Statistical Package for the Social Sciences (SPSS) version 11 (SPSS Inc., Chicago, IL, USA).

Results

Image analysis using Image Scope™

Our previous publication demonstrated the accuracy of quantifying immune cell populations by immunohistochemistry in formalin-fixed paraffin-embedded tissues using a digital image analysis software (Gannon et al., 2006). In the present study, whole-slide high-resolution images were analyzed with the freely available Image Scope™ software from Aperio. By changing the various parameters of the pixel-count algorithm (see Material and Methods section), we were able to accurately differentiate between specific and non-specific staining (Figure 1a, 1b). Prior to analysis with the Image Scope™ software, a visual assessment of every digital specimen is necessary to manually remove tissue artifacts (corpus amylacea, staining of the surgical margins) and staining debris that interfere with the pixel-count analysis (Figure 1c). Furthermore, during this visual assessment it is important for the observer to quantify crudely the immune cell density (absent, low, intermediate, high) present within the tissue. This visual quantification of immune cell abundance is then compared to the output value from the software's analysis. In a small proportion of tissues, when the immunohistochemical staining produced non-specific background, the visual assessment was essential to fine-tune the software's parameters in order to minimize false-positive results caused by this non-specific staining. As presented in Figure 1, the output values of the software's analyses correlated with the visual assessments of the prostatic inflammation (Figure 1d, 1e, 1f).

Increased density of T lymphocytes and macrophages in ADT patients.

In order to provide a detailed understanding of the density of the inflammatory infiltrate following ADT, we quantified the abundance of innate immune cell populations ($CD56^+$ Natural Killer cells and $CD68^+$ macrophages), adaptive immune cell population ($CD20^+$ B lymphocytes, $CD3^+$ and $CD8^+$ T lymphocytes) and $Foxp3^+$ lymphocytes. Using paraffin-embedded formalin-fixed lymph nodes as positive controls, all antibodies were carefully optimized in order to obtain strong positive signals with minimal background staining (Figure 2).

Unfortunately, we were unable to optimize an anti-CD4 antibody with satisfactory results. Immunohistochemistry using appropriate isotype control antibodies resulted in negative staining (Data not shown).

Our results demonstrate a specific increase in the abundance of T lymphocytes and macrophages in the ADT group. $CD3^+$ T lymphocytes had an average relative density of 0.743 relative units in control patients and 1.391 relative units in ADT patients, a 1.87-fold increase ($p < 0.001$, Mann-U) (Figure 3a). Consequently, the average density of $CD8^+$ T lymphocytes was also increased by ~2.00-fold, from 0.434 relative units in the control group to 0.866 relative units in the ADT group ($p < 0.001$, Mann-U) (Figure 3b). We did not detect statistically significant changes in the average relative abundance of $CD20^+$ B lymphocytes (1.836 relative units vs 2.153 relative units, $p = 0.066$, Mann-U) (Figure 3c) or of $CD56^+$ NK cells (1.554 relative units vs 1.754 relative units, $p = 0.310$, Mann-U) (Figure 3d) in control versus ADT patients, respectively. Patients in the ADT group did however have a 1.78-fold increase in the average relative abundance of $CD68^+$ macrophages compared to control patients (1.066 relative units vs 0.598 relative units, respectively, $p < 0.001$, Mann-U) (Figure 3e). Finally, we evaluated the infiltration of $Foxp3^+$ lymphocytes with regards to ADT. In our cohort, we did not observe significant changes in the average relative density of $Foxp3^+$ cells in control (0.238 relative units) compared to ADT group (0.325 relative units) ($p = 0.196$, Mann-U) (Figure 3f).

Correlations between the relative abundance of immune cell populations

Correlations between the relative abundance of the various immune cell populations were analyzed in both the control group and the ADT group independently (Table 1). In the control group (Table 1, top panel), several significant correlations were observed. Predictably, the infiltration of $CD3^+$ T lymphocytes correlated with the infiltration of $CD8^+$ T lymphocytes (Spearman's Rho = 0.290, $p = 0.015$). A strong $CD3^+$ or $CD8^+$ T lymphocyte infiltration also positively correlated with the $CD68^+$ macrophage infiltration (Spearman's Rho = 0.293, $p = 0.013$; and Spearman's Rho = 0.311, $p = 0.005$, respectively). Interestingly, we noted a positive

correlation between the $CD20^+$ B lymphocyte infiltration and the $CD56^+$ NK cell density (Spearman's Rho = 0.248, p = 0.039).

The hypothesized immunological modulatory properties of ADT were evidenced when looking at the correlations in the ADT group (Table 1, bottom panel). We did not observe the expected correlation between $CD3^+$ and $CD8^+$ T lymphocytes, which highlights a possible dysfunction in immune regulatory pathways. However, we did detect an inverse correlation between the abundance of $CD68^+$ macrophages and $Foxp3^+$ lymphocytes (Spearman's Rho = -0.744, $p < 0.001$).

We also evaluated whether the immune cell infiltrate correlated with clinico-pathological parameters (Table 2). In the control group, we found that $CD3^+$ T lymphocytes positively correlated with extracapsular invasion (Spearman's Rho = 0.397, p = 0.013) and that a high abundance of $CD68^+$ macrophages correlated with positive surgical margins (Spearman's Rho = 0.277, p = 0.044). There was also an inverse correlation between the density of $CD56^+$ NK cells and seminal vesicle invasion (Spearman's Rho = -0.349, p = 0.013). Finally, a high relative abundance of $Foxp3^+$ lymphocytes was associated with elevated pre-operative PSA levels (Spearman's Rho = 0.366, p = 0.009). No correlations were observed within the ADT group (Data not shown).

Immune cell infiltration correlates with biochemical recurrence

We then analyzed whether the relative density of immune cells measured via the image analysis software could predict biochemical recurrence, an indicator of prostate cancer progression (Table 3). Univariate Cox regression analyses revealed that a dense infiltrate of $CD56^+$ NK cells protected control patients from biochemical recurrence (Odd's ratio = 0.213, p = 0.044). On the other hand, control patients with high relative abundance of $CD68^+$ macrophages were at a higher risk of developing biochemical recurrence (Odd's ratio = 4.264, p = 0.011). In the ADT group, we did not identify any immune cell populations which were associated with biochemical recurrence.

Finally, we evaluated the possibility that immune cell density could act as a predictor of biochemical recurrence in a multi-variate model composed of six clinico-

pathological parameters known to predict prostate cancer progression: age at time of surgery, pre-operative PSA levels, Gleason (<7, ≥7), positive surgical margins as well as positive seminal vesicle and lymph node invasion (Supplementary Table 2). To validate the predictive strength of these parameters in our cohort, we performed univariate Cox regression analyses without the immune cell density values. Univariate analyses in the control group revealed that positive surgical margins, seminal vesicle and lymph node invasion were all associated with increased risks of developing biochemical recurrence. Multi-variate analyses combining the six markers showed that, in our cohort, lymph node invasion was the strongest predictor of biochemical recurrence, which can be explain by the relatively high number of lymph node invasion in our cohort. Unfortunately, when the immune cell data was added to the multi-variate model, no immune cell populations were identified as independent predictors of biochemical recurrence (Data not shown). In the ADT group, univariate analyses revealed that pre-operative PSA levels and seminal vesicle invasion were associated with biochemical recurrence. Similar to the control group, our multi-variate model did not identify any immune cell populations as independent predictors of biochemical recurrence (Data not shown).

Discussion

Several studies have associated the degree of immune cell infiltration with prostate cancer progression (Vesalainen et al., 1994; Irani et al., 1999; Sari et al., 1999; Shimura et al., 2000; McArdle et al., 2004; Karja et al., 2005). Increased immune cell infiltrate evaluated by H&E coloration correlates with increased rate of tumor recurrence (Irani et al., 1999) and capsular and perineural invasion (Karja et al., 2005), whereas elevated density of $CD4^+$ T lymphocytes (McArdle et al., 2004) and mast cells (Sari et al., 1999) are associated with poor survival and higher Gleason score, respectively. However, another study did observe that a high TILs density, again evaluated by H&E staining, was protective against disease progression (Vesalainen et al., 1994). Moreover, there are inverse relationships between $CD68^+$ macrophage primary tumor infiltration and disease progression (Shimura et al., 2000). Standardization of the quantification protocol of immune cell abundance is essential in order to better compare independent studies evaluating immune cells within the prostate and establishing conclusions on the pro- or anti-cancer properties of intra-prostatic inflammation.

Together with the fact that several studies reported immune cell density using non-specific H&E staining, a major bias in the previously aforementioned studies was that immune cell abundance was quantified in randomly selected fields. The intra-prostatic inflammation is rather heterogeneous in nature with different immune cell subtypes showing preferential sub-localizations within the tissue. For example, T and B lymphocytes tended to accumulate in inflammatory foci surrounding the glandular epithelium, whereas NK cells and $Foxp3^+$ lymphocytes were found to be distributed throughout the stroma and around the glandular epithelium. To counter this, we utilized a whole-slide scanner which generated high-resolution digital images of the entire tissue. Since it would have been particularly time consuming to manually count positive cells on such large area, the use of an image-analysis software was necessary. A variation of this method was used by Richardsen et al. to quantify infiltration within the prostate (Richardsen et al., 2008), although this analysis was still based on randomly selected fields. We believe that our approach fully eliminates the bias of

randomly selected fields while generating rapid and accurate measurements of immune cell density.

Our results confirmed those obtained by Mercader et al. as to the increased infiltration of T lymphocytes and macrophages in primary tumors of patients treated by ADT. In our small cohort of 40 control patients, we were however unable to demonstrate associations between the density of the lymphocytic infiltration and prostate cancer progression as was previously reported (Vesalainen et al., 1994; Irani et al., 1999; Karja et al., 2005). It is possible that additional associations of statistical significance could have been identified in a larger cohort. However, we did observe an inverse relationship between the density of $CD56^+$ NK cells and the risk of biochemical recurrence as well as an inverse correlation with seminal vesicle invasion. This data support the beneficial roles of NK cells in the anti-tumoral immune response. Conversely, our data suggest, as did that of Shimura et al., that an elevated relative abundance of macrophages could favor prostate cancer progression (Shimura et al., 2000). An important aspect of our data is that we considered the overall inflammation within the primary tumor. All samples contained tumor tissue as confirmed by a pathologist. Again, due to the relatively small size of our cohort, these results should be validated in a larger independent cohort using a similar software-based approach. Future studies could also focused on stratifying the observed differences in immune cell infiltration. Indeed, it would be particularly interesting to quantify stromal inflammation in comparison to inflammation in the proximity of non-malignant or malignant glands, although pathologically this would be challenging in neoadjuvant ADT treated patients.

By taking into consideration the important role of regulatory T cells in the anti-tumoral immune response, our results on Foxp3 expression need to be carefully interpreted. Firstly, our data support published studies describing the presence of $Foxp3^+$ lymphocytes within the prostate (Miller et al., 2006; Fox et al., 2007). Moreover, as it was previously published, $Foxp3^+$ infiltration does seem to not correlate with disease recurrence (Fox et al., 2007; Sfanos et al., 2008). We did however find a novel positive correlation between $Foxp3^+$ lymphocyte density and pre-operative PSA levels. This result raises the interesting question as to whether

regulatory T cells might play a more important role in cancer initiation than tumor progression. Notably, although still recognized as the most specific marker for regulatory T cells, Foxp3 can also be expressed by activated human T lymphocytes (Walker et al., 2003; Morgan et al., 2005; Shevach, 2006). It is thus difficult to draw conclusions on the phenotype of $Foxp3^+$ lymphocytes in an immunohistochemical study. Thus, further detailed studies on the hormonal regulation of $Foxp3^+$ regulatory T cell numbers and functions are warranted.

Finally, although post-ADT inflammation has been observed for several decades, its causes remain elusive. Two main hypotheses are proposed in the literature. On one hand, ADT via anti-LHRH will reduces testosterone levels systemically, which was proposed to increase thymopoiesis and consequently increase the ratio of naïve to memory T lymphocytes (Olsen et al., 1991; Sutherland et al., 2005). On the other hand, increased release of tumor antigen during the ADT-induced apoptotic involution of the prostate combined with an elevated abundance of antigen-presenting cells (macrophages, dendritic cells) could favor the development of a pro-inflammatory microenvironment (Mercader et al., 2001; Haverkamp et al., 2008). Furthermore, there could be immunosuppressive mechanisms expressed by the prostate epithelium cells, which may be dampened following ADT (Drake et al., 2005). Our results, and those of other groups, warrant further studies on the hormonal regulation of the anti-tumoral immune response in prostate cancer. Such studies will shed light on whether androgens (or ADT) modulate the activation potential of specific immune cell subtypes. Moreover, it would be interesting to determine the immunosuppressive potential of prostate cancer cells in an androgen-deprived environment.

In conclusion, this present study details a standardized approach for the rapid and accurate quantification of immune cell density within the primary tumor of prostate cancer patients. We found that ADT increases the relative abundance of $CD3^+$ and $CD8^+$ T lymphocytes as well as $CD68^+$ macrophages. Although not identified as independent predictors of biochemical recurrence in a multi-variate model, we did observe that $CD56^+$ NK cells and $CD68^+$ macrophages infiltration was associated with prostate cancer progression. Our data demonstrate that the relative

density of specific immune cell subtypes is modulated by ADT. Finally, this technique could also be used in prospective studies. Combined with flow cytometry analysis, this technique offers the possibility to visualize the in situ localization of the immune cells, something that cannot be observed during flow cytometry analysis.

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References

Civantos, F., Soloway, M.S. and Pinto, J.E. (1996) Histopathological effects of androgen deprivation in prostatic cancer. Semin Urol Oncol 14, 22-31.

Diallo, J.S., Aldejmah, A., Mouhim, A.F., Fahmy, M.A., Koumakpayi, I.H., Sircar, K., Begin, L.R., Mes-Masson, A.M. and Saad, F. (2008) Co-assessment of cytoplasmic and nuclear androgen receptor location in prostate specimens: potential implications for prostate cancer development and prognosis. BJU Int 101, 1302-9.

Diallo, J.S., Aldejmah, A., Mouhim, A.F., Peant, B., Fahmy, M.A., Koumakpayi, I.H., Sircar, K., Begin, L.R., Mes-Masson, A.M. and Saad, F. (2007) NOXA and PUMA expression add to clinical markers in predicting biochemical recurrence of prostate cancer patients in a survival tree model. Clin Cancer Res 13, 7044-52.

Drake, C.G., Doody, A.D., Mihalyo, M.A., Huang, C.T., Kelleher, E., Ravi, S., Hipkiss, E.L., Flies, D.B., Kennedy, E.P., Long, M., McGary, P.W., Coryell, L., Nelson, W.G., Pardoll, D.M. and Adler, A.J. (2005) Androgen ablation mitigates tolerance to a prostate/prostate cancer-restricted antigen. Cancer Cell 7, 239-49.

Fox, S.B., Launchbury, R., Bates, G.J., Han, C., Shaida, N., Malone, P.R., Harris, A.L. and Banham, A.H. (2007) The number of regulatory T cells in prostate cancer is associated with the androgen receptor and hypoxia-inducible factor (HIF)-2alpha but not HIF-1alpha. Prostate 67, 623-9.

Fradet, V., Lessard, L., Begin, L.R., Karakiewicz, P., Masson, A.M. and Saad, F. (2004) Nuclear factor-kappaB nuclear localization is predictive of biochemical recurrence in patients with positive margin prostate cancer. Clin Cancer Res 10, 8460-4.

Gannon, P.O., Alam Fahmy, M., Begin, L.R., Djoukhadjian, A., Filali-Mouhim, A., Lapointe, R., Mes-Masson, A.M. and Saad, F. (2006) Presence of prostate cancer metastasis correlates with lower lymph node reactivity. Prostate 66, 1710-20.

Grossmann, M.E., Huang, H. and Tindall, D.J. (2001) Androgen receptor signaling in androgen-refractory prostate cancer. J Natl Cancer Inst 93, 1687-97.

Guinan, P., Didomenico, D., Brown, J., Shaw, M., Sharifi, R., Ray, V., Shott, S. and Rubenstein, M. (1997) The effect of androgen deprivation on malignant and benign prostate tissue. Med Oncol 14, 145-52.

Haverkamp, J., Charbonneau, B. and Ratliff, T.L. (2008) Prostate inflammation and its potential impact on prostate cancer: a current review. J Cell Biochem 103, 1344-53.

Irani, J., Goujon, J.M., Ragni, E., Peyrat, L., Hubert, J., Saint, F. and Mottet, N. (1999) High-grade inflammation in prostate cancer as a prognostic factor for biochemical recurrence after radical prostatectomy. Pathologist Multi Center Study Group. Urology 54, 467-72.

Jemal, A., Siegel, R., Ward, E., Hao, Y., Xu, J., Murray, T. and Thun, M.J. (2008) Cancer statistics, 2008. CA Cancer J Clin 58, 71-96.

Karja, V., Aaltomaa, S., Lipponen, P., Isotalo, T., Talja, M. and Mokka, R. (2005) Tumour-infiltrating lymphocytes: A prognostic factor of PSA-free survival in patients with local prostate carcinoma treated by radical prostatectomy. Anticancer Res 25, 4435-8.

Koumakpayi, I.H., Diallo, J.S., Le Page, C., Lessard, L., Filali-Mouhim, A., Begin, L.R., Mes-Masson, A.M. and Saad, F. (2007) Low nuclear ErbB3 predicts biochemical recurrence in patients with prostate cancer. BJU Int 100, 303-9.

Koumakpayi, I.H., Diallo, J.S., Le Page, C., Lessard, L., Gleave, M., Begin, L.R., Mes-Masson, A.M. and Saad, F. (2006) Expression and nuclear localization of ErbB3 in prostate cancer. Clin Cancer Res 12, 2730-7.

Le Page, C., Koumakpayi, I.H., Alam-Fahmy, M., Mes-Masson, A.M. and Saad, F. (2006) Expression and localisation of Akt-1, Akt-2 and Akt-3 correlate with clinical outcome of prostate cancer patients. Br J Cancer 94, 1906-12.

Lessard, L., Mes-Masson, A.M., Lamarre, L., Wall, L., Lattouf, J.B. and Saad, F. (2003) NF-kappa B nuclear localization and its prognostic significance in prostate cancer. BJU Int 91, 417-20.

McArdle, P.A., Canna, K., McMillan, D.C., McNicol, A.M., Campbell, R. and Underwood, M.A. (2004) The relationship between T-lymphocyte subset infiltration and survival in patients with prostate cancer. Br J Cancer 91, 541-3.

Mercader, M., Bodner, B.K., Moser, M.T., Kwon, P.S., Park, E.S., Manecke, R.G., Ellis, T.M., Wojcik, E.M., Yang, D., Flanigan, R.C., Waters, W.B., Kast, W.M. and Kwon, E.D. (2001) T cell infiltration of the prostate induced by androgen withdrawal in patients with prostate cancer. Proc Natl Acad Sci U S A 98, 14565-70.

Mercader, M., Sengupta, S., Bodner, B.K., Manecke, R.G., Cosar, E.F., Moser, M.T., Ballman, K.V., Wojcik, E.M. and Kwon, E.D. (2007) Early effects of pharmacological androgen deprivation in human prostate cancer. BJU Int 99, 60-7.

Miller, A.M., Lundberg, K., Ozenci, V., Banham, A.H., Hellstrom, M., Egevad, L. and Pisa, P. (2006) $CD4^+CD25$high T cells are enriched in the tumor and peripheral blood of prostate cancer patients. J Immunol 177, 7398-405.

Miller, A.M. and Pisa, P. (2007) Tumor escape mechanisms in prostate cancer. Cancer Immunol Immunother 56, 81-7.

Montironi, R. and Schulman, C.C. (1998) Pathological changes in prostate lesions after androgen manipulation. J Clin Pathol 51, 5-12.

Morgan, M.E., van Bilsen, J.H., Bakker, A.M., Heemskerk, B., Schilham, M.W., Hartgers, F.C., Elferink, B.G., van der Zanden, L., de Vries, R.R., Huizinga, T.W., Ottenhoff, T.H. and Toes, R.E. (2005) Expression of FOXP3 mRNA is not confined to $CD4^+CD25^+$ T regulatory cells in humans. Hum Immunol 66, 13-20.

Oefelein, M.G., Ricchiuti, V.S., Conrad, P.W., Goldman, H., Bodner, D., Resnick, M.I. and Seftel, A. (2002) Clinical predictors of androgen-independent prostate cancer and survival in the prostate-specific antigen era. Urology 60, 120-4.

Ohlson, N., Wikstrom, P., Stattin, P. and Bergh, A. (2005) Cell proliferation and apoptosis in prostate tumors and adjacent non-malignant prostate tissue in patients at different time-points after castration treatment. Prostate 62, 307-15.

Olsen, N.J., Watson, M.B., Henderson, G.S. and Kovacs, W.J. (1991) Androgen deprivation induces phenotypic and functional changes in the thymus of adult male mice. Endocrinology 129, 2471-6.

Richardsen, E., Uglehus, R.D., Due, J., Busch, C. and Busund, L.T. (2008) The prognostic impact of M-CSF, CSF-1 receptor, CD68 and CD3 in prostatic carcinoma. Histopathology 53, 30-8.

Sari, A., Serel, T.A., Candir, O., Ozturk, A. and Kosar, A. (1999) Mast cell variations in tumour tissue and with histopathological grading in specimens of prostatic adenocarcinoma. BJU Int 84, 851-3.

Sfanos, K.S., Bruno, T.C., Maris, C.H., Xu, L., Thoburn, C.J., DeMarzo, A.M., Meeker, A.K., Isaacs, W.B. and Drake, C.G. (2008) Phenotypic analysis of prostate-infiltrating lymphocytes reveals TH17 and Treg skewing. Clin Cancer Res 14, 3254-61.

Shevach, E.M. (2006) From vanilla to 28 flavors: multiple varieties of T regulatory cells. Immunity 25, 195-201.

Shimura, S., Yang, G., Ebara, S., Wheeler, T.M., Frolov, A. and Thompson, T.C. (2000) Reduced infiltration of tumor-associated macrophages in human prostate cancer: association with cancer progression. Cancer Res 60, 5857-61.

Sutherland, J.S., Goldberg, G.L., Hammett, M.V., Uldrich, A.P., Berzins, S.P., Heng, T.S., Blazar, B.R., Millar, J.L., Malin, M.A., Chidgey, A.P. and Boyd, R.L. (2005) Activation of thymic regeneration in mice and humans following androgen blockade. J Immunol 175, 2741-53.

Tannock, I.F., de Wit, R., Berry, W.R., Horti, J., Pluzanska, A., Chi, K.N., Oudard, S., Theodore, C., James, N.D., Turesson, I., Rosenthal, M.A. and Eisenberger, M.A. (2004) Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. N Engl J Med 351, 1502-12.

Vesalainen, S., Lipponen, P., Talja, M. and Syrjanen, K. (1994) Histological grade, perineural infiltration, tumour-infiltrating lymphocytes and apoptosis as determinants of long-term prognosis in prostatic adenocarcinoma. Eur J Cancer 30A, 1797-803.

Walker, M.R., Kasprowicz, D.J., Gersuk, V.H., Benard, A., Van Landeghen, M., Buckner, J.H. and Ziegler, S.F. (2003) Induction of FoxP3 and acquisition of T regulatory activity by stimulated human $CD4^{+}CD25$- T cells. J Clin Invest 112, 1437-43.

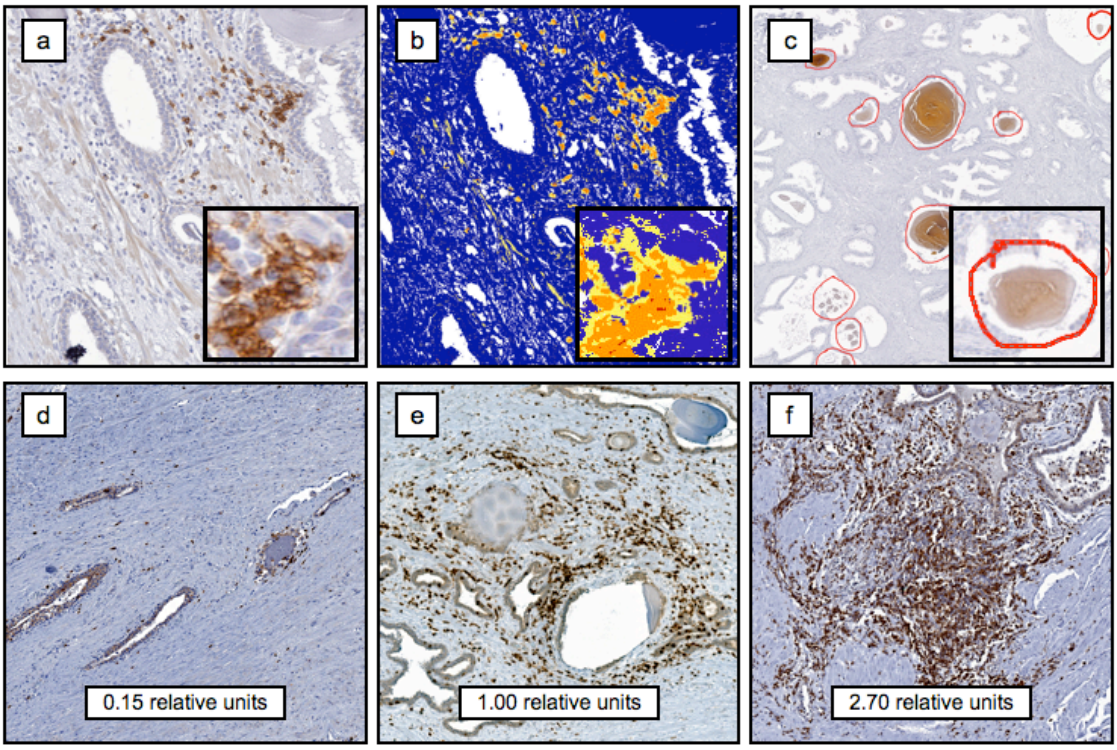

Figure 1. Image analysis with Image Scope and the Pixel-Count Algorithm.

Figure 1. Image analysis with Image Scope and the Pixel-Count Algorithm.

A) Example of a tissue specimen stained with anti-CD20 antibodies and with high non-specific background. B) Pseudo-colored image of panel A illustrating the four output values of the Pixel-Count algorithm: haematoxylin or negative signal (blue), weak positive (yellow), positive (orange) and strong non-specific (brown). C) Representation of areas with corpus amylacea, which were manually removed from the analysis (red lines). Insets are higher magnification images. Representative images of various densities of $CD3^+$ T lymphocytes in primary prostate demonstrating that the image-analysis output matches visual evaluation. D) 0.15 relative units. E) 1.00 relative units. F) 2.70 relative units.

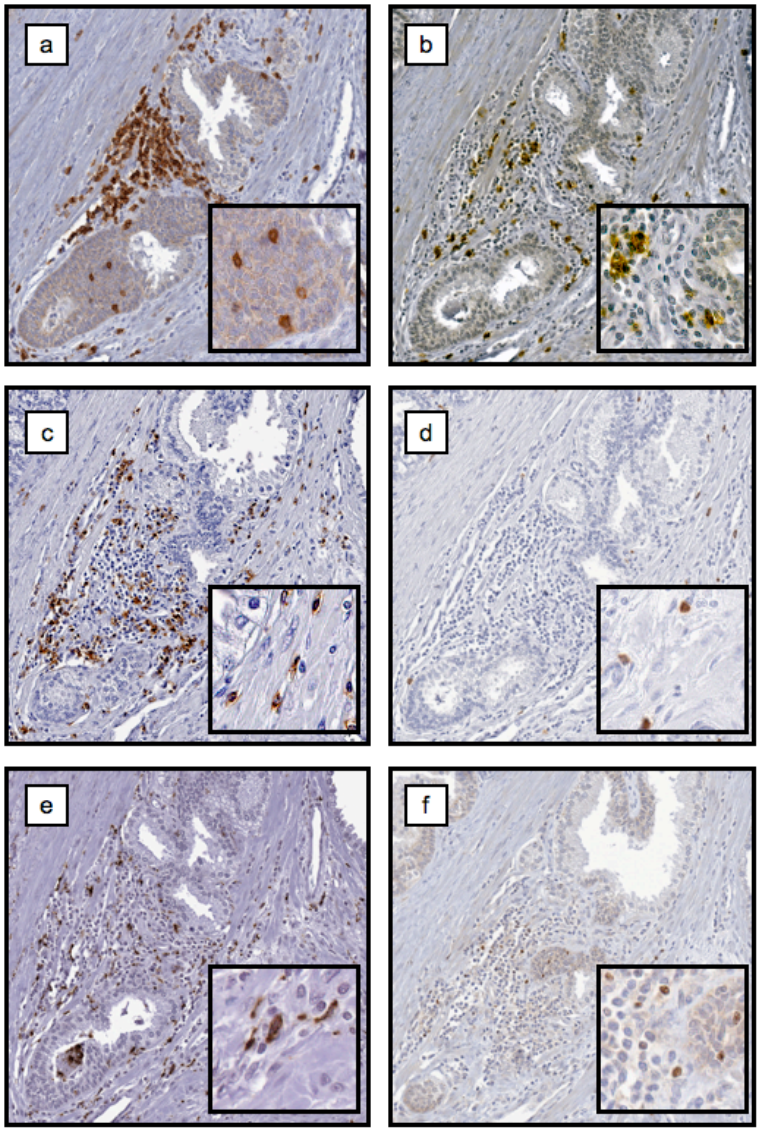

Figure 2. Immunohistochemical staining of immune cells in paraffin-embedded prostate primary tumors.

Figure 2. Immunohistochemical staining of immune cells in paraffin-embedded prostate primary tumors.

A) $CD3^+$ T lymphocytes. B) $CD8^+$ T lymphocytes. C) $CD20^+$ B lymphocytes. D) $CD56^+$ Natural Killer cells. E) $CD68^+$ macrophages. F) $Foxp3^+$ lymphocytes. All images were taken from the same tissue section. Insets are higher magnification images clearly illustrating the positively stained cells in brown. Immunohistochemisty with appropriate isotype control antibodies were negative.

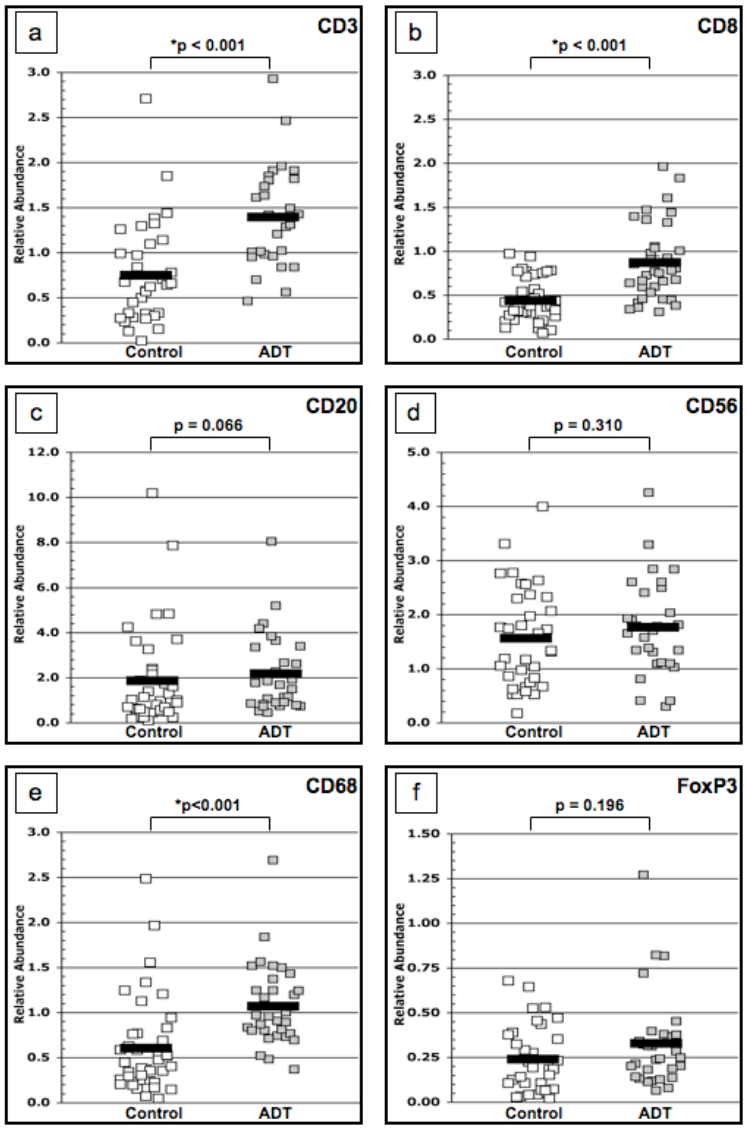

Figure 3. Increased abundance of immune cells in ADT patients.

Figure 3. Increased abundance of immune cells in ADT patients.

Control group is represented in white and the ADT group in gray with the average for each group as the black line. Each square represents the relative abundance for one patient. A) $CD3^+$ T lymphocytes (0.743 units vs 1.391, $p < 0.001$, Mann-U). B) $CD8^+$ T lymphocytes (0.434 units vs 0.866, $p < 0.001$, Mann-U). C) $CD20^+$ B lymphocytes (1.836 units vs 2.153, $p = 0.066$, Mann-U). D) $CD56^+$ Natural Killer cells (1.554 units vs 1.754, $p = 0.310$, Mann-U). E) $CD68^+$ macrophages (0.598 units vs 1.066, $p < 0.001$, Mann-U). F) $Foxp3^+$ lymphocytes (0.261 units vs 0.384, $p = 0.1963$, Mann-U).

Correlations‡ between immune cell populations

Top panel: Control Group							
		CD3	**CD8**	**CD20**	CD56	CD68	FoxP3
CD3	Correlation	1.000	0.290	0.159	-0.100	0.293	0.198
	P value	.	0.015	0.193	0.411	0.013	0.187
CD8	**Correlation**	**0.290**	1.000	0.175	0.057	0.311	0.262
	P value	**0.015**	.	0.122	0.624	0.005	0.055
CD20	Correlation	0.159	0.175	1.000	0.248	0.092	0.147
	P value	0.193	0.122	.	0.039	0.414	0.304
CD56	**Correlation**	-0.100	0.057	**0.248**	1.000	0.159	0.007
	P value	0.411	0.624	**0.039**	.	0.173	0.963
CD68	**Correlation**	**0.293**	**0.311**	0.092	0.159	1.000	0.157
	P value	**0.013**	**0.005**	0.414	0.173	.	0.252
FoxP3	Correlation	0.198	0.262	0.147	0.007	0.157	1.000
	P value	0.187	0.055	0.304	0.963	0.252	.

‡ Spearman's non-parametric correlations

Bottom panel: ADT Group							
		CD3	CD8	CD20	CD56	**CD68**	FoxP3
CD3	Correlation	1.000	0.109	0.047	0.033	-0.040	0.031
	P value	.	0.409	0.747	0.823	0.767	0.890
CD8	Correlation	0.109	1.000	0.090	0.121	0.186	-0.231
	P value	0.409	.	0.487	0.358	0.129	0.250
CD20	Correlation	0.047	0.090	1.000	-0.017	0.027	-0.018
	P value	0.747	0.487	.	0.907	0.843	0.938
CD56	Correlation	0.033	0.121	-0.017	1.000	0.011	-0.127
	P value	0.823	0.358	0.907	.	0.934	0.586
CD68	Correlation	-0.040	0.186	0.027	0.011	1.000	-0.744
	P value	0.767	0.129	0.843	0.934	.	0.000
FoxP3	**Correlation**	0.031	-0.231	-0.018	-0.127	**-0.744**	1.000
	P value	0.890	0.250	0.938	0.586	**< 0.001**	.

‡ Spearman's non-parametric correlations

Table 1. Correlations between immune cell populations

Correlations‡ between immune cell populations and clinical markers

Control Group					
		CD3	CD56	CD68	FoxP3
Extracapsular invasion	Correlation	0.397	.	.	.
	P value	0.013	.	.	.
Seminal vesicle invasion	Correlation	.	-0.349	.	.
	P value	.	0.013	.	.
Positive surgical margins	Correlation	.	.	0.277	.
	P value	.	.	0.044	.
Pre-operative PSA	Correlation	.	.	.	0.366
	P value	.	.	.	0.009

‡ Spearman's non-parametric correlations

* Only statistically significant correlations are shown.

** No statistically significant correlations were noted in the ADT group.

Table 2. Correlations between immune cell populations and clinical markers

Univariate Cox regression analyses† of biochemical recurrence

Top panel: Control Group						
	B	Wald	P Value	Exp(B)	95% CI of Exp(B) Lower	Upper
CD3	0.562	1.182	0.277	1.754	0.637	4.831
CD8	-1.132	0.490	0.484	0.322	0.014	7.681
CD20	0.058	0.162	0.687	1.059	0.800	1.402
CD56	**-1.545**	**4.053**	**0.044**	**0.213**	**0.047**	**0.960**
CD68	**1.450**	**6.441**	**0.011**	**4.264**	**1.391**	**13.068**
FoxP3	0.321	1.713	0.191	1.379	0.852	2.231

† Statistical analysis using continuous variables

Bottom panel: ADT Group						
	B	Wald	P Value	Exp(B)	95% CI of Exp(B) Lower	Upper
CD3	-0.079	0.020	0.888	0.924	0.310	2.758
CD8	0.575	1.228	0.268	1.778	0.643	4.919
CD20	-0.188	0.858	0.354	0.829	0.557	1.233
CD56	-0.163	0.307	0.579	0.849	0.477	1.513
CD68	0.258	0.184	0.668	1.294	0.398	4.206
FoxP3	-0.267	1.584	0.208	0.766	0.506	1.160

† Statistical analysis using continuous variables

Table 3. Univariate Cox regression analyses of biochemical recurrence

Clinico-pathological characteristics of prostate cancer patients

		Control	ADT
# patients		40	35
Age at surgery (± s.d.)		63.4 ± 4.7	62.1 ± 5.1
Pre-op PSA (ng/ml ± s.d.)		11.4 ± 9.2	12.4 ± 11.2
Pre-op ADT	*Cyproterone*	- -	21 (60.0%)
	Leuprolide + Flutamide	- -	9 (25.7%)
	Other		5 (14.3%)
Gleason	*< 7*	19 (47.5%)	17 (48.6%)
	= 7	14 (35.0%)	12 (34.3%)
	> 7	4 (10.0%)	2 (5.7%)
	?	3 (7.5%)	4 (11.4%)
+ Surgical margins		12 (30.0%)	2 (5.7%)
Seminal Vesical Invasion		8 (30.0%)	4 (11.4%)
Lymph Node Invasion		16 (40.0%)	6 (17.1%)
Biochemical Relapse		8 (20.0%)	18 (51.4%)
Time to Relapse (mths ± s.d.)		19.4 ± 8.1	22.4 ± 17.2
Death		6 (15.0%)	3 (8.6%)

Supplementary Table 1. Clinico-pathological characteristics of prostate cancer patients

Univariate and Multi-variate Cox regression analyses of biochemical recurrence

Top panel: Control Group						
					95% CI of Exp(B)	
	B	Wald	P Value	Exp(B)	Lower	Upper
Age	0.033	0.177	0.674	1.033	0.887	1.203
Pre-operative PSA	0.032	0.939	0.332	1.033	0.967	1.103
Gleason[‡,†]	-1.017	1.478	0.224	0.362	0.070	1.864
Positive surgical margins[‡]	**-2.022**	**5.766**	**0.016**	**0.132**	**0.025**	**0.690**
Seminal vesicle invasion[‡]	**-1.679**	**5.560**	**0.018**	**0.187**	**0.046**	**0.753**
Lymph node invasion[‡]	**-2.737**	**6.510**	**0.011**	**0.065**	**0.008**	**0.530**
Multi-variate analysis[§] with all six clinico-pathological parameters						
					95% CI of Exp(B)	
	B	Wald	P Value	Exp(B)	Lower	Upper
Lymph node invasion[‡]	**-2.544**	**5.501**	**0.019**	**0.079**	**0.009**	**0.658**

‡ Denotes variables analyzed as categorical data

† Gleason Score was categorized as <7 and ≥ 7

§ Multi-variate Cox regression analyses using the Forward Wald model

Bottom panel: ADT Group						
					95% CI of Exp(B)	
	B	Wald	P Value	Exp(B)	Lower	Upper
Age	0.012	0.068	0.794	1.012	0.926	1.106
Pre-Operative PSA	**0.065**	**6.833**	**0.009**	**1.067**	**1.016**	**1.120**
Gleason[‡,†]	-0.822	2.715	0.099	0.440	0.165	1.169
Positive surgical margins	3.195	0.787	0.375	24.400	0.021	28395.0
Seminal vesicle invasion	**-1.561**	**4.967**	**0.026**	**0.210**	**0.053**	**0.828**
Lymph node invasion	-1.003	3.571	0.059	0.367	0.130	1.038
Multi-variate analysis[§] with all six clinico-pathological parameters						
					95% CI of Exp(B)	
	B	Wald	P Value	Exp(B)	Lower	Upper
Pre-Op PSA	**0.099**	**6.849**	**0.009**	**1.104**	**1.025**	**1.189**

‡ Denotes variables analyzed as categorical data

† Gleason Score was categorized as <7 and ≥ 7

§ Multi-variate Cox regression analyses using the Forward Wald model

Supplementary Table 2. Univariate and Multi-variate Cox regression analyses of biochemical recurrence

CHAPTER V

ANDROGEN-REGULATED EXPRESSION OF ARGINASE 1, ARGINASE 2 AND INTERLEUKIN-8 IN HUMAN PROSTATE CANCER

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Author contributions:

For this paper, I did the majority of the experiments and wrote the manuscript in its entirety, although all co-authors played a role in the editing process. Jessica Godin-Ethier participated in the experimental design. Nathalie Delvoye did real-time PCR analyses. Meghan Aversa and Alexis Poisson optimized and scored immunohistochemical stainings. Benjamin Péant did ELISAs and Mona Alam Fahmy designed and constructed the tissue micro-arrays.

Keywords: Arginase; androgen; LNCaP cells; Interleukin-8

Abstract

Background: Prostate cancer (PCa) is the most frequently diagnosed cancer in North American men. Androgen-deprivation therapy (ADT) accentuates the infiltration of immune cells within the prostate. However, the immunosuppressive pathways regulated by androgens in PCa are not well characterized. Arginase 2 (ARG2) expression by PCa cells leads to a reduced activation of tumor-specific T cells. Our hypothesis was that androgens could regulate the expression of ARG2 by PCa cells.

Methodology/Principal Findings: In this report, we demonstrate that both ARG1 and ARG2 are expressed by hormone-sensitive (HS) and hormone-refractory (HR) PCa cell lines, with the LNCaP cells having the highest arginase activity. In prostate tissue samples, ARG2 was more expressed in normal and non-malignant prostatic tissues compared to tumor tissues. Following androgen stimulation of LNCaP cells with 10 nM R1881, both ARG1 and ARG2 were overexpressed. The regulation of arginase expression following androgen stimulation was dependent on the androgen receptor (AR), as a siRNA treatment targeting the AR inhibited both ARG1 and ARG2 overexpression. This observation was correlated in vivo in patients by immunohistochemistry. Patients treated by ADT prior to surgery had lower ARG2 expression in both non-malignant and malignant tissues. Furthermore, ARG1 and ARG2 were enzymatically active and their decreased expression by siRNA resulted in reduced overall arginase activity and L-arginine metabolism. The decreased ARG1 and ARG2 expression also translated to diminished LNCaP cells cell growth and increased PBMC activation following exposure to LNCaP cells conditioned media. Finally, we found that interleukin-8 (IL-8) was also upregulated following androgen stimulation and that it directly increased the expression of ARG1 and ARG2 in the absence of androgens.

Conclusion/Significance: Our data provides the first detailed in vitro and in vivo account of an androgen-regulated immunosuppressive pathway in human PCa through the expression of ARG1, ARG2 and IL-8.

Introduction

Prostate cancer (PCa) is the most frequently diagnosed cancer and third leading cause of cancer related deaths for North American men [1]. The prostate's organogenesis and carcinogenesis rely on the presence of androgens [2]. As such, the most common treatment modality for men with an advanced stage or recurrent PCa is androgen-deprivation therapy (ADT). ADT leads to the apoptosis of hormone sensitive prostate epithelial cells [3]. Unfortunately, within one to five years following ADT initiation, most patients develop hormone refractory PCa (HRPC), whose treatment remains palliative [4]. New treatment modalities, such as immunotherapy, attempt to tackle these later stages of PCa. However, current immunotherapies against PCa have resulted in limited success in the clinical settings. A detailed understanding of the tumor immunological microenvironment should provide new insights on how to improve current immune-based protocols.

Recent data demonstrate that various immunosuppressive mechanisms are present within the prostate and may hamper the anti-tumoral immune response in the context of an immunotherapy (reviewed in [5]). Arginase 2 (ARG2) is expressed in human PCa [6] and its inhibition, concomitant with iNOS, increases the activation of tumor-infiltrating lymphocytes (TILs) [7]. While the immunosuppressive properties of arginases through the metabolism of L-arginine are well documented (reviewed in [8]), the regulation of human arginase expression, however, is currently undefined.

Androgens are known to have immunosuppressive properties, which is illustrated by the intra-prostatic inflammation following androgen deprivation therapy [9,10]. Gene expression analyses and murine studies suggest that androgens regulate the expression of ARG2 and other enzymes of the polyamine pathway [11,12,13]. Thus, considering the fundamental roles of androgens in prostate carcinogenesis and in the sculpting of the prostate's microenvironment, we evaluated whether androgens could regulate the expression of arginases by PCa cells in vitro and in vivo.

In this study, we report that PCa cell lines express both functionally active ARG1 and ARG2. Interestingly, hormone sensitive (HS) and hormone refractory (HR) tissues expressed less ARG2 than non-malignant tissues. In the HS LNCaP cell

line, androgen stimulation led to the increased expression of both ARG1 and ARG2 in an androgen receptor (AR) dependant manner. This androgen-regulated expression was also observed in the primary tumor of ADT-treated patients who expressed less ARG2 in both the non-malignant tissues adjacent to the tumor and the tumor tissues. Finally, we discovered that IL-8 was also regulated by androgens under the control of the AR, and participated in the regulation of ARG2 expression. Altogether, our data provides the first detailed account *in vitro* and *in vivo* of an androgen-regulated immunosuppressive pathway in human PCa.

Materials and methods

Cell Culture

LNCaP, 22Rv1, DU145 and PC3 cell lines were obtained from ATCC (MD, USA). All cell lines were maintained as previously described by our group [30]. For R1881 stimulation, cells were plated at 600,000 cells per 60 mm petri dish and incubated for an initial 72 hours in 10% (v/v) charcoal-stripped fetal calf serum (FCS)-supplemented RPMI 1640, which eliminates all steroid hormones from the serum. Afterwards, the cells were washed with PBS and cultured in fresh 10% charcoal-stripped FCS-supplemented RPMI 1640, with either 10 nM R1881 or ethanol (control) [30]. Conditioned media, protein and RNA were extracted at 0, 24, 48 or 72 hours following the R1881 stimulation. For IL-8 stimulation, LNCaP cells were plated in charcoal-stripped serum supplemented media for 72 hours followed by 24 hours in serum-free RPMI. Cells were then stimulated for 72 hours with either 10 nM R1881 or with 100 ng/ml IL-8 (PeproTech, Rocky Hill, NJ) in serum-free RPMI. siRNA targeting the AR, ARG1, ARG2 and IL-8 as well as the RISC-free siGLO fluorescent siRNA control were purchased from Dharmacon (Chicago, IL). When LNCaP cells reached 80% confluence in a 100 mm petri dish they were transfected as recommended by the manufacturer using the Dharmafect 2 transfection reagent. Cells were incubated for 24 hrs after which they were seeded as described for the R1881 stimulation.

Antibodies

The following antibodies were purchased from Santa Cruz (Santa Cruz, CA): anti-ARG1 (BC9, sc-47715), anti-ARG2 (L-20, sc18357), anti-PSA (C-19, sc-7638), anti-RAN (C-20, sc-1146). The anti-AR (Ab-1) was purchased from LabVision/NeoMarkers (Fermont, CA).

Gene and Protein Expression

Quantitative real-time PCR (qPCR) analyses were performed as previously described by our group [31]. RAN served as the housekeeping gene as we found that its expression was not sensitive to R1881 stimulation. Relative mRNA of candidate gene/RAN ratios were calculated using the method described by Pfaffl et al. [32]. Fold change was calculated relative to the mock treated control. Western blotting of proteins extracted in non-denaturing buffer was performed as previously described by our group [33].

Arginase Activity

Arginase activity was quantified as previously described [34]. Briefly, a solution of 10 mM $MnCl_2$ / 50 mM Tris / HCl at pH 7.5 was added to whole cell extracts. Following an incubation at 55 °C for 60 mins, 25 µl of 0.5 M arginine pH 9.7 was added to the samples and further incubated for 60 mins at 37 °C. The arginine hydrolysis reaction was stopped by adding $H_2SO_4/H_3PO_4/H_2O$ at a ratio of (1:3:7, v/v/v). The samples were then boiled at 100 °C for 15 mins following the addition of 9% ISPF and read at 540 nm. Using a standard curve, arginase activity was reported as mUnits / mg of protein.

Immunohistochemistry on PCa TMAs

Four different tissue microarrays (TMAs) were used in this study. The first TMA contained 50 normal prostate specimens obtained from 39 autopsied patients without PCa. The second TMA contained non-malignant tissue adjacent to tumor (n=55), prostate intra-epithelial neoplasic (PIN) tissue (n=32) and HS tumor tissue (n=63) from 63 patients who had undergone radical prostatectomy [35]. The third TMA contained HR tumor tissues obtained by trans-urethral resection of the prostate (TURP) from 36 patients collected subsequent to hormone therapy failure [36,37]. Finally, the fourth TMA contained prostate specimens obtained from 35 patients who were treated by ADT prior to radical prostatectomy (ADT group) and 40 Gleason-matched control patients who were only treated by radical prostatectomy, as previously described [10]. For each patient, a total of four tumor cores and two normal adjacent cores were spotted on duplicate TMAs. Cell pellets of each PCa cell

line (RWPE, LNCaP, 22Rv1, DU145 and PC3) were spotted on each array and served as internal staining controls. Ethics approval for this study was obtained from the local ethics review committee.

Immunohistochemical staining was done as previously described by our group [37,38,39]. Briefly, the 90 min primary antibody incubation was followed by 30 min incubation with an anti-mouse HRP-coupled secondary antibody (sc-2005, Santa Cruz). Positive signals were developed with diaminobenzidine (DAB) (Dako Cytomation, Mississauga, On, Canada) and the nuclei were counterstained with haematoxylin. High-resolution digital images of each TMA were generated using a whole-slide scanner (SanScope XT automated high-throughput scanning system) from Aperio (Vista, CA). Two independent observers evaluated the intensity (0, 1+, 2+, 3+) and the percentage of positively stained cells. For each core a value corresponding to the intensity multiplied by the percentage of stained cells was calculated and reported for statistical analysis.

Quantification of L-Arginine concentration by HPLC.

Perchloric acid (150 µl) was added to conditioned media (150 µl), which was then vortexed and shook for 10 min. The samples were then centrifuged (13,000 rpm) for 20 min and 240 µl of supernatant were transferred into an amber eppendorff tube. This solution containing the L-arginine was thus essentially cleared of cellular proteins [40]. The supernatant was then neutralized with 60 µl of 3 M NaOH and buffered to pH 9.0 using 180 µl of borate buffer. To this solution, 10 µl of 0.1 M NaCN and Naphthalene-2,3-dicarboxaldehyde (NDA) were added and shaken for 20 min before injection into the HPLC. All samples were run on a Varian Pursuit C18 column 250 x 4.6 mm with the following three solvents: Solvent A: 100 mM triethylammonium acetate (TEAA) buffered to pH 7.0 with 5% acetonitrile (ACN) in milli-Q water; Solvent B: 60% ACN in Solvent A; Solvent C: 100% ACN. A series of L-arginine standards were made ranging from 0 to 2.58 x 10-4 g/ml. Each standard was done in triplicate and was functionalized with NDA to determine the retention time of L-arginine and the area under the peak corresponding to L-arginine at specific concentrations. Samples were monitored at 260 nM and 420 nM to identify which

samples had been functionalized with the NDA. Peaks that appear at 420 nM correspond to substances that have a primary amine available to react.

Lymphocyte activation

PBMCs from healthy donors were isolated from whole blood by Ficoll gradient using lymphocyte-separating medium (Wisent, St-Bruno, Qc, Canada). PBMCs (150,000) were incubated in a 96-well flat-bottomed plate with 1 μg/ml of anti-CD3 (OKT3, eBioscience, San Diego, CA) or an isotype control. Supernatants were harvested for cytokine quantification by enzyme-linked immunosorbent assay (ELISA). For proliferation assays, bromodeoxyuridine (BrDU) was added in the last 12 hrs according to the manufacturer's instruction.

ELISA

The ELISA kit for IL-8 was purchased from R&D Systems (Minneapolis, MN) and the cell proliferation BrDU ELISA kit from Roche (Mississauga, ON, Canada). ELISAs were done according to the manufacturer's instruction. The IFN-γ ELISA was completed as previously described [41].

Statistics

Statistical analysis was performed using SPSS software 11.0 (SPSS Inc., Chicago, Il). The non-parametric Mann-Whitney U test was used to show statistically significant differences.

Results

ARG1 and ARG2 expression in PCa

Our data demonstrate that PCa cell lines express both ARG1 and ARG2. Gene expression analyses by qPCR demonstrated that *ARG1* mRNA was more expressed by the 22Rv1 cell line (Figure 1a). ARG1 protein was slightly more expressed by the two HR PCa cell lines (Du145 and PC3) than in the LNCaP cells (Figure 1b). ARG1 protein expression did not correlate with the gene expression analysis results suggesting possible post-transcriptional regulation prior to ARG1 protein expression. As for ARG2 expression, the LNCaP cell line expressed the highest levels of *ARG2* mRNA (Figure 1a). Minimal expression of *ARG2* mRNA was detected in the two HR cell lines DU145 and PC3. ARG2 protein expression correlated with the gene expression results with LNCaP cells expressing significantly more ARG2 than the other three cell lines (Figure 1b). Furthermore, LNCaP cells had the highest arginase activity suggesting that ARG2 is the predominant enzyme with regards to arginase activity of PCA cells (Figure 1c).

Expression of the ARG2 protein was evaluated in clinical samples by immunohistochemistry on three different TMAs regrouping prostate samples from a cohort of 99 PCa patients and 50 normal prostate obtained from autopsies. We did not evaluate ARG1 protein expression as, in our hands, anti-ARG1 antibodies tested were not suitable for immunohistochemistry on archived formalin-fixed paraffin-embedded tissues. We observed that ARG2 expression was restricted to the prostate epithelium and absent from the stroma (Figure 1d). ARG2 was statistically significantly less expressed in tumor tissues compared to normal ($p<0.001$, Mann-U), to non-malignant normal adjacent ($p<0.01$, Mann-U) and to PIN tissues ($p<0.001$, Mann-U) (Figure 1e). HR tissues also expressed less ARG2, although only significantly different from PIN tissues ($p=0.033$, Mann-U). There was no correlation between ARG2 expression within the normal adjacent and tumor tissues (Supplementary Table 1). Finally, we evaluated if the ARG2 expression correlated with clinico-pathological parameters such as Gleason Score, pre-operative PSA and biochemical recurrence. Our results show that ARG2 expression within the normal

adjacent tissue inversely correlated with vesicle seminal invasion (Supplementary Table 1). Altogether, these in vitro and in vivo data demonstrate the differential expression of ARG1 and ARG2 between various stages of PCa progression.

Androgen-regulated expression of ARG1 and ARG2

The differential expression of ARG1 and ARG2 between the HS and HR PCa cell lines led us to investigate the regulatory roles of androgens in arginase expression. *ARG1* mRNA expression was not statistically significantly upregulated in either LNCaP or 22RV1 cell lines following R1881 stimulation (Figure 2a). However, in LNCaP cells, *ARG2* mRNA expression was increased at 48 hours ($p=0.002$, Mann-U) and at 72 hours ($p=0.016$, Mann-U) following the R1881 stimulation (Left panel, Figure 2b). The overexpression of ARG2 in 22RV1 was not statistically significant ($p=0.248$, Mann-U) (Right panel, Figure 2b). In fact, ARG2 expression correlated with the higher androgen sensibility of LNCaP cells compared to 22RV1 (Data not shown but available to reviewer in Supplementary Figure 1a). As such, LNCaP cells were used for further experiments. Corroborating the PCR data, Western blots from LNCaP cells demonstrated that the R1881 stimulation increased ARG2 protein expression (Figure 2d). Interestingly, although no significant changes were observed in ARG1 mRNA expression in LNCaP cells treated with R1881, ARG1 protein expression was significantly increased. We did not any increases in ARG1 or ARG2 protein expression in DU145 and PC3 stimulated with 10 nM of R1881 (Data not shown but available to reviewer in Supplementary Figure 1b).

Implication of the AR in ARG1 and ARG2 expression

As our results suggest that androgens regulate arginase expression, we evaluated the contribution of the AR. We inhibited AR activity with the non-steroidal anti-androgen bicalutamide (Casodex) (Figure 2d). We noted a decreased expression of ARG1 with the highest concentration (40 μM) of bicalutamide following R1881 stimulation. The androgen induction of ARG2 was not blocked, even at the highest concentration of bicalutamide. As previously documented [14], we observed that bicalutamide had AR-agonist activity in LNCaP cells cultured in the absence of

androgens. There was an R1881-independant induction of PSA and ARG2 expression in LNCaP cells stimulated with 20 μM and 40 μM of bicalutamide in the absence of androgens. In this same condition, bicalutamide caused a decreased in ARG1 expression. These results suggest that ARG1 expression may be more sensitive to AR inhibition than ARG2, whose expression was induced by the agnostic effect of the AR inhibitor.

We decided to further inhibit the AR by blocking the AR expression in LNCaP cells using siRNA. The presence of siRNA against the AR resulted in a significant inhibition of AR expression and in a reduced PSA expression following R1881 stimulation (Figure 2e). Both the ARG1 and ARG2 induction following R1881 stimulation were inhibited by the siRNA treatment, which translated in the absence of an upregulation in arginase activity (Figure 2f). These results suggest that the AR regulates the expression of ARG1 and ARG2 although differentially.

Diminished ARG2 expression in PCa patients following ADT

Based on our in vitro data, we hypothesized that androgens might modulate ARG2 protein expression in PCa patients as well. We observed that, compared to control patients (surgery only), ADT-treated patients (ADT prior to surgery) had significantly lower ARG2 expression in both the non-malignant tissues adjacent to the tumor (46.4 vs 23.5 relative units; $p<0.001$, Mann-U) and the tumor tissues (41.7 vs 31.5 relative units; $p<0.01$, Mann-U) (Figure 3a). We also observed that androgen deprivation in vitro could decrease ARG2, but not ARG1 protein expression, in LNCaP and 22RV1 cells cultured for seven days in the absence of androgens (Figure 3b). Taken together, these results suggest that androgens regulate the expression of ARG2 in vivo in PCa patients as ADT reduces ARG2 expression.

ARG1 and ARG2 are metabolically active

To evaluate whether ARG1 and ARG2 expressed by LNCaP cells were metabolically active, we inhibited the expression of either ARG1 or ARG2 by siRNA. Compared to a siCTRL, both siRNA significantly inhibited ARG1 or ARG2 expression (Figure 4a). Inhibition of either ARG1 or ARG2 resulted in diminished

arginase enzymatic activity (Figure 4b). By HPLC, we then determined the impact of the inhibition of ARG1 and ARG2 expression on the metabolism of L-arginine by LNCaP cells. The absence of either ARG1 or ARG2 led to higher concentrations of L-arginine in the conditioned media suggesting a lower metabolism of L-arginine by LNCaP cells (Figure 4c). Moreover, we noted that R1881 stimulation led to a decrease concentration of extracellular L-arginine, which corroborates our results demonstrating an increased arginase expression following androgen stimulation. The expression of nitric oxide synthase (NOS), also known to metabolize L-arginine was also evaluated. We did not observe the expression of iNOS or nNOS, as well as no production of NO in our model (Data not shown).

As arginases are implicated in the polyamine synthesis pathway necessary for cellular proliferation, we evaluated the impact of ARG1 and ARG2 on cell growth. We observed that inhibition of either ARG1 or ARG2 expression resulted in a lower proliferation of LNCaP cells maintained in complete media ($p=0.02$ and $p=0.01$, respectively for siARG1 and siARG2) (Figure 4d). Furthermore, in order to study whether ARG1 and ARG2 expression by LNCaP cells affected their immunosuppressive potential, PBMCs from healthy donors were activated in the presence of conditioned media from LNCaP+siCTRL or LNCaP+siARG1 or LNCaP+siARG2. The inhibition of either ARG1 or ARG2 translated into increased PBMC proliferation as quantified by BrdU incorporation (Figure 4e, left panel). This increased proliferation was associated with an increase in IFN-γ secretion by PBMCs as measured by ELISA (Figure 4e, right panel). No significant variations in the secretion of IL-2 or IL-10 were observed (Data not shown). Finally, we correlated whether the ARG2 expression correlated with the immune cell infiltrate of the primary tumor that we recently published [10]. We noted that ARG2 expression did inversely correlate with the infiltration of T lymphocytes and macrophages within the prostate (Supplementary Table 2). Collectively, these results suggest that ARG1 and ARG2 expressed by LNCaP cells are enzymatically active and participate in important physiological processes such as cellular proliferation and tumor-derived immunosuppression.

Cytokine-induced ARG2 expression

As cytokines are known to induce arginase expression in murine models, we assessed whether this could also occur in human PCa. The cytokine expression profile of LNCaP cells stimulated with 10 nM of R1881 was evaluated using a Proteome Profiler (R&D Systems) cytokine array (Figure 5a). The proteomic data illustrated that the R1881-stimulated LNCaP cells had increased expression of IL-8 and Serpin E1 (Densitometry as data not shown, but available to reviewers in Supplementary Figure 2a). We further investigated the role of IL-8 in arginase expression as IL-8 has been recently linked to the expression of androgen-regulated genes in PCa [15]. By ELISA, we confirmed that R1881 stimulation increased the expression of IL-8 in LNCaP cells (Figure 5b). This IL-8 induction was dependent on the AR. AR expression by siRNA prevented IL-8 secretion following androgen stimulation (Figure 5c). We then evaluated whether inhibition of IL-8 could diminish ARG1 and ARG2 expression following R1881 stimulation. Using a siRNA against IL-8, we could significantly diminish IL-8 secretion (Figure 5d). This reduced IL-8 production was associated with a reduction of ARG1 and ARG2 without and with R1881 stimulation (Figure 5e). The treatment of LNCaP cells with siIL-8 also translated to a decrease in arginase activity (Data not shown, but available to reviewers in Supplementary Figure 2b). Finally, we stimulated androgen-deprived LNCaP cells with increasing concentration of exogenous IL-8 for 72 hrs and monitored the expression of ARG1 and ARG2. By Western blot analysis, we observed that both 50 ng/ml and 100 ng/ml of IL-8 induced the expression of ARG1 and ARG2 when compared to control LNCaP cells (Figure 5f). The decrease in ARG1 and ARG2 protein expression with 250 ng/ml of IL-8 correlated with IL-8 induced cellular toxicity. We also observed an induction of ARG2, but not ARG1, gene expression after a 24 hr stimulation (Data not shown, but available to reviewers in Supplementary Figure 2c). Taken together, the data clearly shows that androgens regulate the expression IL-8, which on its own can induce the expression of both ARG1 and ARG2.

Discussion

A more thorough understanding of the prostate immunological microenvironment mechanisms may improve the clinical efficacy of current immunotherapies against PCa. We and others have shown that ADT leads to drastic changes in the prostate immunological microenvironment [9,10]. The arginase pathway participates in the development of an immunosuppressive state within the primary tumor of PCa patients [7]. However, the regulation of arginase expression by PCa cells remains undefined.

In this report, we observed that androgens induced the expression of both ARG1 and ARG2 in HS PCa cell lines. The AR was implicated in this regulation as both bicalutamide and siAR transfection prevented ARG1 and ARG2 overexpression following R1881 stimulation. Reciprocally, androgen deprivation and ADT reduced ARG2 expression *in vitro* and in the primary tumor of PCa patients, respectively. LNCaP cells expressed enzymatically functional ARG1 and ARG2 which, once their protein expression was inhibited, caused a decrease in cellular proliferation and in their immunosuppressive potential. Finally, we showed that IL-8 was also regulated by R1881 and could stimulate the expression of ARG1 and ARG2 independently of androgen. Altogether, our results provide the first mechanistic evidence of an androgen-driven immunosuppressive pathway in PCa through the expression of ARG1, ARG2 and IL-8 by PCa cells.

We demonstrate that PCa cells express both ARG1 and ARG2. ARG2 was predominantly expressed by HS PCa cell lines and by non-malignant prostate tissues. These results corroborate published data describing a lower ARG2 expression in androgen-insensitive PCa cell lines (DU145 and PC3) and in the tumor and HR tissues of PCa patients [6,16]. However, to our knowledge, we are the first group to study the expression of ARG1 by PCa cells. Similar to ARG2, inhibition of ARG1 expression led to decreased tumor cell proliferation, reduced L-arginine metabolism and reduction of their immunosuppressive potential. Based protein expression (Figure 1b) and on the arginine activity of PCa cells (Figure 1c), our data suggest that

ARG2 may nonetheless have a more prominent role than ARG1 in PCa cells arginase activity [17].

Furthermore, our data showed that ARG1 and ARG2 were differentially regulated by androgens. Contrary to *ARG2* gene and protein expression, we clearly demonstrated that the gene and protein expression of ARG1 do not correlate. This suggests that androgen may influence a post-transcriptional regulation of ARG1 as it was previously reported in xenopus [18] and in yeast models [19]. Since ARG2 expression is localized to mitochondria, we evaluated whether cellular proliferation independent of androgens could induce ARG2 expression in LNCaP cells. In a proliferation assay with EGF instead of R1881, no ARG2 induction was observed (Data not shown). Collectively, our results reveal that, although both induced by R1881, the signaling pathways leading to ARG1 and ARG2 expression differs for the two enzymes and needs to be further examined.

The implication of an androgen-regulated expression of ARG1 and ARG2 in prostate carcinogenesis requires further investigation. Arginase expression and polyamine synthesis are elevated in PCa [20,21] and associated with tumor grade [22]. A high arginase activity correlates with increased proliferation of breast cancer [23], colon cancer [24] and kidney cell lines [25]. However, we observed that tumor or HR tissues express less ARG2 than non-malignant tissues. It is possible that tumor cells do not acquire the expression of these immunosuppressive enzymes as a mean to further their immunosuppressive potential, an aspect associated with tumor progression. In fact, since the prostate is the organ with the highest polyamine production, arginase expression by prostate cells may precede the development of cancer, as polyamine production is essential for the proliferation of prostate cells. Thus, the immunosuppressive advantage gained by prostate cells may be secondary to the proliferative role played by the arginases. From our data and that of others, we hypothesize that arginase may be implicated in the earlier hormone-sensitive stages of prostate carcinogenesis by promoting cancer cell proliferation and the development of an androgen-regulated immunosuppressive environment.

Finally, we observed that IL-8 was upregulated following androgen stimulation and could induce the expression of ARG1 and ARG2. IL-8 mediates its

effects through the activation of two high-affinity G-protein coupled receptors, CXCR1 and CXCR2 [26], both of which are expressed by LNCaP cells [27,28]. It is important to note that expression of ARG1 and ARG2 following IL-8 stimulation was not as substantial as with R1881 stimulation suggesting that other androgen-regulated pathways could be involved. Altogether, this is the first indication that the expression of IL-8 is regulated by androgens and that arginases can be regulated by a TH2 cytokine in human cancer cells.

Conclusion

Our data demonstrate that androgens regulate the expression of both ARG1 and ARG2 in HS PCa cell lines and in PCa patients in an AR-dependent manner. ARG1 and ARG2 are enzymatically active and their inhibition results in reduced L-arginine metabolism, cell growth and immunosuppressive potential. We found that IL-8 secreted by LNCaP cells was also regulated by androgens and could on its own promote the expression of ARG1 and ARG2. Collectively, the results presented in this report suggest that androgens actively participate in the development of an immunosuppressive microenvironment within the prostate through the expression of ARG1 and ARG2. A better understanding of the expression of immunosuppressive pathways at specific stages of PCa progression may eventually provide new insights for improving current immunotherapeutic strategies.

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References

1. Jemal A, Siegel R, Ward E, Hao Y, Xu J, et al. (2008) Cancer statistics, 2008. CA Cancer J Clin 58: 71-96.

2. Grossmann ME, Huang H, Tindall DJ (2001) Androgen receptor signaling in androgen-refractory prostate cancer. J Natl Cancer Inst 93: 1687-1697.

3. Montironi R, Schulman CC (1998) Pathological changes in prostate lesions after androgen manipulation. J Clin Pathol 51: 5-12.

4. Chang SS, Kibel AS (2009) The role of systemic cytotoxic therapy for prostate cancer. BJU Int 103: 8-17.

5. Miller AM, Pisa P (2007) Tumor escape mechanisms in prostate cancer. Cancer Immunol Immunother 56: 81-87.

6. Mumenthaler SM, Yu H, Tze S, Cederbaum SD, Pegg AE, et al. (2008) Expression of arginase II in prostate cancer. Int J Oncol 32: 357-365.

7. Bronte V, Kasic T, Gri G, Gallana K, Borsellino G, et al. (2005) Boosting antitumor responses of T lymphocytes infiltrating human prostate cancers. J Exp Med 201: 1257-1268.

8. Bronte V, Zanovello P (2005) Regulation of immune responses by L-arginine metabolism. Nat Rev Immunol 5: 641-654.

9. Mercader M, Bodner BK, Moser MT, Kwon PS, Park ES, et al. (2001) T cell infiltration of the prostate induced by androgen withdrawal in patients with prostate cancer. Proc Natl Acad Sci U S A 98: 14565-14570.

10. Gannon PO, Poisson AO, Delvoye N, Lapointe R, Mes-Masson AM, et al. (2009) Characterization of the intra-prostatic immune cell infiltration in androgen-deprived prostate cancer patients. J Immunol Methods 348: 9-17.

11. Yamanaka H, Kirdani RY, Saroff J, Murphy GP, Sandberg AA (1975) Effects of testosterone and prolactin on rat prostatic weight, 5alpha-reductase, and arginase. Am J Physiol 229: 1102-1109.

12. Manteuffel-Cymborowska M, Chmurzynska W, Peska M, Grzelakowska-Sztabert B (1995) Arginine and ornithine metabolizing enzymes in testosterone-induced hypertrophic mouse kidney. Int J Biochem Cell Biol 27: 287-295.
13. Levillain O, Diaz JJ, Blanchard O, Dechaud H (2005) Testosterone down-regulates ornithine aminotransferase gene and up-regulates arginase II and ornithine decarboxylase genes for polyamines synthesis in the murine kidney. Endocrinology 146: 950-959.
14. Lu S, Wang A, Dong Z (2007) A novel synthetic compound that interrupts androgen receptor signaling in human prostate cancer cells. Mol Cancer Ther 6: 2057-2064.
15. Seaton A, Scullin P, Maxwell PJ, Wilson C, Pettigrew J, et al. (2008) Interleukin-8 signaling promotes androgen-independent proliferation of prostate cancer cells via induction of androgen receptor expression and activation. Carcinogenesis 29: 1148-1156.
16. Mumenthaler SM, Rozengurt N, Livesay JC, Sabaghian A, Cederbaum SD, et al. (2008) Disruption of arginase II alters prostate tumor formation in TRAMP mice. Prostate.
17. Kee K, Vujcic S, Merali S, Diegelman P, Kisiel N, et al. (2004) Metabolic and antiproliferative consequences of activated polyamine catabolism in LNCaP prostate carcinoma cells. J Biol Chem 279: 27050-27058.
18. Xu Q, Baker BS, Tata JR (1993) Developmental and hormonal regulation of the Xenopus liver-type arginase gene. Eur J Biochem 211: 891-898.
19. Olszewska A, Krol K, Weglenski P, Dzikowska A (2007) Arginine catabolism in Aspergillus nidulans is regulated by the rrmA gene coding for the RNA-binding protein. Fungal Genet Biol 44: 1285-1297.
20. Harris BE, Pretlow TP, Bradley EL, Jr., Whitehurst GB, Pretlow TG, 2nd (1983) Arginase activity in prostatic tissue of patients with benign prostatic hyperplasia and prostatic carcinoma. Cancer Res 43: 3008-3012.
21. Keskinege A, Elgun S, Yilmaz E (2001) Possible implications of arginase and diamine oxidase in prostatic carcinoma. Cancer Detect Prev 25: 76-79.

22. Pretlow TG, 2nd, Harris BE, Bradley EL, Jr., Bueschen AJ, Lloyd KL, et al. (1985) Enzyme activities in prostatic carcinoma related to Gleason grades. Cancer Res 45: 442-446.

23. Singh R, Pervin S, Karimi A, Cederbaum S, Chaudhuri G (2000) Arginase activity in human breast cancer cell lines: N(omega)-hydroxy-L-arginine selectively inhibits cell proliferation and induces apoptosis in MDA-MB-468 cells. Cancer Res 60: 3305-3312.

24. Buga GM, Wei LH, Bauer PM, Fukuto JM, Ignarro LJ (1998) NG-hydroxy-L-arginine and nitric oxide inhibit Caco-2 tumor cell proliferation by distinct mechanisms. Am J Physiol 275: R1256-1264.

25. Tate DJ, Jr., Vonderhaar DJ, Caldas YA, Metoyer T, Patterson JRt, et al. (2008) Effect of arginase II on L-arginine depletion and cell growth in murine cell lines of renal cell carcinoma. J Hematol Oncol 1: 14.

26. Brat DJ, Bellail AC, Van Meir EG (2005) The role of interleukin-8 and its receptors in gliomagenesis and tumoral angiogenesis. Neuro Oncol 7: 122-133.

27. Araki S, Omori Y, Lyn D, Singh RK, Meinbach DM, et al. (2007) Interleukin-8 is a molecular determinant of androgen independence and progression in prostate cancer. Cancer Res 67: 6854-6862.

28. Murphy C, McGurk M, Pettigrew J, Santinelli A, Mazzucchelli R, et al. (2005) Nonapical and cytoplasmic expression of interleukin-8, CXCR1, and CXCR2 correlates with cell proliferation and microvessel density in prostate cancer. Clin Cancer Res 11: 4117-4127.

29. Heisler LE, Evangelou A, Lew AM, Trachtenberg J, Elsholtz HP, et al. (1997) Androgen-dependent cell cycle arrest and apoptotic death in PC-3 prostatic cell cultures expressing a full-length human androgen receptor. Mol Cell Endocrinol 126: 59-73.

30. Lessard L, Saad F, Le Page C, Diallo JS, Peant B, et al. (2007) NF-kappaB2 processing and p52 nuclear accumulation after androgenic stimulation of LNCaP prostate cancer cells. Cell Signal 19: 1093-1100.

31. Diallo JS, Betton B, Parent N, Peant B, Lessard L, et al. (2008) Enhanced killing of androgen-independent prostate cancer cells using inositol hexakisphosphate in combination with proteasome inhibitors. Br J Cancer 99: 1613-1622.

32. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29: e45.

33. Le Page C, Koumakpayi IH, Lessard L, Saad F, Mes-Masson AM (2005) Independent role of phosphoinositol-3-kinase (PI3K) and casein kinase II (CK-2) in EGFR and Her-2-mediated constitutive NF-kappaB activation in prostate cancer cells. Prostate 65: 306-315.

34. Grandvaux N, Gaboriau F, Harris J, tenOever BR, Lin R, et al. (2005) Regulation of arginase II by interferon regulatory factor 3 and the involvement of polyamines in the antiviral response. FEBS J 272: 3120-3131.

35. Le Page C, Koumakpayi IH, Alam-Fahmy M, Mes-Masson AM, Saad F (2006) Expression and localisation of Akt-1, Akt-2 and Akt-3 correlate with clinical outcome of prostate cancer patients. Br J Cancer 94: 1906-1912.

36. Diallo JS, Aldejmah A, Mouhim AF, Peant B, Fahmy MA, et al. (2007) NOXA and PUMA expression add to clinical markers in predicting biochemical recurrence of prostate cancer patients in a survival tree model. Clin Cancer Res 13: 7044-7052.

37. Gannon PO, Koumakpayi IH, Le Page C, Karakiewicz PI, Mes-Masson AM, et al. (2008) Ebp1 expression in benign and malignant prostate. Cancer Cell Int 8: 18.

38. Koumakpayi IH, Diallo JS, Le Page C, Lessard L, Gleave M, et al. (2006) Expression and nuclear localization of ErbB3 in prostate cancer. Clin Cancer Res 12: 2730-2737.

39. Gannon PO, Alam Fahmy M, Begin LR, Djoukhadjian A, Filali-Mouhim A, et al. (2006) Presence of prostate cancer metastasis correlates with lower lymph node reactivity. Prostate 66: 1710-1720.

40. Gopalakrishnan V, Burton PJ, Blaschke TF (1996) High-performance liquid chromatographic assay for the quantitation of L-arginine in human plasma. Anal Chem 68: 3520-3523.

41. Godin-Ethier J, Pelletier S, Hanafi LA, Gannon PO, Forget MA, et al. (2009) Human activated T lymphocytes modulate IDO expression in tumors through Th1/Th2 balance. J Immunol 183: 7752-7760.

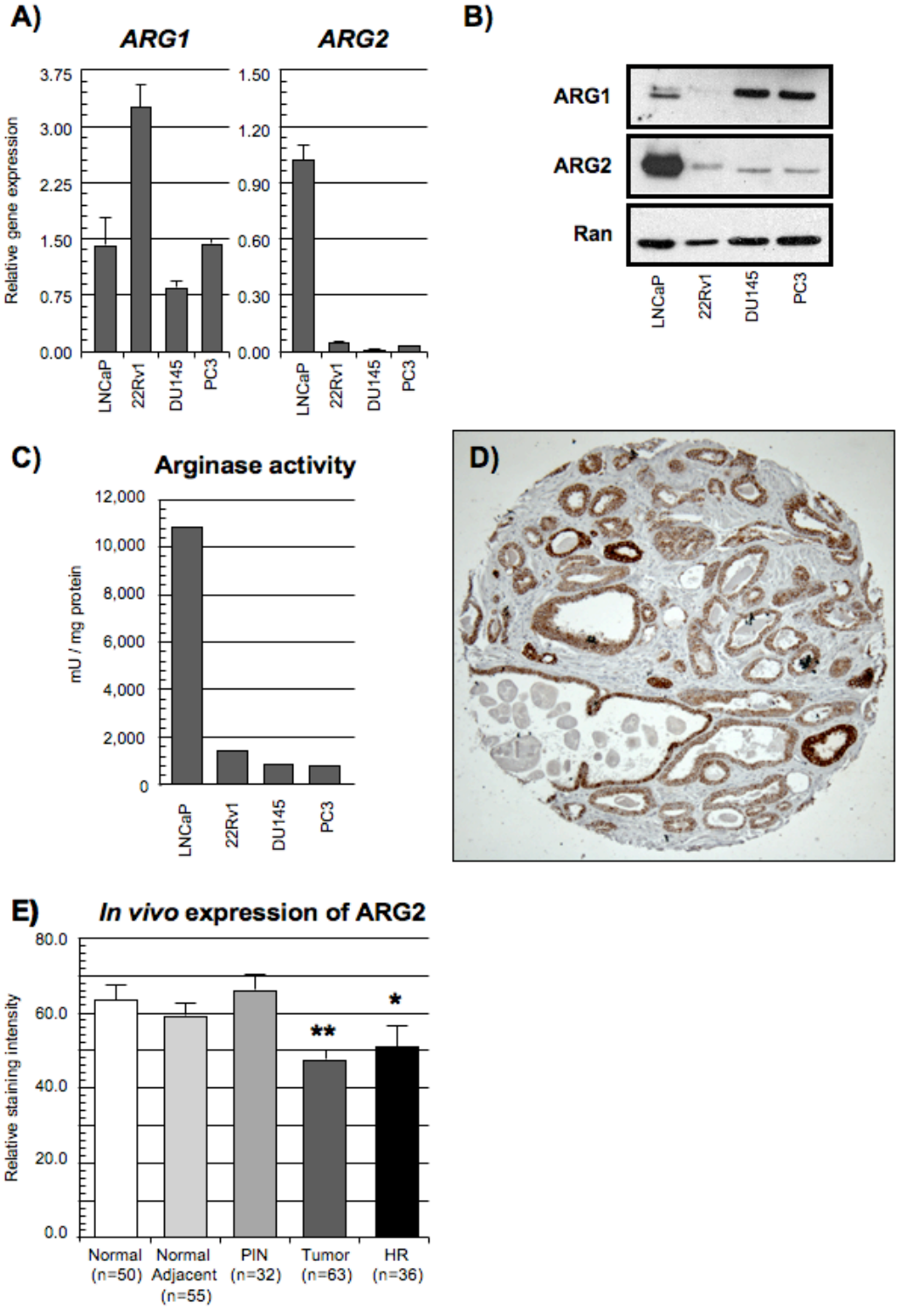


Figure 1. In vitro and in vivo expression of ARG1 and ARG2 in PCa

Figure 1: *In vitro* and *in vivo* expression of ARG1 and ARG2 in PCa

PCa cell lines (LNCaP, 22Rv1, DU145 and PC3) were maintained in RPMI supplemented with 10% FBS. A) Gene expression of ARG1 (left panel) and ARG2 (right panel). Mean relative expression (n=3) with standard error of the mean (error bars). B) Western blot of ARG1 and ARG2. Ran served as loading control. C) Arginase activity of PCa cell lines quantified in mU/mg of proteins. D) Representative image of immunohistochemistry staining of ARG2 expression in prostatic tissue. Note that the expression of ARG2 was confined to the epithelial cells with no stromal staining. E) Quantification of ARG2 expression by immunohistochemistry in prostate specimens. *Statistically significant difference in ARG2 expression between PIN and HR tissues ($p=0.033$, Mann-U). **Statistically significant difference in ARG2 expression between tumor tissues and normal tissues ($p<0.001$, Mann-U), non-malignant tissues adjacent to tumor ($p<0.01$, Mann-U) and PIN tissues ($p<0.001$, Mann-U).

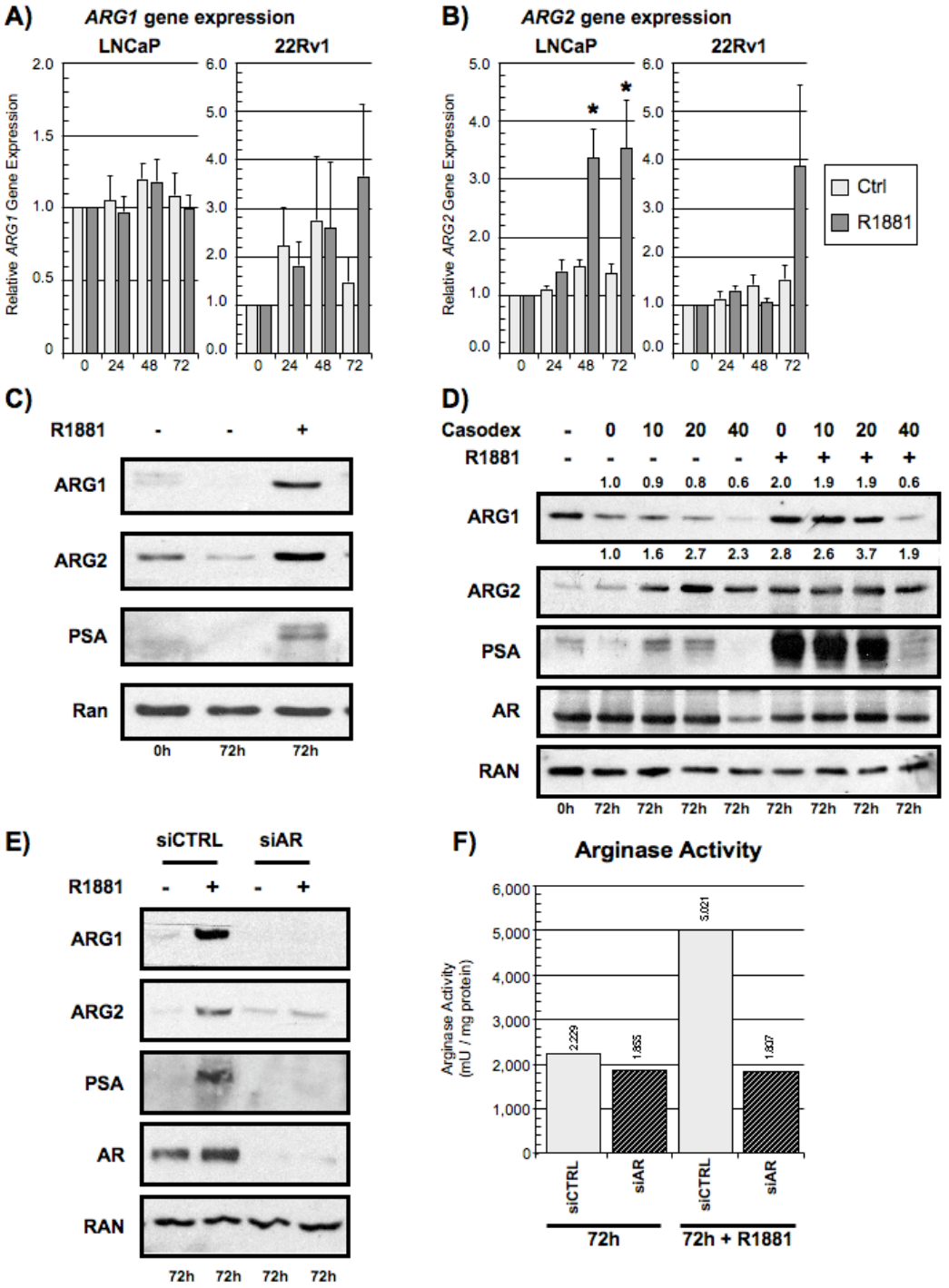


Figure 2. Androgen-regulated expression of ARG1 and ARG2

Figure 2. Androgen-regulated expression of ARG1 and ARG2

A-B) LNCaP cells (left panels) and 22RV1 (right panels) were stimulated over a period of 72 hours with 10 nM R1881 following a 72 hour incubation period in charcoal-stripped media and the gene expression of A) ARG1 and B) ARG2 analyzed by qPCR. Control (gray bars) and R1881-stimulated (black bars). *Statistically significant difference ($p<0.05$, Mann-U). Mean relative expression (n=4) with standard error (error bars). C) Increased protein expression of both ARG1 and ARG2 following R1881 stimulation by Western blot. LNCaP cells were stimulated with 10 nM R1881 as previously described. PSA served as positive control. Representative experiment, (n=6). D) Inhibition of AR activity with bicalutamide. LNCaP cells were stimulated with R1881 as previously described in the presence of increasing doses of bicalutamide (0, 10, 20 and 40 μM). ARG1 and ARG2 expression levels were evaluated by Western blot. Representative experiment shown, (n=3). Note the agonist effect of bicalutamide in the absence of R1881 illustrated by an increased PSA and ARG2 expression. E) Inhibition of AR expression by siRNA. LNCaP cells were transfected as previously described. AR, ARG1 and ARG2 expression levels were evaluated by Western blot. Representative experiment, (n=4). F) Arginase activity of LNCaP cells transfected with siCTRL or siAR and then stimulated with R1881 was quantified in mU/mg of proteins. Representative experiment, (n=3).

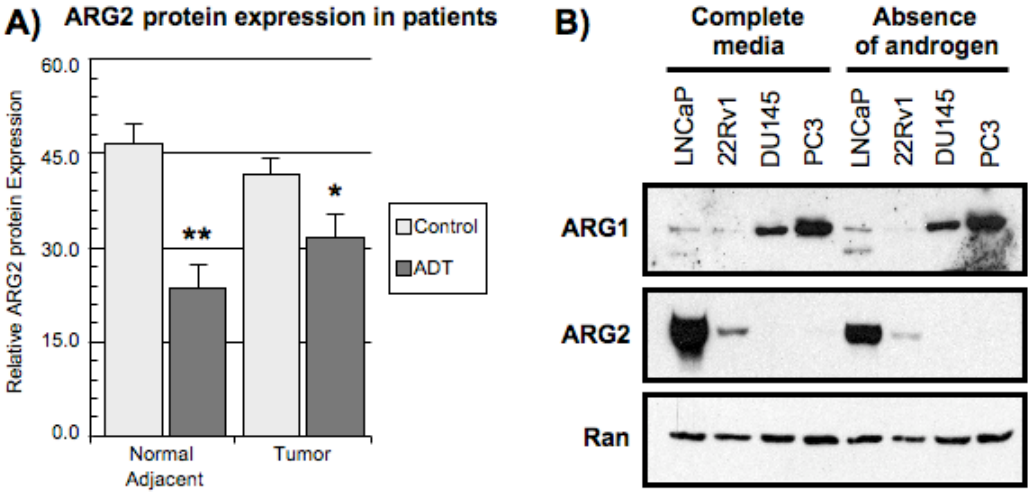


Figure 3. Reduced ARG2 expression following ADT

Figure 3. Reduced ARG2 expression following ADT

A) Analysis of androgen-regulated ARG2 expression in PCa patients by immunohistochemistry. Control patients (Gray bars, n=40) and ADT-treated patients (Black bars, n=35). B) Decreased ARG2 protein expression in the absence of androgens in vitro determined by Western blot. Ran served as loading control. PCa cell lines (LNCaP, 22Rv1, DU145 and PC3) were maintained in RPMI 10% FBS or in RPMI supplemented with 10% charcoal stripped FBS for 7 days (n=3). Note that ARG1 expression did not vary but that ARG2 was reduced in LNCaP and 22Rv1 cells in the absence of androgen.

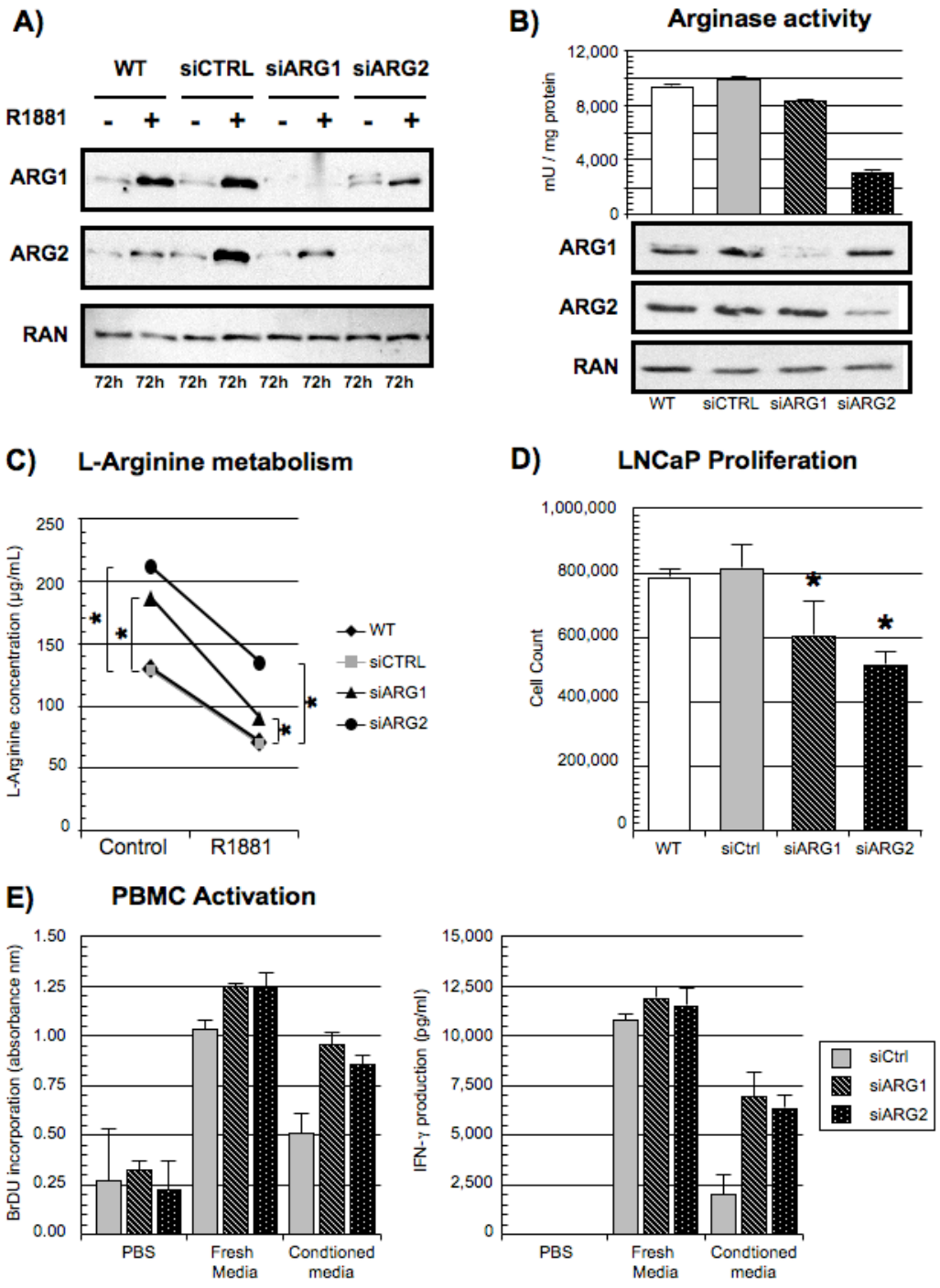


Figure 4. ARG1 and ARG2 are metabolically active

Figure 4. ARG1 and ARG2 are metabolically active

LNCaP cells were transfected with either a siCTRL or a cocktail of three siRNA against the ARG1 or ARG2. Post-transfection (24 hours), cells were plated in charcoal-stripped serum supplemented media for 72 hours and then stimulated for 72 hours with 10 nM R1881. A) siRNA inhibition of ARG1 and ARG2 expression was evaluated by Western Blot. Representative experiment shown, (n=4). B) Decreased arginase activity following transfection with siARG1 or siARG2 in LNCaP cells. The corresponding Western blot is shown in the bottom panel. Representative experiment shown, (n=3). C) Decreased metabolism of L-arginine in the absence of arginase expression. Conditioned media of LNCaP cells transfected with siCTRL, siARG1 or siARG2 were analyzed by HPLC for L-arginine concentration. The conditioned media analyzed by HPLC were from the LNCaP cells presented in Figure 4a. *Statistically significant difference ($p<0.05$, Mann-U). D) Decreased proliferation of LNCaP cells in the absence of arginase expression. LNCaP cells were transfected as previously described. Proliferation was measured by cell count 96 hours post-transfection. *Statistically significant difference ($p<0.05$, Mann-U), (n=3). E) Inhibition of ARG2 expression causes increased PBMC proliferation and activation. PBMCs from normal donors were activated with anti-CD3 (OKT3, 1 μg/ml) with or without IL-2 in the presence of fresh media or conditioned media of LNCaP cells transfected with either control, siCTRL, siARG1 or siARG2 as previously described. Left panel: PBMC proliferation was quantified by BrdU incorporation following 120 hours of OKT3 and IL-2 stimulation. Mean absorbance (n=4) is shown with standard error (error bars). Right panel: PBMC secretion of IFN-γ quantified by ELISA. Same experiment as previously described, but the PBMCs were activated for 24 hours without IL-2. Representative expression is shown (n=4) with standard error of the mean (error bars).

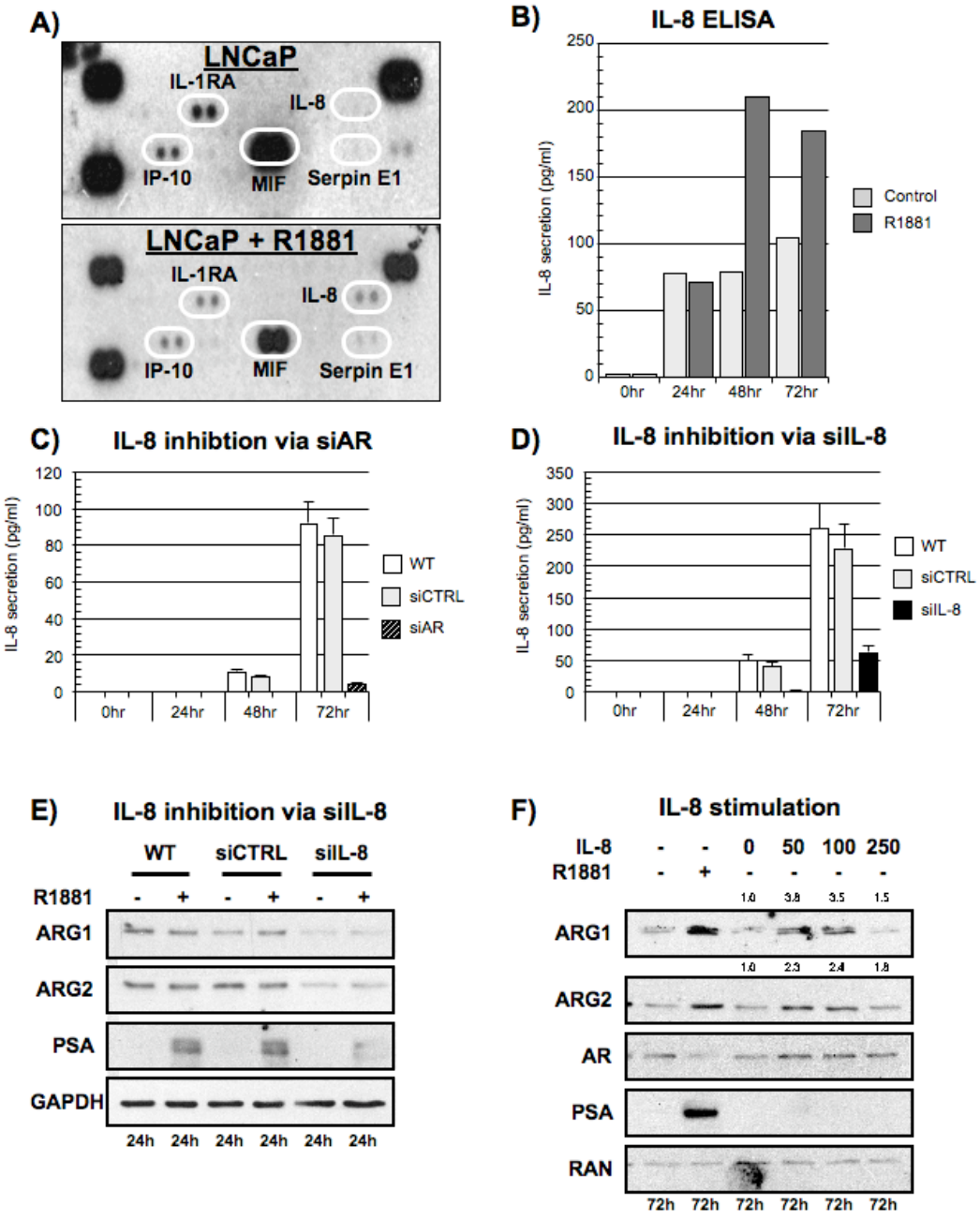


Figure 5. Androgens induced Interleukin-8, which in turn promotes ARG1 and ARG2 expression

Figure 5. Androgens induced Interleukin-8, which in turn promotes ARG1 and ARG2 expression

Evaluation of the cytokine expression profile of LNCaP cells following R1881 stimulation. A) Conditioned media of LNCaP cells stimulated as previously described were analyzed with a Proteome Profiler™ (R&D Systems). B) Conditioned media of LNCaP cells stimulated over time with either ethanol control (gray bars) and R1881 (black bars) were analyzed for the production of IL-8 by ELISA. The representative experiment shown was performed with the same conditioned media used for the Proteome Profiler analysis in 5a, (n=3). C) Quantification of IL-8 secretion by LNCaP cells transfected with siAR and stimulated with R1881 as previously described. Representative experiment shown, (n=3). D) Quantification of IL-8 secretion by LNCaP cells transfected with siIL-8 and stimulated with R1881 as previously described. Representative experiment shown, (n=3). For 5b and 5c, there was no IL-8 secretion detected in the absence of R1881 stimulation. E) Expression of ARG1 and ARG2 in LNCaP cells following transfection of siIL-8 and R1881 stimulation. Representative experiment shown, (n=3). F) LNCaP cells were plated in charcoal-stripped serum supplemented media for 72 hours and for 24 hours in serum-free RPMI. Cells were then stimulated for 72 hours with 10 nM R1881 or with 50, 100 or 250 ng/ml of IL-8 in serum-free RPMI. ARG1 and ARG2 expression levels were detected by Western blot. Representative experiment, (n=3). Note the induction of both ARG1 and ARG2 at 50 and 100 ng/ml of IL-8 concentration in the absence of R1881.

Supplementary Table 1. Correlations between ARG2 expression and clinico-pathological markers

Clinico-Pathological Parameters	ARG2 expression in Normal Adjacent tissues			ARG2 expression in Tumor tissues		
	Correlation*	P value	n	Correlation*	P value	n
Age at Surgery	0.100	0.402	72	0.114	0.334	74
Pre-Operative PSA	-0.065	0.600	68	0.079	0.521	69
Gleason Score	-0.080	0.524	66	-0.037	0.769	67
Positive Surgical Margins	-0.057	0.653	65	0.106	0.397	66
Prostate Weight	0.006	0.967	60	-0.032	0.809	61
Tumor Grade	-0.030	0.810	68	-0.065	0.598	69
Vesicle Seminal Invasion	-0.271	0.026	68	-0.101	0.410	69
Biochemical Recurrence	-0.207	0.081	72	-0.117	0.321	74
Development of Metastasis	0.095	0.435	69	-0.048	0.692	71
Death from Prostate Cancer	-0.099	0.408	72	0.063	0.594	74
ARG2 expression in Normal Adjacent	-	-	-	0.032	0.793	71
ARG2 exression in Tumor	0.032	0.793	71	-	-	-

* Spearman's Rho non-parametric correlations

Supplementary Table 1. Correlations between ARG2 expression and clinico-pathological parameters.

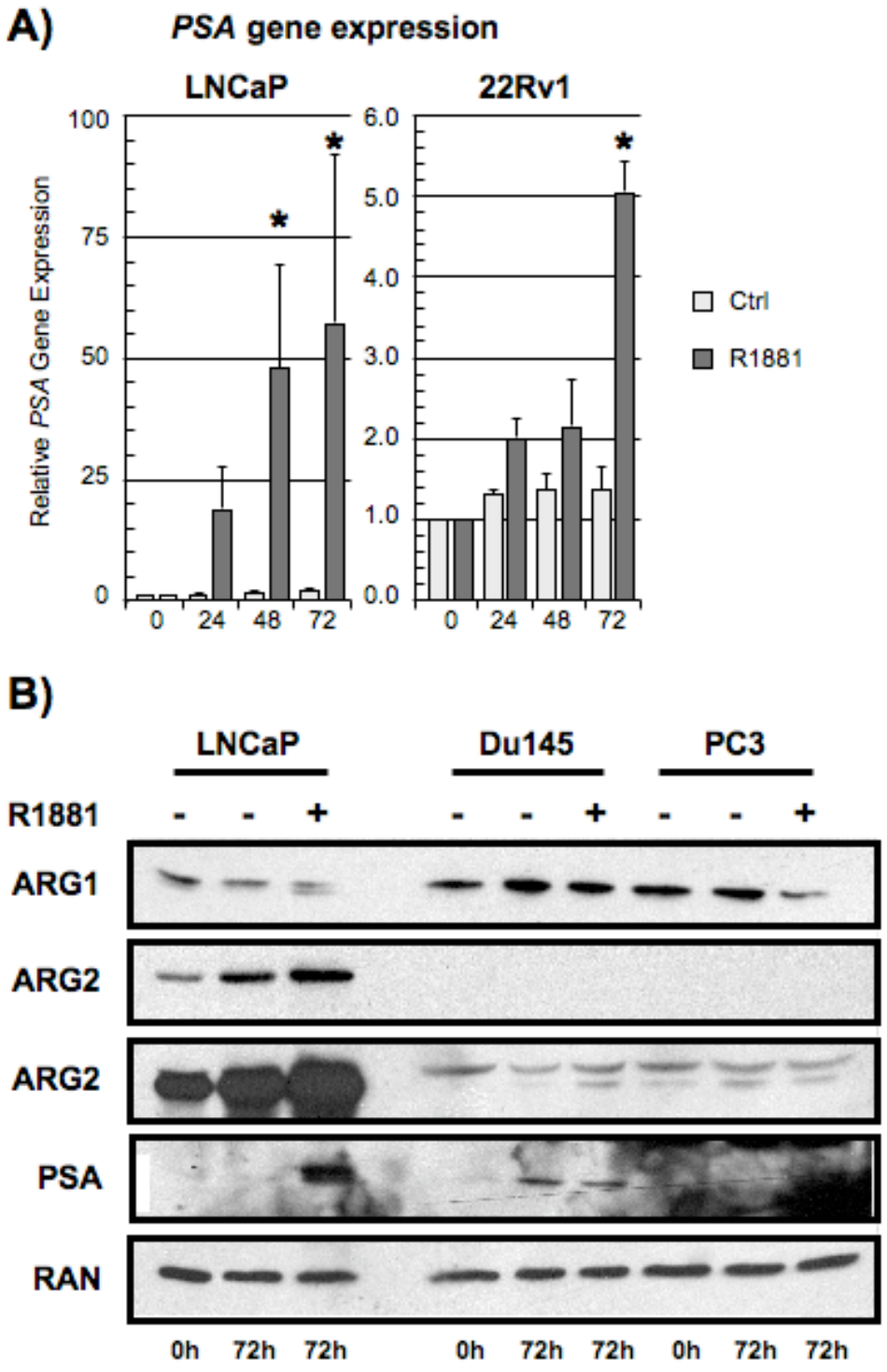
Supplementary Table 2. Correlations between ARG2 expression and immune cell infiltration‡

	ARG2 expression in Normal Adjacent tissues			ARG2 expression in Tumor tissues		
Clinico-Pathological Parameters	Correlation*	P value	n	Correlation*	P value	n
CD3+ T Lymphocytes	-0.246	0.056	61	-0.355	0.004	63
CD8+ T Lymphocytes	-0.328	0.005	72	-0.199	0.090	74
CD20+ B Lymphocytes	-0.007	0.957	65	-0.130	0.293	67
CD56+ Natural Killer cells	0.019	0.886	62	-0.164	0.194	64
CD68+ Macrophages	-0.364	0.002	70	-0.135	0.254	73
FoxP3+ T lymphocytes	-0.228	0.075	62	0.039	0.762	64

* Spearman's Rho non-parametric correlations

‡ Immune cell infiltration data from our previous study [10]

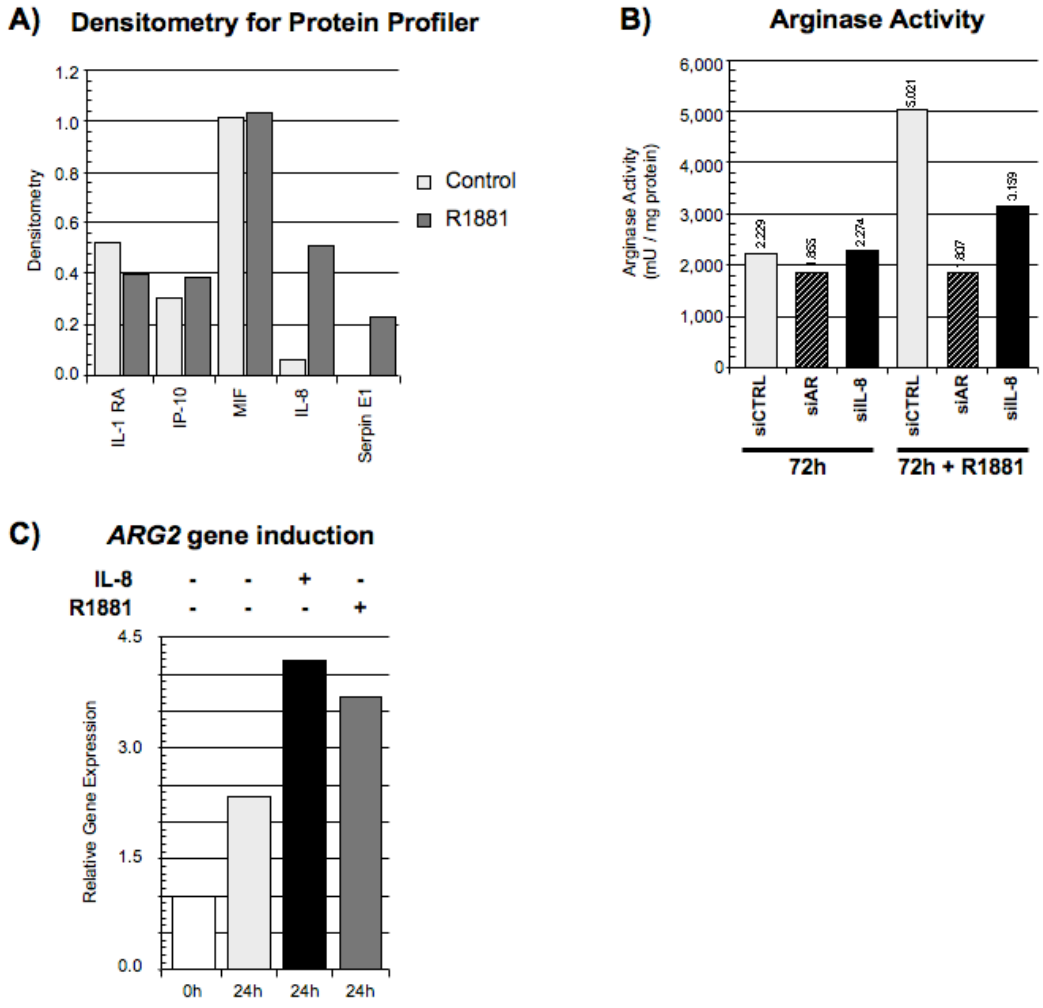
Supplementary Table 2. Correlations between ARG2 expression and immune cell infiltration in the primary tumor.



Supplementary Figure 1. Androgen stimulation of PCa cells

Supplementary Figure 1: Androgen stimulation of PCa cells

A) LNCaP cells (left panels) and 22RV1 (right panels) were stimulated over a period of 72 hours with 10 nM R1881 following a 72 hour incubation period in charcoal-stripped media and the gene expression of Prostate-Specific Antigen (PSA), a positive control for R1881 stimulation, was analyzed by qPCR. Note that the ARG2 gene expression correlated presented in Figure 1b correlated with the higher androgen sensibility of LNCaP cells compared to 22RV1 as exemplified by the mRNA expression of Prostate-Specific Antigen (PSA). B) Expression of ARG1 and ARG2 determined by Western blot in LNCaP, Du145 and PC3 cells stimulated with R1881 for 72 hours as previously described. Note the absence of ARG1 and ARG2 in the two HR PCa cell lines, DU145 and PC3.



Supplementary Figure 2: ARG1 and ARG2 induction following IL-8 stimulation.

Supplementary Figure 2: ARG1 and ARG2 induction following IL-8 stimulation.

A) Positive signals from the Proteome Profiler™ were quantified by densitometry using Quantity One software (Bio-Rad). Ethanol control (gray bars) and R1881 (black bars). B) Arginase activity of LNCaP cells transfected with siCTRL, siAR or siIL-8 and then stimulated with R1881 was quantified in mU/mg of proteins. Same representative experiment as presented in Figure 2f, (n=3). C) Increased ARG2 gene expression at 24 hours following IL-8 stimulation. LNCaP were stimulated with IL-8 as previously described. Ran served as the loading control.

CHAPTER VI

DISCUSSION

The all-encompassing goal of this doctoral thesis was to further our understanding of the tumor immunological microenvironment in human prostate cancer. The results presented in the previous two chapters demonstrate that androgens play determining roles in the sculpting of a unique local immunological microenvironment. Our data also highlights several key aspects that should be further studied. It would be insightful to precisely characterize the activation/maturation phenotype of immune cells present in tumor microenvironments (primary tumor and metastatic tissues). To achieve this, novel analysis methods need to be optimized and antibodies against activation/maturation markers suited for immunohistochemistry on FFPE specimens need to be further developed. Furthermore, a strong collaborative understanding between the hospital pathology department and the fundamental research unit is essential to provide fresh clinical samples for research. A second objective would be to evaluate in greater detail the immunological environment in metastatic and HRPC patients. The uniqueness of the immunological microenvironment of HRPC patients needs to be considered in the optimization of immunotherapies for prostate cancer. Immunotherapy is currently considered as a second-line therapeutic option. As such, it is important to validate whether prostate cancer patients who have received and failed previous therapies still have the "immunological strength" that is required for a successful immunotherapy.

6.1 IMMUNOSUPPRESSION IN TUMOR DRAINING LYMPH NODES OF PROSTATE CANCER PATIENTS

In chapter III, we explored the immunological microenvironment of metastatic LNs of prostate cancer patients. Tumor-draining LNs are necessary for the activation of $CD8^+$ T lymphocytes and the development of a cell-mediated anti-tumoral immune response, such as expected during an immunotherapy. It is thus essential to determine the LN's immunological status in prostate cancer patients. Our results

provide cellular and histopathological evidence suggesting a reduced LN reactivity specifically within metastatic LNs. Compared to non-metastatic LNs of the same patient, metastatic LNs had significantly less activated $CD38^+$ T lymphocytes, less $CD68^+$ macrophages, more $Ki67^+$ proliferating lymphocytes as well as less follicular and sinus hyperplasia. Other groups also observed an increase in lymphocyte proliferation in tumor draining LNs. However, although they do proliferate more, these lymphocytes are anergic, fail to fully develop in mature effectors (446) and remain inefficient at mounting an anti-tumoral immune response against the invading tumor cells (103). This is consistent with our result showing no difference in the expression of CD45RA (antigen naïve lymphocyte) and CD45RO (antigen experienced lymphocyte) between non-metastatic and metastatic LNs. The lower incidence of follicular and sinus hyperplasia within metastatic LNs also support published data highlighting a reduced immunological reactivity within the paracortex of tumor draining LNs (447). Furthermore, we observed that the metastatic masses were mostly free of immune cells (unpublished data). This observation suggests a possible defect in lymphocyte migration within the tumor. *In vitro* studies demonstrate that macrophages induce the expression of vascular cell adhesion molecule-1 (VCAM-1) by prostate cancer cell lines (448). VCAM-1 is implicated in immune evasion by decreasing tumor trafficking of T lymphocytes (449). Our study was centered upon the characterization of lymphocyte populations within the LN tissue and not within the metastasis itself. It would nevertheless be interesting to evaluate the activation status of the immune cells that are able to invade the metastatic mass as well as to quantify VCAM-1 expression in the primary tumor and LN metastasis of prostate cancer patients. This type of study could be part of the larger objective of evaluating the immunoregulatory environment in metastatic and HRPC patients.

From our results, we were however unable to answer the question as to whether LNs are rendered anergic prior to invasion or if the invading cells actively inhibit the LNs concomitantly to their lymphatic colonization. In a breast cancer study, sentinel LNs exhibited signs of immunosuppression, such as decreased abundance $CD4^+$, $CD8^+$ T lymphocytes and $CD1a^+$ DCs (450), prior to tumor invasion (112). In a mouse model, B16 melanoma cells directly implanted in LNs

elicit the activation of $CD8^+$ T lymphocytes and are effectively rejected. However, when the same B16 cells are implanted in extralymphatic sites, tumor draining LNs are rendered anergic. This LN immunosuppression further allowa for the growth of B16 tumors subsequently injected within LNs (103). Our results do show comparable variations in $CD20^+$ B lymphocytes, $CD8^+$ T lymphocytes abundance and in LN size between non-metastatic and metastatic LNs compared to control LNs from non-metastatic patients, therefore suggesting similar variations between sentinel LNs. In line with this, sentinel LNs closest to the primary tumor are reported to be more immunosuppressed and more frequently invaded by tumor cells (451, 452). These data suggest that an immunosuppressive field effect may emanate from the primary tumor. Interestingly, metastatic LNs, LNs closest, as well as those furthest away from the primary tumor are found to be less immunologically reactive compared to LNs in intermediate positions (453) [reviewed in (447)]. Although it would have been interesting to evaluate an immunosuppressive field effect in our cohort of prostate cancer patients, the pathology reports lacked information regarding LN localization and prevented us from doing so. Altogether, these results suggest that the primary tumor modulates the activity of all sentinel LNs. Similar to the "soil-and-seed" hypothesis (454, 455), only the LNs that are effectively rendered anergic would be the ones invaded by metastatic cells. This is exemplified by our data illustrating lower lymphocyte activation, a reduced macrophage population and diminished paracortex reactivity within metastatic LNs.

What mechanisms are implicated in this LN immunosuppression? Several studies associate LN anergy with DC dysfunctions (456-458). DCs present TAA peptides to $CD8^+$ T lymphocyte by cross-presentation. For $CD8^+$ T lymphocytes to be properly activated, cross-presentating DCs also need to activate $CD4^+$ T helper lymphocytes. In a tumor mouse model, DCs were shown to lack adequate MHC-II peptide expression, required for $CD4^+$ T lymphocyte activation, which results in the improper activation of $CD8^+$ T lymphocytes (103). The absence of MHC-II peptide complexes at the surface of DCs is associated with elevated IL-10 concentration within sentinel LNs. IL-10 inhibits the surface expression of MHC-II as well as DC maturation and migration (459). DC expressing IDO also accumulate in tumor

draining LNs further preventing the activation and promoting the apoptosis of TAA-specific lymphocyte (220).

A first step towards determining what mechanisms are implicated in LN immunosuppression would be to accurately characterize the phenotype of immune cells residing in tumor draining LNs in order to denote activation and maturation defects. From our results, we did not observe significant difference in the expression of CD45RA and CD45RO between non-metastatic and metastatic LNs. It is possible that the presence of prostate cancer LN metastasis is not accompanied by an increasing number of antigen-experienced lymphocytes, which could be explained by the lower number of APCs (macrophage and B lymphocytes) within metastatic LNs. Although we did not detect changes in DC numbers between non-metastatic and metastatic LNs, it is conceivable that a more thorough analysis of the DC phenotype could have highlighted significant differences regarding the DCs activation and maturation status with regards the LN's metastatic status. Since fresh clinical LNs samples suitable for FACS analyses are hard to acquire, such an analysis would need to be completed using archived pathological tissue samples. By standard immunohistochemistry or tissue immunofluoresence protocols, it is however difficult to perform the double or triple stainings essential for the proper characterization of the activation/maturation status of specific immune cell population. Novel techniques would thus need to be optimized. By using mRNA extracted by tissue laser-microdissection following an initial DC marker staining, the expression of activation/maturation markers could be analyzed by real-time PCR. In a more sophisticated approach, pathological samples could be analyzed by laser scanning cytometry (LSC) [reviewed in (460)]. LSC allows the *in situ* imaging and quantitative analysis of individual cells using *x-y* tissue mapping and with up to nine colors. In a recent publication, LSC was successfully applied at quantifying the density of $CD4^+$/$FoxP3^+$ T lymphocytes in FFPE biopsy samples (461). Overall, results from these studies would provide insightful data on the precise phenotype of immune cells in tumor draining LNs of prostate cancer patients.

The second step towards identifying the causes of LN immunosuppression would be to look directly at the invading metastatic cells as potential player in the

induction or the maintenance of LN hyporesponsiveness. To achieve this, a high-throughput genomic or proteomic approach could generate significant results. In a collaborative effort with the pathology department, researcher could be given access to fresh LNs. From the pathological sample, metastatic cells could be enriched using positive selection columns. The RNA and protein material from these cells could then be used for genomic and proteomic analyses. As controls, fresh malignant cells from tumor biopsies or from radical prostatectomy could be analyzed to compare to immunoregulatory phenotype of malignant cells with different tissue origins.

Taken as a whole, the limited clinical success of current immunotherapies in prostate cancer could result from an inadequate activation of TAA-specific $CD8^+$ T lymphocytes within tumor draining LNs. It is thus important to determine the causes of the specific immunosuppression associated with human prostate cancer. However, our results do not eliminate the possibility that, earlier in prostate cancer progression, an effective immune response could have developed within the tumor draining LNs. When considering the immune editing theory, it is plausible that the immunosuppression of sentinel LN represent an additional obstacle that the tumor needs to overcome in order to spread beyond the confine of the prostate. Finally, there is the possibility that LN positive prostate cancer patients may be less responsive to immunotherapies due to lower LN reactivity. As such, the metastatic status of prostate cancer patients should be taken into consideration when selecting immunotherapies. With additional knowledge on the kinetics of LN immunosuppression with regards to disease progression, it might be possible to discover the adequate timing for the initiation of immunotherapy.

6.2 ANDROGEN DEPRIVATION THERAPY PROMOTES THE INFILTRATION OF T LYMPHOCYTES AND MACROPHAGES WITHIN THE PRIMARY TUMOR

In chapter IV, we explored the impact of ADT on the immune cell infiltrate within the prostate. This study addressed two important points. First, immunotherapy is preferentially given to hormone refractory prostate cancer (HRPC) patients who

have received and failed ADT. However, to our knowledge, only one study characterized the prostate's immunological microenvironment following medical castration in prostate cancer patients (31). There is also the possibility that ADT could allow for a temporary immunotherapeutic window (439). As such, it is essential to characterize the immunological status of post-ADT prostate cancer patients to evaluate whether these patients would be well-suited for immunotherapy. Second, the literature contains several studies quantifying immune cells infiltration within primary tumors. However, these studies often derived conflicting conclusions as to the implication of immune cell density in disease progression. One point of concern is that the different analysis methods of limited accuracy utilized may account for these varying conclusions. We thus proposed a novel computer-based approach to standardize the quantification of immune cell density within large pathological tissue samples. The use of a precise, rapid and comprehensible evaluation method of the immune cell population could remove interpretation bias and facilitate the interpretation of independent studies. Furthermore, the digital images generated from the whole-slide scanner allow for the virtual dissection of the tissue, thus offering the possibility to compare immune cell density between non-malignant and malignant tissue. Although this was not done during our study, it would be interesting to evaluate, with the assistance of a pathologist, the immune cell abundance in non-malignant and malignant tissue of ADT-treated prostate cancer patients. Such an analysis would allow us to determine if non-malignant tissue adjacent to the tumor is subjected to the immunoregulatory effects emanating from the malignant tissue thereby helping to characterize the tumor immunological field-effect.

In our immunohistochemical analysis, we demonstrate that ADT favors the infiltration of specific immune cell populations. While no changes were observed in the relative densities of B lymphocytes, NK cells and $Foxp3^+$ T lymphocytes, the relative densities of $CD3^+$ T lymphocytes, $CD8^+$ T lymphocytes and $CD68^+$ macrophages were significantly increased in ADT patients. These results support previously published data suggesting that the post-ADT inflammatory response favors an increased abundance in T lymphocytes and macrophages (31). Furthermore, we identified a statistical correlation between prostate cancer progression and the

infiltration of $CD56^+$ NK cells and $CD68^+$ macrophages. We found that patients with a high density of NK cells had a lower risk of developing biochemical recurrence. This result supports the anti-tumoral role of NK cells. NK cells are one of the first cellular effectors to recognize and eliminate tumor cells. It is encouraging to observe that NK cells could still influence prostate cancer progression in patients with clinically-detectable tumors. In line with this observation, pre-clinical trials in mouse models are in fact demonstrating an essential role for NK cells in prostate cancer immunotherapy (462, 463). Conversely, a high density of $CD68^+$ macrophages was associated with an increased prostate cancer progression corroborating accumulating data on the tumor-promoting roles of tumor-associated macrophages (TAMs) in prostate cancer. Again, considering the immunoregulatory potential of macrophages, a detailed characterization of the macrophage phenotype within the primary tumor of prostate cancer patient is greatly needed (more details on macrophages in prostate cancer later on page 193).

The characterization of the immunological microenvironment in clinically detectable tumors could also be useful in prognostication. The activation status of immune cells varies according to the tumor's immunosuppressive potential, which can potentially be related to disease severity, *i.e.* would more aggressive tumors have stronger immunosuppressive potential? As such, analysis of immune cell activation status could allow for the identification of prostate cancer patients at risk of disease progression as well as identifying patients better suited for immunotherapy. In a possible scenario, it might be determined that when a patient reaches a specific level of immunosuppression (elevated density of T_{REGs} or immature DCs), then immunotherapy should not be considered. Furthermore, a better understanding of the immunological status during the earlier stages of the disease is also needed. This is of importance as it may be advantageous to treat patients by immunotherapy prior to the establishment of a potent tumor-derived immunosuppressive microenvironment.

Independent of the causes of the post-ADT inflammatory boost, it is also important to understand the role of this prostatic infiltrate in ADT patients. Prostate cancer patients almost unilaterally fail ADT within 24 months after treatment initiation. This clinical reality suggests that, although significantly denser, the post-

ADT immune cell infiltration remains unable to eliminate residual prostate cancer cells. There are several hypotheses for the apparent ineffectiveness of the post-ADT inflammation. For instance, the remaining ADT-resistant prostate cancer cells could be potent immunosuppressant. According to the cancer stem cell theory in prostate cancer, the residual hormone refractory prostate cancer cells, which repopulate the prostate following ADT, may have a more primitive cell fate than the hormone sensitive prostate epithelial cells. Reports in the literature suggest that these hormone refractory cells have a cancer stem cell phenotype and are derived from the basal cell layer. Without entering into a discussion on the biology of cancer stem cells, proponent of this theory should evaluate the immunosuppressive potential of these cells. One of the arguments for the existence of cancer stem cells is that the injection of relatively low numbers of cancer stem cells leads to the growth of detectable tumor masses. Considering that immune evasion is the seventh hallmark of cancer (197), it is thus of interest to evaluate whether these cancer stem cells can promote the development of an immunosuppressive microenvironment.

Furthermore, there is also the possibility that, similar to the pre-ADT immune cell infiltrate, immune cells in the post-ADT prostate remain anergic and incapable of differentiating into potent cytotoxic effectors targeting the tumor cells. We, and others (31), have shown that $CD8^+$ T lymphocytes and macrophages massively infiltrate the prostate following ADT. The fact that these cells, even at higher numbers, are not clinically beneficial for the patients further argues that a simple evaluation of immune cell numbers cannot be correlated with the biological effects of immune cells with regards to tumor progression. This is substantiated by our previous results demonstrating that metastatic LNs had increased abundance of $CD8^+$ T lymphocytes and elevated lymphocytic proliferation (Ki67+ lymphocytes). These observations could be associated with an immune reaction against the invading cells and considered as favorable markers against disease progression. However, the increase in $CD8^+$ T lymphocytes and in lymphocyte proliferation has limited clinical benefit as metastatic cells are not eradicated and are able to grow into metastases rendering them pathologically detectable. Similarly, it is possible that the higher numbers of infiltrating $CD8^+$ T lymphocytes within the primary tumor of ADT

patients are unable to eradicate the remaining tumor cells due to activation and maturation defects. As previously stated, it would thus be advantageous to analyze in greater details the activation status of the T lymphocyte and APC populations infiltrating the prostate of ADT patients to identify which mechanisms fails. To address this, flow cytometry analyses using fresh clinical samples (detailed phenotypic characterization) in combination with immunohistochemistry (*in situ* localization) would provide a precise characterization of the immune infiltrate.

Finally, as previously stated, the macrophage population infiltrating the post-ADT prostate microenvironment should be more closely evaluated. Since ADT causes the apoptosis of hormone sensitive prostate epithelial cells, it is proposed that an elevated number of macrophages are recruited to the prostate in order to phagocytose apoptotic cells. Macrophages are attracted to apoptotic cells following to release of chemoatractant, such as the ribosomal protein S19 (464) phospholipids lysophosphatidylcholine (LPC) (465) and CX3CL1/fractalkine (466). Phagocytosis of apoptotic bodies by macrophages temporally enhances TAA presentation to T lymphocytes and could be associated with the increased T lymphocyte density (439). However, following phagocytosis, macrophages also secrete elevated quantities of anti-inflammatory molecules, such as TGF-β, PGE_2, platelet-activating facvtor and IL-10 (467-470), which hinders the development of T lymphocyte effector functions. There is also evidence that ADT causes an increase in the number of circulating HLA-DR^{low} monocytes (163). HLA-DR^{low} monocytes are documented to have immunosuppressive function through the production of IL-10 and TGF-β as well as through the inhibition of DC differentiation and T lymphocyte proliferation (163). Finally, although the abundance of $Foxp3^+$ T lymphocytes was not increased in the primary tumors of ADT patients, it is conceivable that the suppressive functions of $Foxp3^+$ T_{REGs} are increased by a local TGF-β production by tumor-associated macrophages. *Ex vivo* functional assays on the immunosuppressive potential of these cells in prostate cancer patients should be evaluated. Altogether, there is accumulating evidence regarding the immunosuppressive potential of TAMs, which raises the

possibility that, similar to the removal of Foxp3$^+$ T_{REGs}, TAMs depletion may be beneficial to improve the efficacy of immunotherapies.

6.3 ANDROGEN REGULATED IMMUNOSUPPRESSION THROUGH ARGINASE EXPRESSION

The previous section discussed the immunoregulatory impact of ADT on the prostate microenvironment. From a cellular point of view, ADT promotes as significant increase in the infiltration of T lymphocytes and macrophages within the prostate. Several non-exclusive pathways may be involved in this elevated immune cell density that follows ADT. Already mentioned is the fact that the apoptosis of hormone sensitive prostate epithelial cells caused by ADT may induce the recruitment of macrophages. These macrophages phagocytose apoptotic tumor cells thereby increasing TAAs presentation to T lymphocytes and possibly cause their elevated influx within the prostate. Three other androgen-regulated pathways could also be associated with the post-ADT inflammatory boost: (i) an increased thymic output, (ii) the elimination of an androgen-regulated immunosuppressive network based on the direct immunosuppressive action of androgens on immune cells and (iii) on the expression of immunosuppressive molecules by prostate cancer cells. Concerning thymic atrophy, as previously stated, medical castration reverses age-related thymic atrophy, promotes thymopoiesis and increases the pool of naïve T lymphocytes. LHRH agonists also increase the number of lymphoid and myeloid progenitors in the bone marrow (471). We did not test this hypothesis and it cannot be ruled out as a possible cause of the increase in T lymphocyte and macrophage density within the prostate following ADT.

Concerning the direct immunosuppressive action of androgens on immune cells, we did begin preliminary work using PBMCs and macrophages from healthy donors. We stimulated PBMCs with anti-CD3 (OKT3) in the presence of 10 nM of R1881 (methyltienolone, a synthetic analog of testosterone) and evaluated their proliferation by BrdU incorporation and cytokine production by ELISA (Supplementary Figure 1 on page ii). We observe that, compared to PBMCs activated

in control charcoal-stripped serum supplemented media, PBMCs exposed to R1881 had decreased proliferation and decreased IFN-γ secretion. This data suggest that exposure to androgens inhibits T lymphocyte proliferation and activation. In another study, we evaluated the suppressive potential of macrophages exposed to androgens (Supplementary Figure 2 on page iv). We designed an *in vitro* mixed-lymphocyte reaction (MLR) system allowing for the physiological activation of $CD4^+$ T lymphocytes. For this assay, blood monocytes from a healthy donor were differentiated into macrophages for five days in the presence of 10 nM of R1881 or control media (charcoal-stripped serum supplemented media). $CD4^+$ T lymphocytes from a second healthy donor were than added to the macrophage cultures. After an additional five-day incubation period, proliferation was measured by BrdU incorporation. Our results demonstrate that macrophages derived in the presence of androgens induce significantly lower $CD4^+$ T lymphocyte proliferation. These data suggest that androgens render macrophages less apt to activate $CD4^+$ T lymphocytes possibly through the development of androgen-regulated immunosuppressive functions. Further work is necessary to identify the androgen-regulated molecular machinery rendering macrophage more immunosuppressive. ELISAs against IFN-γ, IL-2 and IL-10 did not demonstrate variations in cytokine production between the various culture conditions suggesting that other pathways could be involved. Lastly, we evaluated the expression of the iAR by monocyte-derived macrophages (Supplementary Figure 3 on page v). We did not detect protein expression of the classical iAR in the macrophages populations tested supporting previous report that macrophages respond to androgens by non-genomic signaling or through non-classical AR. Overall, these preliminary results using human immune cells from healthy donors suggest that androgens can reduce T lymphocytes proliferation and activation as well as decrease the activating potential of macrophages.

In parallel, androgens could also positively regulate the expression of immunosuppressive molecules by prostate cancer cells. The decision to study the expression of immunosuppressive molecules by prostate cancer epithelial cells was based on our previous results and on experimental model consideration. From our study on the immunological characterization of tumor draining LNs in prostate

cancer patients, we observed that metastatic LNs were more immunosuppressed than non-metastatic LNs. We hypothesized that metastatic prostate cancer cells could have direct immunosuppressive effects in tumor draining LNs. As such, we wanted to further understand the contribution of the tumor cells to the tumor's immunosuppressive microenvironment. In terms of the choice of the cell line studied, we decided to use prostate cancer hormone sensitive cell lines (LNCaP and 22rv1) for our *in vitro* work. It is probable that stromal cells are important players in the androgen-regulated immunosuppressive microenvironment. As a source of several growth factors, it would have been interesting to characterize the immunoregulatory potential of prostatic stromal cells in the presence and absence of androgens. We tried to obtain one of the only prostate stroma cell lines described in the literature without success (472). The contribution of stromal cells to the prostate's immunological microenvironment should nonetheless be further studied. To compliment our limitation regarding the number of cell lines in our *in vitro* experiments, we evaluated protein expression in prostate tissue micro-arrays by immunohistochemistry. From our immunohistochemistry observation, we observed that ARG2 expression was restricted to prostate epithelial cells and was absent from the stroma thereby supporting the use of prostate epithelial cells in our *in vitro* studies.

Initially, our work began with an extensive literature review. We established a list of possible candidates described to be expressed by prostate cancer cells and to have documented immunosuppressive properties. Using real-time PCRs and Western blots, we evaluated whether androgen stimulation increased a given candidate's gene and protein expression. This approach led us to discover the androgen-regulated expression of ARG1, ARG2 and IL-8 (discussed further below). We also discovered that the COX-2 – PGES – PGE_2 pathway was also induced by androgens (Supplementary Figure 4 on page vi). This finding is of interest since COX-2 is regarded as a major inflammatory player in the prostate. Furthermore, PGE_2 is associated with the regulation of ARG1 expression in murine macrophage (312) and in human MDSCs in cancer patients (170). When LNCaP cells were stimulated with 10 nM R1881, *COX-2* gene expression was significantly induced in conjunction with *MPGES-1*. As previously described in section 1.3.4.4 on page 55, only *MPGES-1,* and

not *MPGES-2* or *CPGES*, demonstrates an inducible expression during inflammation. This work is preliminary and needs to be validated through Western blots and ELISA in order to evaluate if the increased expression of COX-2 and mPGES-1 leads to elevated PGE_2 secretion.

Our approach also led to the observation that *TGF-β* expression by prostate cancer cells was also induced by androgens, which supports previously published data (473). A report in the literature also suggests that androgens, in this case DHT, inhibit TGF-β signaling by downregulating the expression of TGF-βRII in PC3 cells thereby protecting the cells from the pro-apoptotic action of TGF-β (474). Altogether, this preliminary work supports the hypothesis that androgens may regulate the prostate's immunosuppressive microenvironment by inducing the expression of several immunosuppressive candidates.

Our results presented in Chapter V further demonstrate that ARG1 and ARG2 expressions are induced by androgens. *In vitro*, androgen stimulated LNCaP cells express higher levels of both ARG1 and ARG2 proteins. The androgen-regulated expression of ARG2 was also observed in a cohort of 75 prostate cancer patients. Prostate cancer patients treated by ADT prior to surgery expressed significantly lower ARG2 levels in non-malignant and malignant tissues than control patients treated by surgery only. This result is interesting as, on one hand, it implies that the absence of androgens causes a reduction of ARG2 expression. Our *in vitro* data supports such a hypothesis as LNCaP cells cultivated in androgen-deprived media had lower basal ARG2 expression. On the other hand, there is the possibility that ADT eliminates most of the hormone-sensitive, ARG2 expressing cells. This idea is also consistent with our observation that hormone-refractory prostate cancer cells do not express ARG2. Furthermore, by considering the essential roles of androgens during the prostate's organogenesis and the earlier stages of carcinogenesis, it is plausible that androgen-regulated immunosuppressive molecules are also expressed prior to neoplastic transformation. This was in fact the case for ARG2 expression in prostate cancer patients. We observed a stronger ARG2 expression in normal and non-malignant prostate tissues compared to malignant and hormone-refractory specimens.

Altogether, these results suggest that in the case that ARG2 should be targeted during an immunotherapy, patients with hormone-sensitive disease may respond better than patients with advance HRPC. Further work needs to be initiated to evaluate the expression of ARG1 by immunohistochemistry in pathological prostate specimens.

We also noticed clear differences between ARG1 and ARG2 expression in our *in vitro* model. For ARG2, the increased protein expression correlated with an augmented *ARG2* gene expression. However, ARG1 protein up-regulation was not associated with an increase in *ARG1* gene expression. As previously discussed in Chapter V, data from the literature suggest that ARG1 expression is subject to post-transcriptional regulation. Our data suggests that these post-transcriptional modifications would be influenced by the presence of androgens. Furthermore, during promoter analysis studies, we noted that the proximal promoter regions of *ARG1* contained only two putative AR response elements (AREs) (at -849bp and $^{+}$301bp relative to the initiation site), whereas the proximal promoter of *ARG2* contained four AREs (at -99bp, -75bp, -49bp and -25bp relative to the initiation site). There is the possibility that the *ARG1* promoter does not respond, or respond poorly, to androgen. We are currently evaluating the contribution of these ARE to the regulation of *ARG1* and *ARG2* expression by promoter deletion studies. Altogether, these results highlight differential regulatory pathways leading to the androgen-regulated expression between ARG1 and ARG2.

The physiological effects of ARG1 and ARG2 expression by prostate cancer cell were evaluated. Inhibition of ARG1 and ARG2 protein expression by siRNA caused a decrease in LNCaP cell proliferation, which may be attributed to a reduction in polyamine synthesis. Similar to our results, inhibition of ODC (a key enzyme involved in polyamine synthesis) in LNCaP cells (475) and PC3 cells (476) caused a reduction of polyamine production and a growth arrest in G1. This growth arrest could be rescued by the addition of exogenous putrescine. HPLC analyses offered a second proof that ARG1 and ARG2 were enzymatically active. Following protein inhibition, we observed an elevated concentration of L-arginine in the conditioned media. From our results, it is however difficult to determine whether ARG1 or ARG2 has a more potent L-arginine metabolizing activity in LNCaP cells. Finally, we

demonstrated that ARG2 participated in the immunosuppressive potential of LNCaP cells. The inhibition of ARG2 expression was associated with an elevated PBMC proliferation and IFN-γ secretion, which is associated with increased lymphocyte activation. These results confirm previously published data on the immunosuppressive role of arginase in prostate cancer. Altogether, these results demonstrate that LNCaP cells express enzymatically active ARG1 and ARG2. It is noteworthy that we are the first group to demonstrate the expression of ARG1 by human cancer cells.

As several publications have linked T_H1 and T_H2 cytokines with the expression of ARG1 and ARG2, we characterized the cytokine expression profile of LNCaP cells exposed to androgens. We observed that LNCaP cells expressed a limited number of cytokines, none of which were initially associated with arginase expression. We identified two molecules upregulated following androgen stimulation: Serpin E1 and IL-8. Serpin E1, also known as plasminogen activator inhibitor-1, is the primary physiologic inhibitor of plasminogen activation to plasmin (477). Plasmin is a serine proteinase that digests fibrin, fibronectin and laminin in the extracellular matrix and activates other matrix-degrading proteinases, such as matrix metalloproteinases (MMPs). An elevated Serpin E1 would thus be protective against tumor invasion. Paradoxically, Serpin E1 is however associated with a worse prognosis in several cancers including prostate cancer (478), breast cancer (479) and glioblastomas (480). In colon cancer patients, Serpin E1 is overexpressed at the invasive front of the tumor (481) and by the peripheral cells of colon cancer liver metastasis (482). Moreover, the absence of Serpin E1 is associated with impaired tumor vascularization (483, 484). From these results, it was proposed that Serpin E1 could protect the tumor from excessive extracellular matrix degradation. Serpin E1 can also protect endothelial cells from FasL mediated apoptosis caused by the pro-apoptotic cleavage of FasL following excessive plasmin activity (484). In terms of the androgen regulation of Serpin E1, two studies found that prostate cancer patients receiving ADT had no changes in Serpin E1 plasma levels (485, 486) This suggests that our result is either an artifact of *in vitro* studies on prostate cancer cell lines or that the elevated secretion of Serpin E1 by prostate cancer cells does not reach the systemic

circulation. Interestingly, IL-8 was found to upregulate the expression of Serpin E1 in human umbilical vein endothelial cells (HUVECs) (487). Conversely, Serpin E1 stabilizes the chemoattractant form of IL-8 at the cell surface of HUVECs (488). Altogether, Serpin E1 may represent an interesting candidate involved in prostate cancer progression.

IL-8, or CXCL8, is a pro-inflammatory T_H1 cytokine implicated in the recruitment of neutrophils. In cancer, IL-8 is associated with tumor angiogenesis, metastasis and poor prognosis (489). In prostate cancer, IL-8 overexpression results in prostate epithelial hyperplasia and a reactive stroma phenotype (490). Serum levels of IL-8 are elevated in metastatic prostate cancer patients (491) and in patients with hormone refractory tumors (52). In mouse models, overexpression of IL-8 correlates with elevated angiogenesis and the development of LN metastasis (120, 492). As for the sexual hormone regulation of IL-8 expression, published data are conflicting. Estrogen inhibits IL-8 expression by epithelial cells (493), but favors IL-8 secretion by monocyte-derived DCs (494). There is also a positive correlation between estradiol and IL-8 secretion in the normal breast tissue *in vivo* (495). We are the first group to report that androgens increase the expression of IL-8. In terms of the association between IL-8 and arginase expression, one study demonstrates that IL-8 secretion by non-small cell lung tumor cells induces the exocytosis of ARG1 by neutrophils (496). Although we observed an increased of *ARG2* and not *ARG1* gene expression following IL-8 stimulation of LNCaP cells, this study does nonetheless provide a second example of arginase regulation through IL-8. It would be of interest to further study the signaling pathways activated by IL-8 leading to arginase expression and determine whether they are similar to those involved in androgen signaling.

Finally, the contribution of estrogens in the sculpting of the prostate's immunological environment was not studied. It would nevertheless be interesting to evaluate the impact of estrogen on the expression of pro- and anti-inflammatory molecules by prostate cancer cells. The incidence of prostate cancer increases with age when there is a parallel decrease in testosterone levels (497). Conversely, estrogen levels remain unchanged with age through increased aromatization of adrenal androgens in the adipose tissue (498), which also increases in older male. Hence, the

ratio of androgens to estrogens decreases and is related to the onset of prostate cancer (367). Furthermore, as detailed previously, contrary to the immunosuppressive functions of androgens, estrogens induce prostatic inflammation. In a mouse model overexpresssing the aromatase gene, the prostate develops normally, but the tissue shows extensive inflammation (367, 499). The pro-inflammatory impact of estrogens is also independent of local androgens as mouse models with low levels (gonadotropin-deficient hypogonadal mouse (500)) or high levels (aromatase deficient mouse (501)) of androgens show similar pro-inflammatory responses to estrogens (502). The roles of estrogens should thus be evaluated on the activation/maturation status of immune cells from prostate cancer patients as well as on the development of an immunosuppressive microenvironment within the prostate.

CONCLUSION

Immunotherapy is now at the doorstep of the urologist's office. To improve its clinical efficacy, more knowledge needs to be gained on the immunological status of prostate cancer patients. The fundamental goal of this doctoral thesis was thus to study the immunological microenvironment in prostate cancer patients. To realize this project we established two main working objectives: (i) to precisely characterize the immune cell populations in tumor draining LNs and in the primary tumor of prostate cancer patients; (ii) to identify and to study the immunosuppressive pathways induced by prostate cancer cells.

For the first objectives, we developed novel software-based approaches allowing for the precise quantification of immune cell density within large pathological samples. We hope that our approach will standardize the reporting of immune cell abundance therefore facilitating the interpretation of independent studies. The results from our first immunohistochemical study argue for a direct association between lymphatic immunosuppression and disease progression. This study also raises the question as to whether patients with LN metastasis would be less responsive to immunotherapy. In our second study, we demonstrate that ADT induces the specific infiltration of T lymphocytes and macrophages within the primary tumor. Preliminary work also suggests that T lymphocytes and macrophages are sensitive to androgens *in vitro*. Further work should focus on the androgenic regulation of T lymphocyte activation as well as on the immunoregulatory functions of human macrophages in the presence of androgens.

For the second objective, we wanted to determine which factors were implicated in the unique immunological microenvironment of the prostate. As such, we studied the regulation by androgens of immunosuppressive molecules expressed by prostate cancer cells. Our results demonstrate that androgens regulate the expression of ARG1, ARG2 and IL-8 suggesting that hormone sensitive prostate cancer cells are directly involved in the development of an immunosuppressive tumor bed.

In conclusion, this doctoral thesis explored important questions to improve the treatment of prostate cancer by working with clinical samples and by trying to answer present clinical priorities. Our work proposes several long-term clinical and fundamental perspectives. Clinically, the immunological status of prostate cancer patients could be evaluated prior to administrating immunotherapy as some patients may have a strong immunosuppressive microenvironment preventing the activation of anti-tumoral response. Moreover, the quantification of immune cell density may offer novel prognosis markers for prostate cancer progression. Furthermore, arginase may represent only one of several immunosuppressive molecules positively regulated by the presence of androgens. Specifically, the immunosuppressive potential of HRPC cells should be evaluated, as it is for this stage of the disease that novel curative therapies are strongly needed. Discovery-based research of novel strategies that block tumor-driven immunosuppression will certainly improve the clinical efficacy of immunotherapies. Finally, integrative collaborative work between clinicians, pathology department and fundamental researchers is necessary in order to better understand to disease and provide effective novel therapy to prostate cancer patients.

REFERENCES

1. Canadian Cancer Society, National Cancer Institute of Canada. Canadian Cancer Statistics 2008. 2009.
2. Public Health Agency of Canada, Statistics Canada, Canadian Institute for Health Information. Leading Causes of Death and Hospitalization in Canada. http://wwwphac-aspcgcca/publicat/lcd-pcd97/index-engphp 2008.
3. Jemal A, Clegg LX, Ward E, et al. Annual report to the nation on the status of cancer, 1975-2001, with a special feature regarding survival. Cancer 2004;101:3-27.
4. Costello LC, Franklin RB. Prostatic fluid electrolyte composition for the screening of prostate cancer: a potential solution to a major problem. Prostate Cancer Prostatic Dis 2009;12:17-24.
5. Thomson AA, Marker PC. Branching morphogenesis in the prostate gland and seminal vesicles. Differentiation 2006;74:382-92.
6. De Marzo AM, Platz EA, Sutcliffe S, et al. Inflammation in prostate carcinogenesis. Nat Rev Cancer 2007;7:256-69.
7. Haverkamp J, Charbonneau B, Ratliff TL. Prostate inflammation and its potential impact on prostate cancer: a current review. J Cell Biochem 2008;103:1344-53.
8. Palapattu GS, Sutcliffe S, Bastian PJ, et al. Prostate carcinogenesis and inflammation: emerging insights. Carcinogenesis 2005;26:1170-81.
9. Hua VN, Schaeffer AJ. Acute and chronic prostatitis. Med Clin North Am 2004;88:483-94.
10. De Marzo AM, Marchi VL, Epstein JI, Nelson WG. Proliferative inflammatory atrophy of the prostate: implications for prostatic carcinogenesis. Am J Pathol 1999;155:1985-92.
11. Carter HB, Coffey DS. The prostate: an increasing medical problem. Prostate 1990;16:39-48.

12. Sakr WA, Grignon DJ, Haas GP, Heilbrun LK, Pontes JE, Crissman JD. Age and racial distribution of prostatic intraepithelial neoplasia. Eur Urol 1996;30:138-44.
13. Damber JE, Aus G. Prostate cancer. Lancet 2008;371:1710-21.
14. Neill MG, Fleshner NE. An update on chemoprevention strategies in prostate cancer for 2006. Curr Opin Urol 2006;16:132-7.
15. Nelson WG, De Marzo AM, Isaacs WB. Prostate cancer. N Engl J Med 2003;349:366-81.
16. Langeberg WJ, Isaacs WB, Stanford JL. Genetic etiology of hereditary prostate cancer. Front Biosci 2007;12:4101-10.
17. Schaid DJ. The complex genetic epidemiology of prostate cancer. Hum Mol Genet 2004;13 Spec No 1:R103-21.
18. Hsing AW, Tsao L, Devesa SS. International trends and patterns of prostate cancer incidence and mortality. Int J Cancer 2000;85:60-7.
19. Mettlin CJ, Murphy GP, Ho R, Menck HR. The National Cancer Data Base report on longitudinal observations on prostate cancer. Cancer 1996;77:2162-6.
20. Morgan TO, Jacobsen SJ, McCarthy WF, Jacobson DJ, McLeod DG, Moul JW. Age-specific reference ranges for prostate-specific antigen in black men. N Engl J Med 1996;335:304-10.
21. Moul JW, Sesterhenn IA, Connelly RR, et al. Prostate-specific antigen values at the time of prostate cancer diagnosis in African-American men. JAMA 1995;274:1277-81.
22. Chu LW, Reichardt JK, Hsing AW. Androgens and the molecular epidemiology of prostate cancer. Curr Opin Endocrinol Diabetes Obes 2008;15:261-70.
23. Grino PB, Griffin JE, Wilson JD. Testosterone at high concentrations interacts with the human androgen receptor similarly to dihydrotestosterone. Endocrinology 1990;126:1165-72.
24. Thompson IM, Goodman PJ, Tangen CM, et al. The influence of finasteride on the development of prostate cancer. N Engl J Med 2003;349:215-24.
25. Sarvis JA, Thompson IM. Androgens and prevention of prostate cancer. Curr Opin Endocrinol Diabetes Obes 2008;15:271-7.

26. Coffey DS. Similarities of prostate and breast cancer: Evolution, diet, and estrogens. Urology 2001;57:31-8.
27. Harkonen PL, Makela SI. Role of estrogens in development of prostate cancer. J Steroid Biochem Mol Biol 2004;92:297-305.
28. Dennis LK, Lynch CF, Torner JC. Epidemiologic association between prostatitis and prostate cancer. Urology 2002;60:78-83.
29. Smith MJ. The lymphatics of the prostate. Invest Urol 1966;3:439-44.
30. Gittes RF, McCullough DL. Occult carcinoma of the prostate: an oversight of immune surveillance--a working hypothesis. J Urol 1974;112:241-4.
31. Mercader M, Bodner BK, Moser MT, et al. T cell infiltration of the prostate induced by androgen withdrawal in patients with prostate cancer. Proc Natl Acad Sci U S A 2001;98:14565-70.
32. Roden AC, Moser MT, Tri SD, et al. Augmentation of T cell levels and responses induced by androgen deprivation. J Immunol 2004;173:6098-108.
33. Steiner GE, Newman ME, Paikl D, et al. Expression and function of pro-inflammatory interleukin IL-17 and IL-17 receptor in normal, benign hyperplastic, and malignant prostate. Prostate 2003;56:171-82.
34. Elkahwaji JE, Zhong W, Hopkins WJ, Bushman W. Chronic bacterial infection and inflammation incite reactive hyperplasia in a mouse model of chronic prostatitis. Prostate 2007;67:14-21.
35. Schlaberga R, Choeb DJ, Browna KR, Thakerb HM, Singha IR. XMRV is present in malignant prostatic epithelium and is associated with prostate cancer, especially high-grade tumors. Proc Natl Acad Sci U S A 2009.
36. Gilleran JP, Putz O, DeJong M, et al. The role of prolactin in the prostatic inflammatory response to neonatal estrogen. Endocrinology 2003;144:2046-54.
37. MacLennan GT, Eisenberg R, Fleshman RL, et al. The influence of chronic inflammation in prostatic carcinogenesis: a 5-year followup study. J Urol 2006;176:1012-6.
38. Sutcliffe S, Giovannucci E, De Marzo AM, Leitzmann MF, Willett WC, Platz EA. Gonorrhea, syphilis, clinical prostatitis, and the risk of prostate cancer. Cancer Epidemiol Biomarkers Prev 2006;15:2160-6.

39. Irani J, Goujon JM, Ragni E, et al. High-grade inflammation in prostate cancer as a prognostic factor for biochemical recurrence after radical prostatectomy. Pathologist Multi Center Study Group. Urology 1999;54:467-72.
40. McArdle PA, Canna K, McMillan DC, McNicol AM, Campbell R, Underwood MA. The relationship between T-lymphocyte subset infiltration and survival in patients with prostate cancer. Br J Cancer 2004;91:541-3.
41. Sari A, Serel TA, Candir O, Ozturk A, Kosar A. Mast cell variations in tumour tissue and with histopathological grading in specimens of prostatic adenocarcinoma. BJU Int 1999;84:851-3.
42. Karja V, Aaltomaa S, Lipponen P, Isotalo T, Talja M, Mokka R. Tumour-infiltrating lymphocytes: A prognostic factor of PSA-free survival in patients with local prostate carcinoma treated by radical prostatectomy. Anticancer Res 2005;25:4435-8.
43. Vesalainen S, Lipponen P, Talja M, Syrjanen K. Histological grade, perineural infiltration, tumour-infiltrating lymphocytes and apoptosis as determinants of long-term prognosis in prostatic adenocarcinoma. Eur J Cancer 1994;30A:1797-803.
44. Ebelt K, Babaryka G, Figel AM, et al. Dominance of CD4+ lymphocytic infiltrates with disturbed effector cell characteristics in the tumor microenvironment of prostate carcinoma. Prostate 2008;68:1-10.
45. Getnet D, Maris CH, Hipkiss EL, et al. Tumor recognition and self-recognition induce distinct transcriptional profiles in antigen-specific CD4 T cells. J Immunol 2009;182:4675-85.
46. Ebelt K, Babaryka G, Frankenberger B, et al. Prostate cancer lesions are surrounded by FOXP3+, PD-1+ and B7-H1+ lymphocyte clusters. Eur J Cancer 2009;45:1664-72.
47. Yokokawa J, Cereda V, Remondo C, et al. Enhanced functionality of CD4+CD25(high)FoxP3+ regulatory T cells in the peripheral blood of patients with prostate cancer. Clin Cancer Res 2008;14:1032-40.

48. Miller AM, Lundberg K, Ozenci V, et al. CD4+CD25high T cells are enriched in the tumor and peripheral blood of prostate cancer patients. J Immunol 2006;177:7398-405.
49. Gannon PO, Poisson AO, Delvoye N, Lapointe R, Mes-Masson AM, Saad F. Characterization of the intra-prostatic immune cell infiltration in androgen-deprived prostate cancer patients. J Immunol Methods 2009;348:9-17.
50. Sfanos KS, Bruno TC, Maris CH, et al. Phenotypic analysis of prostate-infiltrating lymphocytes reveals TH17 and Treg skewing. Clin Cancer Res 2008;14:3254-61.
51. Michalaki V, Syrigos K, Charles P, Waxman J. Serum levels of IL-6 and TNF-alpha correlate with clinicopathological features and patient survival in patients with prostate cancer. Br J Cancer 2004;90:2312-6.
52. Uehara H, Troncoso P, Johnston D, et al. Expression of interleukin-8 gene in radical prostatectomy specimens is associated with advanced pathologic stage. Prostate 2005;64:40-9.
53. Poutahidis T, Rao VP, Olipitz W, et al. CD4+ lymphocytes modulate prostate cancer progression in mice. Int J Cancer 2009.
54. Denmeade SR, Isaacs JT. A history of prostate cancer treatment. Nat Rev Cancer 2002;2:389-96.
55. Moul JW. The evolving definition of advanced prostate cancer. Rev Urol 2004;6 Suppl 8:S10-7.
56. Oefelein MG, Agarwal PK, Resnick MI. Survival of patients with hormone refractory prostate cancer in the prostate specific antigen era. J Urol 2004;171:1525-8.
57. Bill-Axelson A, Holmberg L, Ruutu M, et al. Radical prostatectomy versus watchful waiting in early prostate cancer. N Engl J Med 2005;352:1977-84.
58. Huggins C, Hodges C. The effect of castration of estrogen and androgen injection on serum phosphatases in metastatic carcinoma of the prostate. Cancer Res 1941;1:93-7.
59. Montironi R, Schulman CC. Pathological changes in prostate lesions after androgen manipulation. J Clin Pathol 1998;51:5-12.

60. Berthold DR, Sternberg CN, Tannock IF. Management of advanced prostate cancer after first-line chemotherapy. J Clin Oncol 2005;23:8247-52.
61. Petrylak DP. Docetaxel-based chemotherapy trials in androgen-independent prostate cancer: first demonstration of a survival benefit. Curr Oncol Rep 2005;7:205-6.
62. Bolla M, Gonzalez D, Warde P, et al. Improved survival in patients with locally advanced prostate cancer treated with radiotherapy and goserelin. N Engl J Med 1997;337:295-300.
63. Messing EM, Manola J, Sarosdy M, Wilding G, Crawford ED, Trump D. Immediate hormonal therapy compared with observation after radical prostatectomy and pelvic lymphadenectomy in men with node-positive prostate cancer. N Engl J Med 1999;341:1781-8.
64. Lucas A, Petrylak DP. The case for early chemotherapy for the treatment of metastatic disease. J Urol 2006;176:S72-5.
65. Tannock IF, de Wit R, Berry WR, et al. Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. N Engl J Med 2004;351:1502-12.
66. Petrylak DP, Tangen CM, Hussain MH, et al. Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer. N Engl J Med 2004;351:1513-20.
67. Berthold DR, Pond GR, Soban F, de Wit R, Eisenberger M, Tannock IF. Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer: updated survival in the TAX 327 study. J Clin Oncol 2008;26:242-5.
68. Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy: moving beyond current vaccines. Nat Med 2004;10:909-15.
69. Drake CG, Jaffee E, Pardoll DM. Mechanisms of immune evasion by tumors. Adv Immunol 2006;90:51-81.
70. Rhodes DR, Barrette TR, Rubin MA, Ghosh D, Chinnaiyan AM. Meta-analysis of microarrays: interstudy validation of gene expression profiles reveals pathway dysregulation in prostate cancer. Cancer Res 2002;62:4427-33.

71. Taylor BS, Varambally S, Chinnaiyan AM. Differential proteomic alterations between localised and metastatic prostate cancer. Br J Cancer 2006;95:425-30.
72. Lilja H. A kallikrein-like serine protease in prostatic fluid cleaves the predominant seminal vesicle protein. J Clin Invest 1985;76:1899-903.
73. Lilja H, Ulmert D, Vickers AJ. Prostate-specific antigen and prostate cancer: prediction, detection and monitoring. Nat Rev Cancer 2008;8:268-78.
74. Qiu SD, Young CY, Bilhartz DL, et al. In situ hybridization of prostate-specific antigen mRNA in human prostate. J Urol 1990;144:1550-6.
75. Klyushnenkova EN, Ponniah S, Rodriguez A, et al. CD4 and CD8 T-lymphocyte recognition of prostate specific antigen in granulomatous prostatitis. J Immunother 2004;27:136-46.
76. Elkord E, Rowbottom AW, Kynaston H, Williams PE. Correlation between CD8+ T cells specific for prostate-specific antigen and level of disease in patients with prostate cancer. Clin Immunol 2006;120:91-8.
77. Kiessling A, Schmitz M, Stevanovic S, et al. Prostate stem cell antigen: Identification of immunogenic peptides and assessment of reactive CD8+ T cells in prostate cancer patients. Int J Cancer 2002;102:390-7.
78. Peshwa MV, Shi JD, Ruegg C, Laus R, van Schooten WC. Induction of prostate tumor-specific CD8+ cytotoxic T-lymphocytes in vitro using antigen-presenting cells pulsed with prostatic acid phosphatase peptide. Prostate 1998;36:129-38.
79. Vihko P, Virkkunen P, Henttu P, Roiko K, Solin T, Huhtala ML. Molecular cloning and sequence analysis of cDNA encoding human prostatic acid phosphatase. FEBS Lett 1988;236:275-81.
80. Patel PH, Kockler DR. Sipuleucel-T: a vaccine for metastatic, asymptomatic, androgen-independent prostate cancer. Ann Pharmacother 2008;42:91-8.
81. Waller EK. The role of sargramostim (rhGM-CSF) as immunotherapy. Oncologist 2007;12 Suppl 2:22-6.
82. Small EJ, Schellhammer PF, Higano CS, et al. Placebo-controlled phase III trial of immunologic therapy with sipuleucel-T (APC8015) in patients with

metastatic, asymptomatic hormone refractory prostate cancer. J Clin Oncol 2006;24:3089-94.

83. Chang SS, Kibel AS. The role of systemic cytotoxic therapy for prostate cancer. BJU Int 2009;103:8-17.
84. Simons JW, Sacks N. Granulocyte-macrophage colony-stimulating factor-transduced allogeneic cancer cellular immunotherapy: the GVAX vaccine for prostate cancer. Urol Oncol 2006;24:419-24.
85. Fong L, Small EJ. Immunotherapy for prostate cancer. Semin Oncol 2003;30:649-58.
86. Kantoff PW, Schuetz TJ, Blumenstein BA, et al. Overall survival analysis of a phase II randomized controlled trial of a Poxviral-based PSA-targeted immunotherapy in metastatic castration-resistant prostate cancer. J Clin Oncol;28:1099-105.
87. Small EJ, Tchekmedyian NS, Rini BI, Fong L, Lowy I, Allison JP. A pilot trial of CTLA-4 blockade with human anti-CTLA-4 in patients with hormone-refractory prostate cancer. Clin Cancer Res 2007;13:1810-5.
88. Fong L, Kwek SS, O'Brien S, et al. Potentiating endogenous antitumor immunity to prostate cancer through combination immunotherapy with CTLA4 blockade and GM-CSF. Cancer Res 2009;69:609-15.
89. Gulley JL, Arlen PM, Bastian A, et al. Combining a recombinant cancer vaccine with standard definitive radiotherapy in patients with localized prostate cancer. Clin Cancer Res 2005;11:3353-62.
90. Sharp HJ, Wansley EK, Garnett CT, et al. Synergistic antitumor activity of immune strategies combined with radiation. Front Biosci 2007;12:4900-10.
91. Gelbard A, Garnett CT, Abrams SI, et al. Combination chemotherapy and radiation of human squamous cell carcinoma of the head and neck augments CTL-mediated lysis. Clin Cancer Res 2006;12:1897-905.
92. Emens LA, Jaffee EM. Leveraging the activity of tumor vaccines with cytotoxic chemotherapy. Cancer Res 2005;65:8059-64.
93. Nowak AK, Lake RA, Robinson BW. Combined chemoimmunotherapy of solid tumours: improving vaccines? Adv Drug Deliv Rev 2006;58:975-90.

94. Wada S, Yoshimura K, Hipkiss EL, et al. Cyclophosphamide augments antitumor immunity: studies in an autochthonous prostate cancer model. Cancer Res 2009;69:4309-18.
95. Arlen PM, Gulley JL, Parker C, et al. A randomized phase II study of concurrent docetaxel plus vaccine versus vaccine alone in metastatic androgen-independent prostate cancer. Clin Cancer Res 2006;12:1260-9.
96. Arlen PM, Gulley JL, Todd N, et al. Antiandrogen, vaccine and combination therapy in patients with nonmetastatic hormone refractory prostate cancer. J Urol 2005;174:539-46.
97. Gray A, Raff AB, Chiriva-Internati M, Chen SY, Kast WM. A paradigm shift in therapeutic vaccination of cancer patients: the need to apply therapeutic vaccination strategies in the preventive setting. Immunol Rev 2008;222:316-27.
98. Koh YT, Gray A, Higgins SA, Hubby B, Kast WM. Androgen ablation augments prostate cancer vaccine immunogenicity only when applied after immunization. Prostate 2009.
99. Balch CM, Riley LB, Bae YJ, et al. Patterns of human tumor-infiltrating lymphocytes in 120 human cancers. Arch Surg 1990;125:200-5.
100. Mestas J, Hughes CC. Of mice and not men: differences between mouse and human immunology. J Immunol 2004;172:2731-8.
101. Dranoff G. Cytokines in cancer pathogenesis and cancer therapy. Nat Rev Cancer 2004;4:11-22.
102. Rock KL, Shen L. Cross-presentation: underlying mechanisms and role in immune surveillance. Immunol Rev 2005;207:166-83.
103. Preynat-Seauve O, Contassot E, Schuler P, Piguet V, French LE, Huard B. Extralymphatic tumors prepare draining lymph nodes to invasion via a T-cell cross-tolerance process. Cancer Res 2007;67:5009-16.
104. den Haan JM, Lehar SM, Bevan MJ. CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo. J Exp Med 2000;192:1685-96.
105. Belz GT, Behrens GM, Smith CM, et al. The CD8alpha(+) dendritic cell is responsible for inducing peripheral self-tolerance to tissue-associated antigens. J Exp Med 2002;196:1099-104.

106. Dudziak D, Kamphorst AO, Heidkamp GF, et al. Differential antigen processing by dendritic cell subsets in vivo. Science 2007;315:107-11.
107. Carey TE, Takahashi T, Resnick LA, Oettgen HF, Old LJ. Cell surface antigens of human malignant melanoma: mixed hemadsorption assays for humoral immunity to cultured autologous melanoma cells. Proc Natl Acad Sci U S A 1976;73:3278-82.
108. Knuth A, Danowski B, Oettgen HF, Old LJ. T-cell-mediated cytotoxicity against autologous malignant melanoma: analysis with interleukin 2-dependent T-cell cultures. Proc Natl Acad Sci U S A 1984;81:3511-5.
109. Wang RF, Rosenberg SA. Human tumor antigens for cancer vaccine development. Immunol Rev 1999;170:85-100.
110. de Visser KE, Eichten A, Coussens LM. Paradoxical roles of the immune system during cancer development. Nat Rev Cancer 2006;6:24-37.
111. Henson PM. Dampening inflammation. Nat Immunol 2005;6:1179-81.
112. Serhan CN. Resolution phase of inflammation: novel endogenous anti-inflammatory and proresolving lipid mediators and pathways. Annu Rev Immunol 2007;25:101-37.
113. Dvorak HF. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. N Engl J Med 1986;315:1650-9.
114. Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? Lancet 2001;357:539-45.
115. Chisari FV. Rous-Whipple Award Lecture. Viruses, immunity, and cancer: lessons from hepatitis B. Am J Pathol 2000;156:1117-32.
116. Langowski JL, Zhang X, Wu L, et al. IL-23 promotes tumour incidence and growth. Nature 2006;442:461-5.
117. Voronov E, Shouval DS, Krelin Y, et al. IL-1 is required for tumor invasiveness and angiogenesis. Proc Natl Acad Sci U S A 2003;100:2645-50.
118. Chung TD, Yu JJ, Spiotto MT, Bartkowski M, Simons JW. Characterization of the role of IL-6 in the progression of prostate cancer. Prostate 1999;38:199-207.

119. Culig Z, Steiner H, Bartsch G, Hobisch A. Interleukin-6 regulation of prostate cancer cell growth. J Cell Biochem 2005;95:497-505.
120. Inoue K, Slaton JW, Eve BY, et al. Interleukin 8 expression regulates tumorigenicity and metastases in androgen-independent prostate cancer. Clin Cancer Res 2000;6:2104-19.
121. de Waal Malefyt R, Haanen J, Spits H, et al. Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. J Exp Med 1991;174:915-24.
122. Howard M, O'Garra A. Biological properties of interleukin 10. Immunol Today 1992;13:198-200.
123. Delves PJ, Roitt IM. The immune system. Second of two parts. N Engl J Med 2000;343:108-17.
124. Yong KL, Linch DC. Granulocyte-macrophage-colony-stimulating factor differentially regulates neutrophil migration across IL-1-activated and nonactivated human endothelium. J Immunol 1993;150:2449-56.
125. Grote D, Cattaneo R, Fielding AK. Neutrophils contribute to the measles virus-induced antitumor effect: enhancement by granulocyte macrophage colony-stimulating factor expression. Cancer Res 2003;63:6463-8.
126. Astigiano S, Morandi B, Costa R, et al. Eosinophil granulocytes account for indoleamine 2,3-dioxygenase-mediated immune escape in human non-small cell lung cancer. Neoplasia 2005;7:390-6.
127. van Egmond M. Neutrophils in antibody-based immunotherapy of cancer. Expert Opin Biol Ther 2008;8:83-94.
128. Stoppacciaro A, Melani C, Parenza M, et al. Regression of an established tumor genetically modified to release granulocyte colony-stimulating factor requires granulocyte-T cell cooperation and T cell-produced interferon gamma. J Exp Med 1993;178:151-61.
129. Lozupone F, Luciani F, Venditti M, et al. Murine granulocytes control human tumor growth in SCID mice. Int J Cancer 2000;87:569-73.

130. Pericle F, Kirken RA, Epling-Burnette PK, Blanchard DK, Djeu JY. Direct killing of interleukin-2-transfected tumor cells by human neutrophils. Int J Cancer 1996;66:367-73.
131. Noffz G, Qin Z, Kopf M, Blankenstein T. Neutrophils but not eosinophils are involved in growth suppression of IL-4-secreting tumors. J Immunol 1998;160:345-50.
132. Morikawa K, Kamegaya S, Yamazaki M, Mizuno D. Hydrogen peroxide as a tumoricidal mediator of murine polymorphonuclear leukocytes induced by a linear beta-1,3-D-glucan and some other immunomodulators. Cancer Res 1985;45:3482-6.
133. Dallegri F, Ottonello L, Ballestrero A, et al. Tumor cell lysis by activated human neutrophils: analysis of neutrophil-delivered oxidative attack and role of leukocyte function-associated antigen 1. Inflammation 1991;15:15-30.
134. Learn DB, Thomas EL. Inhibition of tumor cell glutamine uptake by isolated neutrophils. J Clin Invest 1988;82:789-96.
135. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. Nat Immunol 2008;9:503-10.
136. Smyth MJ, Swann J, Cretney E, Zerafa N, Yokoyama WM, Hayakawa Y. NKG2D function protects the host from tumor initiation. J Exp Med 2005;202:583-8.
137. Street SE, Hayakawa Y, Zhan Y, et al. Innate immune surveillance of spontaneous B cell lymphomas by natural killer cells and gammadelta T cells. J Exp Med 2004;199:879-84.
138. Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. Nat Rev Immunol 2005;5:953-64.
139. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. Nat Rev Immunol 2008;8:958-69.
140. Martinez FO, Sica A, Mantovani A, Locati M. Macrophage activation and polarization. Front Biosci 2008;13:453-61.

141. Roca H, Varsos ZS, Sud S, Craig MJ, Ying C, Pienta KJ. CCL2 and interleukin-6 promote survival of human CD11b+ peripheral blood mononuclear cells and induce M2-type macrophage polarization. J Biol Chem 2009;284:34342-54.
142. Loke P, Gallagher I, Nair MG, et al. Alternative activation is an innate response to injury that requires CD4+ T cells to be sustained during chronic infection. J Immunol 2007;179:3926-36.
143. Brandt E, Woerly G, Younes AB, Loiseau S, Capron M. IL-4 production by human polymorphonuclear neutrophils. J Leukoc Biol 2000;68:125-30.
144. Sternberg EM. Neural regulation of innate immunity: a coordinated nonspecific host response to pathogens. Nat Rev Immunol 2006;6:318-28.
145. Franchimont D. Overview of the actions of glucocorticoids on the immune response: a good model to characterize new pathways of immunosuppression for new treatment strategies. Ann N Y Acad Sci 2004;1024:124-37.
146. Edwards JP, Zhang X, Frauwirth KA, Mosser DM. Biochemical and functional characterization of three activated macrophage populations. J Leukoc Biol 2006;80:1298-307.
147. Klimp AH, de Vries EG, Scherphof GL, Daemen T. A potential role of macrophage activation in the treatment of cancer. Crit Rev Oncol Hematol 2002;44:143-61.
148. Romieu-Mourez R, Solis M, Nardin A, et al. Distinct roles for IFN regulatory factor (IRF)-3 and IRF-7 in the activation of antitumor properties of human macrophages. Cancer Res 2006;66:10576-85.
149. Lin EY, Pollard JW. Tumor-associated macrophages press the angiogenic switch in breast cancer. Cancer Res 2007;67:5064-6.
150. Pollard JW. Macrophages define the invasive microenvironment in breast cancer. J Leukoc Biol 2008;84:623-30.
151. Condeelis J, Pollard JW. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. Cell 2006;124:263-6.
152. Coussens LM, Werb Z. Inflammation and cancer. Nature 2002;420:860-7.
153. Pollard JW. Tumour-educated macrophages promote tumour progression and metastasis. Nat Rev Cancer 2004;4:71-8.

154. Lissbrant IF, Stattin P, Wikstrom P, Damber JE, Egevad L, Bergh A. Tumor associated macrophages in human prostate cancer: relation to clinicopathological variables and survival. Int J Oncol 2000;17:445-51.
155. Loberg RD, Ying C, Craig M, Yan L, Snyder LA, Pienta KJ. CCL2 as an important mediator of prostate cancer growth in vivo through the regulation of macrophage infiltration. Neoplasia 2007;9:556-62.
156. Halin S, Rudolfsson SH, Van Rooijen N, Bergh A. Extratumoral macrophages promote tumor and vascular growth in an orthotopic rat prostate tumor model. Neoplasia 2009;11:177-86.
157. Shimura S, Yang G, Ebara S, Wheeler TM, Frolov A, Thompson TC. Reduced infiltration of tumor-associated macrophages in human prostate cancer: association with cancer progression. Cancer Res 2000;60:5857-61.
158. Steinman RM, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. J Exp Med 1973;137:1142-62.
159. Guery JC, Adorini L. Dendritic cells are the most efficient in presenting endogenous naturally processed self-epitopes to class II-restricted T cells. J Immunol 1995;154:536-44.
160. Steinman RM. The dendritic cell system and its role in immunogenicity. Annu Rev Immunol 1991;9:271-96.
161. Yang L, Carbone DP. Tumor-host immune interactions and dendritic cell dysfunction. Adv Cancer Res 2004;92:13-27.
162. Sciarra A, Lichtner M, Autran GA, et al. Characterization of circulating blood dendritic cell subsets DC123+ (lymphoid) and DC11C+ (myeloid) in prostate adenocarcinoma patients. Prostate 2007;67:1-7.
163. Vuk-Pavlovic S, Bulur PA, Lin Y, et al. Immunosuppressive CD14+HLA-DRlow/- monocytes in prostate cancer. Prostate;70:443-55.
164. Waeckerle-Men Y, Allmen EU, von Moos R, et al. Dendritic cells generated from patients with androgen-independent prostate cancer are not impaired in migration and T-cell stimulation. Prostate 2005.

165. Wilkinson R, Kassianos AJ, Swindle P, Hart DN, Radford KJ. Numerical and functional assessment of blood dendritic cells in prostate cancer patients. Prostate 2006;66:180-92.

166. Westphal E. Uber mastzellen (About Mast Cells). In Ehrlich P, ed Farbenanalytische Utersuchuugen Zur Histologie und Klinik des Plutes: Gesammelte Mitt(h)eilungen 1891;1:17.

167. Coussens LM, Raymond WW, Bergers G, et al. Inflammatory mast cells up-regulate angiogenesis during squamous epithelial carcinogenesis. Genes Dev 1999;13:1382-97.

168. Coussens LM, Werb Z. Inflammatory cells and cancer: think different! J Exp Med 2001;193:F23-6.

169. Bronte V, Zanovello P. Regulation of immune responses by L-arginine metabolism. Nat Rev Immunol 2005;5:641-54.

170. Ochoa AC, Zea AH, Hernandez C, Rodriguez PC. Arginase, prostaglandins, and myeloid-derived suppressor cells in renal cell carcinoma. Clin Cancer Res 2007;13:721s-6s.

171. Peranzoni E, Marigo I, Dolcetti L, et al. Role of arginine metabolism in immunity and immunopathology. Immunobiology 2007;212:795-812.

172. Serafini P, Borrello I, Bronte V. Myeloid suppressor cells in cancer: recruitment, phenotype, properties, and mechanisms of immune suppression. Semin Cancer Biol 2006;16:53-65.

173. Ciavarra RP, Holterman DA, Brown RR, et al. Prostate tumor microenvironment alters immune cells and prevents long-term survival in an orthotopic mouse model following flt3-ligand/CD40-ligand immunotherapy. J Immunother 2004;27:13-26.

174. Swain SL, Bradley LM, Croft M, et al. Helper T-cell subsets: phenotype, function and the role of lymphokines in regulating their development. Immunol Rev 1991;123:115-44.

175. Weaver CT, Harrington LE, Mangan PR, Gavrieli M, Murphy KM. Th17: an effector CD4 T cell lineage with regulatory T cell ties. Immunity 2006;24:677-88.

176. Steiner GE, Stix U, Handisurya A, et al. Cytokine expression pattern in benign prostatic hyperplasia infiltrating T cells and impact of lymphocytic infiltration on cytokine mRNA profile in prostatic tissue. Lab Invest 2003;83:1131-46.
177. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science 2003;299:1057-61.
178. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. Cell 2008;133:775-87.
179. Tang Q, Bluestone JA. The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. Nat Immunol 2008;9:239-44.
180. Onishi Y, Fehervari Z, Yamaguchi T, Sakaguchi S. Foxp3+ natural regulatory T cells preferentially form aggregates on dendritic cells in vitro and actively inhibit their maturation. Proc Natl Acad Sci U S A 2008;105:10113-8.
181. Antony PA, Restifo NP. Do CD4+ CD25+ immunoregulatory T cells hinder tumor immunotherapy? J Immunother 2002;25:202-6.
182. Tien AH, Xu L, Helgason CD. Altered immunity accompanies disease progression in a mouse model of prostate dysplasia. Cancer Res 2005;65:2947-55.
183. Kiniwa Y, Miyahara Y, Wang HY, et al. CD8+ Foxp3+ regulatory T cells mediate immunosuppression in prostate cancer. Clin Cancer Res 2007;13:6947-58.
184. Curiel TJ, Coukos G, Zou L, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med 2004;10:942-9.
185. Russell JH, Ley TJ. Lymphocyte-mediated cytotoxicity. Annu Rev Immunol 2002;20:323-70.
186. Sfanos KS, Bruno TC, Meeker AK, De Marzo AM, Isaacs WB, Drake CG. Human prostate-infiltrating CD8(+) T lymphocytes are oligoclonal and PD-1(+). Prostate 2009;69:1694-703.
187. Shafer-Weaver KA, Anderson MJ, Stagliano K, Malyguine A, Greenberg NM, Hurwitz AA. Cutting Edge: Tumor-specific CD8+ T cells infiltrating prostatic tumors are induced to become suppressor cells. J Immunol 2009;183:4848-52.

188. Fagarasan S, Honjo T. T-Independent immune response: new aspects of B cell biology. Science 2000;290:89-92.
189. Rodriguez-Pinto D, Moreno J. B cells can prime naive CD4+ T cells in vivo in the absence of other professional antigen-presenting cells in a CD154-CD40-dependent manner. Eur J Immunol 2005;35:1097-105.
190. Ahmadi T, Flies A, Efebera Y, Sherr DH. CD40 Ligand-activated, antigen-specific B cells are comparable to mature dendritic cells in presenting protein antigens and major histocompatibility complex class I- and class II-binding peptides. Immunology 2008;124:129-40.
191. Kasaian MT, Casali P. Autoimmunity-prone B-1 (CD5 B) cells, natural antibodies and self recognition. Autoimmunity 1993;15:315-29.
192. Ehrlich P. Ueber den jetzigen Stand der Karzinomforschung. (About the current state of cancer research). Ned Tijdschr Geneeskd 1909;5 (Part 1):273-90.
193. Thomas L. Discussion. In Cellular and Humoral Aspects of the Hypersensitive States. New York: Hoeber-Harper; 1959.
194. Burnet FM. The concept of immunological surveillance. Prog Exp Tumor Res 1970;13:1-27.
195. Stutman O. Tumor development after 3-methylcholanthrene in immunologically deficient athymic-nude mice. Science 1974;183:534-6.
196. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. Nat Immunol 2002;3:991-8.
197. Dunn GP, Old LJ, Schreiber RD. The three Es of cancer immunoediting. Annu Rev Immunol 2004;22:329-60.
198. Dighe AS, Richards E, Old LJ, Schreiber RD. Enhanced in vivo growth and resistance to rejection of tumor cells expressing dominant negative IFN gamma receptors. Immunity 1994;1:447-56.
199. van den Broek ME, Kagi D, Ossendorp F, et al. Decreased tumor surveillance in perforin-deficient mice. J Exp Med 1996;184:1781-90.

200. Shinkai Y, Rathbun G, Lam KP, et al. RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. Cell 1992;68:855-67.

201. Gatti RA, Good RA. Occurrence of malignancy in immunodeficiency diseases. A literature review. Cancer 1971;28:89-98.

202. Birkeland SA, Storm HH, Lamm LU, et al. Cancer risk after renal transplantation in the Nordic countries, 1964-1986. Int J Cancer 1995;60:183-9.

203. Pham SM, Kormos RL, Landreneau RJ, et al. Solid tumors after heart transplantation: lethality of lung cancer. Ann Thorac Surg 1995;60:1623-6.

204. Clemente CG, Mihm MC, Jr., Bufalino R, Zurrida S, Collini P, Cascinelli N. Prognostic value of tumor infiltrating lymphocytes in the vertical growth phase of primary cutaneous melanoma. Cancer 1996;77:1303-10.

205. Zhang L, Conejo-Garcia JR, Katsaros D, et al. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. N Engl J Med 2003;348:203-13.

206. Naito Y, Saito K, Shiiba K, et al. CD8+ T cells infiltrated within cancer cell nests as a prognostic factor in human colorectal cancer. Cancer Res 1998;58:3491-4.

207. Schumacher K, Haensch W, Roefzaad C, Schlag PM. Prognostic significance of activated CD8(+) T cell infiltrations within esophageal carcinomas. Cancer Res 2001;61:3932-6.

208. Ishigami S, Natsugoe S, Tokuda K, et al. Prognostic value of intratumoral natural killer cells in gastric carcinoma. Cancer 2000;88:577-83.

209. Villegas FR, Coca S, Villarrubia VG, et al. Prognostic significance of tumor infiltrating natural killer cells subset CD57 in patients with squamous cell lung cancer. Lung Cancer 2002;35:23-8.

210. Coca S, Perez-Piqueras J, Martinez D, et al. The prognostic significance of intratumoral natural killer cells in patients with colorectal carcinoma. Cancer 1997;79:2320-8.

211. Darnell RB. Onconeural antigens and the paraneoplastic neurologic disorders: at the intersection of cancer, immunity, and the brain. Proc Natl Acad Sci U S A 1996;93:4529-36.

212. Teng MW, Swann JB, Koebel CM, Schreiber RD, Smyth MJ. Immune-mediated dormancy: an equilibrium with cancer. J Leukoc Biol 2008;84:988-93.
213. Granziero L, Krajewski S, Farness P, et al. Adoptive immunotherapy prevents prostate cancer in a transgenic animal model. Eur J Immunol 1999;29:1127-38.
214. Koebel CM, Vermi W, Swann JB, et al. Adaptive immunity maintains occult cancer in an equilibrium state. Nature 2007;450:903-7.
215. Meng S, Tripathy D, Frenkel EP, et al. Circulating tumor cells in patients with breast cancer dormancy. Clin Cancer Res 2004;10:8152-62.
216. MacKie RM, Reid R, Junor B. Fatal melanoma transferred in a donated kidney 16 years after melanoma surgery. N Engl J Med 2003;348:567-8.
217. Penn I. Donor transmitted disease: cancer. Transplant Proc 1991;23:2629-31.
218. Suranyi MG, Hogan PG, Falk MC, et al. Advanced donor-origin melanoma in a renal transplant recipient: immunotherapy, cure, and retransplantation. Transplantation 1998;66:655-61.
219. Elder GJ, Hersey P, Branley P. Remission of transplanted melanoma--clinical course and tumour cell characterisation. Clin Transplant 1997;11:565-8.
220. Anderson MJ, Shafer-Weaver K, Greenberg NM, Hurwitz AA. Tolerization of tumor-specific T cells despite efficient initial priming in a primary murine model of prostate cancer. J Immunol 2007;178:1268-76.
221. Muller AJ, Scherle PA. Targeting the mechanisms of tumoral immune tolerance with small-molecule inhibitors. Nat Rev Cancer 2006;6:613-25.
222. Gannon PO, Alam Fahmy M, Begin LR, et al. Presence of prostate cancer metastasis correlates with lower lymph node reactivity. Prostate 2006;66:1710-20.
223. Miller AM, Pisa P. Tumor escape mechanisms in prostate cancer. Cancer Immunol Immunother 2007;56:81-7.
224. Natali PG, Nicotra MR, Bigotti A, et al. Selective changes in expression of HLA class I polymorphic determinants in human solid tumors. Proc Natl Acad Sci U S A 1989;86:6719-23.

225. Blades RA, Keating PJ, McWilliam LJ, George NJ, Stern PL. Loss of HLA class I expression in prostate cancer: implications for immunotherapy. Urology 1995;46:681-6; discussion 6-7.

226. Bander NH, Yao D, Liu H, et al. MHC class I and II expression in prostate carcinoma and modulation by interferon-alpha and -gamma. Prostate 1997;33:233-9.

227. Zhang H, Melamed J, Wei P, et al. Concordant down-regulation of proto-oncogene PML and major histocompatibility antigen HLA class I expression in high-grade prostate cancer. Cancer Immun 2003;3:2.

228. Filella X, Alcover J, Zarco MA, Beardo P, Molina R, Ballesta AM. Analysis of type T1 and T2 cytokines in patients with prostate cancer. Prostate 2000;44:271-4.

229. Wise GJ, Marella VK, Talluri G, Shirazian D. Cytokine variations in patients with hormone treated prostate cancer. J Urol 2000;164:722-5.

230. Elsasser-Beile U, Gierschner D, Jantscheff P, Schultze-Seemann W, Katzenwadel A, Wetterauer U. Different basal expression of type T1 and T2 cytokines in peripheral lymphocytes of patients with adenocarcinomas and benign hyperplasia of the prostate. Anticancer Res 2003;23:4027-31.

231. Cardillo MR, Petrangeli E, Perracchio L, Salvatori L, Ravenna L, Di Silverio F. Transforming growth factor-beta expression in prostate neoplasia. Anal Quant Cytol Histol 2000;22:1-10.

232. Nakashima J, Tachibana M, Horiguchi Y, et al. Serum interleukin 6 as a prognostic factor in patients with prostate cancer. Clin Cancer Res 2000;6:2702-6.

233. Elsasser-Beile U, Kolble N, Grussenmeyer T, et al. Th1 and Th2 cytokine response patterns in leukocyte cultures of patients with urinary bladder, renal cell and prostate carcinomas. Tumour Biol 1998;19:470-6.

234. Gokmen SS, Aygit AC, Ayhan MS, Yorulmaz F, Gulen S. Significance of arginase and ornithine in malignant tumors of the human skin. J Lab Clin Med 2001;137:340-4.

235. Polat MF, Taysi S, Polat S, Boyuk A, Bakan E. Elevated serum arginase activity levels in patients with breast cancer. Surg Today 2003;33:655-61.

236. Singh R, Pervin S, Karimi A, Cederbaum S, Chaudhuri G. Arginase activity in human breast cancer cell lines: N(omega)-hydroxy-L-arginine selectively inhibits cell proliferation and induces apoptosis in MDA-MB-468 cells. Cancer Res 2000;60:3305-12.

237. Porembska Z, Skwarek A, Mielczarek M, Baranczyk-Kuzma A. Serum arginase activity in postsurgical monitoring of patients with colorectal carcinoma. Cancer 2002;94:2930-4.

238. Grabon W, Mielczarek-Puta M, Chrzanowska A, Baranczyk-Kuzma A. l-Arginine as a factor increasing arginase significance in diagnosis of primary and metastatic colorectal cancer. Clin Biochem 2009;42:353-7.

239. Lexander H, Franzen B, Hirschberg D, et al. Differential protein expression in anatomical zones of the prostate. Proteomics 2005;5:2570-6.

240. Harris BE, Pretlow TP, Bradley EL, Jr., Whitehurst GB, Pretlow TG, 2nd. Arginase activity in prostatic tissue of patients with benign prostatic hyperplasia and prostatic carcinoma. Cancer Res 1983;43:3008-12.

241. Keskinege A, Elgun S, Yilmaz E. Possible implications of arginase and diamine oxidase in prostatic carcinoma. Cancer Detect Prev 2001;25:76-9.

242. Reschner A, Harlin H, Laven B, Eriksson F, Pisa P, Egevad L. Expression of immunomodulating genes in prostate cancer and benign prostatic tissue. Anal Quant Cytol Histol 2009;31:74-82.

243. Elgun S, Keskinege A, Yilmaz E, Baltaci S, Beduk Y. Evaluation of serum arginase activity in benign prostatic hypertrophy and prostatic cancer. Int Urol Nephrol 1999;31:95-9.

244. Mumenthaler SM, Yu H, Tze S, et al. Expression of arginase II in prostate cancer. Int J Oncol 2008;32:357-65.

245. Pretlow TG, 2nd, Harris BE, Bradley EL, Jr., Bueschen AJ, Lloyd KL, Pretlow TP. Enzyme activities in prostatic carcinoma related to Gleason grades. Cancer Res 1985;45:442-6.

246. Kee K, Vujcic S, Merali S, et al. Metabolic and antiproliferative consequences of activated polyamine catabolism in LNCaP prostate carcinoma cells. J Biol Chem 2004;279:27050-8.

247. Klotz T, Bloch W, Volberg C, Engelmann U, Addicks K. Selective expression of inducible nitric oxide synthase in human prostate carcinoma. Cancer 1998;82:1897-903.

248. Uotila P, Valve E, Martikainen P, Nevalainen M, Nurmi M, Harkonen P. Increased expression of cyclooxygenase-2 and nitric oxide synthase-2 in human prostate cancer. Urol Res 2001;29:23-8.

249. Wang J, Torbenson M, Wang Q, Ro JY, Becich M. Expression of inducible nitric oxide synthase in paired neoplastic and non-neoplastic primary prostate cell cultures and prostatectomy specimen. Urol Oncol 2003;21:117-22.

250. Aaltoma SH, Lipponen PK, Kosma VM. Inducible nitric oxide synthase (iNOS) expression and its prognostic value in prostate cancer. Anticancer Res 2001;21:3101-6.

251. Mellor A. Indoleamine 2,3 dioxygenase and regulation of T cell immunity. Biochem Biophys Res Commun 2005;338:20-4.

252. Feder-Mengus C, Wyler S, Hudolin T, et al. High expression of indoleamine 2,3-dioxygenase gene in prostate cancer. Eur J Cancer 2008;44:2266-75.

253. Pereg D, Lishner M. Non-steroidal anti-inflammatory drugs for the prevention and treatment of cancer. J Intern Med 2005;258:115-23.

254. Wang W, Bergh A, Damber JE. Cyclooxygenase-2 expression correlates with local chronic inflammation and tumor neovascularization in human prostate cancer. Clin Cancer Res 2005;11:3250-6.

255. Kirschenbaum A, Liu X, Yao S, Levine AC. The role of cyclooxygenase-2 in prostate cancer. Urology 2001;58:127-31.

256. Mahmud S, Franco E, Aprikian A. Prostate cancer and use of nonsteroidal anti-inflammatory drugs: systematic review and meta-analysis. Br J Cancer 2004;90:93-9.

257. Badawi AF. The role of prostaglandin synthesis in prostate cancer. BJU Int 2000;85:451-62.

258. Sharma S, Yang SC, Zhu L, et al. Tumor cyclooxygenase-2/prostaglandin E2-dependent promotion of FOXP3 expression and CD4+ CD25+ T regulatory cell activities in lung cancer. Cancer Res 2005;65:5211-20.

259. Baratelli F, Lin Y, Zhu L, et al. Prostaglandin E2 induces FOXP3 gene expression and T regulatory cell function in human CD4+ T cells. J Immunol 2005;175:1483-90.

260. Furuya Y, Fuse H, Masai M. Serum soluble Fas level for detection and staging of prostate cancer. Anticancer Res 2001;21:3595-8.

261. Liu QY, Rubin MA, Omene C, Lederman S, Stein CA. Fas ligand is constitutively secreted by prostate cancer cells in vitro. Clin Cancer Res 1998;4:1803-11.

262. Wu G, Bazer FW, Davis TA, et al. Arginine metabolism and nutrition in growth, health and disease. Amino Acids 2008.

263. Wu G, Bazer FW, Cudd TA, et al. Pharmacokinetics and safety of arginine supplementation in animals. J Nutr 2007;137:1673S-80S.

264. Castillo L, Chapman TE, Yu YM, Ajami A, Burke JF, Young VR. Dietary arginine uptake by the splanchnic region in adult humans. Am J Physiol 1993;265:E532-9.

265. Wu G, Morris SM, Jr. Arginine metabolism: nitric oxide and beyond. Biochem J 1998;336 (Pt 1):1-17.

266. Castillo L, Chapman TE, Sanchez M, et al. Plasma arginine and citrulline kinetics in adults given adequate and arginine-free diets. Proc Natl Acad Sci U S A 1993;90:7749-53.

267. Schipper RG, Romijn JC, Cuijpers VM, Verhofstad AA. Polyamines and prostatic cancer. Biochem Soc Trans 2003;31:375-80.

268. Shantz LM, Levin VA. Regulation of ornithine decarboxylase during oncogenic transformation: mechanisms and therapeutic potential. Amino Acids 2007;33:213-23.

269. Gerner EW, Meyskens FL, Jr. Polyamines and cancer: old molecules, new understanding. Nat Rev Cancer 2004;4:781-92.

270. Pegg AE. Polyamine metabolism and its importance in neoplastic growth and a target for chemotherapy. Cancer Res 1988;48:759-74.
271. Cederbaum SD, Yu H, Grody WW, Kern RM, Yoo P, Iyer RK. Arginases I and II: do their functions overlap? Mol Genet Metab 2004;81 Suppl 1:S38-44.
272. Lam TL, Wong GK, Chong HC, et al. Recombinant human arginase inhibits proliferation of human hepatocellular carcinoma by inducing cell cycle arrest. Cancer Lett 2009.
273. Sugimura K, Ohno T, Kusuyama T, Azuma I. High sensitivity of human melanoma cell lines to the growth inhibitory activity of mycoplasmal arginine deiminase in vitro. Melanoma Res 1992;2:191-6.
274. Cheng PN, Leung YC, Lo WH, Tsui SM, Lam KC. Remission of hepatocellular carcinoma with arginine depletion induced by systemic release of endogenous hepatic arginase due to transhepatic arterial embolisation, augmented by high-dose insulin: arginase as a potential drug candidate for hepatocellular carcinoma. Cancer Lett 2005;224:67-80.
275. Grillo MA, Lanza A, Colombatto S. Transport of amino acids through the placenta and their role. Amino Acids 2008;34:517-23.
276. Deves R, Boyd CA. Transporters for cationic amino acids in animal cells: discovery, structure, and function. Physiol Rev 1998;78:487-545.
277. Nicholson B, Manner CK, Kleeman J, MacLeod CL. Sustained nitric oxide production in macrophages requires the arginine transporter CAT2. J Biol Chem 2001;276:15881-5.
278. Closs EI, Boissel JP, Habermeier A, Rotmann A. Structure and function of cationic amino acid transporters (CATs). J Membr Biol 2006;213:67-77.
279. Kakuda DK, Sweet MJ, Mac Leod CL, Hume DA, Markovich D. CAT2-mediated L-arginine transport and nitric oxide production in activated macrophages. Biochem J 1999;340 (Pt 2):549-53.
280. Yeramian A, Martin L, Serrat N, et al. Arginine transport via cationic amino acid transporter 2 plays a critical regulatory role in classical or alternative activation of macrophages. J Immunol 2006;176:5918-24.

281. McCord N, Ayuk P, McMahon M, Boyd RC, Sargent I, Redman C. System y+ arginine transport and NO production in peripheral blood mononuclear cells in pregnancy and preeclampsia. Hypertension 2006;47:109-15.

282. Heird WC, Nicholson JF, Driscoll JM, Jr., Schullinger JN, Winters RW. Hyperammonemia resulting from intravenous alimentation using a mixture of synthetic l-amino acids: a preliminary report. J Pediatr 1972;81:162-5.

283. Zea AH, Culotta KS, Ali J, et al. Decreased expression of CD3zeta and nuclear transcription factor kappa B in patients with pulmonary tuberculosis: potential mechanisms and reversibility with treatment. J Infect Dis 2006;194:1385-93.

284. Flynn NE, Meininger CJ, Kelly K, Ing NH, Morris SM, Jr., Wu G. Glucocorticoids mediate the enhanced expression of intestinal type II arginase and argininosuccinate lyase in postweaning pigs. J Nutr 1999;129:799-803.

285. Flynn NE, Bird JG, Guthrie AS. Glucocorticoid regulation of amino acid and polyamine metabolism in the small intestine. Amino Acids 2008.

286. Shi W, Meininger CJ, Haynes TE, Hatakeyama K, Wu G. Regulation of tetrahydrobiopterin synthesis and bioavailability in endothelial cells. Cell Biochem Biophys 2004;41:415-34.

287. Morris SM, Jr. Arginine metabolism: boundaries of our knowledge. J Nutr 2007;137:1602S-9S.

288. Wu G, Flynn NE, Flynn SP, Jolly CA, Davis PK. Dietary protein or arginine deficiency impairs constitutive and inducible nitric oxide synthesis by young rats. J Nutr 1999;129:1347-54.

289. Wang B, Xiong Q, Shi Q, Le X, Xie K. Genetic disruption of host interferon-gamma drastically enhances the metastasis of pancreatic adenocarcinoma through impaired expression of inducible nitric oxide synthase. Oncogene 2001;20:6930-7.

290. Thomas DD, Ridnour LA, Isenberg JS, et al. The chemical biology of nitric oxide: implications in cellular signaling. Free Radic Biol Med 2008;45:18-31.

291. Mocellin S, Bronte V, Nitti D. Nitric oxide, a double edged sword in cancer biology: searching for therapeutic opportunities. Med Res Rev 2007;27:317-52.

292. De Ridder M, Verellen D, Verovski V, Storme G. Hypoxic tumor cell radiosensitization through nitric oxide. Nitric Oxide 2008;19:164-9.

293. Parajuli N, Muller-Holzner E, Bock G, Werner ER, Villunger A, Doppler W. Infiltrating CD11b+CD11c+ cells have the potential to mediate inducible nitric oxide synthase-dependent cell death in mammary carcinomas of HER-2/neu transgenic mice. Int J Cancer;126:896-908.

294. Heller A. Apoptosis-inducing high (.)NO concentrations are not sustained either in nascent or in developed cancers. ChemMedChem 2008;3:1493-9.

295. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol 2002;23:549-55.

296. Umemura N, Saio M, Suwa T, et al. Tumor-infiltrating myeloid-derived suppressor cells are pleiotropic-inflamed monocytes/macrophages that bear M1- and M2-type characteristics. J Leukoc Biol 2008;83:1136-44.

297. Melichar B, Hu W, Patenia R, Melicharova K, Gallardo ST, Freedman R. rIFN-gamma-mediated growth suppression of platinum-sensitive and -resistant ovarian tumor cell lines not dependent upon arginase inhibition. J Transl Med 2003;1:5.

298. Iyer RK, Yoo PK, Kern RM, et al. Mouse model for human arginase deficiency. Mol Cell Biol 2002;22:4491-8.

299. Huynh NN, Andrews KL, Head GA, et al. Arginase II knockout mouse displays a hypertensive phenotype despite a decreased vasoconstrictory profile. Hypertension 2009;54:294-301.

300. Vercelli D. Arginase: marker, effector, or candidate gene for asthma? J Clin Invest 2003;111:1815-7.

301. Bruch-Gerharz D, Schnorr O, Suschek C, et al. Arginase 1 overexpression in psoriasis: limitation of inducible nitric oxide synthase activity as a molecular mechanism for keratinocyte hyperproliferation. Am J Pathol 2003;162:203-11.

302. Vincendeau P, Gobert AP, Daulouede S, Moynet D, Mossalayi MD. Arginases in parasitic diseases. Trends Parasitol 2003;19:9-12.

303. Kropf P, Fuentes JM, Fahnrich E, et al. Arginase and polyamine synthesis are key factors in the regulation of experimental leishmaniasis in vivo. FASEB J 2005;19:1000-2.

304. Munder M, Schneider H, Luckner C, et al. Suppression of T-cell functions by human granulocyte arginase. Blood 2006;108:1627-34.

305. Reid KM, Tsung A, Kaizu T, et al. Liver I/R injury is improved by the arginase inhibitor, N(omega)-hydroxy-nor-L-arginine (nor-NOHA). Am J Physiol Gastrointest Liver Physiol 2007;292:G512-7.

306. Kashyap SR, Lara A, Zhang R, Park YM, DeFronzo RA. Insulin reduces plasma arginase activity in type 2 diabetic patients. Diabetes Care 2008;31:134-9.

307. Morris CR, Poljakovic M, Lavrisha L, Machado L, Kuypers FA, Morris SM, Jr. Decreased arginine bioavailability and increased serum arginase activity in asthma. Am J Respir Crit Care Med 2004;170:148-53.

308. Yu YM, Ryan CM, Castillo L, et al. Arginine and ornithine kinetics in severely burned patients: increased rate of arginine disposal. Am J Physiol Endocrinol Metab 2001;280:E509-17.

309. Morris CR, Kato GJ, Poljakovic M, et al. Dysregulated arginine metabolism, hemolysis-associated pulmonary hypertension, and mortality in sickle cell disease. JAMA 2005;294:81-90.

310. Munder M, Eichmann K, Moran JM, Centeno F, Soler G, Modolell M. Th1/Th2-regulated expression of arginase isoforms in murine macrophages and dendritic cells. J Immunol 1999;163:3771-7.

311. Jost MM, Ninci E, Meder B, et al. Divergent effects of GM-CSF and TGFbeta1 on bone marrow-derived macrophage arginase-1 activity, MCP-1 expression, and matrix metalloproteinase-12: a potential role during arteriogenesis. FASEB J 2003;17:2281-3.

312. Corraliza IM, Soler G, Eichmann K, Modolell M. Arginase induction by suppressors of nitric oxide synthesis (IL-4, IL-10 and PGE2) in murine bone-marrow-derived macrophages. Biochem Biophys Res Commun 1995;206:667-73.

313. Bernard AC, Fitzpatrick EA, Maley ME, et al. Beta adrenoceptor regulation of macrophage arginase activity. Surgery 2000;127:412-8.
314. Gobert AP, Cheng Y, Wang JY, et al. Helicobacter pylori induces macrophage apoptosis by activation of arginase II. J Immunol 2002;168:4692-700.
315. Rutschman R, Lang R, Hesse M, Ihle JN, Wynn TA, Murray PJ. Cutting edge: Stat6-dependent substrate depletion regulates nitric oxide production. J Immunol 2001;166:2173-7.
316. Morris SM, Jr. Regulation of enzymes of the urea cycle and arginine metabolism. Annu Rev Nutr 2002;22:87-105.
317. Marathe C, Bradley MN, Hong C, et al. The arginase II gene is an anti-inflammatory target of liver X receptor in macrophages. J Biol Chem 2006;281:32197-206.
318. Nathan C. Natural resistance and nitric oxide. Cell 1995;82:873-6.
319. Modolell M, Corraliza IM, Link F, Soler G, Eichmann K. Reciprocal regulation of the nitric oxide synthase/arginase balance in mouse bone marrow-derived macrophages by TH1 and TH2 cytokines. Eur J Immunol 1995;25:1101-4.
320. Morris SM, Jr. Enzymes of arginine metabolism. J Nutr 2004;134:2743S-7S; discussion 65S-67S.
321. Lee J, Ryu H, Ferrante RJ, Morris SM, Jr., Ratan RR. Translational control of inducible nitric oxide synthase expression by arginine can explain the arginine paradox. Proc Natl Acad Sci U S A 2003;100:4843-8.
322. Lewis ND, Asim M, Barry DP, et al. Arginase II restricts host defense to Helicobacter pylori by attenuating inducible nitric oxide synthase translation in macrophages. J Immunol;184:2572-82.
323. Rui H, Brekke I, Morkas L, Purvis K. Androgen interaction with the polyamine system of the rat prostate. Andrologia 1987;19:134-42.
324. Levillain O, Diaz JJ, Blanchard O, Dechaud H. Testosterone down-regulates ornithine aminotransferase gene and up-regulates arginase II and ornithine decarboxylase genes for polyamines synthesis in the murine kidney. Endocrinology 2005;146:950-9.

325. Luckner-Minden C, Fischer I, Langhans CD, et al. Human eosinophil granulocytes do not express the enzyme arginase. J Leukoc Biol.

326. Munder M, Mollinedo F, Calafat J, et al. Arginase I is constitutively expressed in human granulocytes and participates in fungicidal activity. Blood 2005;105:2549-56.

327. Grandvaux N, Gaboriau F, Harris J, tenOever BR, Lin R, Hiscott J. Regulation of arginase II by interferon regulatory factor 3 and the involvement of polyamines in the antiviral response. FEBS J 2005;272:3120-31.

328. Xu LL, Su YP, Labiche R, et al. Quantitative expression profile of androgen-regulated genes in prostate cancer cells and identification of prostate-specific genes. Int J Cancer 2001;92:322-8.

329. Velasco AM, Gillis KA, Li Y, et al. Identification and validation of novel androgen-regulated genes in prostate cancer. Endocrinology 2004;145:3913-24.

330. Asadi F, Faraj M, Malakouti S, Kukreja SC. Effect of parathyroid hormone related protein, and dihydrotestosterone on proliferation and ornithine decarboxylase mRNA in human prostate cancer cell lines. Int Urol Nephrol 2001;33:417-22.

331. Rodriguez PC, Zea AH, Culotta KS, Zabaleta J, Ochoa JB, Ochoa AC. Regulation of T cell receptor CD3zeta chain expression by L-arginine. J Biol Chem 2002;277:21123-9.

332. Rodriguez PC, Zea AH, DeSalvo J, et al. L-arginine consumption by macrophages modulates the expression of CD3 zeta chain in T lymphocytes. J Immunol 2003;171:1232-9.

333. Rodriguez PC, Quiceno DG, Zabaleta J, et al. Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses. Cancer Res 2004;64:5839-49.

334. Rodriguez PC, Quiceno DG, Ochoa AC. L-arginine availability regulates T-lymphocyte cell-cycle progression. Blood 2007;109:1568-73.

335. Kropf P, Baud D, Marshall SE, et al. Arginase activity mediates reversible T cell hyporesponsiveness in human pregnancy. Eur J Immunol 2007;37:935-45.

336. Rodriguez PC, Hernandez CP, Quiceno D, et al. Arginase I in myeloid suppressor cells is induced by COX-2 in lung carcinoma. J Exp Med 2005;202:931-9.

337. Zea AH, Rodriguez PC, Culotta KS, et al. L-Arginine modulates CD3zeta expression and T cell function in activated human T lymphocytes. Cell Immunol 2004;232:21-31.

338. Zea AH, Rodriguez PC, Atkins MB, et al. Arginase-producing myeloid suppressor cells in renal cell carcinoma patients: a mechanism of tumor evasion. Cancer Res 2005;65:3044-8.

339. Shearer JD, Richards JR, Mills CD, Caldwell MD. Differential regulation of macrophage arginine metabolism: a proposed role in wound healing. Am J Physiol 1997;272:E181-90.

340. Stefani MM, Muller I, Louis JA. Leishmania major-specific CD8+ T cells are inducers and targets of nitric oxide produced by parasitized macrophages. Eur J Immunol 1994;24:746-52.

341. Macphail SE, Gibney CA, Brooks BM, Booth CG, Flanagan BF, Coleman JW. Nitric oxide regulation of human peripheral blood mononuclear cells: critical time dependence and selectivity for cytokine versus chemokine expression. J Immunol 2003;171:4809-15.

342. Duhe RJ, Evans GA, Erwin RA, Kirken RA, Cox GW, Farrar WL. Nitric oxide and thiol redox regulation of Janus kinase activity. Proc Natl Acad Sci U S A 1998;95:126-31.

343. Bingisser RM, Tilbrook PA, Holt PG, Kees UR. Macrophage-derived nitric oxide regulates T cell activation via reversible disruption of the Jak3/STAT5 signaling pathway. J Immunol 1998;160:5729-34.

344. Oberlies J, Watzl C, Giese T, et al. Regulation of NK cell function by human granulocyte arginase. J Immunol 2009;182:5259-67.

345. Choi BS, Martinez-Falero IC, Corset C, et al. Differential impact of L-arginine deprivation on the activation and effector functions of T cells and macrophages. J Leukoc Biol 2009;85:268-77.

346. Hofmann F, Kreusch J, Maier KP, Munder PG, Decker K. The urea cycle in different types of macrophages. Biochem Soc Trans 1978;6:990-3.

347. Wu G, Meininger CJ. Arginine nutrition and cardiovascular function. J Nutr 2000;130:2626-9.

348. Jobgen WS, Fried SK, Fu WJ, Meininger CJ, Wu G. Regulatory role for the arginine-nitric oxide pathway in metabolism of energy substrates. J Nutr Biochem 2006;17:571-88.

349. Kaul DK, Zhang X, Dasgupta T, Fabry ME. Arginine therapy of transgenic-knockout sickle mice improves microvascular function by reducing non-nitric oxide vasodilators, hemolysis, and oxidative stress. Am J Physiol Heart Circ Physiol 2008;295:H39-47.

350. Grasemann H, Kurtz F, Ratjen F. Inhaled L-arginine improves exhaled nitric oxide and pulmonary function in patients with cystic fibrosis. Am J Respir Crit Care Med 2006;174:208-12.

351. Witte MB, Barbul A. Arginine physiology and its implication for wound healing. Wound Repair Regen 2003;11:419-23.

352. Fricke O, Baecker N, Heer M, Tutlewski B, Schoenau E. The effect of L-arginine administration on muscle force and power in postmenopausal women. Clin Physiol Funct Imaging 2008;28:307-11.

353. Li P, Yin YL, Li D, Kim SW, Wu G. Amino acids and immune function. Br J Nutr 2007;98:237-52.

354. Lubec B, Hoeger H, Kremser K, Amann G, Koller DY, Gialamas J. Decreased tumor incidence and increased survival by one year oral low dose arginine supplementation in the mouse. Life Sci 1996;58:2317-25.

355. Kojima M, Morisaki T, Tsukahara Y, et al. Nitric oxide synthase expression and nitric oxide production in human colon carcinoma tissue. J Surg Oncol 1999;70:222-9.

356. Ma Q, Williamson KE, O'Rourke D, Rowlands BJ. The effects of l-arginine on crypt cell hyperproliferation in colorectal cancer. J Surg Res 1999;81:181-8.

357. Ma Q, Wang Y, Gao X, Ma Z, Song Z. L-arginine reduces cell proliferation and ornithine decarboxylase activity in patients with colorectal adenoma and adenocarcinoma. Clin Cancer Res 2007;13:7407-12.

358. Cheng PN, Lam TL, Lam WM, et al. Pegylated recombinant human arginase (rhArg-peg5,000mw) inhibits the in vitro and in vivo proliferation of human hepatocellular carcinoma through arginine depletion. Cancer Res 2007;67:309-17.

359. Ni Y, Schwaneberg U, Sun ZH. Arginine deiminase, a potential anti-tumor drug. Cancer Lett 2008;261:1-11.

360. Devens BH, Weeks RS, Burns MR, Carlson CL, Brawer MK. Polyamine depletion therapy in prostate cancer. Prostate Cancer Prostatic Dis 2000;3:275-9.

361. Tate DJ, Jr., Vonderhaar DJ, Caldas YA, et al. Effect of arginase II on L-arginine depletion and cell growth in murine cell lines of renal cell carcinoma. J Hematol Oncol 2008;1:14.

362. Bronte V, Kasic T, Gri G, et al. Boosting antitumor responses of T lymphocytes infiltrating human prostate cancers. J Exp Med 2005;201:1257-68.

363. De Santo C, Serafini P, Marigo I, et al. Nitroaspirin corrects immune dysfunction in tumor-bearing hosts and promotes tumor eradication by cancer vaccination. Proc Natl Acad Sci U S A 2005;102:4185-90.

364. Hiramatsu M, Maehara I, Ozaki M, Harada N, Orikasa S, Sasano H. Aromatase in hyperplasia and carcinoma of the human prostate. Prostate 1997;31:118-24.

365. Ellem SJ, Schmitt JF, Pedersen JS, Frydenberg M, Risbridger GP. Local aromatase expression in human prostate is altered in malignancy. J Clin Endocrinol Metab 2004;89:2434-41.

366. Kuiper GG, Carlsson B, Grandien K, et al. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology 1997;138:863-70.

367. Ellem SJ, Risbridger GP. The dual, opposing roles of estrogen in the prostate. Ann N Y Acad Sci 2009;1155:174-86.

368. Whitacre CC. Sex differences in autoimmune disease. Nat Immunol 2001;2:777-80.

369. Fish EN. The X-files in immunity: sex-based differences predispose immune responses. Nat Rev Immunol 2008;8:737-44.

370. Bebo BF, Jr., Schuster JC, Vandenbark AA, Offner H. Androgens alter the cytokine profile and reduce encephalitogenicity of myelin-reactive T cells. J Immunol 1999;162:35-40.

371. Mo R, Chen J, Grolleau-Julius A, Murphy HS, Richardson BC, Yung RL. Estrogen regulates CCR gene expression and function in T lymphocytes. J Immunol 2005;174:6023-9.

372. Klein SL. The effects of hormones on sex differences in infection: from genes to behavior. Neurosci Biobehav Rev 2000;24:627-38.

373. Roberts CW, Walker W, Alexander J. Sex-associated hormones and immunity to protozoan parasites. Clin Microbiol Rev 2001;14:476-88.

374. Ansar Ahmed S, Penhale WJ, Talal N. Sex hormones, immune responses, and autoimmune diseases. Mechanisms of sex hormone action. Am J Pathol 1985;121:531-51.

375. Amadori A, Zamarchi R, De Silvestro G, et al. Genetic control of the CD4/CD8 T-cell ratio in humans. Nat Med 1995;1:1279-83.

376. Mackall CL, Granger L, Sheard MA, Cepeda R, Gress RE. T-cell regeneration after bone marrow transplantation: differential CD45 isoform expression on thymic-derived versus thymic-independent progeny. Blood 1993;82:2585-94.

377. Greenstein BD, Fitzpatrick FT, Kendall MD, Wheeler MJ. Regeneration of the thymus in old male rats treated with a stable analogue of LHRH. J Endocrinol 1987;112:345-50.

378. Chidgey A, Dudakov J, Seach N, Boyd R. Impact of niche aging on thymic regeneration and immune reconstitution. Semin Immunol 2007;19:331-40.

379. Gould KA, Shull JD, Gorski J. DES action in the thymus: inhibition of cell proliferation and genetic variation. Mol Cell Endocrinol 2000;170:31-9.

380. Yao G, Hu Y, Liang J, Hou Y. Nonylphenol-induced thymocyte apoptosis is related to Fas/FasL pathway. Life Sci 2005;77:3306-20.

381. Ryan MR, Shepherd R, Leavey JK, et al. An IL-7-dependent rebound in thymic T cell output contributes to the bone loss induced by estrogen deficiency. Proc Natl Acad Sci U S A 2005;102:16735-40.

382. Leposavic G, Perisic M, Kosec D, et al. Neonatal testosterone imprinting affects thymus development and leads to phenotypic rejuvenation and masculinization of the peripheral blood T-cell compartment in adult female rats. Brain Behav Immun 2009;23:294-304.

383. Le Campion A, Lucas B, Dautigny N, Leaument S, Vasseur F, Penit C. Quantitative and qualitative adjustment of thymic T cell production by clonal expansion of premigrant thymocytes. J Immunol 2002;168:1664-71.

384. Ito Y, Arai S, van Oers NS, Aifantis I, von Boehmer H, Miyazaki T. Positive selection by the pre-TCR yields mature CD8+ T cells. J Immunol 2002;169:4913-9.

385. Harkonen PL, Vaananen HK. Monocyte-macrophage system as a target for estrogen and selective estrogen receptor modulators. Ann N Y Acad Sci 2006;1089:218-27.

386. Bouman A, Schipper M, Heineman MJ, Faas MM. Gender difference in the non-specific and specific immune response in humans. Am J Reprod Immunol 2004;52:19-26.

387. Giron-Gonzalez JA, Moral FJ, Elvira J, et al. Consistent production of a higher TH1:TH2 cytokine ratio by stimulated T cells in men compared with women. Eur J Endocrinol 2000;143:31-6.

388. Matalka KZ. The effect of estradiol, but not progesterone, on the production of cytokines in stimulated whole blood, is concentration-dependent. Neuro Endocrinol Lett 2003;24:185-91.

389. Pernis AB. Estrogen and CD4+ T cells. Curr Opin Rheumatol 2007;19:414-20.

390. Grimaldi CM. Sex and systemic lupus erythematosus: the role of the sex hormones estrogen and prolactin on the regulation of autoreactive B cells. Curr Opin Rheumatol 2006;18:456-61.

391. Grimaldi CM, Cleary J, Dagtas AS, Moussai D, Diamond B. Estrogen alters thresholds for B cell apoptosis and activation. J Clin Invest 2002;109:1625-33.

392. Pauklin S, Sernandez IV, Bachmann G, Ramiro AR, Petersen-Mahrt SK. Estrogen directly activates AID transcription and function. J Exp Med 2009;206:99-111.

393. Kanda N, Tsuchida T, Tamaki K. Testosterone inhibits immunoglobulin production by human peripheral blood mononuclear cells. Clin Exp Immunol 1996;106:410-5.

394. Kanda N, Tsuchida T, Tamaki K. Testosterone suppresses anti-DNA antibody production in peripheral blood mononuclear cells from patients with systemic lupus erythematosus. Arthritis Rheum 1997;40:1703-11.

395. Bouman A, Moes H, Heineman MJ, de Leij LF, Faas MM. Cytokine production by natural killer lymphocytes in follicular and luteal phase of the ovarian cycle in humans. Am J Reprod Immunol 2001;45:130-4.

396. Souza SS, Castro FA, Mendonca HC, et al. Influence of menstrual cycle on NK activity. J Reprod Immunol 2001;50:151-9.

397. Ferguson MM, McDonald FG. Oestrogen as an inhibitor of human NK cell cytolysis. FEBS Lett 1985;191:145-8.

398. Hao S, Zhao J, Zhou J, Zhao S, Hu Y, Hou Y. Modulation of 17beta-estradiol on the number and cytotoxicity of NK cells in vivo related to MCM and activating receptors. Int Immunopharmacol 2007;7:1765-75.

399. Rettew JA, Huet YM, Marriott I. Estrogens augment cell surface TLR4 expression on murine macrophages and regulate sepsis susceptibility in vivo. Endocrinology 2009;150:3877-84.

400. Faas M, Bouman A, Moesa H, Heineman MJ, de Leij L, Schuiling G. The immune response during the luteal phase of the ovarian cycle: a Th2-type response? Fertil Steril 2000;74:1008-13.

401. Bouman A, Moes H, Heineman MJ, de Leij LF, Faas MM. The immune response during the luteal phase of the ovarian cycle: increasing sensitivity of human monocytes to endotoxin. Fertil Steril 2001;76:555-9.

402. Trzonkowski P, Mysliwska J, Tukaszuk K, Szmit E, Bryl E, Mysliwski A. Luteal phase of the menstrual cycle in young healthy women is associated with decline in interleukin 2 levels. Horm Metab Res 2001;33:348-53.

403. Konecna L, Yan MS, Miller LE, Scholmerich J, Falk W, Straub RH. Modulation of IL-6 production during the menstrual cycle in vivo and in vitro. Brain Behav Immun 2000;14:49-61.

404. Ostensen M. Sex hormones and pregnancy in rheumatoid arthritis and systemic lupus erythematosus. Ann N Y Acad Sci 1999;876:131-43; discussion 44.

405. Skobeloff EM, Spivey WH, Silverman R, Eskin BA, Harchelroad F, Alessi TV. The effect of the menstrual cycle on asthma presentations in the emergency department. Arch Intern Med 1996;156:1837-40.

406. Viglietta V, Baecher-Allan C, Weiner HL, Hafler DA. Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. J Exp Med 2004;199:971-9.

407. Lawson CA, Brown AK, Bejarano V, et al. Early rheumatoid arthritis is associated with a deficit in the CD4+CD25high regulatory T cell population in peripheral blood. Rheumatology (Oxford) 2006;45:1210-7.

408. Arruvito L, Sanz M, Banham AH, Fainboim L. Expansion of CD4+CD25+and FOXP3+ regulatory T cells during the follicular phase of the menstrual cycle: implications for human reproduction. J Immunol 2007;178:2572-8.

409. Olsen NJ, Kovacs WJ. Gonadal steroids and immunity. Endocr Rev 1996;17:369-84.

410. Cutolo M, Seriolo B, Villaggio B, Pizzorni C, Craviotto C, Sulli A. Androgens and estrogens modulate the immune and inflammatory responses in rheumatoid arthritis. Ann N Y Acad Sci 2002;966:131-42.

411. Asirvatham AJ, Schmidt M, Gao B, Chaudhary J. Androgens regulate the immune/inflammatory response and cell survival pathways in rat ventral prostate epithelial cells. Endocrinology 2006;147:257-71.

412. Desai KV, Michalowska AM, Kondaiah P, Ward JM, Shih JH, Green JE. Gene expression profiling identifies a unique androgen-mediated inflammatory/immune signature and a PTEN (phosphatase and tensin homolog deleted on chromosome 10)-mediated apoptotic response specific to the rat ventral prostate. Mol Endocrinol 2004;18:2895-907.

413. McMurray RW, Suwannaroj S, Ndebele K, Jenkins JK. Differential effects of sex steroids on T and B cells: modulation of cell cycle phase distribution, apoptosis and bcl-2 protein levels. Pathobiology 2001;69:44-58.

414. Liva SM, Voskuhl RR. Testosterone acts directly on CD4+ T lymphocytes to increase IL-10 production. J Immunol 2001;167:2060-7.

415. Rettew JA, Huet-Hudson YM, Marriott I. Testosterone reduces macrophage expression in the mouse of toll-like receptor 4, a trigger for inflammation and innate immunity. Biol Reprod 2008;78:432-7.

416. Posma E, Moes H, Heineman MJ, Faas MM. The effect of testosterone on cytokine production in the specific and non-specific immune response. Am J Reprod Immunol 2004;52:237-43.

417. Bouman A, Heineman MJ, Faas MM. Sex hormones and the immune response in humans. Hum Reprod Update 2005;11:411-23.

418. Calado RT, Yewdell WT, Wilkerson KL, et al. Sex hormones, acting on the TERT gene, increase telomerase activity in human primary hematopoietic cells. Blood 2009;114:2236-43.

419. McDonnell DP, Norris JD. Connections and regulation of the human estrogen receptor. Science 2002;296:1642-4.

420. Mor G, Sapi E, Abrahams VM, et al. Interaction of the estrogen receptors with the Fas ligand promoter in human monocytes. J Immunol 2003;170:114-22.

421. Erlandsson MC, Ohlsson C, Gustafsson JA, Carlsten H. Role of oestrogen receptors alpha and beta in immune organ development and in oestrogen-mediated effects on thymus. Immunology 2001;103:17-25.

422. Tiwari-Woodruff S, Morales LB, Lee R, Voskuhl RR. Differential neuroprotective and antiinflammatory effects of estrogen receptor (ER)alpha and ERbeta ligand treatment. Proc Natl Acad Sci U S A 2007;104:14813-8.

423. Stefano GB, Peter D. Cell surface estrogen receptors coupled to cNOS mediate immune and vascular tissue regulation: therapeutic implications. Med Sci Monit 2001;7:1066-74.

424. Heinlein CA, Chang C. The roles of androgen receptors and androgen-binding proteins in nongenomic androgen actions. Mol Endocrinol 2002;16:2181-7.

425. Peterziel H, Mink S, Schonert A, Becker M, Klocker H, Cato AC. Rapid signalling by androgen receptor in prostate cancer cells. Oncogene 1999;18:6322-9.

426. Chang C. Androgens and Androgen Receptor: Mechanisms, Functions and Clinical Application. 1 edition ed: Springer; 2002.

427. Sun YH, Gao X, Tang YJ, Xu CL, Wang LH. Androgens induce increases in intracellular calcium via a G protein-coupled receptor in LNCaP prostate cancer cells. J Androl 2006;27:671-8.

428. Benten WP, Lieberherr M, Giese G, et al. Functional testosterone receptors in plasma membranes of T cells. Faseb J 1999;13:123-33.

429. Benten WP, Stephan C, Wunderlich F. B cells express intracellular but not surface receptors for testosterone and estradiol. Steroids 2002;67:647-54.

430. Benten WP, Lieberherr M, Stamm O, Wrehlke C, Guo Z, Wunderlich F. Testosterone signaling through internalizable surface receptors in androgen receptor-free macrophages. Mol Biol Cell 1999;10:3113-23.

431. Benten WP, Guo Z, Krucken J, Wunderlich F. Rapid effects of androgens in macrophages. Steroids 2004;69:585-90.

432. Nakhla AM, Khan MS, Romas NP, Rosner W. Estradiol causes the rapid accumulation of cAMP in human prostate. Proc Natl Acad Sci U S A 1994;91:5402-5.

433. Rosner W, Hryb DJ, Khan MS, Nakhla AM, Romas NA. Androgen and estrogen signaling at the cell membrane via G-proteins and cyclic adenosine monophosphate. Steroids 1999;64:100-6.

434. Nakhla AM, Romas NA, Rosner W. Estradiol activates the prostate androgen receptor and prostate-specific antigen secretion through the intermediacy of sex hormone-binding globulin. J Biol Chem 1997;272:6838-41.

435. Grossman CJ. Interactions between the gonadal steroids and the immune system. Science 1985;227:257-61.

436. Viselli SM, Stanziale S, Shults K, Kovacs WJ, Olsen NJ. Castration alters peripheral immune function in normal male mice. Immunology 1995;84:337-42.

437. Frisancho-Kiss S, Coronado MJ, Frisancho JA, et al. Gonadectomy of male BALB/c mice increases Tim-3(+) alternatively activated M2 macrophages, Tim-3(+) T cells, Th2 cells and Treg in the heart during acute coxsackievirus-induced myocarditis. Brain Behav Immun 2009;23:649-57.

438. Hahn S, Nesslinger NJ, Drapala RJ, et al. Castration induces autoantibody and T cell responses that correlate with inferior outcomes in an androgen-dependent murine tumor model. Int J Cancer 2009.

439. Drake CG, Doody AD, Mihalyo MA, et al. Androgen ablation mitigates tolerance to a prostate/prostate cancer-restricted antigen. Cancer Cell 2005;7:239-49.

440. Civantos F, Soloway MS, Pinto JE. Histopathological effects of androgen deprivation in prostatic cancer. Semin Urol Oncol 1996;14:22-31.

441. Guinan P, Didomenico D, Brown J, et al. The effect of androgen deprivation on malignant and benign prostate tissue. Med Oncol 1997;14:145-52.

442. Page ST, Plymate SR, Bremner WJ, et al. Effect of medical castration on CD4+ CD25+ T cells, CD8+ T cell IFN-gamma expression, and NK cells: a physiological role for testosterone and/or its metabolites. Am J Physiol Endocrinol Metab 2006;290:E856-63.

443. Sutherland JS, Goldberg GL, Hammett MV, et al. Activation of thymic regeneration in mice and humans following androgen blockade. J Immunol 2005;175:2741-53.

444. Ismail HA, Lessard L, Mes-Masson AM, Saad F. Expression of NF-kappaB in prostate cancer lymph node metastases. Prostate 2004;58:308-13.

445. Gannon PO, Godin-Ethier J, Hassler M, et al. In vitro and in vivo androgen-regulated expression of arginase I, arginase II and interleukin-8 in prostate cancer. Cancer Res Submitted May 2009.

446. Mortarini R, Piris A, Maurichi A, et al. Lack of terminally differentiated tumor-specific CD8+ T cells at tumor site in spite of antitumor immunity to self-antigens in human metastatic melanoma. Cancer Res 2003;63:2535-45.

447. Cochran AJ, Huang RR, Lee J, Itakura E, Leong SP, Essner R. Tumour-induced immune modulation of sentinel lymph nodes. Nat Rev Immunol 2006;6:659-70.

448. Wong CP, Bray TM, Ho E. Induction of proinflammatory response in prostate cancer epithelial cells by activated macrophages. Cancer Lett 2009;276:38-46.

449. Lin KY, Lu D, Hung CF, et al. Ectopic expression of vascular cell adhesion molecule-1 as a new mechanism for tumor immune evasion. Cancer Res 2007;67:1832-41.

450. Cernadas M, Lu J, Watts G, Brenner MB. CD1a expression defines an interleukin-12 producing population of human dendritic cells. Clin Exp Immunol 2009;155:523-33.

451. Reiss CK, Volenec FJ, Humphrey M, Singla O, Humphrey LJ. The role of the regional lymph node in breast cancer: a comparison between nodal and systemic reactivity. J Surg Oncol 1983;22:249-53.

452. Cochran AJ, Wen DR, Farzad Z, et al. Immunosuppression by melanoma cells as a factor in the generation of metastatic disease. Anticancer Res 1989;9:859-64.

453. Cochran AJ, Pihl E, Wen DR, Hoon DS, Korn EL. Zoned immune suppression of lymph nodes draining malignant melanoma: histologic and immunohistologic studies. J Natl Cancer Inst 1987;78:399-405.

454. Paget S. The distribution of secondary growths in cancer of the breast. The Lancet 1889;1:571-3.

455. Ribatti D, Mangialardi G, Vacca A. Stephen Paget and the 'seed and soil' theory of metastatic dissemination. Clin Exp Med 2006;6:145-9.

456. Cochran AJ, Morton DL, Stern S, Lana AM, Essner R, Wen DR. Sentinel lymph nodes show profound downregulation of antigen-presenting cells of the paracortex: implications for tumor biology and treatment. Mod Pathol 2001;14:604-8.

457. Lana AM, Wen DR, Cochran AJ. The morphology, immunophenotype and distribution of paracortical dendritic leucocytes in lymph nodes regional to cutaneous melanoma. Melanoma Res 2001;11:401-10.

458. Matsuura K, Yamaguchi Y, Ueno H, Osaki A, Arihiro K, Toge T. Maturation of dendritic cells and T-cell responses in sentinel lymph nodes from patients with breast carcinoma. Cancer 2006;106:1227-36.

459. Hoon DS, Korn EL, Cochran AJ. Variations in functional immunocompetence of individual tumor-draining lymph nodes in humans. Cancer Res 1987;47:1740-4.

460. Harnett MM. Laser scanning cytometry: understanding the immune system in situ. Nat Rev Immunol 2007;7:897-904.

461. Takahashi H, Ruiz P, Ricordi C, et al. In situ quantitative immunoprofiling of regulatory T cells using laser scanning cytometry. Transplant Proc 2009;41:238-9.

462. Raja Gabaglia C, Diaz de Durana Y, Graham FL, Gauldie J, Sercarz EE, Braciak TA. Attenuation of the glucocorticoid response during Ad5IL-12 adenovirus vector treatment enhances natural killer cell-mediated killing of MHC class I-negative LNCaP prostate tumors. Cancer Res 2007;67:2290-7.

463. Mossoba ME, Walia JS, Rasaiah VI, et al. Tumor protection following vaccination with low doses of lentivirally transduced DCs expressing the self-antigen erbB2. Mol Ther 2008;16:607-17.

464. Horino K, Nishiura H, Ohsako T, et al. A monocyte chemotactic factor, S19 ribosomal protein dimer, in phagocytic clearance of apoptotic cells. Lab Invest 1998;78:603-17.

465. Lauber K, Bohn E, Krober SM, et al. Apoptotic cells induce migration of phagocytes via caspase-3-mediated release of a lipid attraction signal. Cell 2003;113:717-30.

466. Truman LA, Ford CA, Pasikowska M, et al. CX3CL1/fractalkine is released from apoptotic lymphocytes to stimulate macrophage chemotaxis. Blood 2008;112:5026-36.

467. Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. J Clin Invest 1998;101:890-8.

468. Huynh ML, Fadok VA, Henson PM. Phosphatidylserine-dependent ingestion of apoptotic cells promotes TGF-beta1 secretion and the resolution of inflammation. J Clin Invest 2002;109:41-50.

469. Freire-de-Lima CG, Xiao YQ, Gardai SJ, Bratton DL, Schiemann WP, Henson PM. Apoptotic cells, through transforming growth factor-beta, coordinately induce anti-inflammatory and suppress pro-inflammatory eicosanoid and NO synthesis in murine macrophages. J Biol Chem 2006;281:38376-84.

470. Reidy MF, Wright JR. Surfactant protein A enhances apoptotic cell uptake and TGF-beta1 release by inflammatory alveolar macrophages. Am J Physiol Lung Cell Mol Physiol 2003;285:L854-61.

471. Goldberg GL, King CG, Nejat RA, et al. Luteinizing hormone-releasing hormone enhances T cell recovery following allogeneic bone marrow transplantation. J Immunol 2009;182:5846-54.

472. Ustach CV, Taube ME, Hurst NJ, Jr., et al. A potential oncogenic activity of platelet-derived growth factor d in prostate cancer progression. Cancer Res 2004;64:1722-9.

473. Blanchere M, Saunier E, Mestayer C, Broshuis M, Mowszowicz I. Alterations of expression and regulation of transforming growth factor beta in human cancer prostate cell lines. J Steroid Biochem Mol Biol 2002;82:297-304.

474. Song K, Wang H, Krebs TL, Kim SJ, Danielpour D. Androgenic control of transforming growth factor-beta signaling in prostate epithelial cells through transcriptional suppression of transforming growth factor-beta receptor II. Cancer Res 2008;68:8173-82.

475. Li W, Liu X, Wang W, et al. Effects of antisense RNA targeting of ODC and AdoMetDC on the synthesis of polyamine synthesis and cell growth in prostate cancer cells using a prostatic androgen-dependent promoter in adenovirus. Prostate 2008;68:1354-61.

476. Zhang Y, Liu XX, Zhang B, Hu HY, Gong L. Antitumor effect of antisense ODC adenovirus on human prostate cancer cells. Prostate Cancer Prostatic Dis 2005;8:280-6.

477. Wind T, Hansen M, Jensen JK, Andreasen PA. The molecular basis for anti-proteolytic and non-proteolytic functions of plasminogen activator inhibitor type-1: roles of the reactive centre loop, the shutter region, the flexible joint region and the small serpin fragment. Biol Chem 2002;383:21-36.
478. Gupta A, Lotan Y, Ashfaq R, et al. Predictive Value of the Differential Expression of the Urokinase Plasminogen Activation Axis in Radical Prostatectomy Patients. Eur Urol 2008.
479. Hildenbrand R, Schaaf A. The urokinase-system in tumor tissue stroma of the breast and breast cancer cell invasion. Int J Oncol 2009;34:15-23.
480. Bryan L, Paugh BS, Kapitonov D, et al. Sphingosine-1-phosphate and interleukin-1 independently regulate plasminogen activator inhibitor-1 and urokinase-type plasminogen activator receptor expression in glioblastoma cells: implications for invasiveness. Mol Cancer Res 2008;6:1469-77.
481. Illemann M, Hansen U, Nielsen HJ, et al. Leading-edge myofibroblasts in human colon cancer express plasminogen activator inhibitor-1. Am J Clin Pathol 2004;122:256-65.
482. Illemann M, Bird N, Majeed A, et al. Two distinct expression patterns of urokinase, urokinase receptor and plasminogen activator inhibitor-1 in colon cancer liver metastases. Int J Cancer 2009;124:1860-70.
483. Soff GA, Sanderowitz J, Gately S, et al. Expression of plasminogen activator inhibitor type 1 by human prostate carcinoma cells inhibits primary tumor growth, tumor-associated angiogenesis, and metastasis to lung and liver in an athymic mouse model. J Clin Invest 1995;96:2593-600.
484. Bajou K, Peng H, Laug WE, et al. Plasminogen activator inhibitor-1 protects endothelial cells from FasL-mediated apoptosis. Cancer Cell 2008;14:324-34.
485. Smith MR, Lee H, Fallon MA, Nathan DM. Adipocytokines, obesity, and insulin resistance during combined androgen blockade for prostate cancer. Urology 2008;71:318-22.
486. Agirbasli M, Baykan OA, Tekin A, et al. Short term effects of GnRH agonists on plasma fibrinolytic balance in patients with advanced prostate cancer. J Thromb Thrombolysis 2009;27:172-4.

487. Cheng M, Li Y, Wu J, et al. IL-8 induces imbalances between nitric oxide and endothelin-1, and also between plasminogen activator inhibitor-1 and tissue-type plasminogen activator in cultured endothelial cells. Cytokine 2008;41:9-15.
488. Marshall LJ, Ramdin LS, Brooks T, PC DP, Shute JK. Plasminogen activator inhibitor-1 supports IL-8-mediated neutrophil transendothelial migration by inhibition of the constitutive shedding of endothelial IL-8/heparan sulfate/syndecan-1 complexes. J Immunol 2003;171:2057-65.
489. Xie K. Interleukin-8 and human cancer biology. Cytokine Growth Factor Rev 2001;12:375-91.
490. Schauer IG, Ressler SJ, Rowley DR. Keratinocyte-derived chemokine induces prostate epithelial hyperplasia and reactive stroma in a novel transgenic mouse model. Prostate 2009;69:373-84.
491. Veltri RW, Miller MC, Zhao G, et al. Interleukin-8 serum levels in patients with benign prostatic hyperplasia and prostate cancer. Urology 1999;53:139-47.
492. Kim SJ, Uehara H, Karashima T, McCarty M, Shih N, Fidler IJ. Expression of interleukin-8 correlates with angiogenesis, tumorigenicity, and metastasis of human prostate cancer cells implanted orthotopically in nude mice. Neoplasia 2001;3:33-42.
493. Kanda N, Watanabe S. 17beta-estradiol, progesterone, and dihydrotestosterone suppress the growth of human melanoma by inhibiting interleukin-8 production. J Invest Dermatol 2001;117:274-83.
494. Bengtsson AK, Ryan EJ, Giordano D, Magaletti DM, Clark EA. 17beta-estradiol (E2) modulates cytokine and chemokine expression in human monocyte-derived dendritic cells. Blood 2004;104:1404-10.
495. Bendrik C, Dabrosin C. Estradiol increases IL-8 secretion of normal human breast tissue and breast cancer in vivo. J Immunol 2009;182:371-8.
496. Rotondo R, Barisione G, Mastracci L, et al. IL-8 induces exocytosis of arginase 1 by neutrophil polymorphonuclears in nonsmall cell lung cancer. Int J Cancer 2009.
497. Baulieu EE. Androgens and aging men. Mol Cell Endocrinol 2002;198:41-9.

498. Vermeulen A, Kaufman JM, Goemaere S, van Pottelberg I. Estradiol in elderly men. Aging Male 2002;5:98-102.

499. Ellem SJ, Wang H, Poutanen M, Risbridger GP. Increased endogenous estrogen synthesis leads to the sequential induction of prostatic inflammation (prostatitis) and prostatic pre-malignancy. Am J Pathol 2009;175:1187-99.

500. Cattanach BM, Iddon CA, Charlton HM, Chiappa SA, Fink G. Gonadotrophin-releasing hormone deficiency in a mutant mouse with hypogonadism. Nature 1977;269:338-40.

501. McPherson SJ, Wang H, Jones ME, et al. Elevated androgens and prolactin in aromatase-deficient mice cause enlargement, but not malignancy, of the prostate gland. Endocrinology 2001;142:2458-67.

502. Bianco JJ, Handelsman DJ, Pedersen JS, Risbridger GP. Direct response of the murine prostate gland and seminal vesicles to estradiol. Endocrinology 2002;143:4922-33.

APPENDIX I:

SUPPLEMENTARY RESULTS

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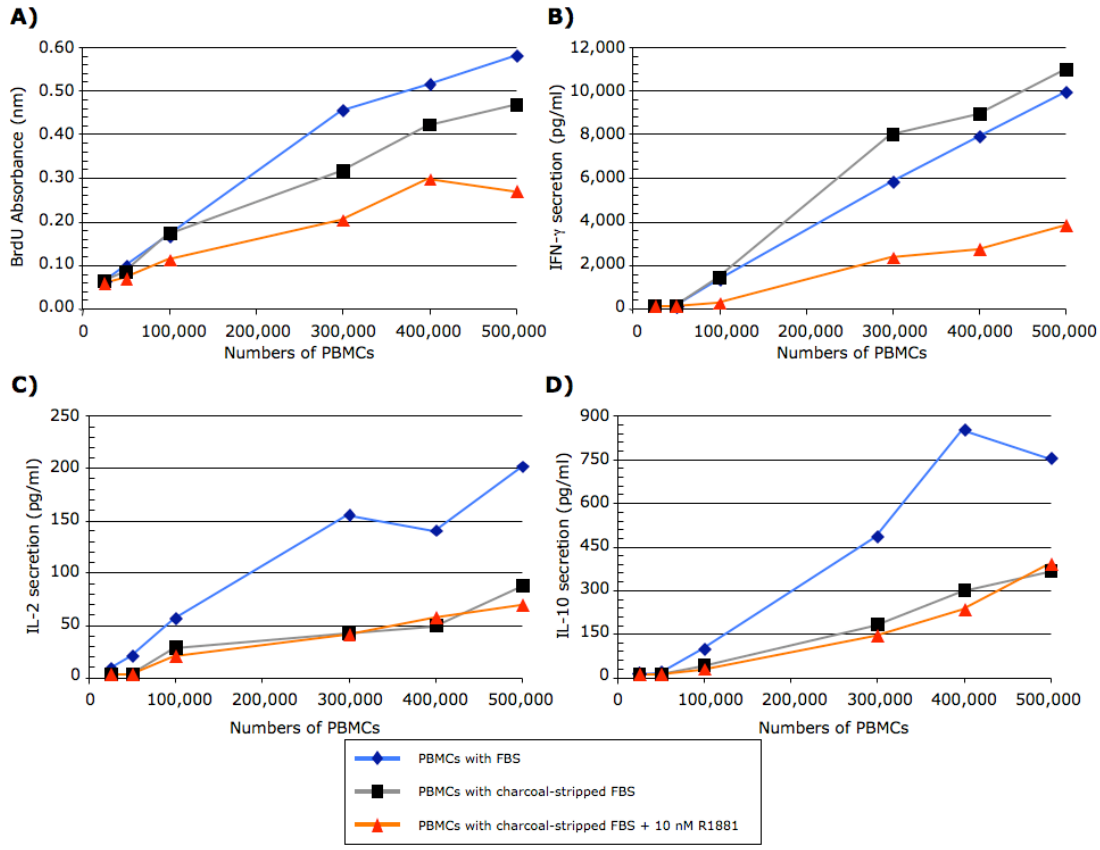
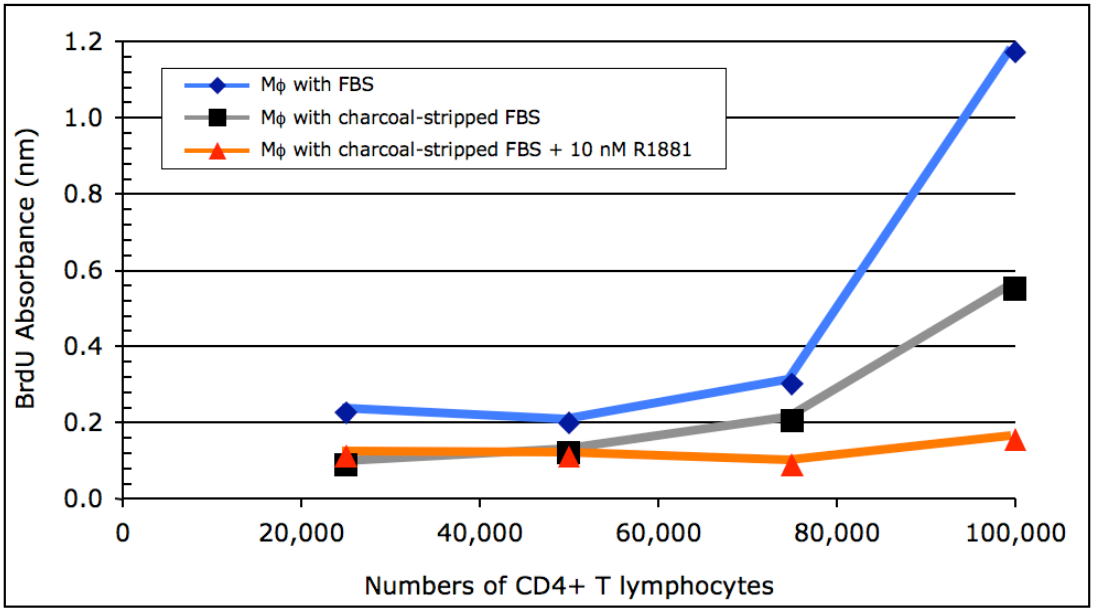


Supplementary Figure 1. Impact of R1881 on the proliferation and activation of human PBMCs

PBMCs of healthy donors were isolated from whole blood by Ficoll gradient using lymphocyte-separating medium. Increasing numbers of PBMCs were incubated in a 96-well flat-bottomed plate with 1 μg/ml of anti-CD3 (OKT3) or an isotype control for 24 hours in the presence of (i) RPMI supplemented with 10% FBS (blue diamond); (ii) RPMI supplemented with 10% charcoal-stripped FBS (black squares); (iii) RPMI supplemented with 10% charcoal-stripped FBS and 10 nM R1881 (red triangles). Supernatants were harvested for cytokine quantification by ELISA. For proliferation assays, bromodeoxyuridine (BrDU) was added in the last 12 hrs according to the manufacturer's instruction. A) PBMCs proliferation. B) IFN-γ secretion. C) IL-2 secretion. D) IL-10 secretion. All panels are from the same representative experiment (n=3).

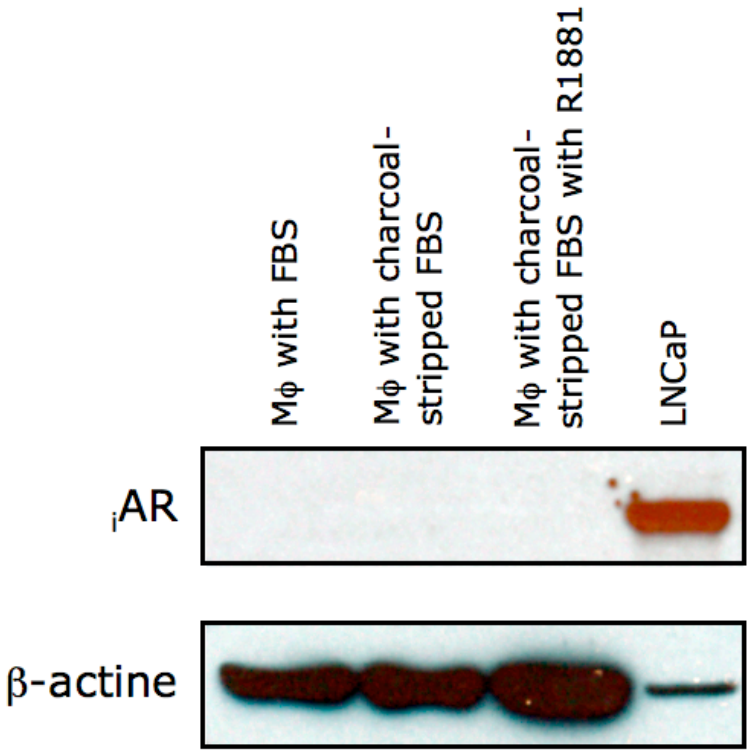
Note that PBMCs incubated with 10 nM R1881 have a lower proliferation and lower IFN-γ secretion with no observable changes in IL-2 and IL-10 secretions.



Supplementary Figure 2. Macrophages differentiated in the presence of R1881 have an immunosuppressive phenotype in a mixed-lymphocyte reaction

PBMCs of a first healthy donor were isolated from whole blood by Ficoll gradient, plated in a 96-well flat-bottom plate and, following a 2 hour incubation at 37°C, non-adherent cells were removed. The remaining adherent cells were maintained in the various media: (i) RPMI supplemented with 10% FBS (blue diamond); (ii) RPMI supplemented with 10% charcoal-stripped FBS (black squares); (iii) RPMI supplemented with 10% charcoal-stripped FBS and 10 nM R1881 (red triangles). Media was changed every two days. After five days, increasing numbers of $CD4^+$ T lymphocytes of a second healthy donor were added to the macrophages and incubated for another five days. Proliferation was measured by BrdU incorporation. A representative experiment is shown (n=4).

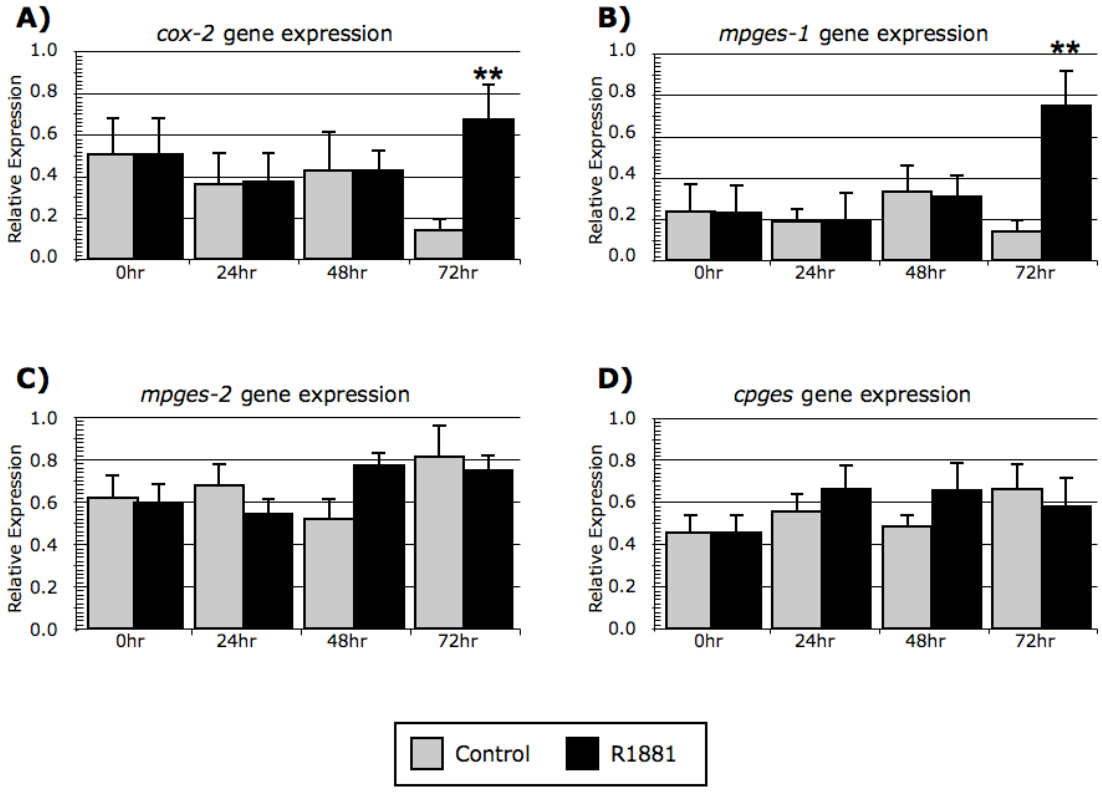
Note that $CD4^+$ T lymphocytes incubated in the presence of macrophages exposed to R1881 had a lower proliferation suggesting that these macrophages had a stronger immunosuppressive potential.



Supplementary Figure 3. Expression of iAR by human monocyte derived macrophages.

PBMCs of a healthy donor were isolated from whole blood by Ficoll gradient, plated in a 96-well flat-bottom plate and, following a 2 hour incubation at 37°C, non-adherent cells were removed. The remaining adherent cells were maintained in the various media: (i) RPMI supplemented with 10% FBS; (ii) RPMI supplemented with 10% charcoal-stripped FBS; (iii) RPMI supplemented with 10% charcoal-stripped FBS and 10 nM R1881. Media was changed every two days. After five days, proteins were extracted. Classical AR ($_i$AR) protein expression was determined by Western blot. LNCaP cells acted as positive controls for $_i$AR protein expression and β-actin was the loading control.

Note that although R1881 stimulation seems to affect the macrophage's phenotype, there is no iAR expression.



Supplementary Figure 4. Analysis of *COX-2*, *MPGES-1*, *MPGES-2* and *CPGES* expression in LNCaP cells following R1881 stimulation

LNCaP cells were stimulated over a period of 72 hours with 10 nM R1881 following a 72 hour incubation period in charcoal-stripped media and the gene expression of A) *COX-2*, B) *MPGES-1* and C) *MPGES-2* and D) *CPGES* were analyzed by qPCR. Control (gray bars) and R1881-stimulated (black bars). **Statistically significant difference ($p<0.05$, Mann-U). Mean relative expression (n=7) with standard error (error bars).

Note the increased *COX-2* and *MPGES-1* gene expression 72 hours following R1881 stimulation.

APPENDIX II:

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