

THE ASSOCIATION OF HUMAN HERPESVIRUS 8 AND A SINGLE NUCLEOTIDE
POLYMORPHISM IN THE GP130 SIGNALING RECEPTOR IN PROSTATE CANCER

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We have previously demonstrated a significant association between human herpesvirus 8 (HHV-8) seroprevalence among men with prostate cancer in the Caribbean island of Tobago compared to cancer free men. HHV-8 DNA has been detected in semen and prostatic tissues in some, but not all reports. I have performed immunohistochemistry (IHC) on prostate tissues from HHV-8 seropositive men from the United States and Tobago with prostate cancer for the expression of viral proteins and determined if expression of these proteins are associated with increased inflammation. My results demonstrate the presence of viral proteins in prostates from seropositive men and among tissues expressing these viral proteins, there is increased inflammation as measured by macrophage infiltrate. I have also looked for the presence of polymorphisms in several genes previously associated with increased risk of prostate cancer to determine if there was a genetic link with the greater risk for prostate cancer in Tobago. We have found single nucleotide polymorphism in the IL-6 gp130 signaling receptor that is associated with increased prostate cancer risk among HHV-8 seropositive men. The public health relevance observed by these results suggests an interaction between HHV-8 infection, increased inflammation and genetic polymorphisms resulting in increased prostate cancer risk. I hypothesize that HHV-8 is a cofactor for prostate cancer in Tobago by establishing a chronic infection which leads to chronic inflammation and ultimately prostate cancer.

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Preface

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1.0 Chapter One: Introduction

1.1 Prostate Cancer

Prostate cancer is the most common cancer among men in the United States and is second to lung cancer as a cause of cancer death ¹. Each year there are 186,320 estimated new cases of prostate cancer, which is equal to 6.5 new cases per 100,000 men a year ¹. Prostate cancer is the leading site of new cancer cases, at 25% of all cancers found in men ¹. The annual incidence of prostate cancer has increased by more than 30% over the last 25 years, primarily due to earlier diagnosis following serum PSA (prostate specific antigen) screening, while the mortality rate has increased around 14% over the last 60 years². It has been said that if a man lives to be 80 he will die with prostate cancer and not from it. Supporting this idea is the statistic that one in 30 men will develop prostate cancer in their life time; and one in six of those men will develop invasive prostate cancer ¹.

1.1.1 Signs and Symptoms

In the early stages of prostate cancer, often times no symptoms occur. In the later, more advanced stages of the cancer, men may experience problems with urine flow such as interrupted, weak, or inability to urinate ².

1.1.2 Risk Factors

Known risk factors include a family history of prostate cancer, age, and ethnicity. Proposed co-factors for prostate cancer include prostatic inflammation, diet, and sexually transmitted agents ³. Risk of prostate cancer is not uniform among the different races. Prostate cancer is most prevalent in African Americans than either Caucasians or Asians, with some cancer registries reporting a 30-fold or greater difference between African Americans and Asians ^{1,4}. This increased risk seen in those of African descent is not limited to the United States. In a recent study from the Caribbean nation of Trinidad & Tobago, the prevalence of screening-detected prostate cancer was 3-fold higher among Tobago men of African descent compared to US Caucasian men ^{5,6}. Our lab has previously reported an elevated seroprevalence of human herpesvirus 8 (HHV-8) among men from Tobago with prostate cancer compared to age-matched controls (P=0.003, OR 2.24, 95% C.I. 1.29-3.90) ⁷. This finding lends support to an infectious agent co-factor for prostate cancer.

While the reason for the population differences in prostate cancer incidence is not known, several studies have suggested a genetic factor ³. A number of prostate cancer candidate genes have been investigated ¹¹, but candidate gene polymorphisms which consistently explain ethnic differences in risk have yet to be identified.

1.1.2.1 Inflammation and Prostate Cancer

A positive association between prostatic inflammation and onset of cancer has been reported ⁸. The results of epidemiologic studies have demonstrated correlations

between prostatitis and sexually transmitted diseases with increased prostate cancer risk ⁹. It has been estimated that up to 15% of all malignancies diagnosed worldwide can be traced to infections ¹⁰. A single infectious agent has not been identified leading Nelson and colleagues to speculate that host immune responses to infections in the prostate (rather than the agent itself) may serve as the risk factor for prostate cancer ⁹. Viruses have the ability to alter the microenvironment in which they are present, therefore it is plausible that inflammation associated with a chronic viral infection may contribute to the development of prostate cancer ¹¹. However, the results from studies investigating several human viruses, notably herpes simplex, cytomegalovirus, and papillomaviruses have not supported this role ¹². In addition, some, but not all, studies on anti-inflammatory drugs have suggested a potential protective effect on prostate cancer development ¹³⁻¹⁵. The idea that chronic inflammation may trigger cancer by providing additional insults to pre-malignant cells is shared among many cancer types. Recent studies depicting chronic inflammatory environments where leukocyte recruitment is persistent show that stimulation through the release from tumor-associated macrophages of factors such as cytokines and chemokines promote angiogenesis, tumor growth and metastasis ^{8,10,16,17}. More recently, tumor associated macrophage density has been associated with poor clinical outcome among many different cancers ¹⁸. Lissbrant and colleagues reported the association of increased tumor associated macrophages in prostate cancer patients with shorter survival time ¹⁹. An example of the model of chronic infection is cervical carcinoma where a chronic inflammatory response, due to the presence of human papilloma virus, promotes epithelial cell proliferation, tissue remodeling and angiogenesis which, over time, leads

to invasive carcinoma ¹⁶. Thus the presence of a chronic prostatic infection resulting in chronic inflammation may serve as a co-factor for prostate cancer development.

1.1.2.2 IL-6 and Prostate Cancer

Interleukin-6 (IL-6) is a cytokine involved in many cellular functions including proliferation, apoptosis, angiogenesis, and differentiation ²⁰. The major source of IL-6 is macrophages, but it can be produced by multiple cell types including, T and B lymphocytes, fibroblasts, endothelial cells, keratinocytes, and mast cells ²¹. IL-6 has a molecular weight of approximately 23kDa ²⁰. It is comprised of 212 amino acids as a propeptide and 190 amino acids as a mature protein ²². IL-6 signalling produces an autocrine feedback loop in cells that it acts upon.

IL-6 binding consists of two receptors: a binding receptor, gp80 (also known as IL-6R α), and a signaling receptor, gp130 ²⁰. IL-6 binds to a soluble form of gp80 with low affinity²³. Glycoprotein 80 has a single transmembrane span and a short cytoplasmic domain. The extracellular domains of gp80 are an immunoglobulin like domain and a common structural domain of hematopoietic growth factor receptor domain, conserved cysteine residues and the WS (tryptophan and serine) sequence motif ²⁴. The gp80:IL-6 complex is tetrameric, two gp80 molecules and two IL-6 molecules ²⁴. Binding of the gp80 receptor alone does not activate IL-6 signaling. The gp80:IL-6 complex must then bind two gp130 molecules, forming a hexameric complex. The binding to gp130 is required for high affinity ²⁴. Binding of the gp130 molecules activates the Janus kinases, JAK, that phosphorylate gp130. Phosphorylation of gp130

induces activation and translocation of signal transducers and activators of transcription three, STAT-3²⁵.

Initially most prostate cancers are androgen-dependent and therefore are responsive to androgen-ablative therapies²⁶. Androgen ablation inhibits tumor growth. Unfortunately, most of these cancers re-emerge as more lethal androgen-insensitive cancers. It has been postulated that these androgen-independent cancers evolve when they develop the ability to regulate their own cell growth through autocrine pathways²⁶. IL-6 has been implicated as an important growth factor for prostate cancer acting in autocrine or paracrine pathways²⁷. After androgen ablation, IL-6 is seen to be elevated along with its receptor in organ-confined tumors²¹. It is noteworthy that prostatic cancer cell lines that are androgen-dependent (e.g. LNCaP) do not constitutively express hIL-6 while the cytokine is expressed in cell lines from androgen-insensitive prostate cancers (e.g. PC3, DU-145)²⁸. Chronic treatment with exogenous IL-6 leads to a positive feedback loop in prostate cancer cells as shown by the cell line LNCaP-IL-6+, a subline of LNCaP, that has greater proliferation in the presence of IL-6²⁹. In addition, IL-6 has been shown to be capable of activating the androgen receptor pathway through the induction of STAT-3 resulting in the expression of androgen receptor sensitive genes^{30,31}. Thus IL-6 may act as a growth factor for prostate cancer replacing the requirement for androgen. Serum levels of IL-6 have been correlated with prostate cancer disease outcome such that higher levels predict poorer outcomes^{32,33}. Taken together these studies implicate IL-6 as an important mediator of human prostate cancer.

1.2 Human Herpesvirus 8 (HHV-8)

Human herpesvirus 8 (HHV-8), also known as Kaposi's sarcoma associated herpesvirus, is the causative agent of Kaposi's sarcoma (KS) as well as a rare primary effusion lymphoma^{34,35}. HHV-8 is the most recently discovered human herpesvirus. HHV-8 was first identified in AIDS-KS tissue in 1994³⁶. It is a member of the lymphotropic (gamma) herpesviruses; it is a gamma-2 herpesvirus or a *radinovirus*, to be specific³⁵. HHV-8 primarily infects endothelial cells and B-lymphocytes³⁵. Transmission of HHV-8 can occur through sexual contact^{37,38} as well as non-sexual routes³⁹⁻⁴¹.

1.2.1 HHV-8 and Associated Cancers

Human herpesvirus 8 is known to cause Kaposi's sarcoma, primary effusion lymphoma, and some forms of multicentric castleman's disease^{35,42}.

1.2.1.1 Kaposi's sarcoma

Kaposi's sarcoma, KS, was first described by as a disease of elderly men late in the 19th century in Europe³⁵. It was initially known to be more prevalent in Mediterranean areas³⁵. Kaposi's sarcoma is characterized by the classic spindle cell shape (figure 1), neo-angiogenesis, inflammation, and edema^{43,44}. Four types of Kaposi's sarcoma have been described: classic, endemic, iatrogenic, and epidemic⁴⁵.

Classic KS is also referred to as European, sporadic, or chronic KS. Kaposi sarcoma is primarily seen, 90% of the cases, in males 60 years old or older of the Ashkenazi

Jewish or Mediterranean origin ⁴⁵. These lesions typically localize to the lower extremities and affect the skin ⁴⁵.

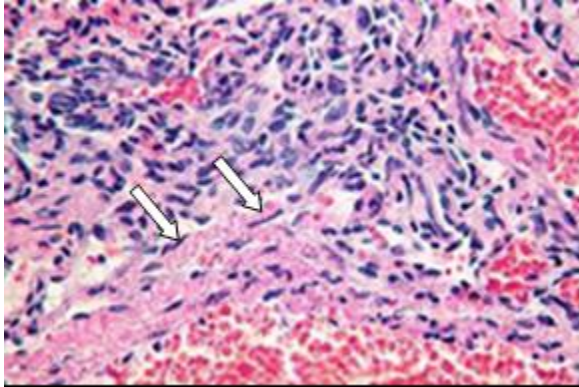


Figure 1: KS Lesion

This is a Hematoxylin & Eosin (H & E) staining of a KS lesion. The picture was taken at 400x. The uniform small spindle cells adjacent to the vessel wall are denoted by an arrow.

Endemic KS is mainly seen in Africa. HHV-8 was prevalent in Africa prior to the HIV epidemic which explained the large prevalence of KS seen on the continent ⁴⁶. Endemic KS is divided into two subtypes: the first seen in adults, ages 25-50, and the second seen in young children under the age of 10⁴⁵. The first subgroup is seen primarily in sub-Saharan countries of Africa. Where KS comprises 17% of all neoplasms in adult males and is the second most common cancer in women. It presents with skin involvement and massive edema⁴⁷. The second subgroup is seen mainly in Bantu children and is characterized by lymphadenopathy that is pronounced in the cervical region. This is a serious condition that often results in death within two years of diagnosis⁴⁷.

Iatrogenic KS is typically associated with solid organ transplant recipients. In the context of immunosuppression, as with organ transplants, both primary infection and reactivation of HHV-8 becomes a potential concern. It has been shown that post-transplant immunosuppression can cause iatrogenic KS to appear ⁴⁷. Post-transplant KS can cause significant clinical manifestations such as rejection of the transplant which can lead to death of the patient. In a study of 356 post-transplant patients with KS, 40% had visceral involvement, and 17% of those with visceral KS died from the tumor ⁴⁷.

Epidemic KS, also known as AIDS-associated KS, is a more aggressive tumor than classic KS and can disseminate into the viscera with a greater likelihood of death ⁴⁸. AIDS-associated KS often presents multifocally and more frequently on the upper body and head regions ⁴⁹. The incidence of KS varies among groups of patients with AIDS, 21-40% of men having sex with men develop KS while only 1.6% of pediatric AIDS cases and 1% of hemophiliac's develop KS ⁴⁵.

1.2.1.2 Primary Effusion Lymphoma

Primary effusion lymphoma (PELs) was first identified as a subset of body-cavity-based lymphomas and have been shown to contain HHV-8 DNA sequences ⁵⁰. The consistent presence of HHV-8 in PELs implies an important role for HHV-8 in the pathogenesis of this rare lymphoma which accounts for only 4.0% of all AIDS malignancies in AIDS patients in the USA ⁵¹. While HHV-8 is always associated with PELs, Epstein-Barr virus is only seen variably ⁵². PELs characteristically lack a solid component and morphologically show immunoblastic and anaplastic large-cell lymphomas with some plasma cell differentiation ⁵¹.

1.2.1.3 Multicentric Castleman's Disease

HHV-8 is also linked to another B cell lymphoproliferative disorder called Multicentric Castleman's Disease (MCD), which can be described by generalized lymphadenopathy with polyclonal hyperimmunoglobulinemia and high levels of serum IL-6^{53 54}. Unlike KS and PELs, HHV-8 DNA sequences have only been detected in a subset of MCD patients, who are usually co-infected with HIV⁵⁴. MCD presents as B-cells with large size and large vesicular nucleus with one or two prominent nucleoli⁵⁵.

1.3 HHV-8 transmission and seroprevalence

The seroprevalence rates of HHV-8 in the general population vary depending on the geographic region. Seroprevalence in the United States is between 5-10%⁵⁶. HHV-8 seroprevalence is higher among HIV-seronegative homosexual subjects compared with the HIV-seronegative heterosexual population, ranging from 20% to 38%^{38,57 58} and is even higher in HIV-seropositive homosexual populations, ranging from 30% to as high as 48%^{56 59 60}. In homosexual men who are seropositive for both HHV-8 and HIV, the presence of HHV-8 DNA in peripheral blood mononuclear cells precedes and predicts the development of KS^{61 41}. Currently virus has been detected in saliva^{41 62}, and occasionally in genital secretions^{41 63 64}. These data shows an important role for salivary virus transmission. Other modes of transmission have also been debated as some large cohorts of intravenous drug users and highly exposed hemophiliacs did not show increased seroprevalence over the general population⁶⁵. Other studies, however have detected a relationship between seropositivity and the frequency or duration of

injection drug use ⁵⁸. In the higher-prevalence societies of the Mediterranean Basin and Africa, a different epidemiologic pattern occurs. Infection is equally prevalent in adult men and women, and infection begins in childhood, often times by the mother chewing the food for the child ⁶⁶. Some infections are acquired from contact with infected parents, but many infections are acquired by spread from child to child during childhood ^{43,67}. Following puberty, seroprevalence continues to slowly rise throughout early to mid-adulthood. This pattern is currently thought to reflect inefficient heterosexual transmission ^{68,69}.

1.4 HHV-8 Genome and Structure

The HHV-8 genome is double-stranded linear DNA of 165 to 170 kilobases (kb) ^{35 70}.

The genome contains a central unique region of 145 kb, which includes all of the viral open reading frames, flanked by a series of highly GC-rich direct terminal repeats ³⁵.

The HHV-8 virion itself is comprised of an electron dense nucleocapsid that is surrounded by a lipid bilayer envelope; in between those two layers is the tegument, a proteinaceous region that is highly organized but morphologically unremarkable ³⁵.

HHV-8 has four major subtypes: A, B, C, and D. Each subtype is found in different regions of the globe. A and C are seen in USA, Asia, Europe, and Middle East ³⁵. B is seen in sub-Saharan Africa, while D is seen in South Asia, Australia, and the Pacific Islands ³⁵.

1.5 HHV-8 Replication Cycle

As is characteristic of herpesviruses, HHV-8 can establish either a latent, non-replicating, or a lytic, actively replicating infection. During latency, the viral genome is maintained as a circular episome whose replication is coupled to cellular replication. The viral gene products that are expressed during latency are important for maintaining the viral genome and for ensuring the survival of an infected population of cells. No infectious virus is produced during latency, but there is a clonal expansion of the infected population due to viral episomal replication being coupled to cellular replication⁷¹. The cells that support latent replication are at risk for tumor development because viral proteins expressed during latency disrupt important regulatory pathways within the cell. HHV-8 latent genes which have wide spread distribution in advanced KS lesions are: latency associated nuclear antigen-1 (LANA-1) (involved in the establishment and maintenance of latency), viral-cyclin (a homologue of cellular cyclin D), viral-FLICE inhibitory protein (v-FLIP) (involved in blocking/recruiting caspase 8), and kaposin which is known to induce tumor formation⁷¹. v-FLIP is associated with anti-apoptotic activity⁷², and activation of NF- κ B by v-FLIP is associated with prolonged survival of HHV-8 infected primary effusion lymphoma cell lines⁷³. LANA-1 can antagonize p53-mediated apoptosis⁷¹, and v-cyclin can inactivate tumor suppressor Rb and cyclin dependent kinase pathway inhibitor p27, thus promoting cell cycle progression⁷⁴. LANA-1 and v-FLIP can also induce IL-6 secretion in HHV-8 infected cells^{75,76}. The kaposin gene also encodes several microRNAs (miRNA), which may regulate gene expression by binding to complementary messenger RNAs⁷⁷. Latently

infected cells have the potential to shift to lytic replication once the replication transactivator (RTA/ORF50) is activated ⁷¹. Lytic replication is accompanied by expression of numerous viral proteins needed for making mature virions. Cells undergoing lytic replication are typically not at risk for malignant transformation because the host cell is usually destroyed when the replication cascade is complete ⁷⁸. This process releases hundreds of viral particles from a single cell ⁷⁹. Lytic replication is critical for transmission to uninfected cells, which includes dissemination to new hosts. Infectious virus egress from the cell is often associated with a viral syndrome that results from multiple factors, including the altered function of newly infected cells, the lysis of infected cells, and the expression of viral gene products such as v-IL-6, and a viral-G protein-coupled receptor (v-GPCR), that have systemic and paracrine effects ⁸⁰.

1.5.1 Proteins of Interest

The experiments outlined in this dissertation focus on three viral proteins, LANA-1, K8.1, and vIL-6 (viral IL-6). LANA-1 is used as the latent phase protein, while K8.1 and vIL-6 are used as the lytic phase proteins.

1.5.1.1 LANA-1

LANA-1 is a widely used as the marker for HHV-8 infection. The protein is approximately 230kDa in size, ⁸¹ and is encoded by open reading frame (ORF) 73 ⁸¹.

1.5.1.2 K8.1

K8.1 is a late lytic gene that encodes for a glycoprotein that is approximately 36kDa in size⁸². K8.1 encodes two transcripts, K8.1A and K8.1B; K8.1A is highly immunogenic and is the transcript chosen for these studies⁸³.

1.5.1.3 vIL-6 (viral Interleukin 6)

HHV-8 encodes a homologue for IL-6, termed viral IL-6 (vIL-6) which is encoded by ORF K2. The protein has 204 amino acids with a predicted size of 23.4 kD and shares 24.7% identity with the human IL-6 and 24.9% identity with murine IL-6⁸⁴. Comparison of the predicted amino acid sequences of vIL-6 and human and mouse IL-6 (hIL-6 and mIL-6) shows strong homology to the region of the cellular IL-6 protein involved in receptor binding⁸⁴. The vIL-6 protein has been shown to be capable of supporting the growth of an IL-6-dependent hybridoma cell line, B9, as well as being capable of binding to the gp80 receptor, activating the IL-6 signaling cascade⁸⁵. Interestingly, the vIL-6 protein can also bind to the gp130 homodimer, in the absence of gp80⁸⁶. Thus the vIL-6 protein can initiate IL-6 signaling without binding to the gp80 receptor. This would permit the vIL-6 protein to trigger signaling in a larger variety of cell types (including those that do not express the gp80 receptor, but do express gp130). It has been demonstrated by several laboratories that prostate cells and prostatic cancer cells express both gp80 and gp130⁸⁷. Since HHV-8 seropositivity is associated with the presence of prostate cancer and the virus encodes a homologue for IL-6 which is capable of binding both gp80 and gp130 receptors, I have hypothesized that HHV-8, through expression of vIL-6, may mediate either the development or progression of prostate cancer.

1.6 HHV-8 Seroprevalence Among Men from Tobago with Prostate Cancer

Our laboratory recently published a case control study with participants from the Caribbean Island of Tobago⁷. The study consisted of 138 men from Tobago with biopsy-proven prostate cancer (Tobago Cancer). There were 140 age matched controls consisting of men from Tobago with no evidence of prostate cancer (Tobago controls). Control men had normal digital rectal exams and PSA values less than 4.0ng/ml. Also included in the study were 174 men from Trinidad with no evidence of prostate cancer (Trinidad controls). The race distribution of the groups were as follows: Tobagonians 97% African, where Trinidadians were 90% Asian Indian descent with the remaining 10% of African descent.

The study revealed that in HHV-8 seroprevalence being higher among the prostate cancer group compared to the corresponding controls (Table 1). Men with prostate cancer were more likely to be HHV-8 seropositive than there age-matched cancer free controls (OR 2.24;95% C.I. 1.29-3.90)⁷.

Table 1 HHV-8 Serological Comparisons

Group	HHV-8 Serology (% positive)	P*	OR (95% C.I.)
Tobago Prostate Cancers	55/138 (39.9)	0.003*	2.24 (1.29 - 3.90)
Tobago Controls	32/140 (22.9)		
Trinidad Controls**	35/174 (20.1)	<0.001**	2.63 (1.54 B 4.50)

* Fisher's Exact Test

** Comparison with Tobago Prostate Cancers

1.7 HHV-8 infection and gp130 polymorphism in prostate cancer.

Recent studies in our laboratory discovering the association of HHV-8 infection and prostate cancer, which may be mediated by vIL-6 signaling through gp130 led to a search of the single nucleotide polymorphism (SNP) database for polymorphism in the gp130 gene that may be associated with increased cancer risk. The gp130 signaling receptor is shared among the members of the four helix cytokines including IL-6, IL-11, and Oncostatin M²⁵. The laboratory of Dr. Robert Ferrell at the University of Pittsburgh, Graduate School of Public Health resequenced nine putative SNPs in gp130. Six (rs3730294 4084522, 4865630, 1063560, 4084824 and 10180) were monomorphic in 24 Tobago samples; one (rs4277867) was minimally informative; and two (rs3730293 and 3729960) were informative, but in complete linkage disequilibrium. Rs 3729960, an arginine to glycine transition at position R148G in the conserved extracellular ligand

binding domain of gp130 was genotyped in 217 cases and 183 controls. The GG genotype was associated with elevated prostate cancer risk, OR=1.5 (95% CI 0.98-2.32) in logistic regression and both gp130 and HHV-8 seropositivity were independently associated with prostate cancer. Further exploration of the six genotype HHV-8 combinations revealed an OR of 3.1 (95% CI 1.2-8.1 p=0.022) for prostate cancer in men who are HHV-8 seropositive and have the GG genotype, compared to the lowest risk group, who are HHV-8 negative and have the CC genotype (Table 2). This effect of gp130 genotype may be mediated through its role in IL-6 signaling in inflammation or through its direct role in vIL-6 signaling.

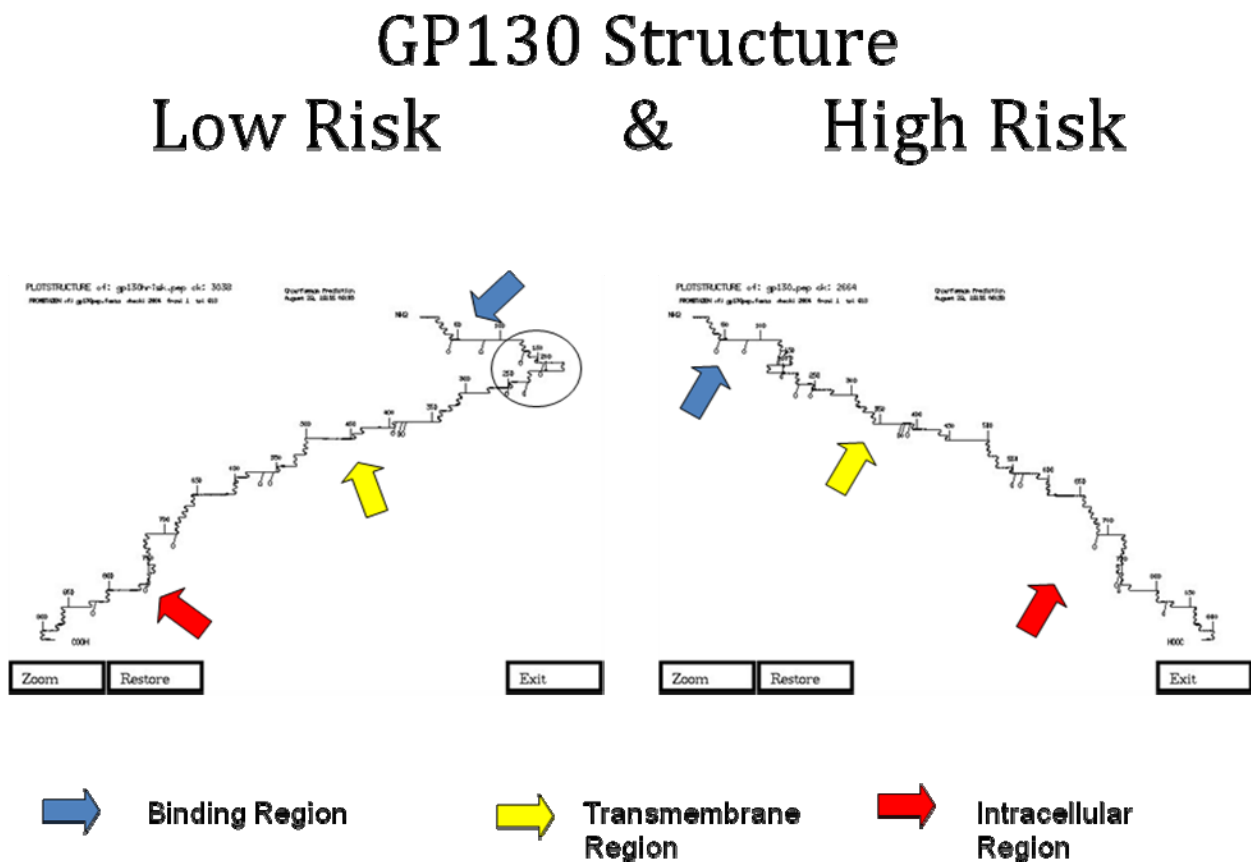


Figure 2: Predicted Protein Secondary Structure of the High and Low Risk Alleles of gp130

Table 2. HHV-8 gp130 Interaction							
		Case		Non-Case			
HHV8	gp130	N	%	N	%	OR	95%CI
-	CC	39	17.9	41	22.4	1.0	
-	CG	65	29.9	61	33.3	1.0	0.57-1.73
-	GG	37	17.1	34	18.6	1.2	0.62-2.25
+	CC	18	8.3	11	6.0	1.5	0.64-3.72
+	CG	37	17.1	29	15.8	1.3	0.69-2.58
+	GG	21	9.7	7	3.9	3.1	1.18-8.11
Total		217		183			

Table 2 shows the HHV-8 gp130 interaction in Tobago.

1.8 Hypothesis

The following research focuses on the presence of HHV-8 in the prostate and how HHV-8 infection in the prostate results in macrophage infiltration to the infection site. As previously stated, increase macrophage infiltrate in the prostate has been associated with poorer clinical outcome^{8,16,17,18}. Following the model outlined by human papilloma virus in cervical carcinoma which results in a chronic inflammatory response due to the presence of the virus that over time leads to invasive carcinoma¹⁶, it is reasonable to look for a chronic prostatic infection resulting in chronic inflammation that may serve as a co-factor for prostate cancer development.

I believe that, since HHV-8 has oncogenic potential, can be spread sexually, and has been detected in prostate tissue⁸⁸, it is a reasonable candidate for an infectious agent cofactor for the development of prostate cancer (Figure 3)

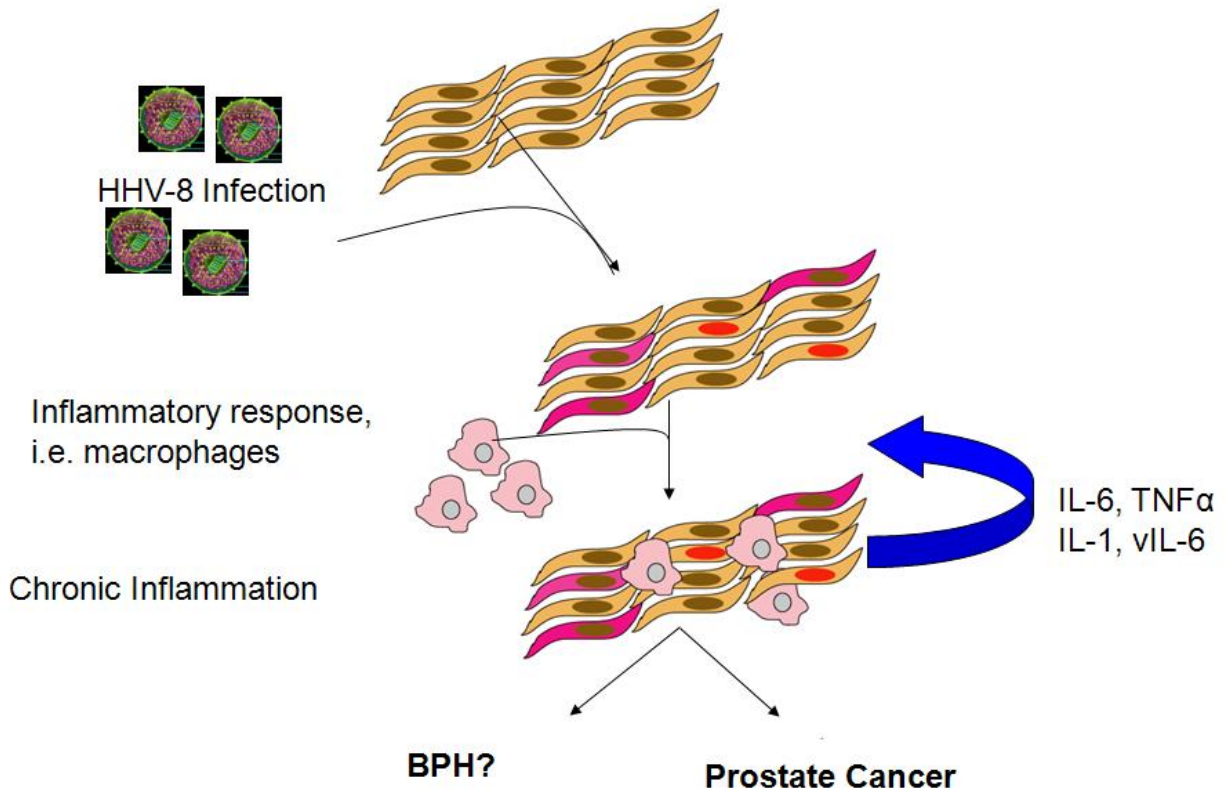


Figure 3: HHV-8 as a Co-Factor in the Development of Prostate Cancer.

HHV-8 infects the cells of the prostate, both lytic (pink cells) and latent (red nuclei) infection is present. The presence of the virus causes an inflammatory response (macrophage infiltrate). The virus sets up a chronic infection leading to a chronic inflammation which can lead to benign prostatic hyperplasia or prostate cancer.

1.9 Specific Aims

This project is built on previous epidemiological work done by Linda Hoffman, Ph.D. in our laboratory ⁷.

1.9.1 Specific Aim 1

Test the hypothesis that HHV-8 is present in the prostates of HHV-8 seropositive men. We will test three distinct cohorts: MACS, Tobago and Pucc.

I performed immunohistochemistry on normal, non-cancerous prostate tissue of men from the MACS (Multicenter AIDS Cohort Study) group. The MACS cohort will have an N=18, 16 seropositive and 2 seronegative. Serial sections were examined for expression of a viral protein expressed during viral latency, the latent nuclear antigen-1 (LANA-1) and two viral proteins expressed during viral replication (vIL-6 and K8.1). The vIL-6 protein is a viral homologue of the cellular cytokine IL-6 while K8.1 is a viral glycoprotein.

Detection of HHV-8 protein expression in cancerous prostates is also necessary to support our hypothesis. We have performed IHC on biopsy material and prostatectomy sections from men with prostate cancer from the Caribbean nation of Trinidad & Tobago. The Tobago cohort consisted of biopsy subjects N=19, 12 seropositive and 7 seronegative, and the prostatectomy subjects N=20, 11 seropositive and 9

seronegative, each subject had areas of cancer and areas that are cancer-free that were stained. Serial sections were examined for LANA-1 and K8.1.

A cohort from the Pucc, Pittsburgh Urologic Cancer Cohort, was also examined for expression of the same proteins. The Pucc cohort had an N=39, 19 seropositive and 20 seronegative.

1.9.2 Specific Aim 2

Test the hypothesis that expression of HHV-8 proteins in prostate tissues is associated with increased inflammation. Inflammation was determined by IHC using antibodies directed against CD68, a macrophage marker, and CD20, a B-cell marker. Differences in levels of inflammation will be determined by percent of total cells positive for a marker. This will be done in all three cohorts: MACS, Tobago, and Pucc.

1.9.3 Specific Aim 3

Test the hypothesis that the polymorphism R148G in the gp130 signaling receptor, which has been associated with an increase risk of prostate cancer in Tobago, results in altered protein properties. I will characterize cell lines expressing the high and low risk gp130 allele (C/C low risk & G/G high risk). I have obtained 10 cell lines from Tobago men with prostate cancer who have one of the three possible genotypes (3 C/C, 5 C/G, 2 G/G). For each cell line determinations will be made on whether the different genotypes affect cellular growth in the presence or absence of the gp130 alleles and the

cytokines IL-6, IL-11, and OSM. The effect of exogenous hIL-6 on cell proliferation will be tested.

I will also determine the functional effect of the gp130 polymorphism by determining downstream signaling effects. I will determine the effects of IL-6 on STAT-3 activation in the different Tobago cell lines.

2.0 Chapter Two: Detection of HHV-8 in the cells of the Prostate: MACS Cohort

Detection of Human Herpesvirus 8 (HHV-8) in Normal Prostates

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2.1 Preface

Inflammation has been indicated as a possible co-factor of prostate cancer. The results of epidemiology studies have demonstrated correlations between prostatitis and sexually transmitted diseases with increased prostate cancer risk ⁹. A single infectious agent has not been identified leading to speculation that host immune responses to infections in the prostate, rather than the agent itself, may serve as the risk factor for prostate cancer. We wished to test the hypothesis that HHV-8 is present in the prostates of seropositive men and that expression of HHV-8 proteins in the prostate tissues is associated with increased inflammation. This cohort is comprised of men who died with AIDS, but had histologically proven cancer-free prostates. We were able to show that HHV-8 is present in the prostates of seropositive men, and the expression of viral proteins is associated with increased inflammation.

The following manuscript, for which I was first author and performed all experiments was published in *The Prostate*.

2.2 Abstract

Background: Human herpesvirus 8 (HHV-8) DNA has been detected in semen and prostatic tissues in some, but not all reports. We have analyzed prostate tissues from

HHV-8 seropositive men for the expression of viral proteins and determined if expression of these proteins are associated with increased inflammation. **Methods:** Paraffin sections of non-cancerous prostates from HHV-8 seropositive (n=16) and seronegative (n=2) men who died with AIDS were screened for expression of three viral proteins by immunohistochemistry. Levels of inflammation were determined by expression of CD68 and CD20. Cellular proliferation was determined by expression of Ki67. **Results:** Among the 16 HHV-8 seropositive cases, 68.9% (11/16) (95% C.I.= 0.41-0.89) were positive for HHV-8 protein expression, while the 2 seronegative patients showed no HHV-8 protein expression. There was increased inflammation among HHV-8 positive prostates. **Conclusions:** These results demonstrate that HHV-8 is present in normal prostates of HIV-infected men and the expression of viral proteins is associated with increased localized inflammation.

2.3 Introduction

Human herpesvirus 8 (HHV-8) is the causative agent of Kaposi's sarcoma (KS) as well as a rare primary effusion lymphoma ³⁵. Transmission of HHV-8 can occur through sexual contact ^{37,38} suggesting that the virus is present in organs and associated ducts of the male reproductive tract such as testes, prostate, urethra, etc. While viral DNA has been found by some laboratories in semen and in both normal and cancerous prostate tissues ⁸⁹⁻⁹³ these findings are controversial since other laboratories have failed to detect viral DNA in any of these tissues ^{64,94-96}. None of these studies determined the

HHV-8 serostatus of the individuals and thus these conflicting results may be due to the low HHV-8 seroprevalence (<10%) in the population ⁶⁵.

HHV-8, like other herpesviruses can establish both lytic and latent infections. During a lytic infection, the majority of the viral genes are expressed and viral replication occurs (reviewed in ³⁵. Two examples of lytic genes are the viral homologue to IL-6 (vIL-6) and K8.1, a late glycoprotein ^{97,98}. During a latent infection a small subset of viral genes is expressed and viral replication does not occur. An example of a latent viral gene is the latency associated nuclear antigen -1 (LANA-1)³⁵. Like most herpesviruses, the normal life cycle of HHV-8 most likely consists of a latent infection interspersed with occasional episodes of reactivation resulting in lytic infections.

In this study, we performed immunohistochemistry on normal, non-cancerous prostate tissue of men from the MACS (Multicenter AIDS Cohort Study) group. Serial sections were examined for expression of a viral protein expressed during latency, the latent nuclear antigen-1 (LANA-1) and two viral proteins expressed during replication of the virus (vIL-6 and K8.1). Localized inflammation was determined by using a macrophage/monocyte marker, CD68, and a B-cell marker, CD20. We also determined if cellular proliferation was evident as measured by expression of Ki67.

2.4 Methods

2.4.1 Immunohistochemistry/ Immunofluorescence

Paraffin sections were cut at 5 μ m and mounted on Surgipath microslides snowcoat X-tra. The serial sections were deparaffinized in 3 xylene washes and rehydrated in a series of ethanol grades (100%, 95%, and 70%) to water. Antigen retrieval was performed using Target retrieval buffer (Dako cytomation cat. # S1699) in a rice steamer for 40 (LANA-1) or 20 (vIL-6, K8.1, CD68, CD20, Ki67 and PSA) min. Blocking of endogenous peroxidases was done in methanol and H₂O₂ for 20 min. Non-specific proteins were blocked with either normal rabbit serum (LANA-1 and CD68) or normal goat serum (vIL-6, K8.1, CD20, Ki67, and PSA) for 30 min. at room temperature. Anti-ORF 73 (anti-LANA-1) rat monoclonal, anti-ORF K8.1 mouse monoclonal, and anti-HHV-8 vIL-6 polyclonal rabbit were used as primary antibodies at a concentration of 1:500. (ABI Cat. # 13-210-100, 13-213-101, 13-214-050, respectively). Anti-CD20 a mouse monoclonal antibody, anti-CD68 a goat monoclonal, and anti-PSA mouse monoclonal (Abcam cat # ab9475-250, Santa Cruz Cat. # sc-7082, and Chemicon Cat. # MAB4082, respectively) were used as primary antibodies at a concentration of 1:250. Anti-Ki67 (Abcam cat # ab6526), a mouse monoclonal antibody was used at a concentration of 1:100. Incubation of primary antibodies occurred overnight at 4°C in a humidified chamber. Biotinylated rabbit anti-rat IgG, goat anti-mouse IgG, goat anti-rabbit IgG, and rabbit anti-goat IgG, were used as secondary antibodies (Vector labs Cat. # BA-400, BA 9200, BA-1000, and BA-5000 respectively). Incubation of the

secondary antibodies occurred for 60 min. at room temperature in a humidified chamber. Vector ABC Elite (Cat. # PK-7200) and Vector NovaRED substrate (Cat. # SK4800) were used to develop the staining for IHC. Hematoxylin was used as the counter stain for immunohistochemistry. The immunofluorescence procedure used rabbit anti-goat IgG FITC and goat anti-mouse IgG FITC as secondary antibodies (Sigma Cat. # F7367 and Roche Cat. # 0311769001). The slide reader was blinded to the HHV-8 serostatus of each subject when viewing the different viral antibody stains.

2.4.1 Prostate Tissues

Prostate tissues taken during autopsy were analyzed by IHC from 18 men, 16 HHV-8 seropositive and 2 HHV-8 seronegative subjects, from the Multicenter AIDS Cohort Study (MACS), a longitudinal study of HIV infection among men who have sex with men in the United States⁹⁹. HHV-8 serostatus was determined using an immunofluorescence assay as described previously¹⁰⁰. All subjects were HIV positive men who have sex with men and who died with AIDS. Hematoxylin and eosin (H&E) staining was performed on all samples to determine cancer status. The type of HHV-8 infection was discerned by the presence of LANA-1 for latent infections and the presence of K8.1 and vIL-6 for lytic infections. Controls consisted of staining serial sections with normal rat, mouse, rabbit, or goat serum as the primary antibody. A TPA-induced BCBL-1/BJAB mixture along with a KS anal lesion were used as positive controls for the viral staining. BCBL-1 is a B cell line that is HHV-8 positive¹⁰¹] while BJAB is a well studied, HHV-8 negative B cell line.

2.5 Results

2.5.1 Subject characteristics

The MACS prostate samples represent men with non-cancerous prostate tissue, taken at autopsy, who are both seropositive (n=16) and seronegative (n=2) for HHV-8. All subjects were HIV positive men who have sex with men and died with AIDS. The prostate samples were stained with hematoxylin and eosin to determine cancer status and all were deemed cancer free by pathological examination. Table three lists the subject characteristics and selected protein expression. The mean age at death was 44.3 years. Seven of the men had KS at death and four of these had documented visceral KS. The duration of KS from time of diagnosis to death of these seven men ranged from 10 mo. to 31 mo. with a mean duration of 18.1 mo.

2.5.2 IHC/ IFA staining

The IHC staining conditions for HHV-8 proteins were established using 2 different controls specimens. One was a KS lesion and the other was a 2:1 mixture of TPA induced BCBL-1 cells and BJAB cells. The KS lesion demonstrated numerous LANA-1 positive cells (Figure 4A), fewer K8.1 positive cells, and a few vIL-6 positive cells (data not shown). The BCBL-1/ BJAB cell mixture demonstrated strong staining for all three HHV-8 proteins (Figure 4 B-D). Negative controls consisted of staining serial sections with normal rat, mouse, or rabbit serum as the primary antibody; there was no staining detected in any of the negative control serial sections (data not shown).

HHV-8 protein expression as detected by IHC in the individual prostates is listed in Table 1 and representative stained sections are shown in Figure 5. For each sample, three serial sections were stained for each of the three viral proteins. Among the HHV-8 seronegative subjects, HHV-8 protein expression was not detected (Table 3). Among the HHV-8 seropositive subjects, we detected viral protein expression in multiple regions of each section. In some instances, the stained regions consisted of both lytic and latent protein expression, while other regions contained only lytic or only latent proteins. Among the HHV-8 seropositive subjects, the majority of samples (68.9% or 11/16) expressed at least one HHV-8 protein (summarized in Figure 6). The combination of all three proteins (LANA-1, vIL-6, and K8.1), suggesting the presence of both lytic and latent viral infections within the same section was seen in 43.8% (7/16) of the samples. vIL-6 expression was detected only in prostate tissues in which LANA-1 and K8.1 expression was seen. In a similar fashion, LANA-1 expression was detected only within sections in which K8.1 expression was also present; and this occurred in 18.8% (3/16) patients. Five of the 16 prostates from HHV-8 seropositive patients (31.3%) did not express any HHV-8 proteins (Figure 6).

Table 3. Subject Characteristics and Protein Expression Results

	Age @ death	KS status	Visceral KS Y or N	Duration of KS	HHV8 serostatus	LANA-1	vIL-6	K8.1	Protein Expression		
									CD68	CD20	Ki67
1	44	N	-	-	-	-	-	-	Focal	None	None
2	57	N	-	-	-	-	-	-	None	None	None
3	41	N	-	-	+	+	+	+	Focal	None	None
4	47	N	-	-	+	+	+	+	Diffuse	None	None
5	40	Y	N	16 mo.	+	+	+	+	Diffuse	None	None
6	49	N	-	-	+	+	+	+	None	None	None
7	46	Y	N	14 mo.	+	+	+	+	Diffuse	None	None
8	30	N	-	-	+	+	+	+	Focal	None	None
9	39	N	-	-	+	+	+	+	None	None	None
10	43	N	-	-	+	+	-	+	None	None	None
11	41	Y	Y	10 mo.	+	+	-	+	None	None	None
12	51	N	-	-	+	+	-	+	None	None	None
13	33	Y	Y	18 mo.	+	-	-	+	Focal	None	None
14	45	N	-	-	+	-	-	-	None	None	None
15	51	Y	Y	31 mo.	+	-	-	-	Focal	None	None
16	48	N	-	-	+	-	-	-	None	None	None
17	33	Y	N	17 mo.	+	-	-	-	None	None	None
18	49	Y	Y	21 mo.	+	-	-	-	None	None	None

To determine the amount of inflammation in each prostate, individual sections were stained with antibodies directed against CD20, a pan-B-cell marker, and CD68, a membrane-bound protein found primarily on macrophages. CD20 was not detectable in any of the sections indicating a lack of B cell infiltrate (Table 3). Three types of staining reactions however, were seen with the anti-CD68 antibody. The lowest level of CD68 staining had an occasional macrophage present in the tissue and was labeled no inflammation (Figure 7B). There were two different CD68 staining patterns reflecting increased inflammation. Sections that exhibited pronounced areas of staining consisting of several cells in each area were labeled as focal staining (Figure 7 D,E). Sections that exhibited many individual or small groups of macrophages located throughout the tissue were labeled diffuse (Figure 7 G,H). The staining results are

listed in Table 3. Among the seropositive samples, 20% (1/5) of the samples that did not express viral proteins exhibited inflammation compared to 54.5% (6/11) of the samples that expressed at least one viral protein. Thus expression of at least one viral protein was associated with increased inflammation.

Upon examination of the prostate sections, expression of viral proteins was not limited to exclusively latent or only lytic antigens. Rather both classes of proteins were seen. The majority of sections expressed both LANA-1 and at least one lytic protein (Table 3). The expression of LANA-1 was separate and distinct from the lytic antigens. Interestingly, the three sections that demonstrated diffuse CD68 staining exhibited diffuse lytic antigen staining. The lytic viral antigen positive cells were seen in clusters throughout the section in both the glandular epithelial and stromal compartments.

Those sections that exhibited focal CD68 staining and were positive for lytic viral antigen, expressed the lytic viral antigen in scattered clusters throughout the tissue. The sections that did not exhibit much CD68 staining still expressed lytic viral antigen in smaller scattered clusters in both the glandular epithelial and the stromal compartments. LANA-1, the representative latent viral antigen, was seen predominately in the glandular epithelial region. We observed that LANA-1 expression was rather consistent throughout the sections irrespective of the CD68 status. Within each distinct viral positive area, there were anywhere from 3-54 positive cells. Sections were measured and the average area of the section was determined to be 36.66 mm².

To determine if cell proliferation was present in the prostate samples, the sections were also stained with an antibody directed against Ki67, a cellular proliferation antigen.

None of the sections demonstrated the presence of Ki67 indicating a lack of cell proliferation in the prostate samples (Table 3).

To determine if the cells expressing viral proteins were infiltrating macrophages or prostatic epithelia, separate sequential, serial sections were stained for CD68, viral antigen (LANA-1, vIL-6 or K8.1) and PSA. Analyses of the stained sections revealed that LANA-1 staining was present only in prostatic cells and not in infiltrating macrophages (Figure 8), while vIL-6 and K8.1 were present in both types of cells. Figure six shows an example of serial sections stained with CD68, vIL-6, and PSA. In this section vIL-6 expression is seen in both CD68-positive macrophages as well as PSA-positive prostatic epithelia. In general, LANA-1 positive cells were found predominately in the prostatic glandular epithelium while lytic cycle proteins were found in both the epithelial and stromal compartments of the prostate.

2.6 Discussion

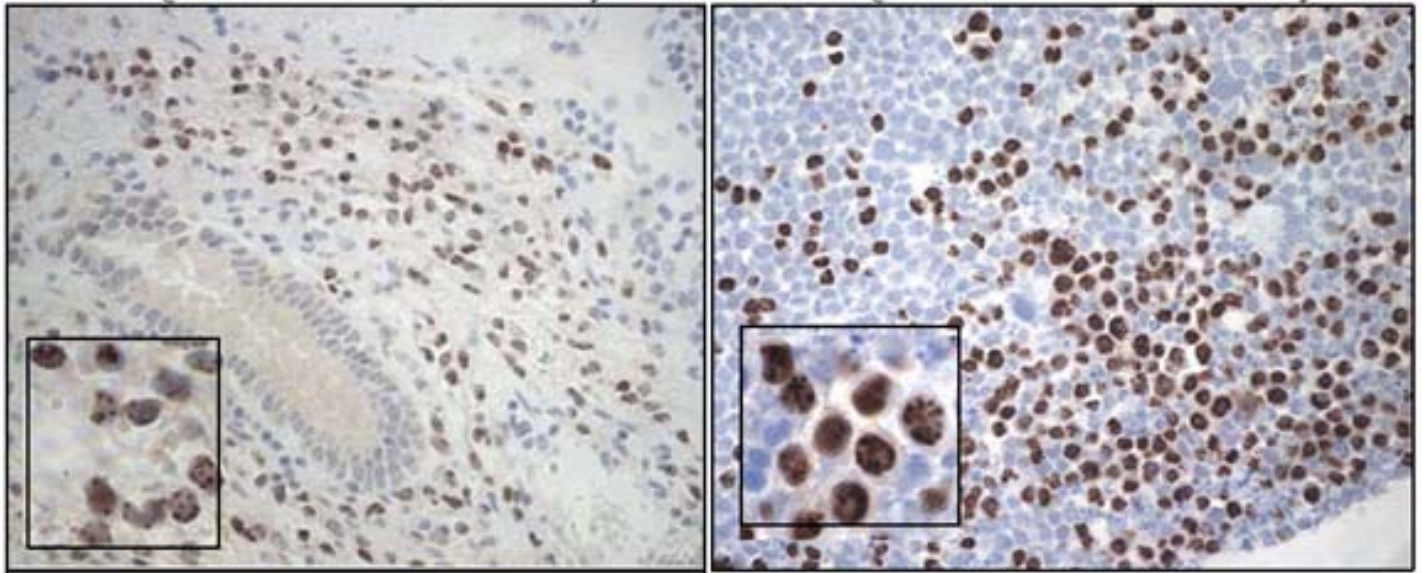
In the present study we addressed whether or not HHV-8 could be detected in normal (non-cancerous) prostate tissues of HIV-infected men using immunohistochemistry and immunofluorescence. We analyzed prostates collected at autopsy from 18 men who were HIV positive and died with AIDS. None of these men had prostate cancer as determined by histological analysis, two were HHV-8 seronegative as demonstrated by serology, and seven were positive for KS, four with visceral KS. The results clearly demonstrate that HHV-8 infected cells are present in a majority of cancer-free prostates of HIV-infected, HHV-8 seropositive men, with the detection of viral proteins not caused

by the presence of KS since a significant number of the samples that were positive for HHV-8 expression were from men with no evidence of KS. In fact, there was no correlation between HHV-8 gene expression and the presence of KS.

Viral gene expression was found in both the infiltrating monocyte/macrophages and in the prostatic glandular epithelium. It is noteworthy that LANA-1 expression (indicating a latent viral infection) was found only in glandular epithelia and not in infiltrating macrophages while gene expression representing a viral lytic cycle (vIL-6 and K8.1) were expressed in both cell types. This suggests that HHV-8 establishes a latent infection in prostatic cells and that infiltrating macrophages do not enter the prostate latently infected. The expression of viral lytic genes in the macrophages may indicate that the macrophages have either become infected with HHV-8 in the prostate or have engulfed cells expressing viral proteins. Previous reports have documented the ability of HHV-8 to infect macrophages¹⁰²⁻¹⁰⁴.

A. Staining for LANA-1 40x, inlay 100x

B. Staining for LANA-1 40x, inlay 100x



C. Staining for K8.1 40x, inlay 100x

D. Staining for vIL-6 40x, inlay 100x

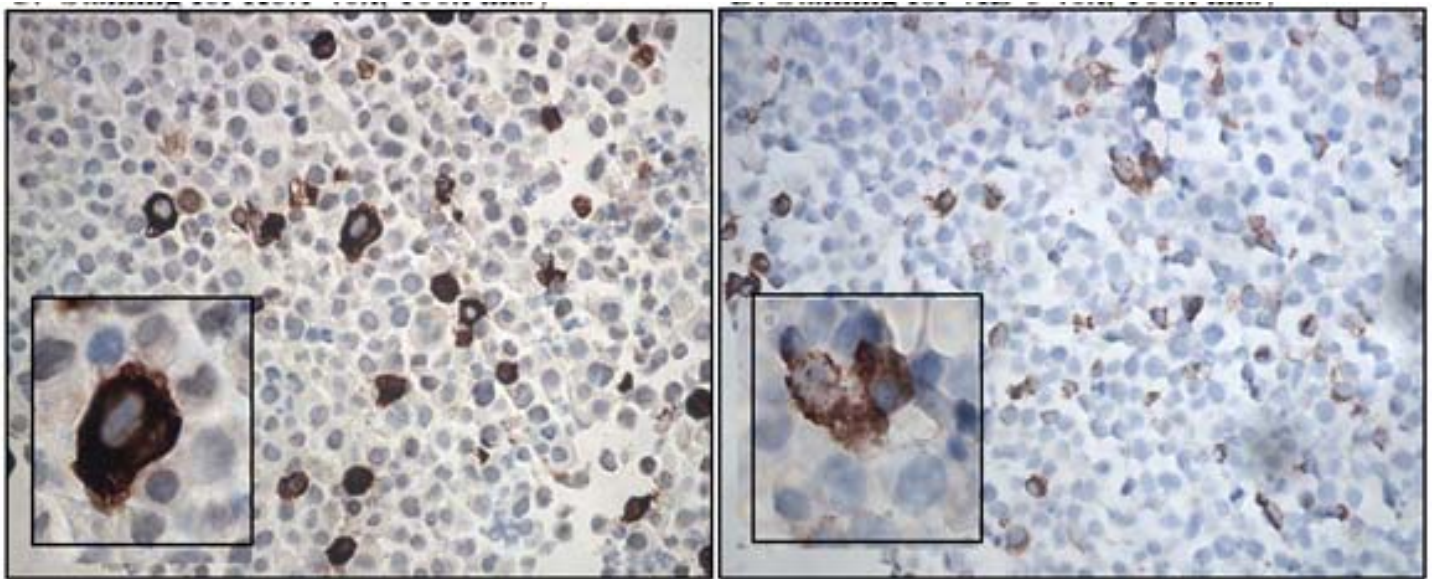
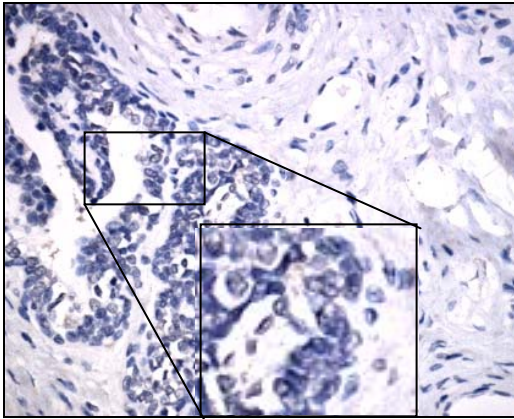


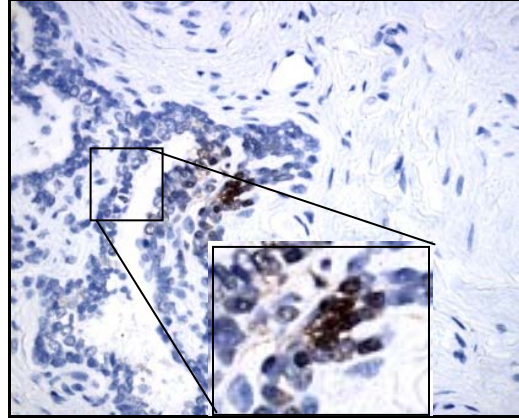
Figure 4: HHV-8 Protein Expression in a KS section and B-cell

A. KS lesion stained for LANA-1. B,C, and D show a 2:1 BCBL-1/BJAB mixture. B is stained for LANA-1, C is stained for K8.1, and D is stained for vIL-6. Pictures were taken at 40x with the insert at 100x.

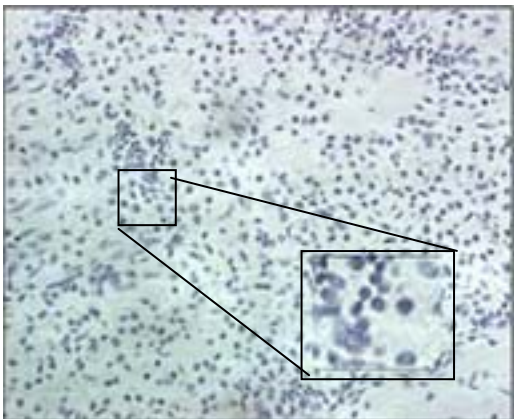
A. Negative control 40x, 100x inlay



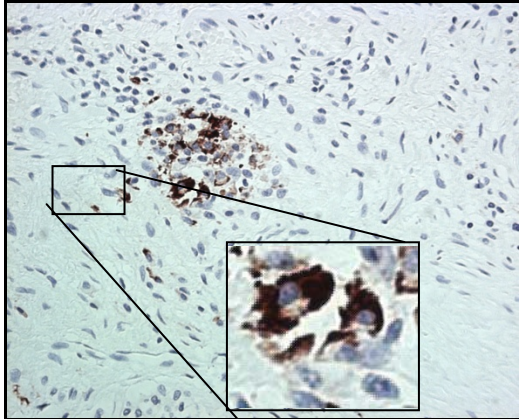
B. Staining for LANA-1 40x, 100x inlay



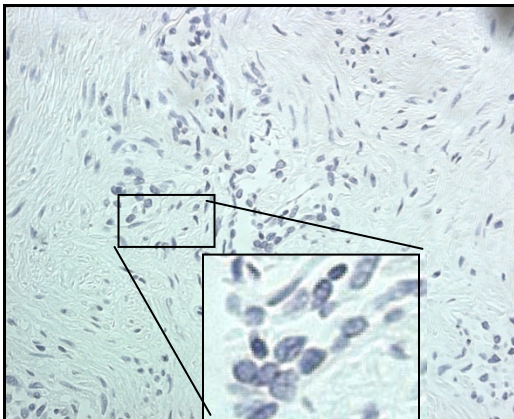
C. Negative control 40x, 100x inlay



D. Staining for K8.1 40x, 100x inlay



E. Negative control 40x, 100x inlay



F. Staining for vIL-6 40x, 100x inlay

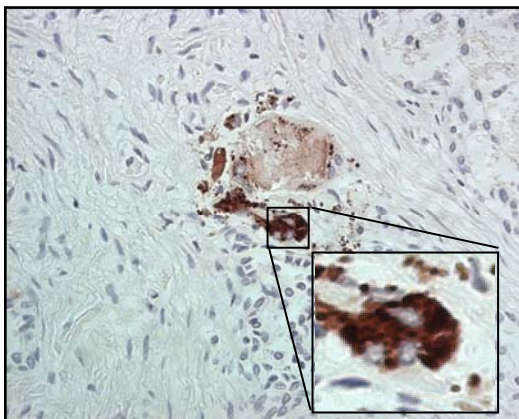


Figure 5: HHV-8 Protein Expression in the Prostatic Tissues of Seropositive Men.

Sections were stained with antibodies directed against LANA-1 (A), K8.1 (C), or vIL-6 (E). Serial sections were stained with normal rat (B), mouse (D), or rabbit (F) as negative control.

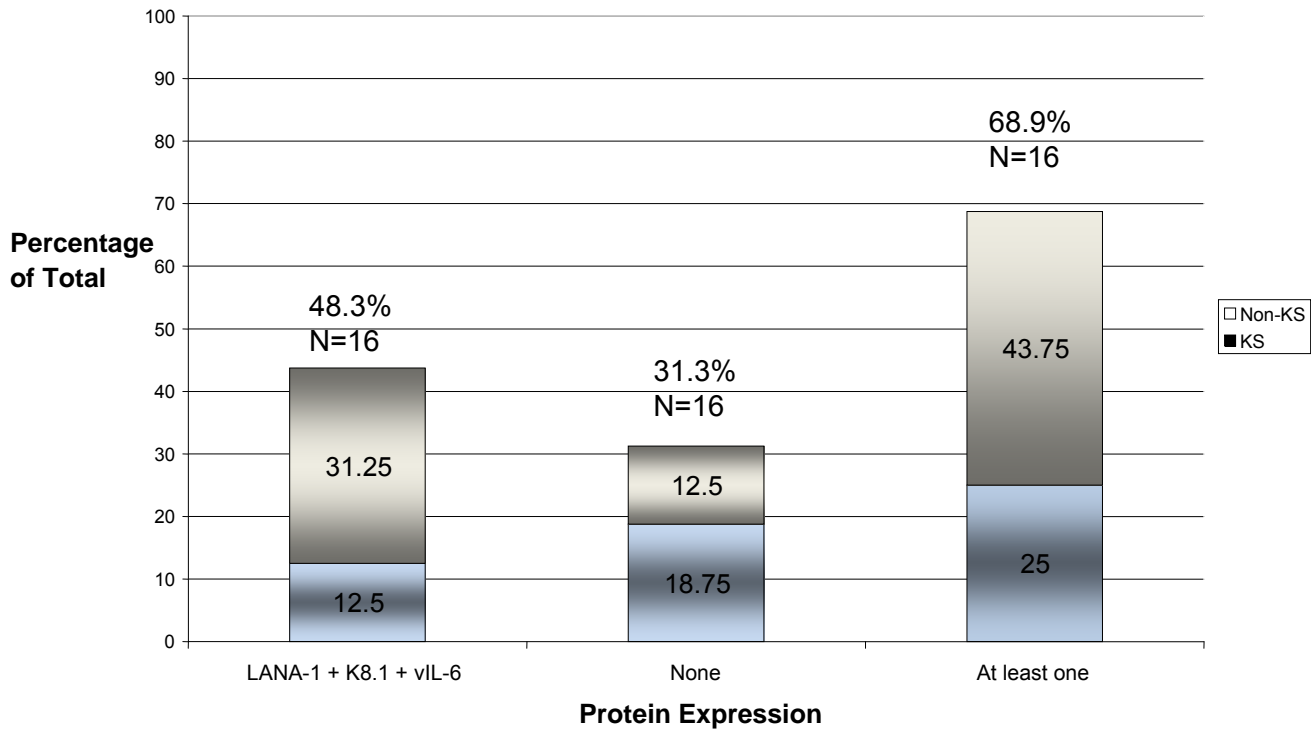


Figure 6: HHV-8 Protein Expression Based on KS Status in Prostates of Seropositive Men

HHV-8 Protein Expression Based on KS status in Prostates of Seropositive Men. The graph shows the percentage of subjects that expressed HHV-8 proteins (LANA-1, K8.1, & vIL-6) together or those that expressed at least one of the HHV-8 proteins. Also shown, are those subjects that expressed no HHV-8 proteins. All subjects represented above were HHV-8 seropositive.

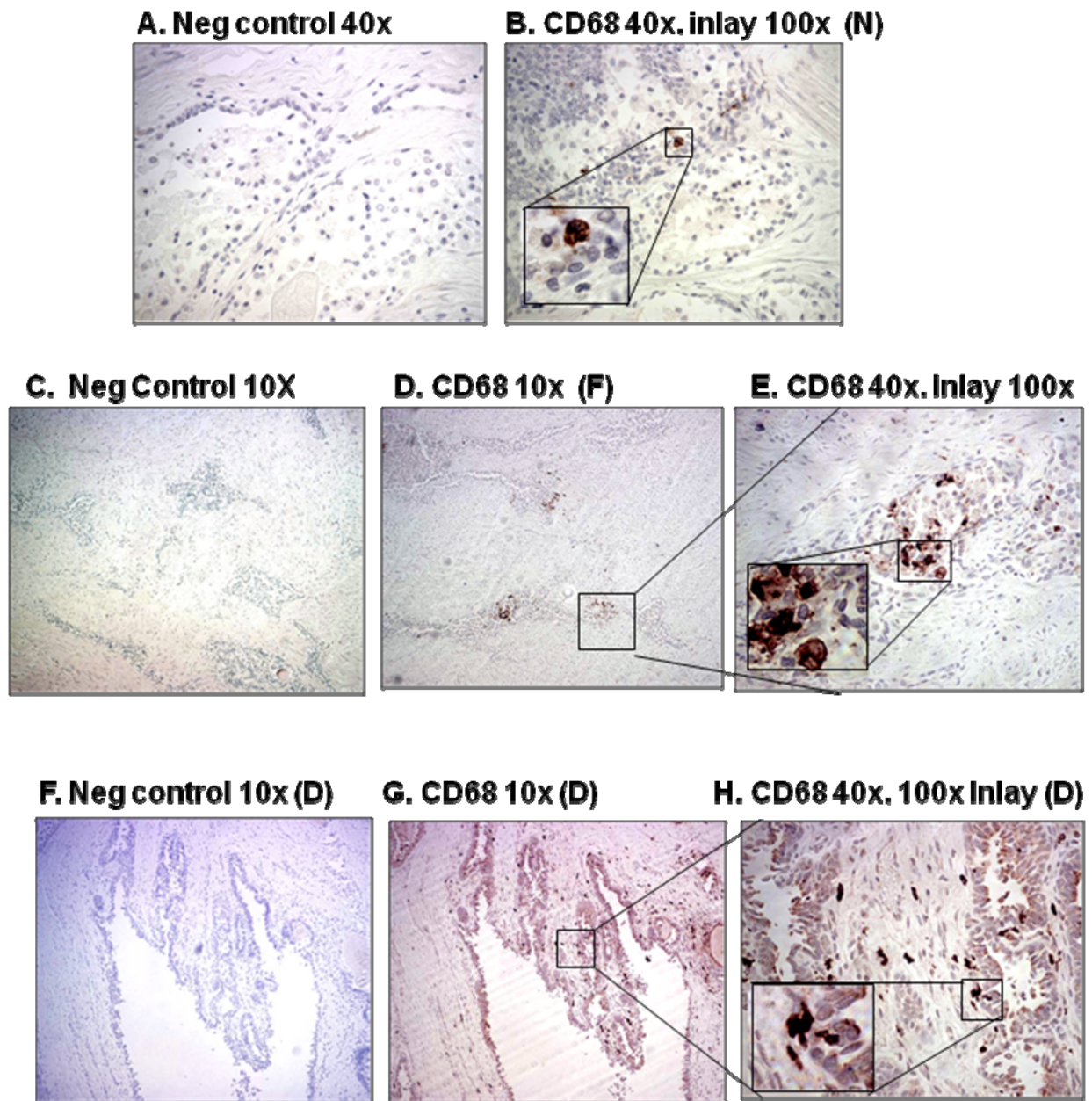
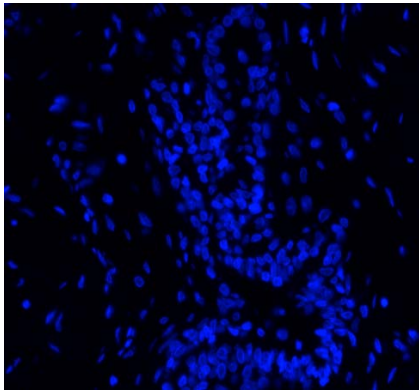


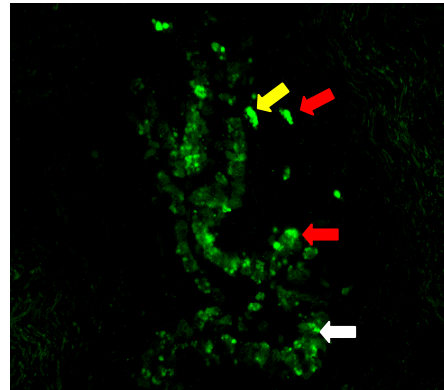
Figure 7: CD68 Expression in the Prostates of Three Representative Subjects.

All negative control slides are stained with goat serum. A and B are serial sections, A shows the negative control slide at 40x and B shows a representative no (N) inflammation staining at 40x with the insert at 100x. C, D, and E are serial sections, C shows the negative control slide at 10x, D shows a representative focal (F) inflammation staining at 10x, and E shows the same slide in D at 40x with an insert at 100x. F, G, and H are serial sections, F shows the negative control slide at 10x, G shows a representative diffuse (D) inflammation staining at 10x, and H shows the same slide in G at 40x with an insert at 100x.

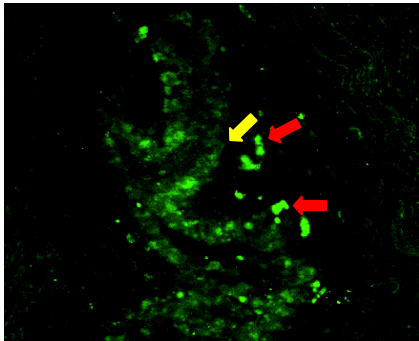
A. Staining for Dapi40x



B. Staining for vIL-6 40x



C. Staining for CD68 40x



D. Staining for PSA 40x

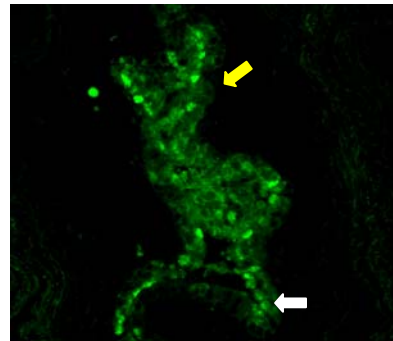


Figure 8: Serial Sections Stained with CD68,vIL-6, and PSA.

A. dapi staining. B. vIL-6 staining. C. CD68 staining. D. staining for PSA. The red arrows represent vIL-6, CD68 positive cells; the white arrow represents a vIL-6, PSA positive cell. The yellow arrow represent a positive vIL-6 cell that is negative for both CD68 and PSA. All pictures were taken at 40x.

We also detected cells present in the prostate that were positive for vIL-6 and negative for both CD68 and PSA. The CD20 staining showed that infiltrating B-cells were not present in the infected prostate. It is possible that these infected cells are prostatic stromal cells. Additional staining will be required to identify the cells. Nonetheless, our results demonstrate that HHV-8 can infect prostate cells and establish a latent infection.

Our results support the results of Diamond *et al*⁹³ who reported that HHV-8 could establish infections in prostate tissue of HIV-infected men. Our data extends their report in that we have detected the expression of multiple viral proteins by IHC while the previous study used *in situ* hybridization to detect viral mRNA, a method that has been criticized for false positive results due to edge artifact. In addition we have analyzed a total of 18 HHV-8 seropositive and seronegative men both with and without KS, while the Diamond study analyzed eight KS patients.

We have shown that prostates of HIV-infected men known to express HHV-8 proteins have increased levels of inflammation as measured by the presence of infiltrating macrophages. This increased inflammation is not due to an increase in the presence of circulating blood since there was no substantial B cell infiltrate (as measured by antibodies to CD20). This suggests the establishment of a chronic inflammation in the prostates of HHV-8 seropositive, HIV-infected men. Interestingly, inflammation and sexually transmitted agents have been suggested as co-factors in the development of prostate cancer. In support of this, we have previously reported an increased seroprevalence of HHV-8 among men with prostate cancer in the Caribbean island of Tobago⁷. The potential link between HHV-8 infection, inflammation, and prostate cancer deserves further study.

2.7 Acknowledgements

Data in this manuscript were collected by the Multicenter AIDS Cohort Study (MACS). With centers (Principal Investigators) located at: The Johns Hopkins Bloomberg School

of Public Health (Joseph Margolick); Howard Brown Health Center and Northwestern University Medical School (John Phair); University of California, Los Angeles (Roger Detels); University of Pittsburgh (Charles Rinaldo); and Data Analysis Center (Lisa Jacobson). The MACS is funded by the National Institute of Allergy and Infectious Diseases, with additional supplemental funding from the National Cancer Institute; and the National Heart, Lung, and Blood Institute: UO1-AI-35042, 5-M01-RR-00052 (GCRC), UO1-AI-35043, UO1-AI-37984, UO1-AI-35039, UO1-AI-35040, UO1-AI-37613, and UO1-AI-35041.

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3.0 Chapter Three: Image Analysis Validation

Validation of a Low Cost Computer-Based Method for Quantification of Immunohistochemistry Stained Sections

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Short Running Title : Low Cost Computer Based Method for Quantification of IHC

Keywords : Immunohistochemistry, Quantification, IHC

3.1 Preface

The majority of the data contained in this dissertation involves immunohistochemical analyses. In order to quantify immunohistochemical data, laboratories have one of two options: they can manually count the cells or they can use a computer based method. Manual counting is laborious and time consuming and introduces the element of human error. Computer based methods are the better option, but most computer image analysis software is expensive and beyond the budgetary reach of most small to medium laboratories. We have the Alpha- Diga Doc RT™ system in the laboratory, making it readily available to us. To solve the problem of expensive image analysis, we developed this study to validate the use of the Alpha-Diga Doc RT™ system for the immunohistochemical image analysis.

This study is In Press in Applied Immuno-histochemistry and Molecular Morphology. I was first author on this paper. I did all of the staining of the slides and served as reader 1, along with developing the technique of using the computer program for cell counting.

3.2 Abstract

The aim of the present study was to determine if the Alpha DigiDoc RT™ system would be an effective method of quantifying immunohistochemical (IHC) staining as compared to a manual counting method, which is considered the gold standard. Two readers were used to count 31 samples by both methods. The results obtained using the Bland-Altman for concordance deemed no statistical difference between the two methods.

Thus, the Alpha DigiDoc RT™ system is an effective, low cost method to quantify IHC data.

3.3 Introduction

A quantitative evaluation of immunohistochemical (IHC) staining is limited to the determination of the percentage of positively stained cells in a given area or within a given number of cells^{105,106}. Levels of staining based on intensity of stain cannot be determined due to the lack of uniform staining. Currently, two options are available to quantify IHC staining; counting the positive cells manually, which is considered to be the gold standard, or using a computer-based counting program¹⁰⁷. Manual counting is laborious, time consuming, and somewhat prone to human error. However, most computer-based methods of counting, which require less time and reduce the possibility of human error, are expensive and beyond the budget of most small to medium laboratories¹⁰⁸. For these reasons, the identification of a low cost computer-based counting program which is effective, determined by being fast, accurate, and reliable, would be of great interest to many laboratories.

The goal of this study was to determine if the Alpha DigiDoc RT™ system is comparable to manual counting in determining the percentage of IHC stained cells in a given area. We chose the Alpha DigiDoc RT™ system for its affordability and ease of use.

3.4 Materials and Methods

3.4.1 Immunohistochemistry

Paraffin sections from 31 cases of prostate cancer were analyzed as described in chapter 2 section 2.4.1. The differences in the procedure are noted as follows. Monoclonal anti CD3 antibody was used as primary antibody at a concentration of 1:500. (Santa Cruz Cat. # sc-1239). Biotinylated goat anti mouse IgG was used as a secondary antibody (Vector labs Cat. # BA 9200). Optimal concentrations for the primary and secondary antibodies were determined using paraffin sections from a leukocyte block. Once the optimal antibody concentrations were determined, the procedure was performed on the human tissue samples.

3.4.2 Image Acquisition

Images were obtained using a Micromaster microscope from Fisher Scientific (cat. # 12-560-48) at one magnification (200x). The image capturing software was Micron 1 (Westover Scientific cat # ZP-MC-0036[A]). This gave an area of 640 x 480 pixels and each pixel occupied 24 bits (8 bits for red, green, and blue, respectively) (Figure 9A). One picture from each of 31 total slides taken at 200x was used for the analysis.

3.4.3 Manual counting method

Images were imported into Microsoft Paint and a grid of 0.062 in² was applied (Figure 9B). Two separate readers were employed to determine the percentage of stained

cells by counting both the number of stained and unstained cells in the grid from each of the 31 pictures (positive cells stained red in color).

3.4.4 Alpha DigiDoc RT™ Counting Method

Images were imported into the Alpha DigiDoc RT™ system (AlphaEase FC software 4.1.0) and equalized using the software. This allowed the colors to sharpen and become more distinct. The images, as described above, were converted to 8-bit grayscale. The grid was selected and threshold was determined by the reader. The threshold function allows the operator to determine the level of staining to be labeled as positive. Using VectorRED, positively stained cells stain as reddish-brown, while negatively stained cells are clear with a light blue nucleus due to hematoxylin counter stain. When this color image is converted to grayscale, the reddish-brown (positive) cells appear darker when compared to the negative cells. By adjusting the threshold the operator can insure that the positive cells are selected for counting. The software's auto count function then determines the number of positive cells within the grid. If the reader disagrees with the computer's determination regarding the staining of a particular cell, then that cell could be removed or added from the count by use of the manual tools. This function permits the removal of erroneous material, such as cellular debris and other staining artifacts that may be present in the section. Next, the total cell count was determined by changing the threshold such that all of the cells in the area are selected as positively stained. From these results the percent positive cells was determined. This progression can be seen in Figure 10.

3.4.5 Statistical Analysis

The degree of concordance between the 2 methods was determined by Bland-Altman plots^{109,110}. Each comparison was subjected to a power analysis, assuming equal variances in readers, to determine if there was significant power to detect the observed bias.

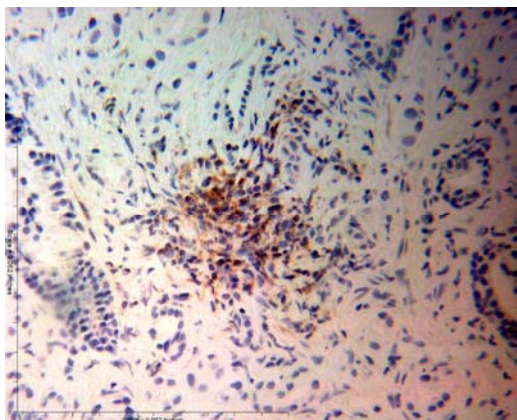
3.5 Results and Discussion

3.5.1 Adaptation of the Digi-Doc RT™ system for analysis of IHC stained sections

To adapt the Alpha DigiDoc RT™ system for IHC stained sections, we analyzed 31 prostatic biopsy sections stained with a monoclonal anti-CD3 antibody which detects the presence of T lymphocytes. Our staining protocol results in the CD3 positive cells staining reddish-brown. Hematoxylin was used as a counterstain so that the nuclei of all cells (both positive and negative stained cells) appear blue. Stained sections were photographed as shown in Figure 9 and a 0.062 in² grid was applied to a representative area (Figure 9B). This image was then imported into the Alpha DigiDoc RT™ program. Figure 10 shows the progression from the original photograph through the selection of positive cells using the Alpha DigiDoc RT™ program. The original image was equalized by the software to allow for better color distinction (Figure 10B). The image was converted to grayscale, the threshold was applied to the grid of interest, and the percent positive cells were determined (Figure 10C-G). Once a percentage was calculated, the

results were plotted and compared by Bland-Altman. This process was performed on 31 samples.

A.



B.

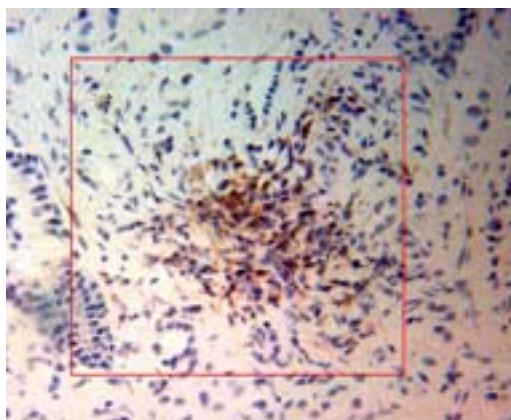


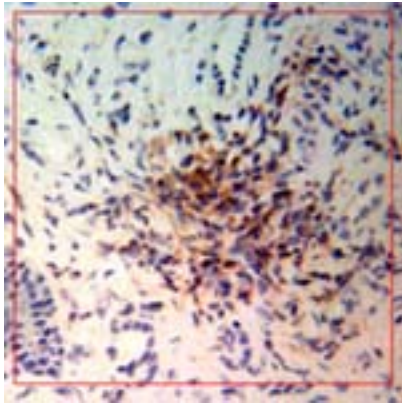
Figure 9: Example of Picture and Grid

A.) Picture of sample taken with the Micron 1 software on the Micromaster microscope
B.) Picture with the 0.062 inch² square added. The square represents the area to be analyze.

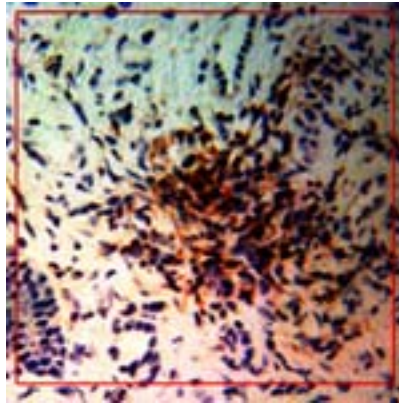
3.5.2 Comparison of Readers Using Manual Counting and Alpha DigiDoc RT™ program

We first determined if there was any significant difference between the two readers using either manual counting or the computer method in determining the percentage of positive cells. As shown in Figure 11A, the comparison between manual counting of 31 separate sections by the two readers using the Bland-Altman plot indicated that there was not significant error. The average percent difference between the two readers using manual counting was -0.13. Further, there was also no significant error found in comparisons between the Alpha DigiDoc RT™ program of the different sections by the two readers. The average percent difference between the two readers using the Alpha DigiDoc RT™ system was 7.04 (Figure 11B).

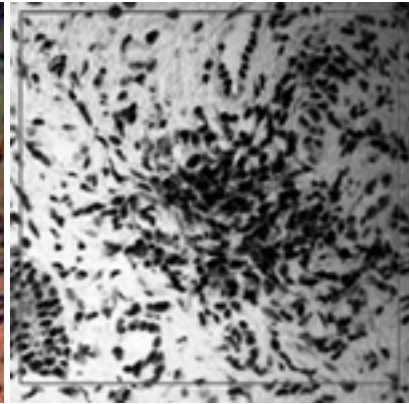
A. Picture from Micron 1



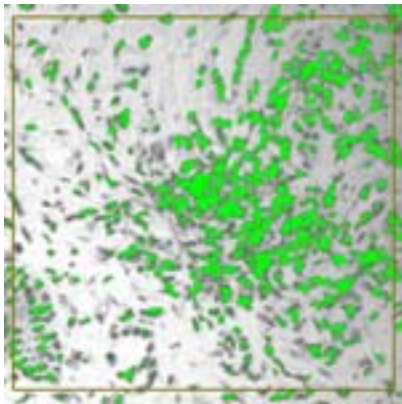
B. Picture Equalized



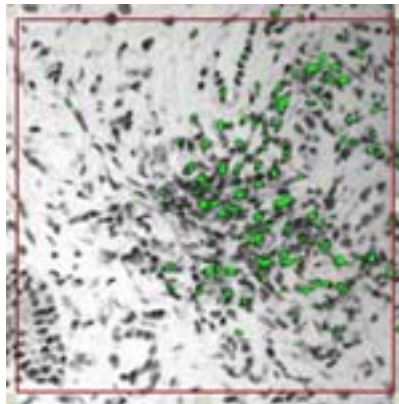
C. Gray Conversion



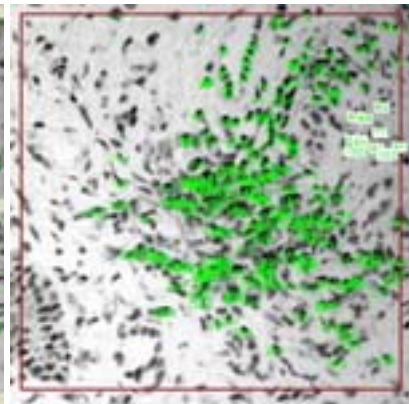
D. Total Cells (Threshold)



E. Positive Cells (Threshold)



F. Erased Cells



G. Final Count of Positive Cells

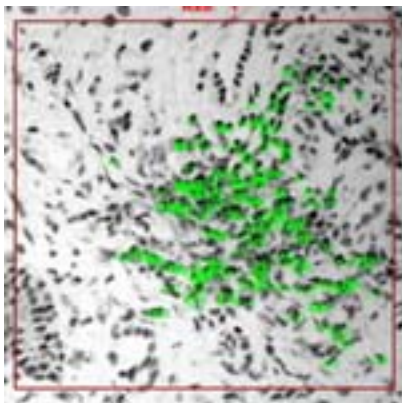


Figure 10: Alpha Digidoc™ Counting Method

A.) Picture of sample taken from the Micromaster microscope using Micron 1 software
B.) Picture equalized in Alpha DigiDoc RT™ C.) Picture converted to grayscale in Alpha DigiDoc RT™ D.) Total cells as determined by Alpha DigiDoc RT™ E.) Positive Cells as determined by Alpha DigiDoc RT™ F.) Erased cells as determined by reader G.) Final count of positive cells

3.5.3 Comparison of Manual Counting and Alpha DigiDoc RT™ program

The comparison of manual and computer-based results are also presented with Bland-Altman plots. As shown in Figure 11C, the two methods were comparable with each reader. The average percent difference between the two methods was 5.26 (Reader 1) and 12.43 (Reader 2).

Using two separate readers to analyze 31 separate IHC stained sections, it was determined that no significant bias was detected between manual counting and Alpha DigiDoc RT™ program. This program was found to be fast, reliable, and cost-effective, enabling small to medium size labs an affordable method for computer based quantification of IHC staining. It is noteworthy that of the two readers used in this study, one had over three years experience reading IHC slides, while the other had less than one month. This demonstrates that the Alpha DigiDoc RT™ program can be used by both experienced and inexperienced individuals for semi-quantitative analysis of IHC stained slides.

The Alpha DigiDoc RT™ was designed for colony counting, but the novel method (described in the present study) of using it for counting cells stained by immunohistochemistry allows labs with restricted budgets to semi-quantitatively analyze their stained slides. In addition, the Alpha DigiDoc RT™ can be used with any image capture software. The authors used the Micron 1 (Westover Scientific cat # ZP-MC-0036[A]) image capturing software, which is included when a Micromaster microscope is purchased for \$2,243 from ThermoFisher. The cost of the Alpha DigiDoc RT™ is \$826; thus, this semi-quantitative method of cell counting can be purchased for

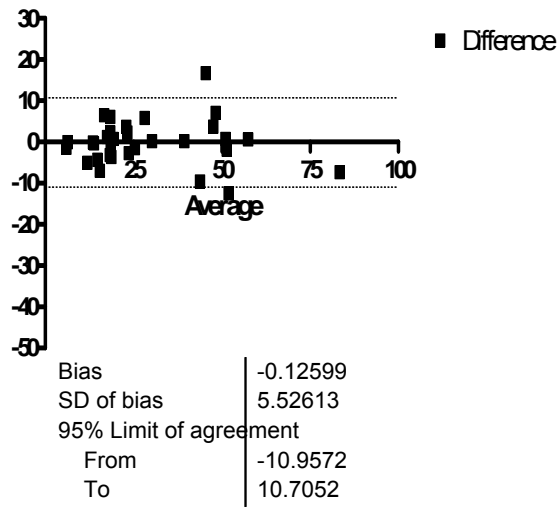
approximately \$3,000. The cost of a more conventional system using software specifically designed for analyzing stained tissue sections (such as Metamorph, Molecular Devices) is approximately \$14,000, almost 5 times the cost of using the Alpha DigiDoc RT™ system. The Alpha DigiDoc RT™ system can be useful for the quantification of many different cell types such as, cytokine producing cells, individual populations as identified by expression of various cellular markers, proliferating cells, and apoptotic cells. Therefore, this system could be a very powerful tool, due to its wide applicability and low-cost, for multiple applications.

3.6 Acknowledgements

The funding for this study was provided by the University of Pittsburgh Cancer Institute.

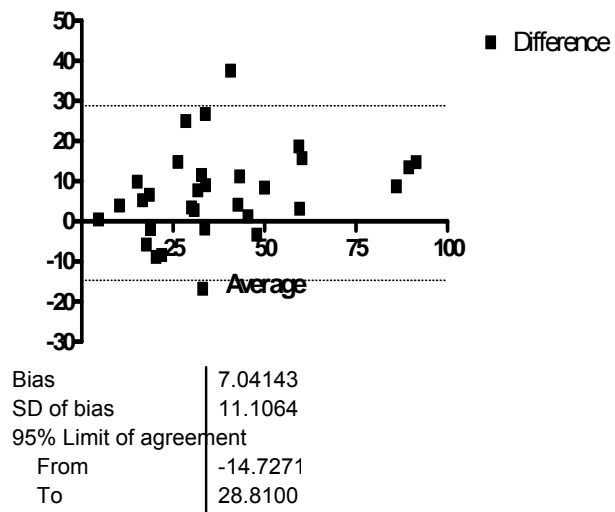
A.

Bland-Altman of Reader 1 Manual vs Reader 2 Manual: Difference vs average



B.

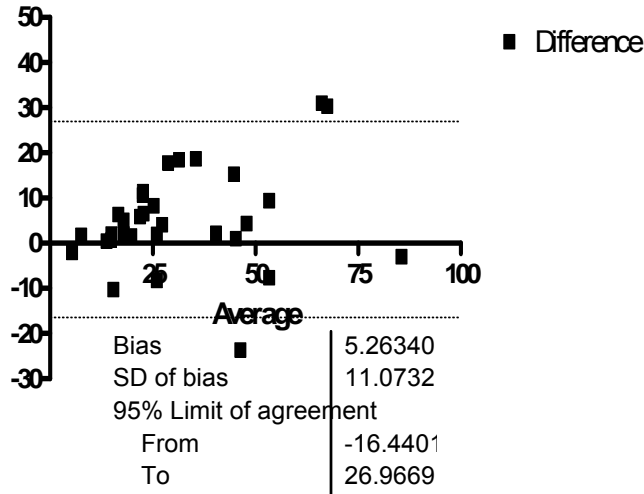
Bland-Altman of Reader 1 Comp vs Reader 2 Comp: Difference vs average



(Figure 11 Continued Below)

C.

Bland-Altman of Reader 1 Manual vs comp: Difference vs average



D.

Bland-Altman of Reader 2 Manual vs Comp: Difference vs average

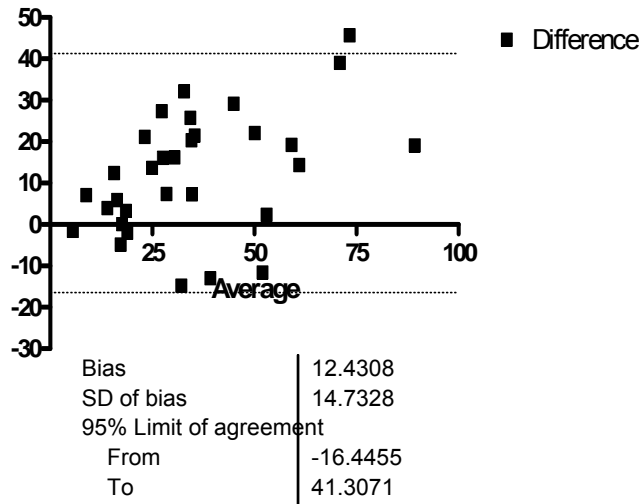


Figure 11: Bland-Altman plots of Concordance

(A) Manual count, reader 1 vs. reader 2 (B) Alpha DigiDoc RT™ use, reader 1 vs. reader 2 (C) Reader 1, manual vs. Alpha DigiDoc RT™ program (D) Reader 2, manual vs. Alpha DigiDoc RT™ program.

4.0 Chapter Four: Detection of HHV-8 in Cancerous Prostates from Tobago.

4.1 Introduction

We have previously shown that HHV-8 is present in normal, non-cancerous prostates and expresses both lytic and latent proteins (Chapter 2, ⁸⁸). We have also shown that the presence of HHV-8 protein expression in the prostate results in an increased inflammation as determined by increased immune infiltrate (Chapter 2;⁸⁸). The study described in Chapter 2 was conducted on non-cancerous prostates from men that died with AIDS. If HHV-8 is an infectious agent cofactor for prostate cancer, then we should also be able to find it in cancerous prostates of seropositive men. In addition, we need to determine if the virus is present in cancerous cells or surrounding normal cells.

In this Chapter, we will describe analyses of prostate samples of men from the Caribbean island of Tobago. The cohort is divided into two study groups, biopsy material from 19 men and prostatectomy material from 20 men. The biopsy group consists of 19 men, 12 seropositive for HHV-8 and 7 seronegative for HHV-8. The prostatectomy group consists of 19 men, 11 seropositive and 9 seronegative for HHV-8; the prostatectomy group has tissue blocks representing areas of the prostate with cancer and areas that are cancer-free (as determined histologically by a pathologist).

In addition, previous work from our laboratory has demonstrated an interaction between a R148G polymorphism in the gp130 signaling receptor of human IL-6 and HHV-8 seropositivity that results in an increased risk for prostate cancer in Tobago (see section

1.7 table 2). As a result, we have selected prostatectomy cases based on both HHV-8 seropositivity and gp130 genotype (Figure 12).

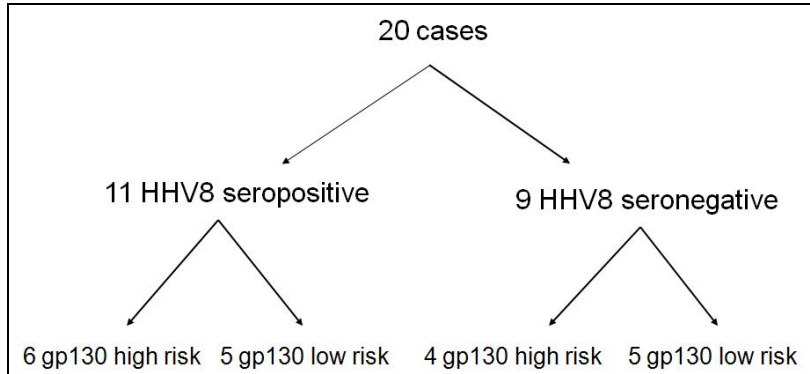


Figure 12: Breakdown of Tobago Prostatectomy Subjects by HHV-8 seropositivity and gp130 allele status.

In this study, we performed immunohistochemistry on cancerous prostates from 39 men, 20 who received prostatectomies and 19 men who received biopsies from Tobago. Serial sections were examined for expression of the latent HHV-8 viral protein, LANA-1, the lytic viral proteins, K8.1 and vIL-6 (for biopsy only). Localized inflammation was determined by using a macrophage/monocyte marker, CD68, and a B-cell marker, CD20 (only on the prostatectomy subgroup, see table 4).

4.2 Methods

4.2.1 Immunohistochemistry

See section 2.4.1 of Chapter 2

The type of HHV-8 infection was discerned by the presence of LANA-1 for a latent infection and the presence of vIL-6 and/or K8.1 for lytic infection. Controls consisted of staining serial sections with normal rat, mouse, or goat serum depending on the species of the primary antibody. A TPA-induced BCBL-1/BJAB mixture was used as positive control for the viral staining. BCBL-1 is a B cell line that is HHV-8 positive¹⁰¹ where TPA induced cells act as a lytic control and uninduced cells act as a latent control. The BJAB line is a well studied, HHV-8 negative B cell line.

4.2.2 Subjects

A full description of the Tobago Prostate Survey is given in Bunker et. al⁶. Briefly, the study is ongoing in the Caribbean island of Tobago. The protocol was approved by both the Institutional Review Boards of the University of Pittsburgh and the Division of Health and Social Services, Tobago House of Assembly. Participants in the study are recruited mostly by public service announcement and by word of mouth. Thus far, 60% of the male residents between the ages of 40-79 are enrolled. Each participant receives a PSA screening and a digital rectal exam (DRE). Those patients having an elevated PSA, 4.0 ng/ml or more, or an abnormal DRE are referred to Tobago Regional Hospital for sextant biopsy. The biopsy prostate tissues were taken during this routine surgery

while the prostatectomy samples were taken during a subsequent surgery. Hematoxylin and eosin staining was performed on all samples to determine cancer status.

4.2.3 HHV-8 Serology

HHV-8 serostatus was determined using an immunofluorescence assay as described previously¹⁰⁰.

4.2.4 Percentage of positive cells

See sections 3.4.2 and 3.4.4.

4.2.5 Statistics

The SPSS statistical package was used for statistical analysis. All populations were assumed to be non-parametric. We used the Wilcoxin signed ranks test and the Mann-Whitney test to determine statistical significance.

4.2.6 gp130 Genotyping

Determination of gp130 genotype was performed by Dr. Robert Ferrell's laboratory in the Department of Human Genetics, Graduate School of Public Health, University of Pittsburgh. The primers used were, forward (5'ccagcaaaaatgactaac 3'), and reverse (gttgattgtgaacgagg). The annealing temperature was 54°C with a Magnesium Chloride salt concentration of 2.0mM. The detection sequence primer was (5'

caagtgtgtttccctccac 3'). All polymerase chain reactions (PCR) were done at 35 cycles of 95°C for 30 sec, annealing temperature for 30 sec, followed by 30 sec of 72°C. Ten microliters of the PCR product was cut with SAP/EXO to remove excess primers and dinucleotide triphosphates, dNTPs. Following digestion of the PCR product, a one base pair extension at position 148 was done with fluorescently labeled dNTPs for detection.

4.3 Results

4.3.1 Characterization of the Tobago Biopsy Subgroup

The Tobago biopsy subgroup represents men with prostate cancer where the tissue was taken at biopsy. All subjects were deemed cancerous by pathological examination. Table 4 lists the subject characteristics and protein expression for the biopsy study group. Figure 13 shows the percentage of seropositive men who demonstrated antigen expression of at least on protein, LANA-1, vIL-6, or K8.1.

Table 4: Subject Characteristics and Protein Expression Results of Cancerous Biopsies of Prostates from Tobago.

Number	Gleason Score	Serostatus	LANA	% LANA	vIL-6	% vIL-6	K8.1	% K8.1	CD68	Ki67
1	6	-	-	0	-	0	-	0	+	+
2	7	-	-	0	-	0	-	0	+	+
3	6	-	-	0	-	0	-	0	+	+
4	8	-	-	0	-	0	-	0	+	+
5	7	-	-	0	-	0	-	0	+	+
6	7	-	-	0	-	0	-	0	+	+
7	9	-	-	0	-	0	-	0	+	+
8	8	+	-	0	-	0	-	0	+	+
9	8	+	-	0	-	0	-	0	+	+
10	8	+	-	0	-	0	-	0	+	+
11	6	+	-	0	-	0	+	0.01	+	+
12	6	+	-	0	-	0	+	10.43	+	+
13	7	+	-	0	+	9.01	-	0	+	+
14	8	+	-	0	+	8.46	-	0	+	+
15	6	+	+	5.56	-	0	-	0	+	+
16	7	+	+	12.12	-	0	-	0	+	+
17	6	+	+	16.85	-	0	-	0	+	+
18	6	+	+	1.75	+	7.25	-	0	+	+
19	6	+	+	7.25	+	4.44	-	0	+	+

Table 4 shows the subject characteristics of the Biopsy subgroup. %protein means the number of positively stained cells in the specified grid. (see chapter 3).

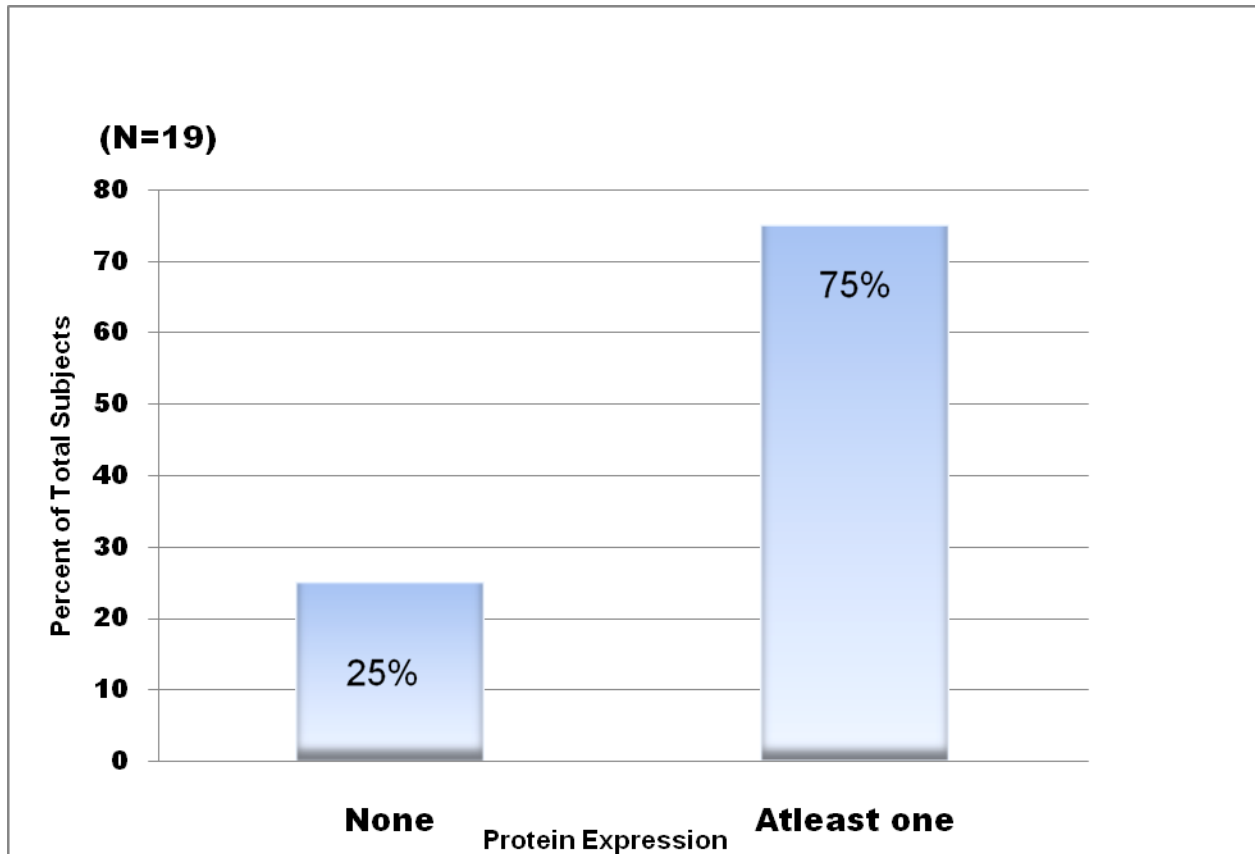


Figure 13: HHV8 Protein Expression in the Cancerous Biopsies.

The graph shows that 75% of the seropositive subjects expressed at least one protein (LANA-1, K8.1, or vIL-6) and that 25% of the seropositive subjects expressed no viral proteins.

4.3.2 Viral Protein Expression by IHC Staining- Tobago Biopsy Subgroup

The conditions for IHC established in section 2.5.2 are the same used in this study. Briefly, we used two different controls to optimize the staining, a KS lesion and a 2:1 BCBL-1: BJAB mix. Figure 14 shows staining for all HHV-8 proteins, LANA-1, vIL-6, and K8.1 in a representative prostate biopsy samples. Negative controls consisted of

staining serial sections with normal rat or mouse serum as the primary antibody. The arrows indicate areas of cancer.

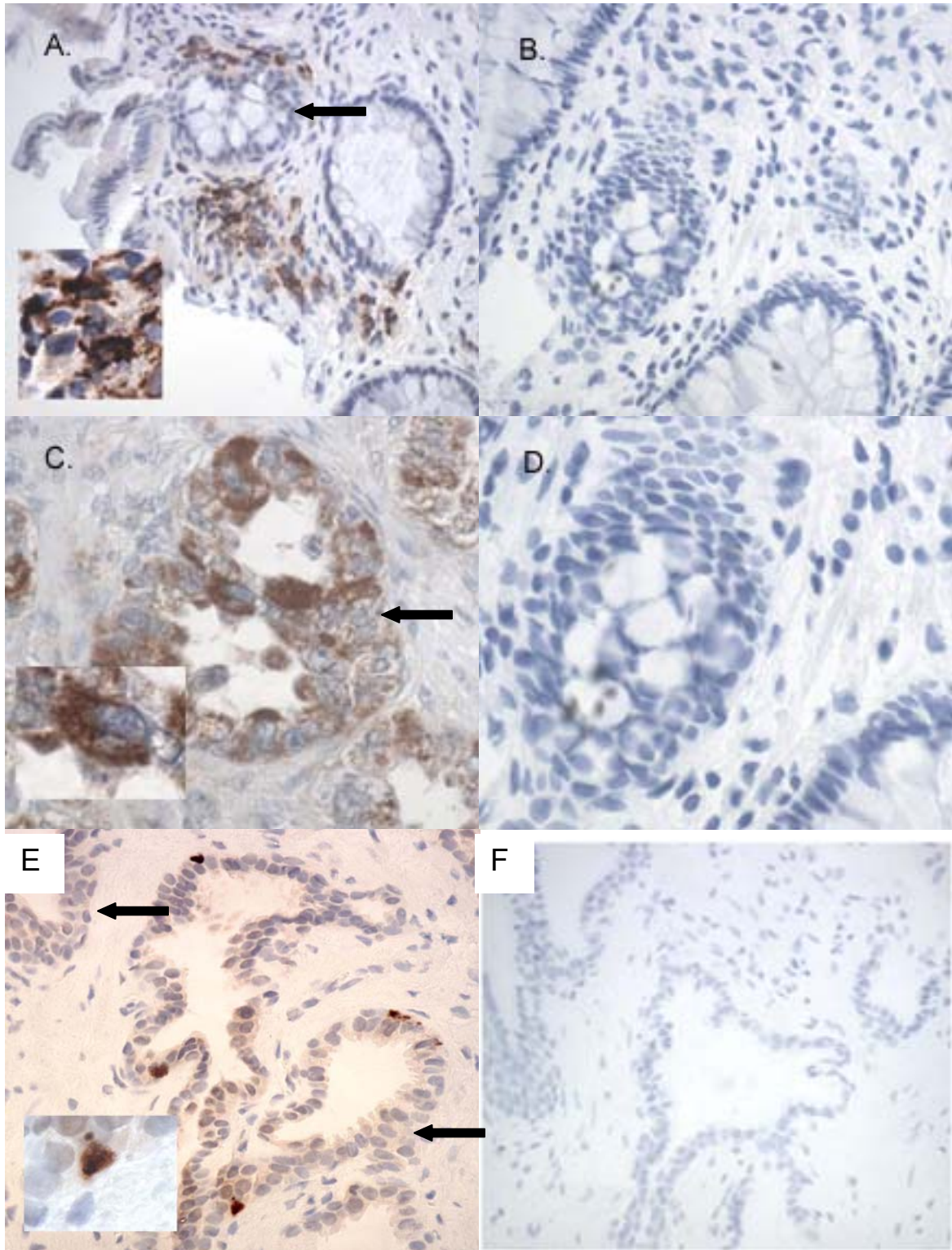


Figure 14: HHV-8 Proteins in the Prostate, but not in the Cancer. Tobago Biopsy Subgroup.

A. Shows staining for K8.1 at 400x, insert at 1000x. B. Shows negative control staining for normal mouse serum at 400x. C. Shows staining for vIL-6 at 400x, insert at 1000x. D. Shows staining for normal rabbit serum at 400x. E. Shows staining for LANA-1 400x, insert 1000x. F. Shows staining for normal rat serum 400x. The arrows indicate areas with cancer.

HHV-8 protein expression as detected by IHC in the biopsy prostates is shown in Table 4. Each subject had serial sections from the paraffin block stained for LANA-1, vIL-6, and K8.1. Among the HHV-8 seropositive subjects, viral protein expression was detected in multiple regions of each section, but not in the cancer. Some of the subjects show both lytic and latent expression in the stained regions, while other regions contained only latent or lytic. HHV-8 protein expression was not seen in any of the HHV-8 seronegative subjects. All subjects stained positive for Ki67, a marker of cellular proliferation which is often used to demonstrate the presence of cancer.

Among the seropositive subjects, HHV-8 protein expression of at least one protein was seen in 75% (9/12) of the biopsies (summarized in figure 13). LANA-1 was expressed in the majority of stained sections, 5/9 or 55.6%. Three of the twelve HHV-8 seropositive men did not express any HHV-8 proteins (Table 4).

In order to determine the amount of inflammation in each prostate, individual sections were stained with antibodies directed against CD68, a macrophage marker. Representative samples can be seen in Figure 15. We used a semi-quantitative image approach developed in our laboratory and described in sections 3.4.2 and 3.4.4 of this dissertation to determine the percentage of macrophages present in a defined area. This analysis produces a total number of positive cells that is adjusted for total cell number. These results are shown in Table 4.

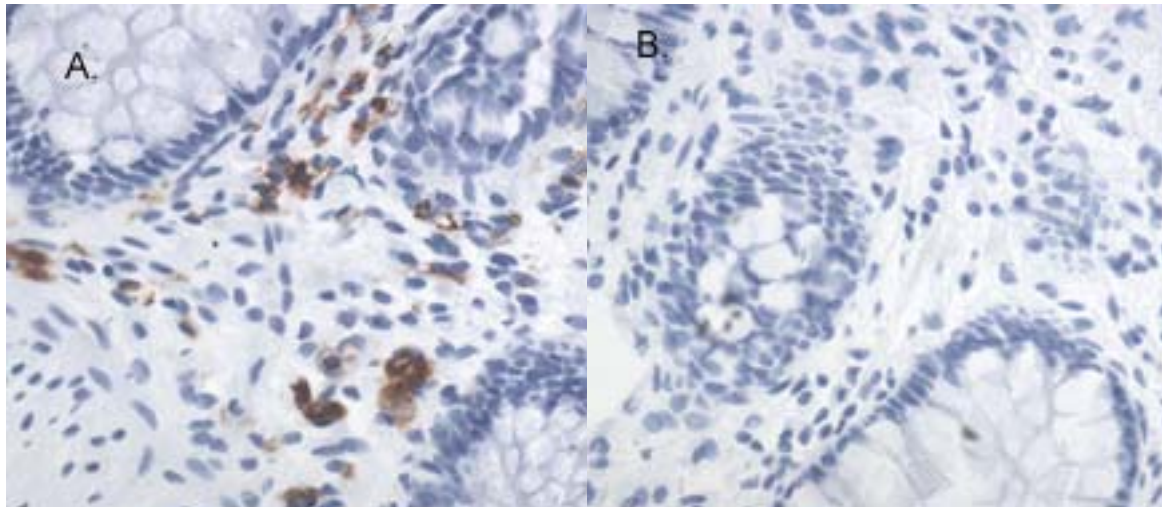


Figure 15: CD68 Staining in the Tobago Biopsy

A. Shows a representative prostate biopsy sample stained with CD68. B. Shows the negative control serial section prostate biopsy stained with normal goat serum.

There was no difference seen in the percentage of CD68-positive cells between the seronegative and seropositive subjects (Tables 5).

Table 5. Differences in CD68 expression in Seropositive and Seronegative Subjects

	%CD68	
	Mean of % positive CD68 staining +/- SD	p*
Seronegative	12.37 +/- 5.71	0.432
Seropositive	12.4 +/- 12.61	

*Mann-Whitney U

4.3.3 Characterization of the Tobago Prostatectomy Subgroup

The Tobago Prostatectomy Subgroup represents men with prostate cancer who had their prostates removed with surgery. The group is comprised of 20 men, all deemed cancerous by pathological examination. The subjects were selected based on their HHV-8 serostatus and gp130 genotype (Figure 12.)

Table 6: Subject Characteristics and Protein Expression-Tobago Prostatectomy

Subject	Cancer (C)/Non-Cancer(NC)	HHV-8 Serostatus	LANA	% LANA	K8.1	% K8.1	CD68	% CD68	CD20	% CD20
1	C	+	+	35.03	+	0.25	+	0.22	+	60.56
1	NC	+	+	0.52	+	3.76	+	2.35	+	19.64
2	C	+	+	0.56	+	0.26	+	9.44	+	40.16
2	NC	+	+	1.35	+	1.01	+	5.91	+	5.36
3	C	+	+	6.04	+	4.08	+	3.93	+	76.34
3	NC	+	+	1.22	+	15.27	+	0.27	+	83.59
4	C	+	+	31.67	+	2.44	+	2.5	+	33.85
4	NC	+	+	11.46	+	6.69	+	37.69	+	60.55
5	C	+	+	0.93	+	2.93	+	2.75	+	70.62
5	NC	+	+	1.52	+	2.47	+	0	+	0.22
6	C	+	+	17.87	+	7.63	+	70.61	+	2.24
6	NC	+	+	2.4	+	6.15	+	0.695	+	3.15
7	C	+	-	0	+	1.35	-	0	+	39.57
7	NC	+	-	0	+	16.32	-	0	+	79.1
8	C	+	-	0	+	0.87	+	0	+	87.54
8	NC	+	-	0	+	0.67	+	0	+	53.12
9	C	+	+	0.35	+	56.77	+	16.95	+	60.03
9	NC	+	-	0	-	0	+	0	+	79.75
10	C	+	-	0	-	0	+	26.56	+	77.12
10	NC	+	-	0	+	2.44	+	3.48	+	26.56
11	C	+	-	0	+	3.41	+	5.21	-	0
11	NC	+	-	0	+	2.23	+	0	+	43.44
12	C	-	+	3.13	+	13.03	+	0	+	31.09
12	NC	-	-	0	-	0	+	0	+	39.83
13	C	-	-	0	+	11.02	-	0	+	11.44
13	NC	-	+	0.5	+	4.16	+	16.67	+	63.03
14	C	-	-	0	-	0	+	4.32	+	60.58
14	NC	-	+	3.66	-	0	+	11.93	+	73.97
15	C	-	+	17.08	+	45.78	+	0.11	+	58.09
15	NC	-	+	5.84	+	0.34	+	0.28	+	22.97
16	C	-	+	5.59	+	0.33	+	1.03	+	44.26
16	NC	-	+	11.09	+	0.99	+	0.51	+	58.45
17	C	-	+	0.81	+	44.85	-	0	+	51.83
17	NC	-	+	1.41	-	0	-	0	+	57.57
18	C	-	-	0	-	0	-	0	+	27.19
18	NC	-	-	0	-	0	-	0	+	11.5
19	C	-	-	0	-	0	-	0	+	28.83
19	NC	-	-	0	-	0	-	0	-	0
20	C	-	-	0	-	0	-	0	+	45.29
20	NC	-	-	0	-	0	-	0	+	45.85

The table shows that for each subject I had a slide of an area with cancer (C) and a slide of an area without cancer (NC). The proteins stained for were, LANA-1, K8.1, CD68, and CD20. The percentages equal the percent of protein positive cells as determined by the threshold analysis described in chapter three.

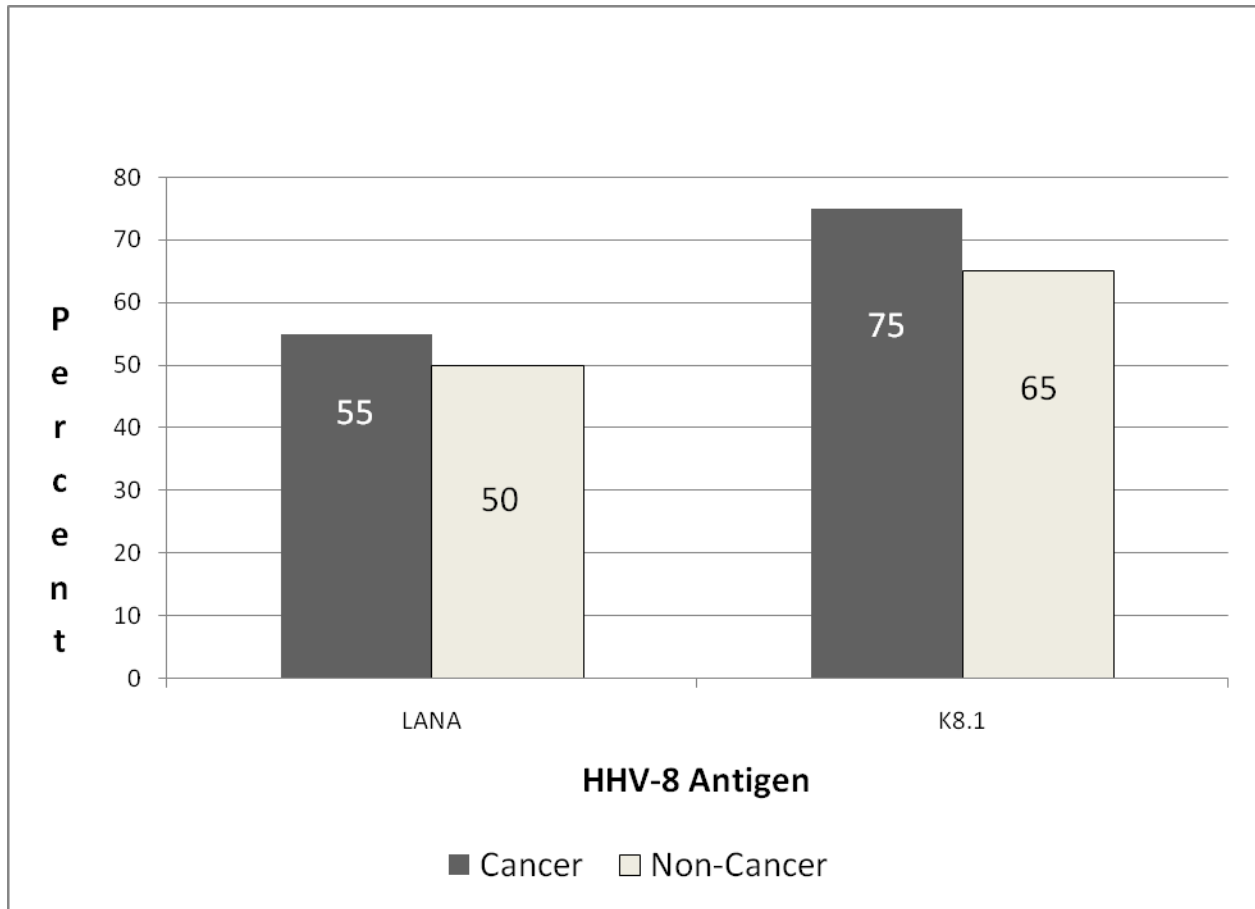


Figure 16: HHV-8 Protein Expression in Prostatectomy Subgroup from Tobago.

The graph shows the percent of subjects with and without cancer that stained positive for LANA-1 and K8.1

4.3.4 IHC Staining- Tobago Prostatectomy Subgroup

For each subject, there were paraffin blocks available representing areas with cancer and areas without cancer. Sections from each block were stained for the viral proteins K8.1 and LANA-1. Viral IL-6 staining was not done because in the previous MACS cohort it was not seen in many subjects and deemed unnecessary. Negative controls were serial sections stained with normal mouse or normal rat as the primary antibody.

HHV-8 protein expression is listed in Table 6 and a representative stain is seen in Figure 17. Each subject had serial sections stained for LANA-1 and K8.1. Among the HHV-8 seropositive subjects, viral protein expression was detected in multiple regions of each section, but not in the cancerous cells. Some of the subjects show both lytic and latent expression in the stained regions, while other regions contained only latent or lytic protein expression. Frequently, if LANA-1 was present then K8.1 was also present (12/13) subjects. Three of the nine seronegative subjects did not express viral proteins (see Table 6).

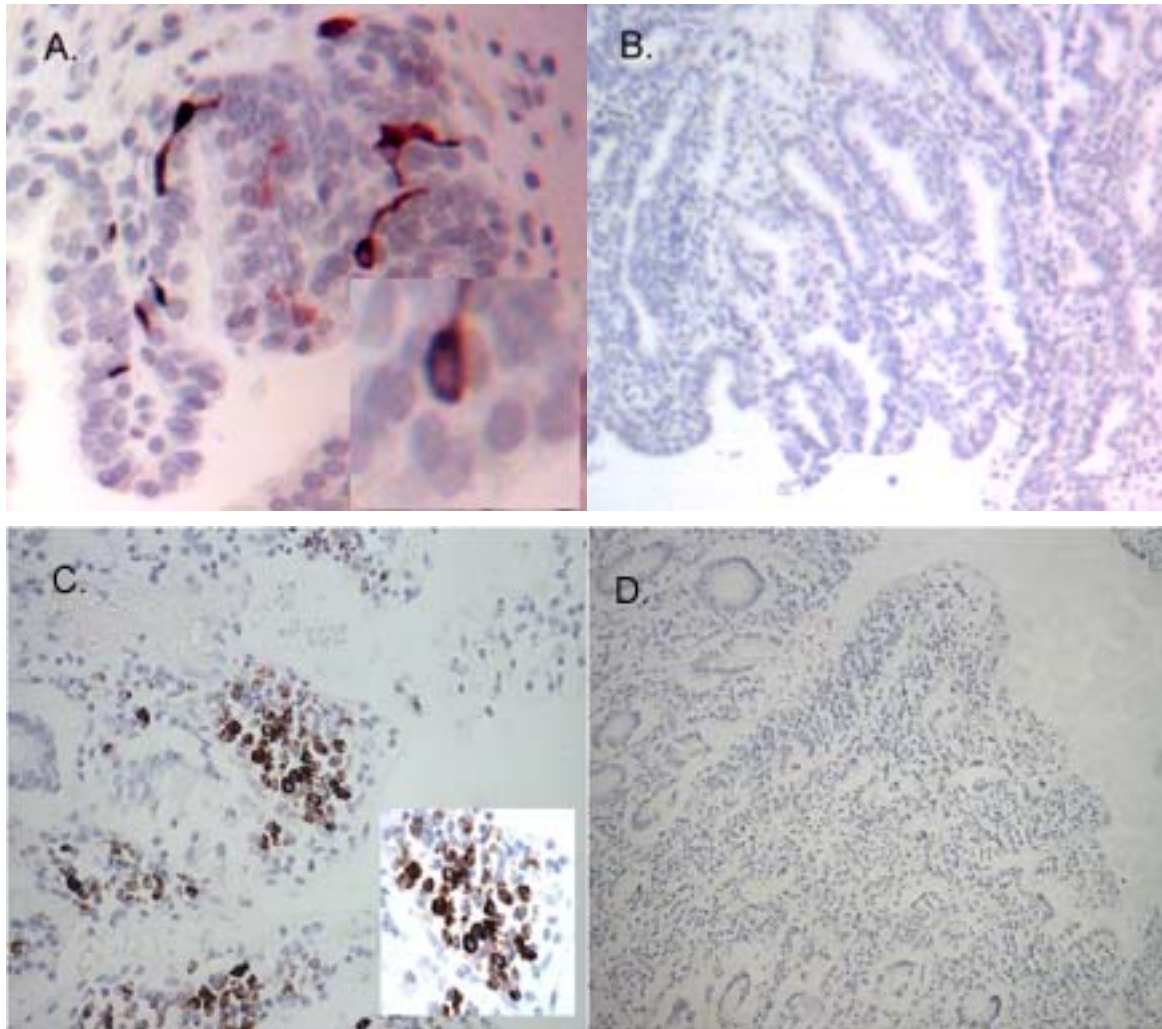


Figure 17: HHV-8 Protein Expression in the Prostates of Seropositive men from Tobago Prostatectomy group.

A. Shows staining for LANA-1 at 400x, inset at 1000x. B. Shows serial section staining for normal rat serum at 400x. C. Shows staining for K8.1 at 400x, inset at 1000x. D. Shows serial section staining for normal mouse serum at 400x.

This subgroup contained tissue blocks representing areas with cancer (C) and areas without cancer (NC) from the same subject. We asked whether there were differences seen in viral antigen expression, as measured by LANA-1 and K8.1 staining, within the same patient. Table 7 shows that there was significant difference seen between areas

with cancer and areas without cancer based on viral antigen expression. The areas with cancer exhibited a higher level of both K8.1 and LANA-1 staining.

Table 7: HHV-8 Protein Expression is Higher in Cancerous Sections

	LANA-1		K8.1	
	Mean of % LANA-1 positive cells +/- SD	p*	Mean of % K8.1 positive cells +/- SD	p*
Non-Cancer	2.91 +/- 4.49	0.001	3.68 +/- 5.02	0.000
Cancer	6.75 +/- 11.13		11.42 +/- 18.55	

*Wilcoxon Signed Ranks Test

This subgroup of the Tobago cohort was genotyped for the gp130 R148G allele that has been previously associated with a high risk for prostate cancer when combined with HHV-8 seropositivity. We asked whether differences were seen in LANA-1 and K8.1 expression based on gp130 status in the non-cancer sections. Table 8 shows the results of the Mann-Whitney test. No difference in viral protein expression was seen based on gp130 allele status in the non-cancerous areas.

Table 8: No Difference seen in HHV-8 Protein Expression Based on gp130 Allele Status in Non-cancerous areas.

	LANA		K8.1	
	Mean of % LANA-1 positive cells +/- SD	p*	Mean of % K8.1 positive cells +/- SD	p*
Non-Cancer High Risk	3.55 +/- 3.98	0.174	3.47 +/- 4.68	0.887
Non-Cancer Low Risk	2.40 +/- 5.01		3.97 +/- 5.85	

*Mann-Whitney Test

In order to determine the amount of inflammation in each prostate, individual sections were stained with antibodies directed against CD68, a macrophage marker and CD20 a B-cell marker. Representative staining can be seen in Figure 17. To determine the total number of positive cells we used a semi-quantitative image analysis developed in our laboratory and described in sections 3.4.2 and 3.4.4 of this dissertation. That analysis produced a total number of positive cells that was adjusted for total cell number. Those values can be seen in Table 6.

We asked whether within the same subject there were differences seen in inflammation based on CD68 and CD20 expression. Table 6 shows that differences were seen in inflammation; the areas with cancer exhibited an increased inflammation over the areas without cancer.

Table 9: Differences were seen in Inflammation between areas with and without Cancer within the same subject

	CD20		CD68	
	Mean of % CD20 positive cells +/- SD	p*	Mean of % CD68 positive cells +/- SD	p*
Non-Cancer	41.38 +/- 28.45	0.000	4.00 +/- 9.10	0.009
Cancer	45.33 +/- 24.43		7.19 +/- 16.40	

* Wilcoxin Signed Ranks Test

We next asked if differences were seen in inflammation based on gp130 allele status. Table 10 shows the results of the non-cancerous sections. Differences were seen in inflammation based on gp130 allele status; the gp130 high risk group exhibited an increase inflammation over the gp130 low risk group based on CD68 staining. No difference was seen in the cancerous sections (data not shown).

Table 10: Differences in Inflammation Based on gp130 Allele Status in Non-Cancerous Sections

	CD20		CD68	
	Mean of % CD20 positive cells +/- SD	p*	Mean of % CD68 positive cells +/- SD	p*
Non-Cancer High Risk	42.76 +/- 29.36	0.739	7.5610 +/- 12.06	0.023
Non-Cancer Low Risk	45.33 +/- 24.43		.4395 +/- 1.09	

*Mann-Whitney Test

4.3.5 Summary of Tobago Prostatectomy Subgroup

We asked whether differences were seen in inflammation, as measured by CD68 and CD20 expression, or viral antigen expression, as measured by LANA-1 or K8.1 expression, between areas with cancer and areas without cancer in the same patient.

Table 11 shows the summary of the data for the Tobago Prostatectomy Subgroup.

Table 11: Summary of the Tobago Prostatectomy Subgroup

	LANA-1		K8.1		CD20		CD68	
	Mean of % positive LANA-1 cells +/- SD	p*	Mean of % positive K8.1 cells +/- SD	p*	Mean of % CD20 positive cells +/- SD	p*	Mean of % CD68 positive cells +/- SD	p*
NC	2.91 +/- 4.49	0.001	3.68 +/- 5.02	0.000	41.38 +/- 28.45	0.000	4.00 +/- 9.10	0.009
C	6.75 +/- 11.13		11.42 +/- 18.55		45.33 +/- 24.43		7.19 +/- 16.40	

* Wilcoxin Signed Ranks Test

4.4 Discussion

The Tobago cohort is comprised of two subgroups, Tobago biopsies and Tobago Prostatectomies. The data presented here, from both groups, shows that HHV-8 protein expression can be detected in the prostates of men from Tobago. Viral protein expression is not present in the cancer cells, but in the surrounding tissues (Figure 14).

4.4.1 Tobago Biopsy subgroup

Among the Tobago biopsy subgroup, no viral antigen expression was seen in the seronegative subjects (table 4). Viral antigen expression, in the seropositive subjects, was similar to what we saw in the MACS cohort (see chapter 2). In the MACS cohort we saw at least one viral antigen expressed in 68.9% of the seropositive subjects, and in the Tobago biopsy subgroup we saw expression of at least on viral antigen in 75% of the seropositive subjects.

We found no significant difference in inflammatory infiltrates, based on CD68 expression, between the seropositive and the seronegative subjects (table 5). This could be a product of our N being so low. We only had 7 seronegative and 12 seropositive patients; perhaps if we were able to increase the N, we would find significant difference. It also may be explained by the cancer. It is well documented that cancer causes inflammation and inflammation is measured by CD68 infiltrates. Another possible explanation is that these men were undergoing a biopsy of the prostate. That is a traumatic injury to the organ, injury is a possible cause of inflammation. For these reasons, it is not unreasonable that we have not found a difference in inflammation, as marked by CD68 staining, in the seropositive and seronegative prostates of the Tobago biopsy subgroup.

4.4.2 Tobago Prostatectomy Subgroup

In this subgroup, all but three subjects expressed viral antigen. There was antigen positive staining detected in the seronegative subjects, six in total. In five of the six

seronegative subjects that expressed viral antigen, both K8.1 and LANA-1 were seen. This suggests that those patients who stained positive for viral antigen but were seronegative possessed titers that were below the detection limit for the serological assay we used. All of the seronegative patients underwent re-testing to reconfirm that they were seronegative. Upon retesting, the subjects remained negative by the serological test. Since the majority of the seronegative subjects expressed both viral proteins, we believe them to be HHV-8 positive by antigen expression, but with low HHV-8 antibody titers. Recent studies in our laboratory have shown that individuals can sero-revert (unpublished data, McDonald, Bunker and Jenkins). Presumably sero-reversion is a result of the antibody titers falling below the sensitivity of the serological assay.

Within the prostatectomy subgroup, LANA-1 and K8.1 were seen together in 12 of the 13 patients that were positive for LANA-1. This suggests that both types of infection are present in these prostates. These data complement what we saw in both the Tobago biopsy subgroup and the MACS cohort.

LANA-1 appeared to be expressed predominantly in the endothelial cells of the prostate. K8.1 appeared to be expressed predominately in what looked like macrophages. These data also complement what we saw in the MACS cohort. Due to difficulties in double staining the same cell by IHC, we were unable to stain the K8.1 positive cells for the macrophage marker. This difficulty was also a problem for determining if the LANA-1 positive cells were indeed endothelial cells. Antigen retrieval time was also a problem for LANA-1 positive cells. In order to see the LANA-1 stain, we

need to do a 40 min retrieval. This made it almost impossible to see any membrane bound or cytoplasmic proteins.

We were able to determine the percentage of positive cells for each marker. This allowed us to determine that there was a significant difference in viral antigen expression within the same patient between cancerous and non-cancerous areas of the prostate. Viral protein expression, both LANA-1 and K8.1, was higher in the cancerous sections versus the non-cancerous sections. When viral protein expression was present we also observed an increased macrophage infiltrate. This suggests that in areas of cancer there is a higher amount of viral replication, due to K8.1 presence, and an increased immune infiltrate.

We also showed that within the same patient, the areas with cancer exhibited an increased immune infiltrate, both CD20 and CD68, over the areas that were cancer-free. The increased immune infiltrate could be a result of just the cancer or a combination of the cancer and the virus. Our earlier results with the cancer-free prostates of the MACS cohort suggest that at least some of the increased in immune infiltrate is due to the presence of the virus. The difference in antigen expression seen in the cancer vs. non-cancer can aid in explaining our hypothesis that the virus infects, establishes a chronic inflammation and sets up a microenvironment that aids in tumor development. We found an increase in macrophage staining in the Tobago cohort over what we saw in the MACS. This may be due to the MACS men having AIDS.

We also determined that the gp130 high risk allele exhibited an increased macrophage infiltrate in the non-cancer subjects when compared to the gp130 low risk allele in the

non-cancer subjects. This suggests that the gp130 high risk allele is associated with an increased inflammation.

We did see more macrophage staining in the Tobago cohort than in the MACS cohort. We believe that this is due to the MACS cohort being immunocompromised in that those men had AIDS.

It is possible that our increased staining seen in the cancerous sections of the Tobago prostatectomy group is simply due to the cancer being present. However, a positive association between prostatic inflammation and onset of cancer has been reported⁸, meaning that the inflammation in some cases occurs before the cancer. It is also possible that the men possessing the high risk gp130 genotype are at a later stage in their cancer development. This may be why we see greater inflammation in the gp130 high risk genotype men.

Further discussion of this cohort, as well as, comparisons between the cohorts will be done in chapter seven of this dissertation.

5.0 Chapter Five: Detection of HHV-8 in the Cancerous Prostates of Men from the United States. PUCC Cohort.

5.1 Introduction

It has been shown that HHV-8 is present in cancerous prostates and produce both lytic and latent proteins (see Chapter 4). We showed a difference in inflammation, within the same patient, between the areas with and without cancer. The study described in Chapter 4 was done with subjects from Tobago where we saw an interaction between a genetic polymorphism in gp130 and HHV-8 infection that produced a 3.1 odds ratio for prostate cancer. The study described in this Chapter was designed to see if, in a population where the gp130 polymorphism and HHV-8 infection are less prevalent, we see interactions resulting in increased cancer risk.

Recent studies from our laboratory have shown seroprevalence of HHV-8 in cohorts of men with prostate cancer from the U.S. to be 17-20% ^{7,111}. This seroprevalence is lower than the 40% seen in Tobago ⁷. The Hoffman et. al. ⁷ paper was the first to be published; this study looked at men from Tobago and the U.S. It suggests that HHV-8 plays a role in the development of prostate cancer. They found that HHV-8 seropositivity is elevated in men with prostate cancer when compared to the controls. The following paper, Sutcliffe and colleagues found an inverse association between HHV-8 seropositivity and prostate cancer (OR, 0.70; 95% CI, 0.52-0.95). They stated that HHV-8 may serve to protect against prostate cancer ¹¹². The most recent paper looked at HHV-8 seroprevalence in two case-control studies; one cohort consisted of

white men from Bologna, Italy and the other cohort was comprised of African American men from Washington, D.C ¹¹¹. This study found a seroprevalence among cases of 40% in Italy and 17% in Washington D.C. The conclusion of this study was that HHV-8 seroprevalence was not significantly different in either study, but the cases exhibited a lower seroprevalence. They stated: “ these data imply that HHV-8 is not a major prevalent cause of prostate cancer” ¹¹¹.

In this study, we strived to replicate our study from Tobago with the U.S. cohort. We performed immunohistochemistry on cancerous prostates from 39 men from the PUGC, Pittsburgh Urologic Cancer Cohort. Nineteen men were HHV-8 seropositive and 20 men were HHV-8 seronegative. Serial paraffin sections were examined for expression of viral proteins to determine if HHV-8 can be found in cancerous prostates. These proteins were LANA-1, a protein expressed during latency, and K8.1, a viral protein expressed during lytic replication. We also wanted to see if localized inflammation was present determined by using a macrophage/monocyte marker, CD68, and a B-cell marker, CD20.

5.2 Methods

5.2.1 Immunohistochemistry

See section 2.4.1 of Chapter 2.

5.2.2 Prostate Tissue

The PUCC, Pittsburgh Urological Cancer Cohort, provided our laboratory with serum samples from 508 men for HHV-8 serological testing. From those 508 men, 39 we identified for this study.

5.2.3 Percentage of Positive Cells

See sections 3.4.2 and 3.4.4.

5.2.4 Statistics

See section 4.2.4

5.2.5 gp130 Genotyping

See section 4.2.6

5.3 Results

The PUCC cohort represented men with prostate cancer from the United States where the tissue was taken at surgery or biopsy. All subjects were deemed cancerous by pathological examination. Among the men tested, 61 (12.01%) were seropositive for HHV-8 upon duplicate serological testing. The seropositive men were age and race-matched to seronegative men. Table 12 shows the age breakdown of the cohort. The race breakdown was 61.5% (24/39) Caucasian and 38.5% (15/39) unknown.

Tables 13 and 14 list the subject characteristics and selected protein expression of the seropositive and seronegative subjects respectively. In figure 18 the percentage of seropositive men who showed antigen expression of at least one protein, LANA-1 or K8.1 is shown, while figure 19 shows a representative sample of LANA-1 and K8.1 staining.

Table 12: Age Range of PUCG Cohort

Age	N	Percent
50-54	10	25.68
55-59	12	30.8
60-64	9	23.1
65-69	4	10.3
70-74	4	10.3

The Table shows the ages of the PUCG cohort subjects.

Table 13: Subject Characteristics and Protein Expression of Seropositive PUCG Subjects

Seropositive	IgA	%IgA	k8.1	%k8.1	CD68	%CD68	CD20	CD20%
1	-	0	+	42.27	+	32.09	+	22.45
2	+	2.58	+	50.35	+	38.52	+	22.23
3	-	0	+	8.68	+	88.48	+	17.25
4	+	0.8	-	0	+	51.19	+	3.15
5	-	0	+	3.27	+	12.62	+	4.11
6	-	0	+	78.84	+	67.45	+	22.97
7	-	0	-	0	+	54.86	+	6.78
8	+	0.68	+	7.94	+	62.19	+	19.64
9	+	1.07	+	66.99	+	31.18	+	39.57
10	+	1.31	-	0	+	15.79	+	5.23
11	+	0.69	+	27.53	+	6.82	+	27.99
12	+	2.48	+	50.43	+	8.29	+	24.56
13	+	1.06	+	25.22	+	4.65	+	5.78
14	-	0	+	0.42	+	91.1	+	60.51
15	-	0	+	30.53	+	37.85	+	59.61
16	-	0	+	25.78	+	16.35	+	34.34
17	-	0	-	0	+	59.09	+	18.74
18	+	0.54	+	50.87	+	7.28	+	3.33
19	-	0	-	0	+	38.67	+	22.99

The table shows the characteristics of the seropositive PUCG subjects. %protein means the number of positively stained cells in the specified grid. (see chapter 3).

Table 14: Subject Characteristics and Protein Expression of Seronegative PUC Subject

Seronegative	lana	%lana	k8.1	%k8.1	CD68	%CD68	CD20	CD20%
20	+	0.79	+	26.04	+	14.78	+	16.18
21	-	0	-	0	+	68.44	+	44.44
22	-	0	+	8.93	+	44.13	+	18.22
23	+	6.17	+	27.37	+	41.12	+	44.26
24	+	7.55	+	88.3	+	61.78	+	64.92
25	+	0.86	+	8.36	+	30.2	+	11.19
26	-	0	-	0	+	40.65	+	43.29
27	-	0	-	0	+	34.49	+	37
28	-	0	-	0	+	53.02	+	37.94
29	-	0	-	0	+	23.16	+	19.78
30	-	0	-	0	+	22.99	+	20.19
31	-	0	-	0	+	60	+	33.99
32	-	0	-	0	+	10.36	+	12.32
33	-	0	-	0	+	54.89	+	70.62
34	+	1.33	+	39.63	+	45.66	+	49.01
35	-	0	-	0	+	62.8	+	55.57
36	-	0	-	0	+	58.65	+	48.61
37	-	0	-	0	+	24.69	+	11.67
38	-	0	-	0	+	31.49	+	38.54
39	-	0	-	0	+	3.04	+	2.11

The table shows the characteristics of the seronegative PUC Subject. %protein means the number of positively stained cells in the specified grid. (see chapter 3).

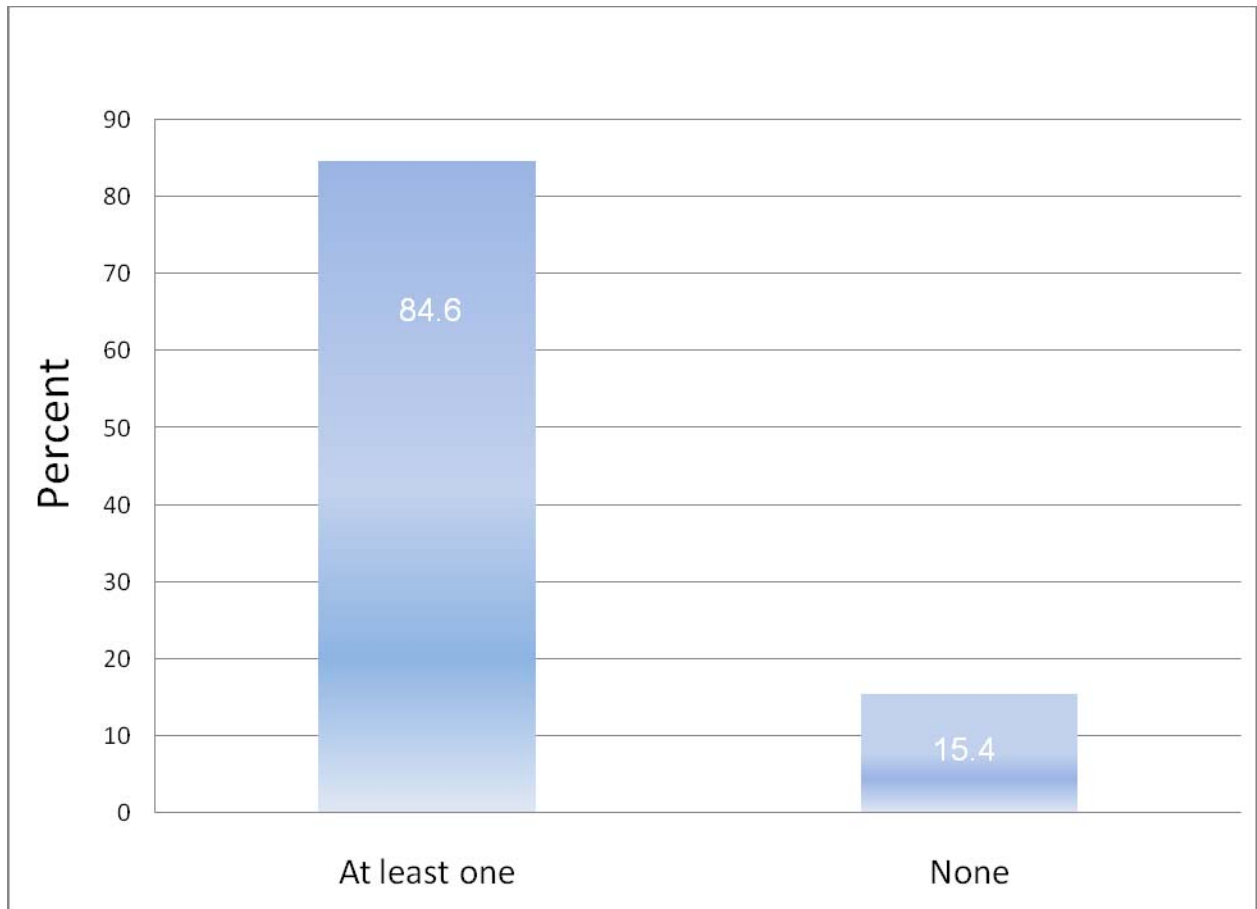


Figure 18: PUCO Cohort HHV-8 Protein Expression

Figure 18 shows that 84.6% of the seropositive subjects expressed at least one protein (LANA-1 or K8.1) and that 15.4% of the seropositive subjects expressed no viral proteins.

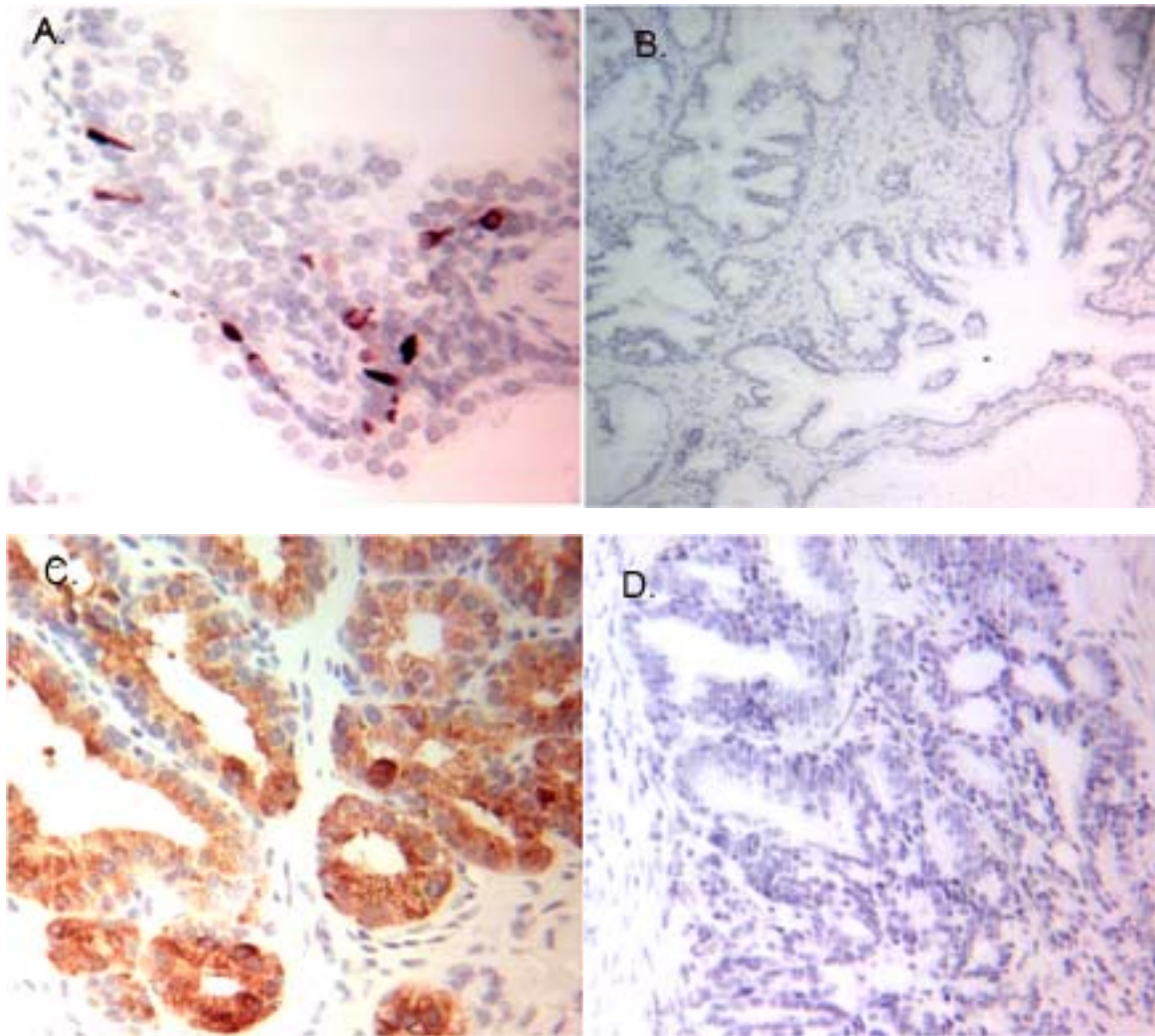


Figure 19: HHV-8 Proteins in the Prostate Tissue of Men from the PUGC Cohort

A. Shows staining for LANA-1 at 400x. B Shows serial section stained with normal rat at 400x. C. Shows staining for K8.1 at 400x. D. Shows serial section stained with normal mouse at 400x.

Among the HHV-8 seropositive subjects, viral protein expression was detected in multiple regions of each section, but not in the cancer. Some of the subjects show both lytic and latent expression in the stained regions, while other regions contained only latent or only lytic. Six of the seronegative subjects showed protein expression (Table 15) with K8.1 expression the more predominate protein expressed. Of the 14 samples that expressed LANA-1, 12 or 85.7% expressed K8.1.

In order to determine the amount of inflammation in each prostate, individual sections were stained with antibodies directed against CD68, a macrophage marker and CD20 a B-cell marker. Representative samples can be seen in Figure 20. To determine the total number of positive cells we used a semi-quantitative image analysis developed and validated in our laboratory and described in sections 3.4.2 and 3.4.4 of this dissertation. The number of positive cells was adjusted for total cell number. Those values are shown in Tables 13 and 14.

We asked whether differences in percentages of CD68 and CD20 positive cells were seen between HHV-8 antigen positive and HHV-8 antigen negative sections. No difference was found in CD68 and CD20 expression based on viral antigen status ($p>0.05$; Mann-Whitney U)

We next asked whether differences were seen between LANA-1 positive and LANA-1 negative sections and K8.1 positive and negative sections with respect to CD68 and CD20 infiltrate. Again we found no differences between LANA-1 positive and negative sections or K8.1 positive and negative sections ($p>0.05$; Mann-Whitney U)

We have unpublished data on the R148G polymorphism (discussed in section 4.1 and section 1.7 table 2) from the Caribbean island of Tobago. That same gp130 SNP analysis was performed on a United States cohort, a combination of MACS and PUCG men, 263 in total. Table 15 shows the data from the SNP analysis. A significant $p=0.000$ difference (chi-square analysis) between the U.S. and Tobago cohorts is seen. The G genotype was present in at least one allele in 71.4% of Tobago men and 29.7% of US men.

The PUCC cohort was tested for gp130 allele status. Table 16 shows the breakdown of allele status in the PUCC cohort. There were no homozygous high risk genotypes (G/G) seen and only 27% of the men were heterozygous (C/G). We asked whether gp130 allele status resulted in increased inflammatory markers. No difference was seen in inflammatory markers between the gp130 heterozygous and C/C homozygous low risk allele.

Table 15: Comparison of Tobago and United States gp130 SNP analyses

	United States		Tobago	
	N	%	N	%
C/C	185	70.4	335	27.5
C/G	68	25.9	598	49.1
G/G	10	3.7	284	23.4

Table 16: gp130 Allele Status of PUCC Cohort

	N	%
C/C	27	73
C/G	10	27
G/G	0	0

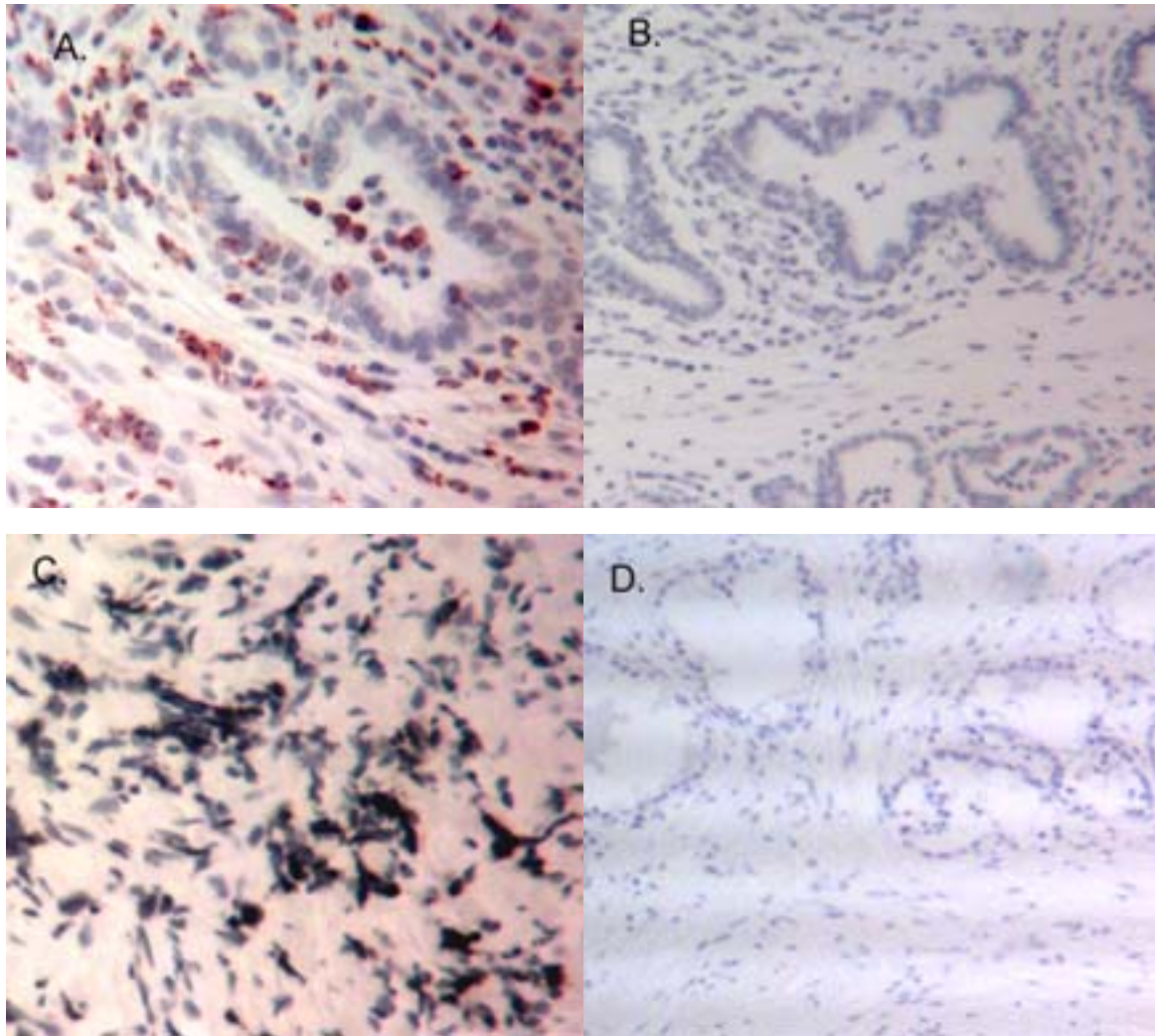


Figure 20: Immune Infiltrate Staining in PUGC Cohort

Figure 20 PUGC Cohort Staining. A. Shows staining for CD68 at 400x. B. Shows serial section stained with normal goat serum. C. Shows staining for CD20 at 400x. D. Shows serial section stained with normal mouse serum.

5.4 Discussion

This cohort was comprised of men from the United States who have prostate cancer. We did see viral antigen expression in the seronegative subjects. Six out of the 20 seronegative subjects expressed viral proteins (see table 14). Of those six, five of them expressed both K8.1 and LANA-1. This, as with the Tobago prostatectomy subgroup, may be a result of the subjects HHV-8 antibody titer being below the detection limit of the serological assay. Subjects underwent re-testing to verify that they were in fact seronegative. Upon retesting, subjects remained seronegative. As with the Tobago prostatectomy subgroup, the majority of the subjects that expressed viral protein expressed both. We believe them to be seropositive by protein expression and below the detection limit of the serological assay.

Within this cohort LANA-1 and K8.1 expression were seen in the majority (12/22) of the subjects expressing antigen. This suggests that both types of infection, latent and lytic, were present.

As with both other cohorts, LANA-1 expression was seen primarily in the endothelial cells of the prostate and K8.1 was seen primarily in what appeared to be the macrophages. As discussed previously, the difficulties of double staining sections prohibited us from verifying these observations. Figure 19 shows K8.1 staining in the endothelial cells; this is not edge artifact the picture was taken in the center of the slide.

We were able to determine total number of positive cells for each antigen. These results showed no differences between the inflammatory markers, CD68 and CD20,

with respect to subjects that were antigen positive, subjects that were LANA-1 positive, and subjects that were K8.1 positive. No difference was seen based on gp130 allele status.

We found a lower HHV-8 seroprevalence than the three other studies out of our laboratory. We saw a seroprevalence of 12.01%, where as the other studies found a seroprevalence of 17-20% in prostate cancer cases. Our n=508 was larger than the other studies. This could be why our seroprevalence was lower. The study conducted on the men from Italy and Washintgon D.C. involved men with advanced metastatic prostate cancer; this could be a confounding issue in their study. In our study, the men did not have such advanced stages of cancer. Also, the study done in Washington D.C. involved more African American men, this may have added to the higher seroprevalence seen in that study. African Americans have a higher seroprevalence for HHV-8.

We saw in this cohort, as we did in the Tobago cohorts, that HHV-8 is present in the prostates of seropositive men. However, the differences in inflammation between seropositive and seronegative were not seen in the Pucc cohort. This could be due to the design of the Pucc study. We were able to have areas with and without cancer from the same patient in the Tobago Prostatectomy subgroup. This allowed us to compare differences in inflammation within the same patient. Those tissues were not available to us with the Pucc cohort. This could explain why we did not see the differences in inflammation in the Pucc cohort, we did not have tissue that was cancer free.

The gp130 genotype is not as prevalent in the U.S. as it is in Tobago (table 15). We know from the Tobago cohort data that HHV-8 combined with the high risk G/G gp130 polymorphism produces an odds ratio for prostate cancer of 3.1. The high risk polymorphism is found in 3.7% of the U.S. cohort compared to the 23.4% seen in the Tobago cohort. Also, in the Tobago cohort, the gp130 high risk polymorphism was associated with an increase inflammation in the areas of non-cancer (section 4.3.4, table 10). This suggests that the absence of the high risk gp130 polymorphism in the U.S. population explains the lack of association seen in the U.S.

Further discussion of this cohort, as well as, comparisons between the cohorts will be done in chapter seven of this dissertation.

6.0 Chapter Six: Functional Characterization of the gp130 R148G Polymorphism

6.1 Introduction

The gp130 cytokine receptor is a member of the hematopoietic cytokine receptors¹¹³. It is expressed on almost all cell types²⁵. These receptors have an extracellular region, a single transmembrane portion, and a cytoplasmic domain. There is a conserved domain on the gp130 receptor that is characterized by four positionally conserved cysteine residues in its amino terminal end and a WSXWS (W=tryptophane, S=serine, X=non conserved amino acid) motif at its carboxyl end¹¹⁴.

The gp130 receptor signals through the Janus kinase- signal transducer and activator of transcription (JAK-STAT) pathway. Of the various cytokine receptors, gp130 is the signal transducer for several ligands. It signals for IL-6, IL-11, and OSM to name a few. Binding of these cytokines to their respective binding receptors leads to binding of gp130. Once gp130 is bound, JAK is activated causing downstream signaling of STATs.

In order for downstream signaling to occur through gp130, the cytokine must first bind to its own receptor. For example, IL-6 and IL-11 must first bind to a nonsignaling α receptor, and then that complex binds to gp130²⁵. In the case of IL-6, two molecules bind to two of the IL-6R α s, which then binds a homodimer of gp130, forming a hexameric complex¹¹⁵. IL-11 uses the same pattern, two molecules of IL-11 bind to two molecules of IL-11R α , which then bind to a homodimer of gp130²⁵. In contrast, the

cytokine OSM does not have a nonsignaling receptor; it binds to the heterodimer of OSMR and gp130.

For the purposes of this dissertation we will focus on the relationships of IL-6, IL-11 and OSM to gp130. The objectives of this study were to determine if the gp130 R148G polymorphism affects the function of the gp130 protein. To this end, we will determine if the polymorphism has an effect on growth of lymphoblastoid B cell lines (LCLs) developed from Tobago men. We have the following LCL lines: 3 homozygous low risk, 5 heterozygous, and 7 homozygous high risk. We will also evaluate the STAT-3 activation induced by stimulation with IL-6, IL11, and OSM.

6.1.1 IL-6

As described above, IL-6 forms a 2:2:2 hexameric complex with gp130 and IL-6R α ¹¹⁶. IL-6 downstream signaling activates JAKs which activate STAT-3. The activated STAT-3 homodimers translocate into the nucleus¹¹⁶. IL-6 is a B-cell growth factor that creates an autocrine feedback loop¹¹⁶.

6.1.2 IL-11

IL-11 also forms a hexameric complex with gp130 and IL-11R α ²⁵. IL-11 downstream signaling activates STAT-3 in a similar fashion as IL-6. Studies have shown that IL-11 is a potent anti-inflammatory cytokine; it down regulates macrophage effector function by inhibiting TNF α ¹¹⁷.

6.1.3 OSM

Oncostatin M uses one OSMR and one gp130 molecule to exhibit its downstream signaling¹¹⁸. OSM is a 28kDa protein that is known to inhibit growth in several types of cells, such as tissue tumor cells, melanoma cells, and plasmacytoma cells¹¹⁸. However, OSM is known as an autocrine growth factor for AIDS-associated Kaposi's sarcoma cells. Downstream signaling of OSM involves the JAK-STAT pathway as well

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6.2 Methods

6.2.1 Cell Culture

Cells were maintained in RPMI 1640 with 10000 U/ml Pen/10000ug/ml Strep and 10% FBS. For cytokine experiments cells were plated 24 hours before in AIM V media.

6.2.2 Generation of LCLs

LCLs were generated by in vitro transformation of peripheral B cells with Epstein Barr Virus (EBV). Briefly, 5×10^5 PBMCs in 1 ml of B-cell media [BCM; 500ml of RPMI 1640, 10% FBS, 5.5ml of 100x L-glutamine (Gibco Cat. # 10-040-CV), 10000 U/ml Pen/10000ug/ml Strep (Gibco), 100mM Sodium Pyruvate (Gibco), 100x MEM Non-essential amino acids (Gibco) , and 1M HEPES (Gibco)] were mixed with 1ml of EBV

virus supernatant (generously provided by Monica Tomaszewski-Flick, Ph.D, University of Pittsburgh) and incubated at 37°C for 20 min in a 15ml conical tube. An additional 8 mls of BCM was added along with cyclosporine (final concentration 1ug/ml). The cells were plated at 100 ul per well in a 96 well plate (final concentration 5000 PBMCs per well of a 96 well plate, approximately 250 B cells). On day 13, 75ul of BCM was added. At 7 weeks, the cells from each well were transferred to a well in a 24 well plate. At 10 weeks the cells from each well in the 24 well plate were transferred to a T-25 flask. Ten of the fifteen LCLs were made by the same protocol in the laboratory of Dr. Giovanna Rappocciolo at the University of Pittsburgh Graduate School of Public Health.

6.2.3 Growth Curves

Growth curves of the LCLs were performed using a 4% trypan blue solution (in phosphate buffered saline ph 7.2) exclusion cell count on a hemocytometer. Cells were diluted in the trypan blue solution at 1:100 and counts were taken. Trypan blue stains dead cells blue, alive cells remain unstained. A count of both alive cells and dead cells was taken at each time point. Cells were then plated at 1×10^4 /well in a 96 well plate in AIM V media and incubated at 37°C overnight. The next day each well was either left untreated or given 25ng of either rhIL-6, rhIL-11 or rhOSM (R&D systems Cat. # 206-IL, 218-IL, and 295-OM respectively). Cells from three wells for each treatment were collected and counted at 0, 12, 24, 36, 48, 60, and 72 hours post-treatment. Graphs were plotted on GraphPad Prism 4 software.

6.2.4 STAT-3 Activation

STAT-3 activation was measured using the CASE Cellular Activation of Signaling ELISA (Super Array, Cat. # FE018) per manufacturer's instructions. Briefly, cells were seeded in a 96 well plate at 1.5×10^4 cells/well in AIM V media 24 hours before experiment. Wells received either 0, 12.5, 25, or 50ng rhIL-6. At 0, 15, 30, 45, and 60 minutes post treatment, cells were fixed with 8% formaldehyde fixing buffer in 1xPBS. Wells were set up in mirror images with half of the plate being treated with anti-PSTAT-3 antibody (which recognizes only phosphorylated (activated) STAT-3) and half of the plate treated with anti-STAT-3 antibody (which recognizes both non-phosphorylated and phosphorylated STAT-3) and incubated for 1 hour at room temperature. The wells were extensively washed and then treated with secondary antibody at room temperature for 1 hour. Following incubation with the secondary antibody, substrate was added to each well and colorimetric detection performed at 450nm on an ELISA plate reader. Determination of relative cell number was done as per the kit. To determine the relative extent of STAT-3 phosphorylation, normalization of the P-STAT-3 antibody to the STAT-3 antibody for the same experimental control was done. Results were plotted using GraphPAD Prism 4 software.

6.2.5 gp130 Genotyping

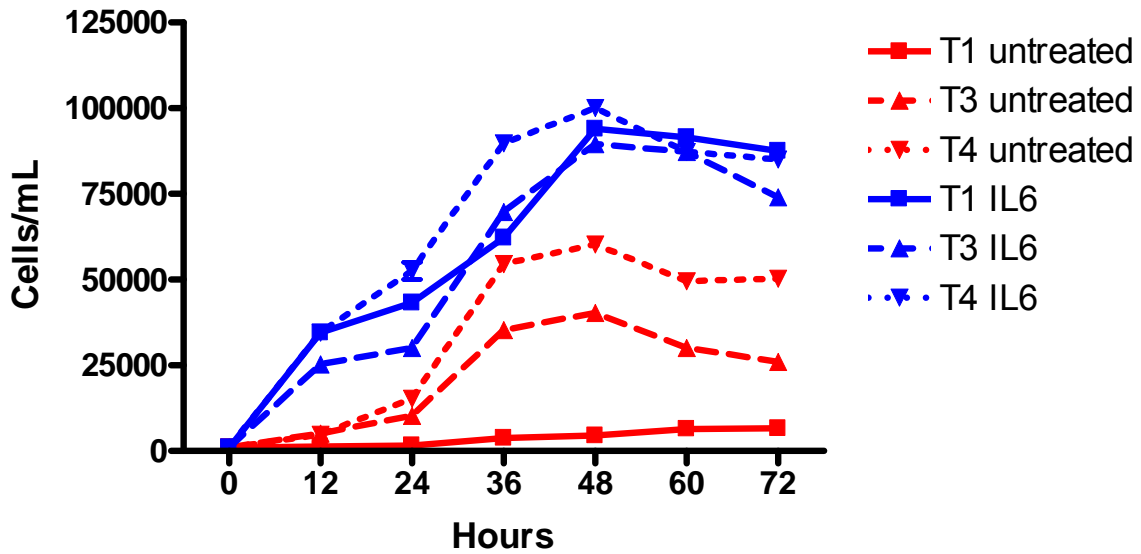
See section 4.2.6.

6.3 Results

As part of our ongoing study in Tobago, we constructed LCLs from 15 Tobago men. The gp130 genotype of each LCL was confirmed by Dr. Ferrell's laboratory (data not shown). We first wanted to determine the effect of IL-6, IL-11, and OSM on cell growth of the different LCLs. We used trypan blue exclusion to assess the effects of 25ng of each cytokine on cellular proliferation and compared those effects to untreated cells, we used 25ng because that most closely resembles physiologic level IL-6.

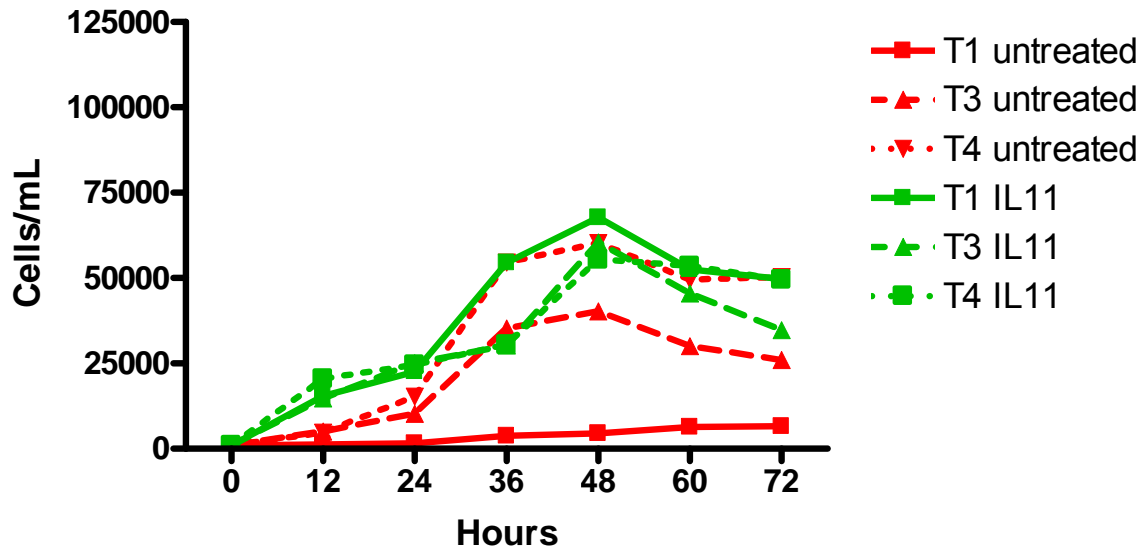
A.

Tobago Low Risk Lines Response to 25ng rhIL-6



B.

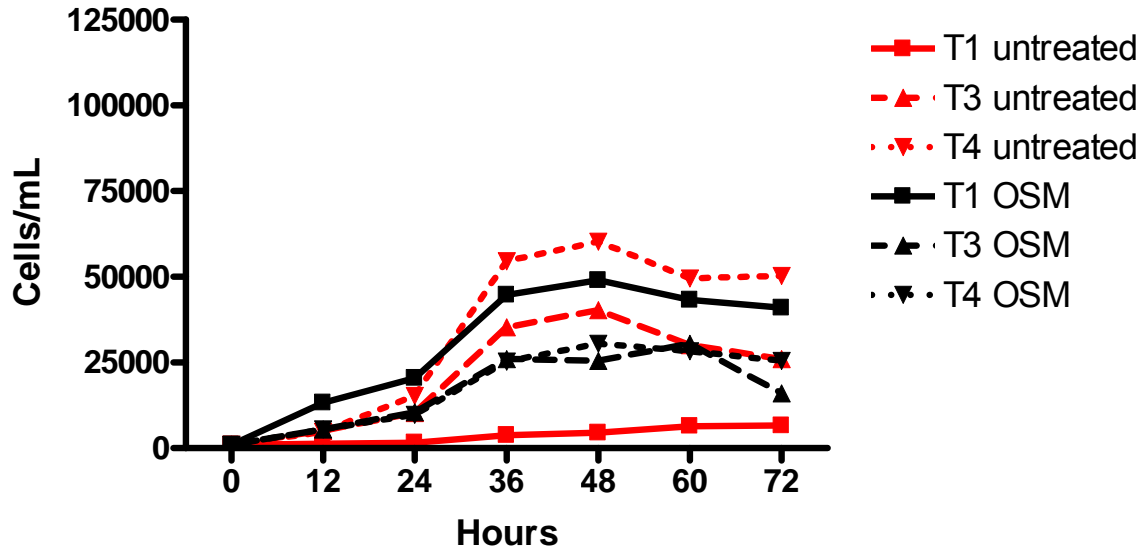
Tobago Low Risk Lines Response to 25ng rhIL-11



(Figure 21 Continued Below)

C.

Tobago Low Risk Lines Response to 25ng rhOSM



D.

Tobago Homozygous Low Risk Lines Response to all Cytokines

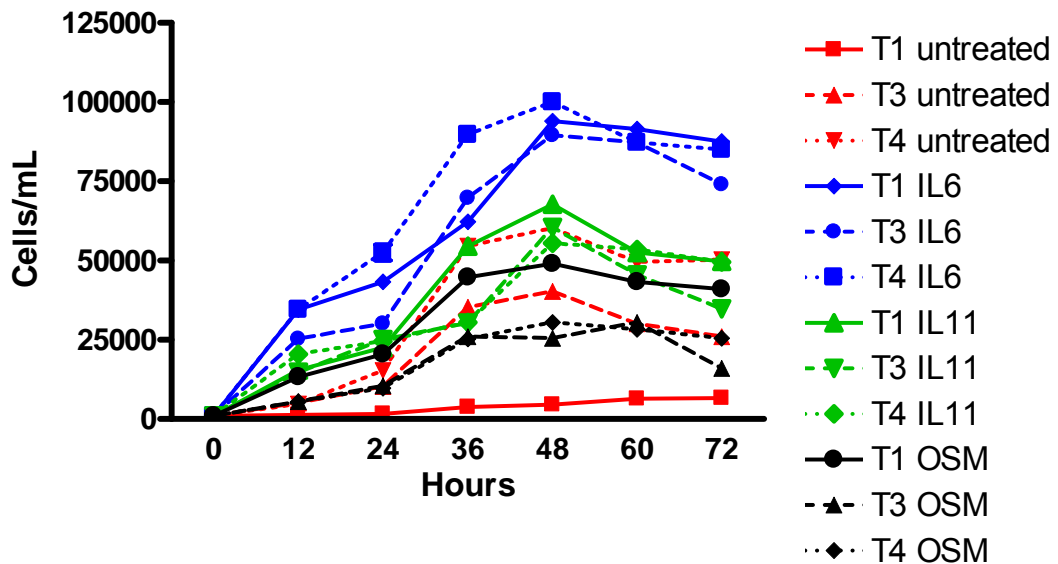
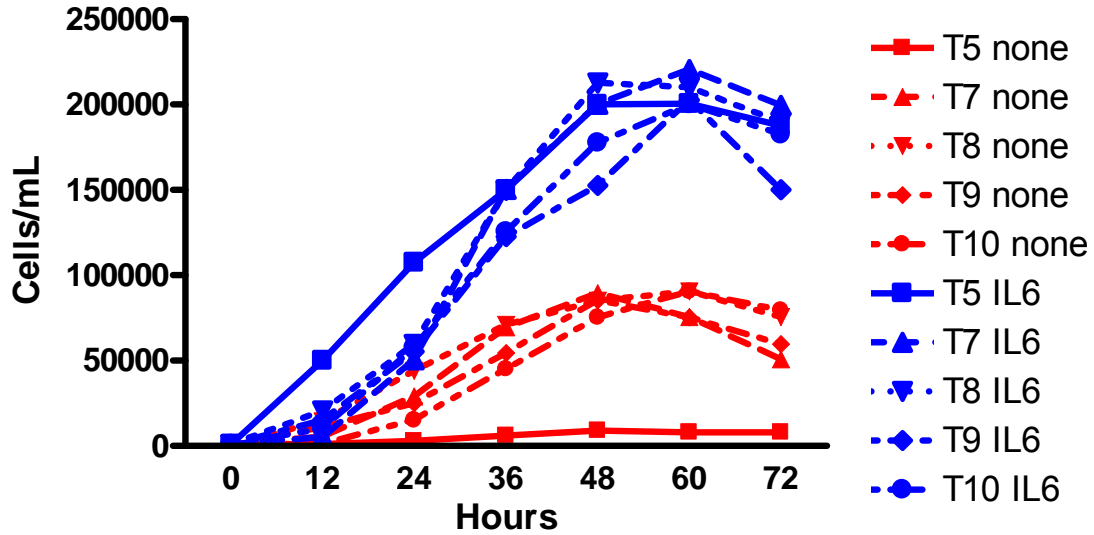


Figure 21: Tobago Low Risk Lines

Growth Curve analysis of Tobago Homozygous Low Risk Lines response to A) 25ng IL-6 B) 25ng IL-11 C) 25ng OSM and D) 25ng of IL-6, IL-11, and OSM. Cells had 25ng of cytokine added at 0 hours and time points were taken every 12 hours. Cell counts were taken and cell numbers were plotted per milliliter.

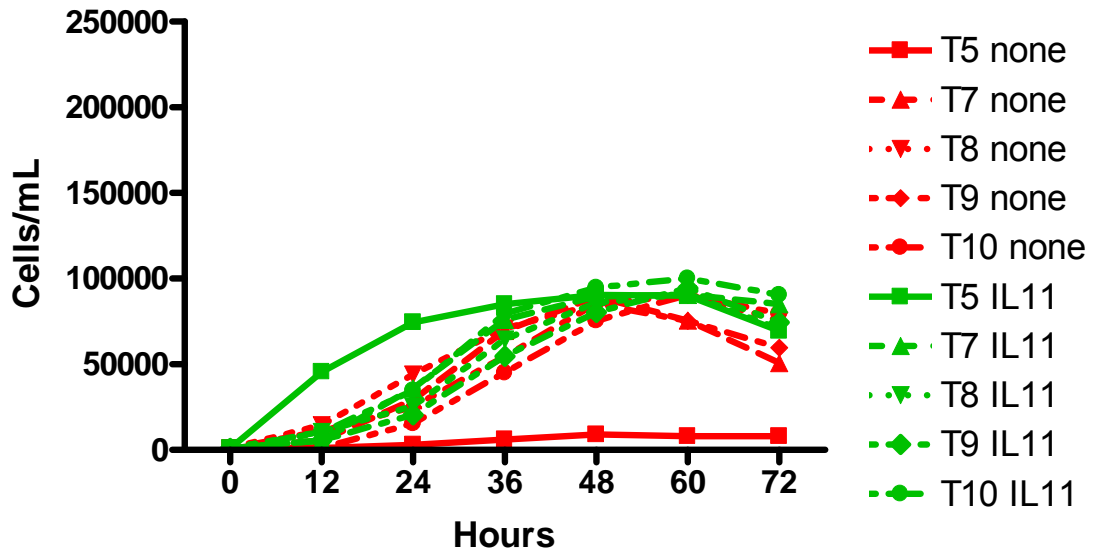
A.

Tobago Heterozygous Lines response to 25ng rhIL6



B.

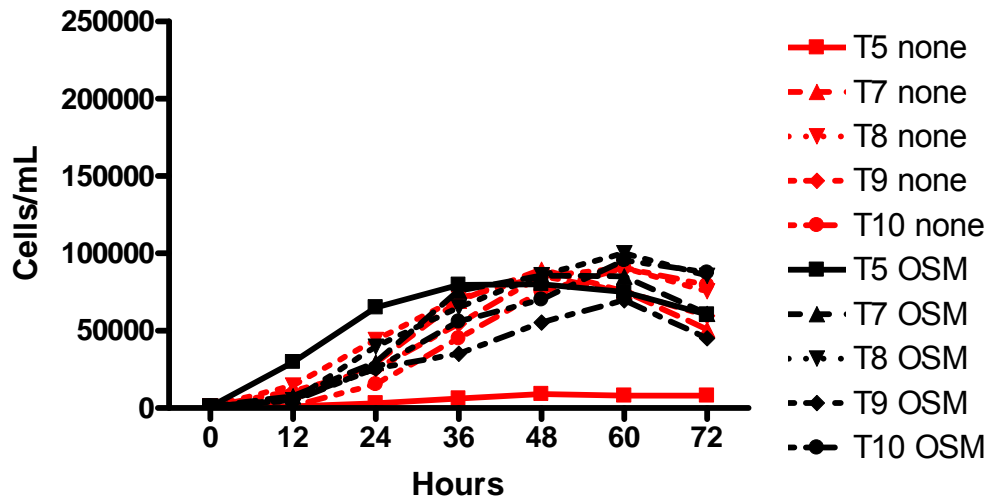
Tobago Heterozygous Line Response to 25ng rhIL-11



(Figure 22 Continued Below)

C.

Tobago Heterozygous Lines Response to 25ng rhOSM



D.

Tobago Heterozygous Lines Response to All Cytokines

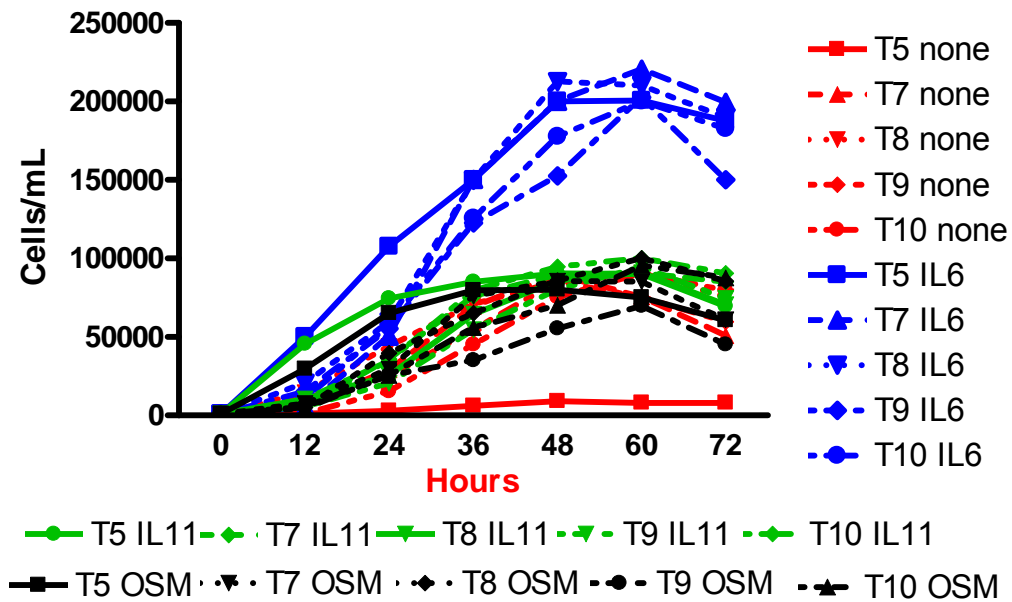
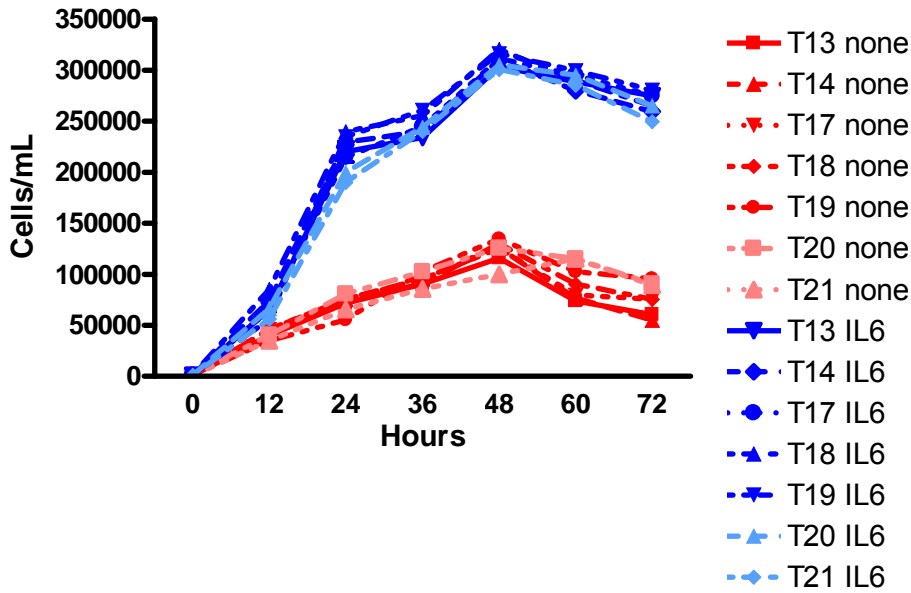


Figure 22: Tobago Heterozygous Lines

Growth Curve analysis of Tobago Heterozygous Lines response to A) 25ng IL-6 B) 25ng IL-11 C) 25ng OSM and D) 25ng of IL-6, IL-11, and OSM. Cells had 25ng of cytokine added at 0 hours and time points were taken every 12 hours. Cell counts were taken and cells per milliter were plotted.

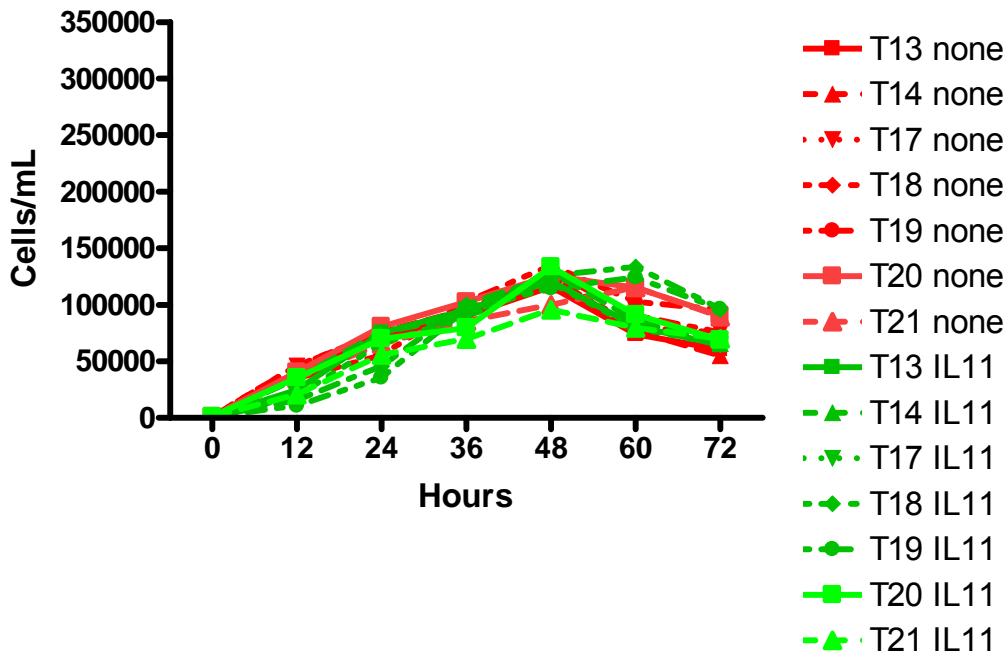
A.

Tobago High Risk Lines Respons to 25ng rhIL6



B.

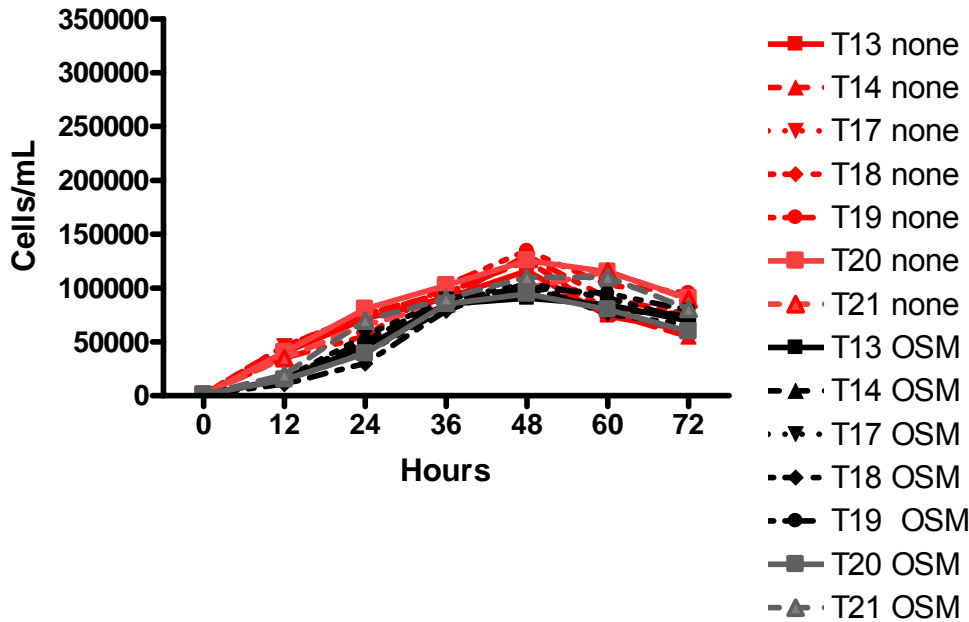
Tobago High Risk Lines Response to 25ng rhIL-11



(Figure 23 Continued Below)

C.

Tobago High Risk Lines Response to 25ng rhOSM



D.

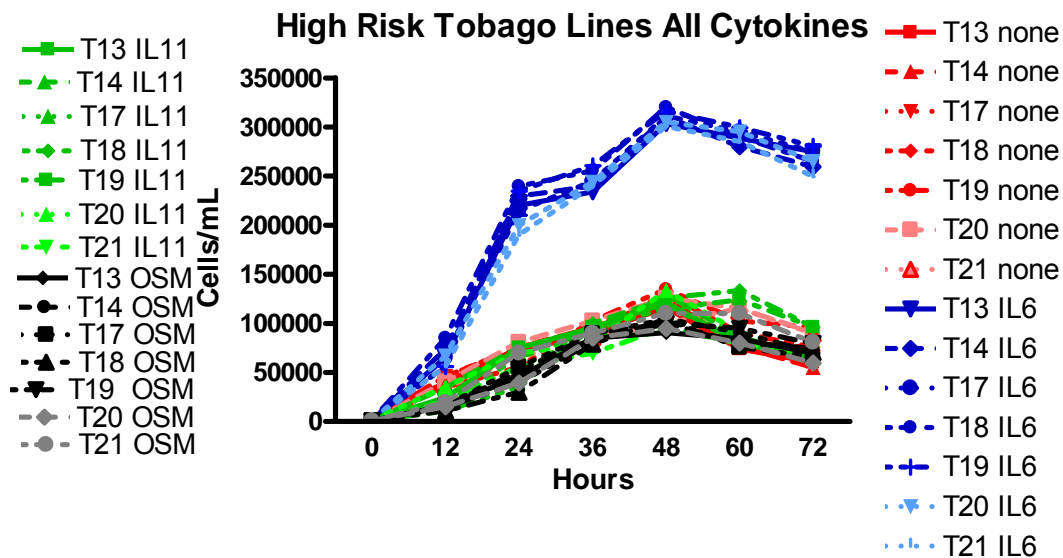


Figure 23: Tobago High Risk Lines

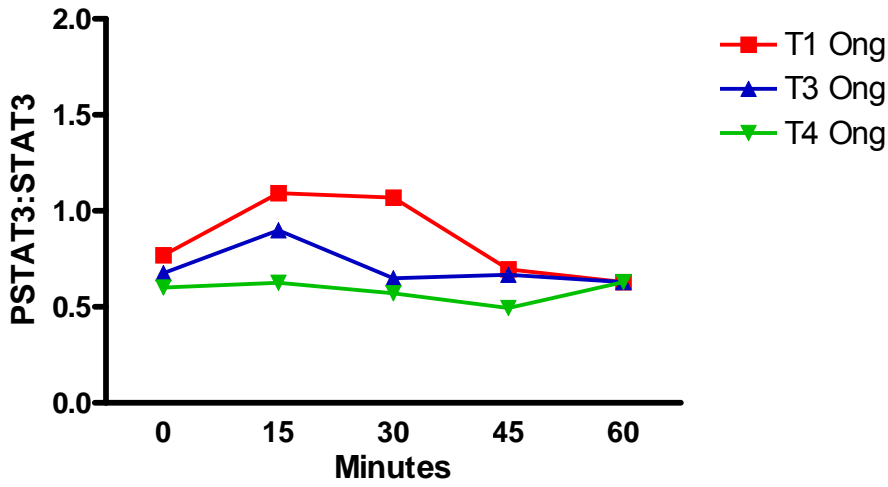
Growth Curve analysis of Tobago Homozygous High Risk Lines response to A) 25ng IL-6 B) 25ng IL-11 C) 25ng OSM and D) 25ng of IL-6, IL-11, and OSM. . Cells had 25ng of cytokine added at 0 hours and time points were taken every 12 hours. Cell counts were taken and cell number was plotted per milliliter.

The cytokine growth curves from the Tobago LCLs showed that all three genotypes exhibited cellular proliferation in the presence of 25ng rhIL-6. The homozygous high risk alleles exhibited a 250% increase over the homozygous low risk lines and a 100% increase over the heterozygous lines in response to 25ng rhIL-6 (Figures 21-23). The heterozygous lines exhibited a 150% increase over the homozygous low risk lines in response to 25ng of rhIL-6 (Figure 22 B, C). In contrast, none of the lines showed increased proliferation to the addition of either IL-11 or OSM, suggesting that the R148G polymorphism is IL-6 specific.

We next asked whether the gp130 R148G polymorphism affected downstream gp130 signalling as measured by STAT-3 phosphorylation. Each LCL was stimulated with 0, 12.5, 25, and 50 ng of rhIL-6, and cells were collected at various time points as measured for STAT-3 activation. The following figures show the results.

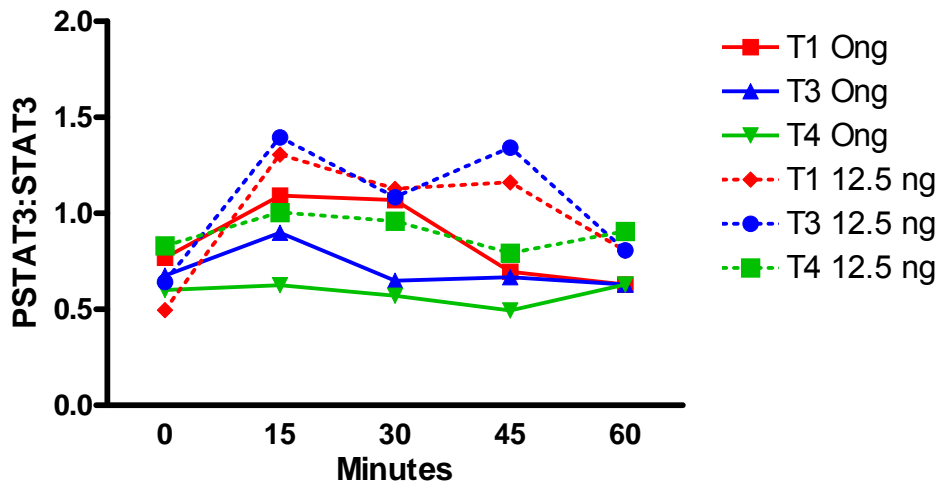
A.

Tobago Homozygous Low Risk Lines: 0ng IL-6



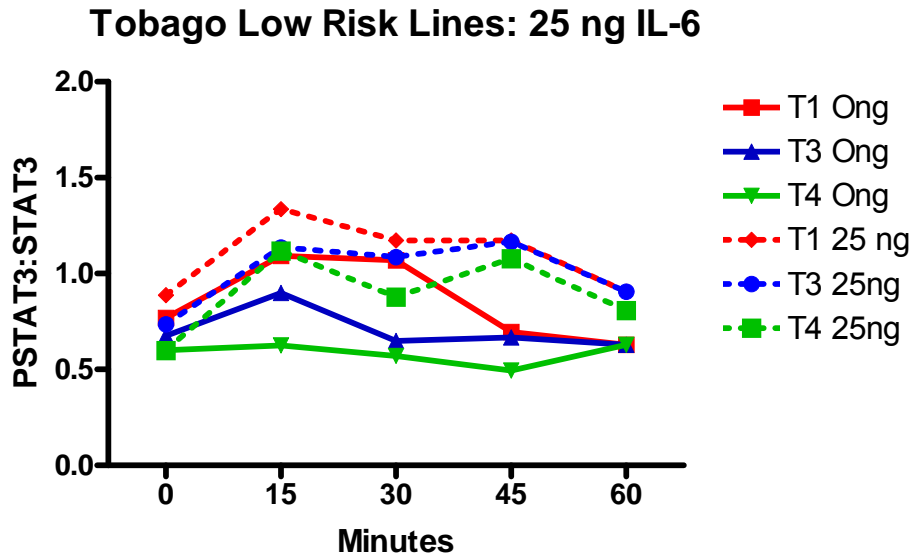
B.

Tobago Low Risk Lines: 12.5 ng IL-6



(Figure 24 Continued Below)

C.



D.

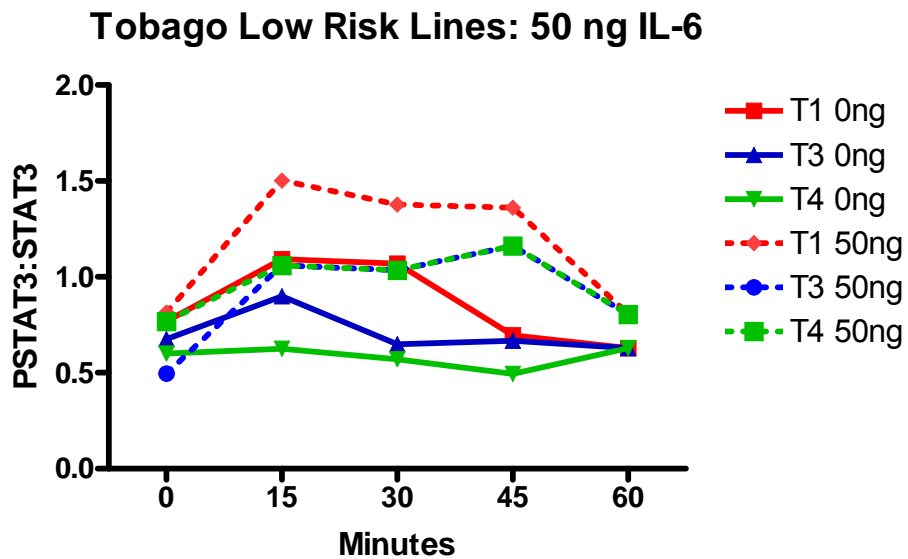
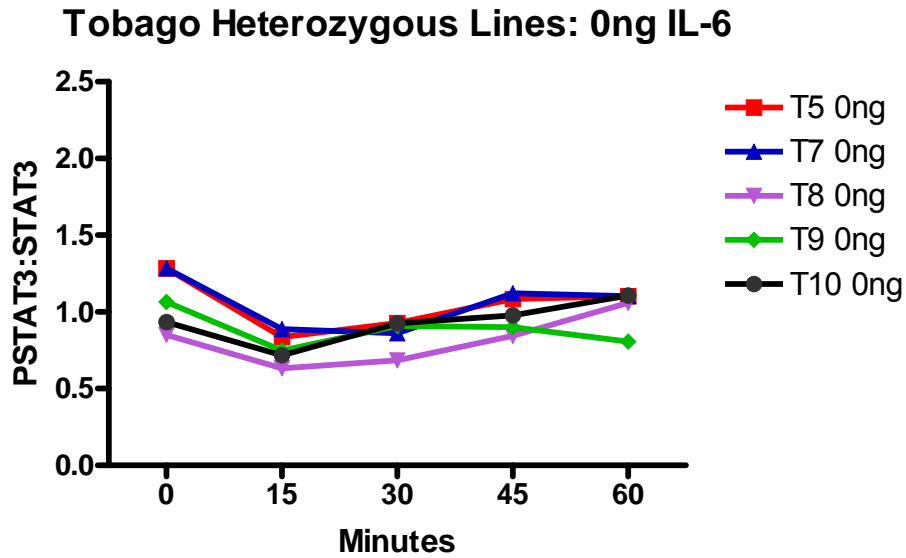


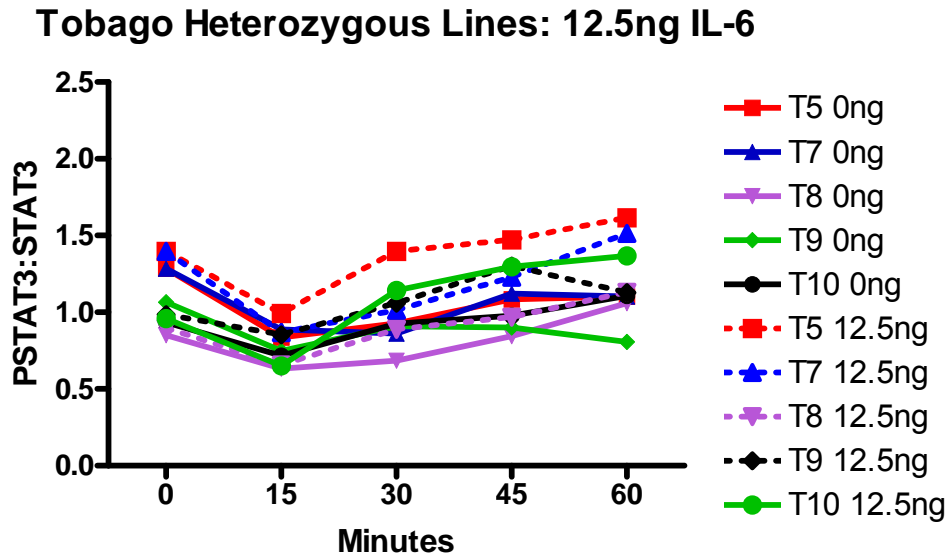
Figure 24: Tobago Homozygous Low Risk Lines STAT-3 Activation

Experiments were done with 1000 cells. A) Shows the C/C lines ratio of activated P-STAT-3 or Total STAT-3 per cell-0ng IL-6. B) shows activation at 12.5ng IL-6 C) shows activation at 25ng IL-6 D) Shows activation at 50ng IL-6.

A.



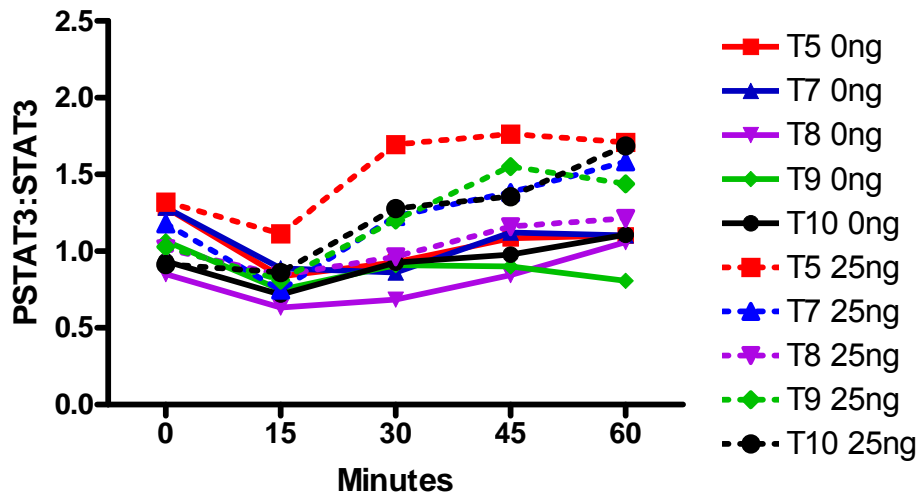
B.



(Figure 25 Continued Below)

C.

Tobago Heterozygous Lines: 25 ng IL-6



D.

Tobago Heterozygous Lines: 50ng IL-6

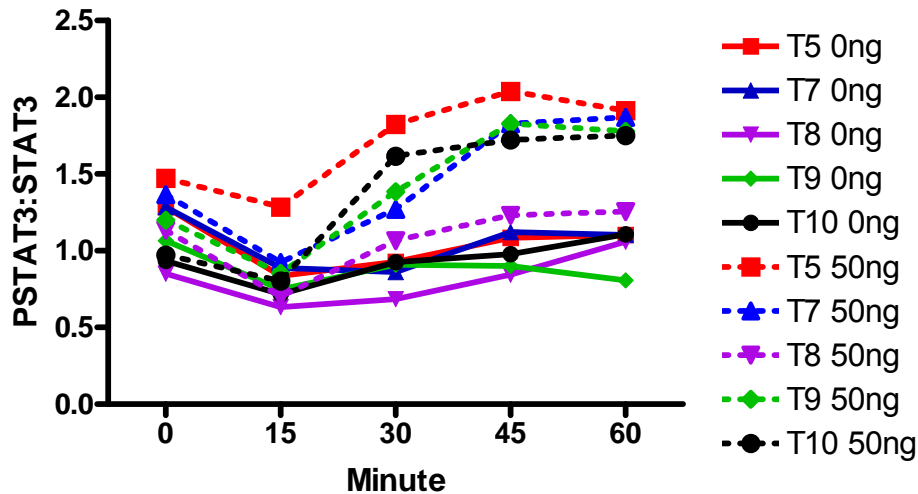
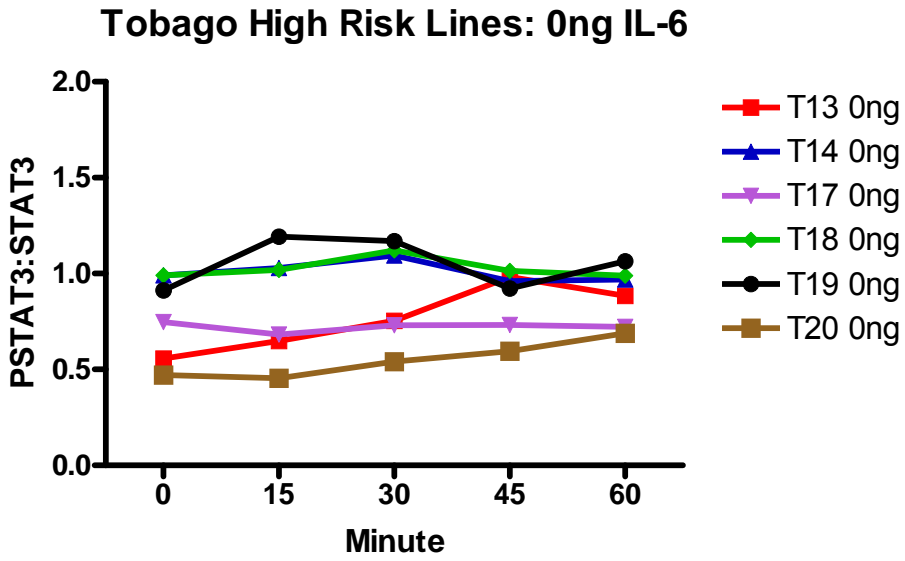


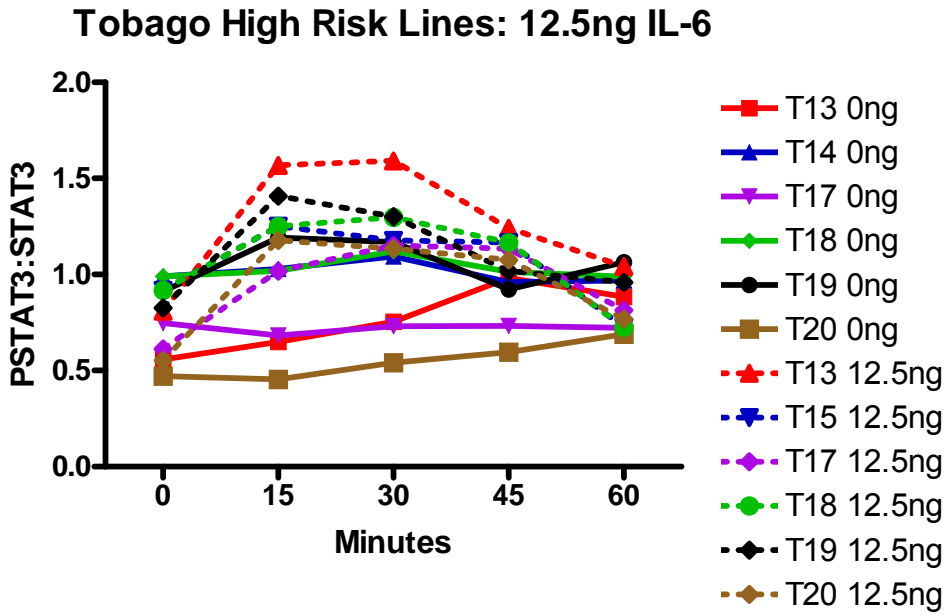
Figure 25: Tobago Heterozygous Lines STAT-3 Activation

Experiment were done with 1000 cells. A) Shows the C/G lines ratio of activated P-STAT-3 or Total STAT-3 per cell-0ng IL-6. B) shows activation at 12.5ng IL-6 C) shows activation at 25ng IL-6 D) Shows activation at 50ng IL-6.

A.

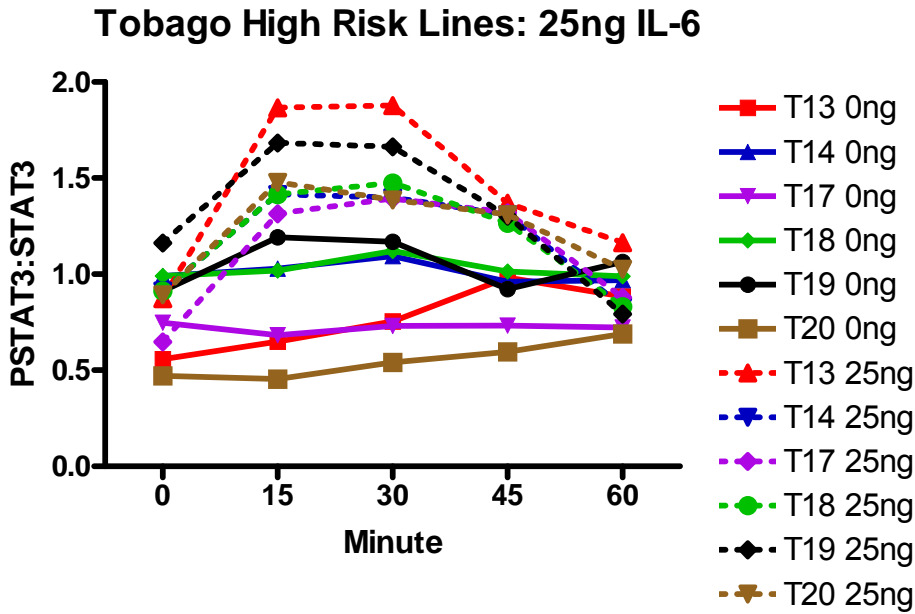


B.



(Figure 26 Continued Below)

C.



D.

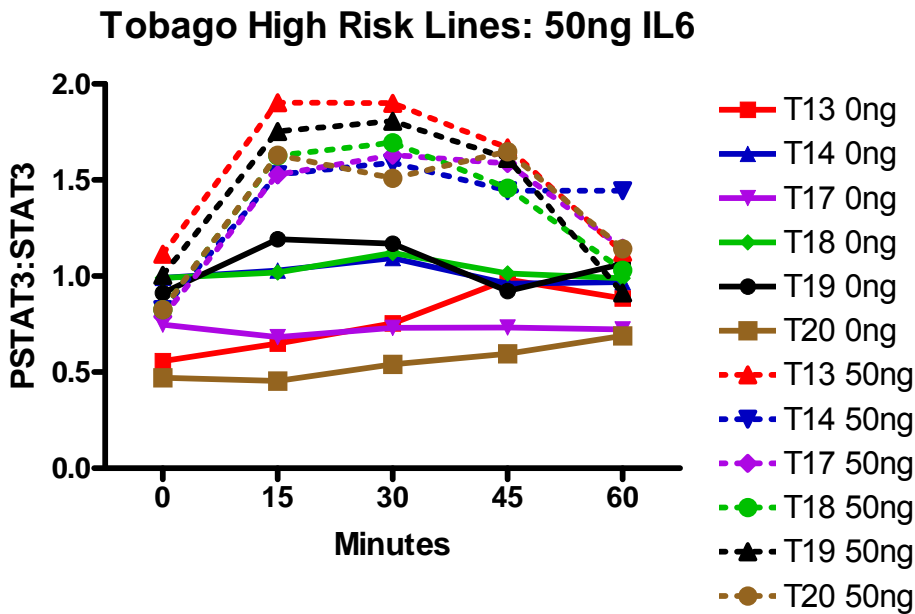


Figure 26: Tobago Homozygous High Risk Lines STAT-3 Activation

Experiment were done with 1000 cells. A) Shows the G/G lines ratio of activated P-STAT-3 or Total STAT-3 per cell-0ng IL-6. B) shows activation at 12.5ng IL-6 C) shows activation at 25ng IL-6 D) Shows activation at 50ng IL-6.

All of the LCLs showed a dose response to the rhIL-6. The homozygous low risk lines showed a weak double peak pattern, with peaks at 15 and 45 minutes. This can best be seen in figure 4D at 50 ng of rhIL-6, negligible activation. The heterozygous lines exhibited a drop in activation with marked increase at 15 minutes. This is most visible at 50ng of rhIL-6 in figure 5D. The high risk lines demonstrated a high level of activation from 15-45 minutes, seen best in figure 6D.

6.4 Discussion

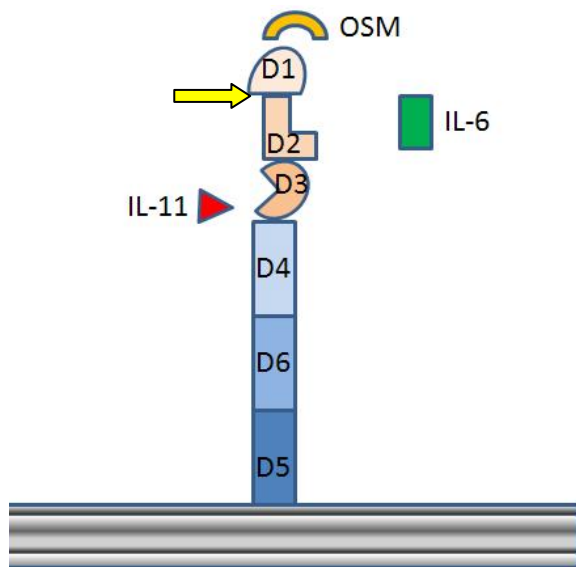


Figure 27: gp130 Binding Regions.

IL-6 is represented in green. IL-11 is represented in red. OSM is represented in orange. The yellow arrow indicates where the gp130 R148G polymorphism is located.

The GG genotype, referred to here as the Tobago homozygous high risk, was associated with elevated prostate cancer risk, OR=1.5 (95% CI 0.98-2.32) in logistic

regression and both gp130 and HHV-8 seropositivity were independently associated with prostate cancer. Further exploration of the six genotype-HHV-8 combinations revealed an OR of 3.1 (95% CI 1.2-8.1 p=0.022) for prostate cancer in men who are HHV-8 seropositive and have the GG genotype, compared to the lowest risk group, who are HHV-8 negative and have the CC genotype (Section 1.7 Table 2). We hypothesized that this effect of gp130 genotype may be mediated through its role in IL-6 signaling.

We have shown that the R148G polymorphism in the gp130 signaling receptor is functional, in that it exhibits an increased proliferative response on LCLs from men with the G/G genotype. In testing the LCL's growth potential when 25ng of rhIL-6, rhIL-11, and rhOSM were added, we saw that the proliferative response appears to be specific to rhIL-6. This suggests that the polymorphism found in gp130 is affecting the binding region of IL-6. Gp130 has six binding regions ¹¹⁹. The gp130 cytokines utilize regions D1-D3 to bind, IL-6 uses D2, IL-11 uses D3, and OSM uses D1 (see Figure 27) ¹¹⁹. The D1 binding region uses the B and D helical faces of the protein; the polymorphism is located at the end of the D1 binding region approximately 40 amino acids below the D helical face ¹¹⁹. This may be why the D1 binding region is not affected. However, our data suggest that the polymorphism may affect the D2 region of gp130. Since the polymorphism is located below the faces that bind in the D1 region, it is possible that the amino acid switch caused by the polymorphism is altering the D2 binding site. This could explain why IL-11 and OSM do not affect the growth of the LCLs in the same manner that IL-6 does.

We have also shown that each genotype shows a distinctly different PSTAT-3:STAT3 response to rhIL-6 at 25ng. The low risk allele shows a double peak pattern with relatively weak increases in activation at 15 and 45 minutes. The heterozygous allele shows a drop early followed by strong activation after 15 minutes. While the high risk allele shows immediate strong activation that remains for 30 minutes.

Together the growth curve and STAT3 activation data suggest that the high risk G/G genotype confers a growth advantage over the other the genotypes in response to IL-6. The continuous activation of STAT-3 beginning at 15 minutes and continuing until 45 minutes suggests that STAT-3 in the high risk allele is more easily activated. Constant activation of STAT3 is known to confer a growth advantage in the activated B-cell subtype of diffuse large B-cell lymphomas ¹²⁰.

The observation that IL-11 and OSM, a cytokine that shares the gp130 homodimer as its signaling receptor with IL-6, did not confer a growth advantage, suggests that this effect is IL-6 specific. We believe that the R148G polymorphism is differentially functional which may explain the increased risk associated with the high risk polymorphism. We have shown that the polymorphism infers a growth advantage to B-cells to which IL-6 has been added. Further functional studies are needed to elucidate the role the polymorphism plays in the inflammatory response, HHV-8 infection and KS.

Further discussion of this can be found in chapter seven of this dissertation.

7.0 Chapter Seven: Discussion

7.1 Summary

While the causation of prostate cancer remains unknown, Nelson and colleagues have suggested a sexually transmitted agent may be a possible cause⁹. It is thought that a man acquires a sexually transmitted agent which sets up a chronic infection that leads to chronic inflammation and ultimately prostate cancer (section 1.8, figure 3). Previous work in our laboratory shows that HHV-8 infection was elevated among men from Tobago with prostate cancer compared to age-matched controls ($P=0.003$, OR 2.24, 95% C.I. 1.29-3.90) (section 1.6, Table 1)⁷. The GG genotype of the gp130 IL-6 signaling receptor was also associated with elevated prostate cancer risk, OR=1.5 (95% CI 0.98-2.32) in logistic regression. Both gp130 and HHV-8 seropositivity were independently associated with prostate cancer. Upon further exploration of the six genotype-HHV-8 combinations, analyses revealed an OR of 3.1 (95% CI 1.2-8.1 $p=0.022$) for prostate cancer in men who are HHV-8 seropositive and have the GG genotype, compared to the lowest risk group, who are HHV-8 negative and have the CC genotype (section 1.7, Table 2).

These data led us to explore the association of HHV-8, gp130, and prostate cancer further. We first sought to determine if HHV-8 was in the prostate, because if HHV-8 is associated with prostate cancer, then it should be found in the prostates of seropositive men. We used three cohorts, the Multicenter AIDS Cohort Study (MACS), Tobago men,

and the Pittsburgh Urologic Cancer Cohort (PUCC). With each of the cohorts, we showed that the virus was present in most of the prostates of seropositive men.

With the MACS cohort, we analyzed prostates collected at autopsy from 18 men who were HIV positive and died with AIDS. None of these men had prostate cancer as determined by histological analysis. Sixteen were HHV-8 seropositive and two were HHV-8 seronegative as demonstrated by serology. Among the HHV-8 seropositive cases, seven had KS, four of which were visceral KS (section 2.5.2, table 4). We detected the expression of at least one viral protein in 68.9% of the seropositive subjects. The results clearly demonstrate that HHV-8 infected cells are present in a majority of cancer-free prostates of HIV-infected, HHV-8 seropositive men, with the detection of viral proteins not caused by the presence of KS since a significant number of the samples that were positive for HHV-8 protein expression were from men with no evidence of KS. In fact, there was no correlation between HHV-8 protein expression and the presence of KS.

The Tobago and PUCC cohorts allowed us to use subjects who have been diagnosed by histological analysis with prostate cancer. This allowed us to see if the virus was present in the cancer, or in the surrounding tissues.

The Tobago cohort was comprised of two subgroups, Tobago biopsies and Tobago prostatectomies. These data presented, from both groups, show that HHV-8 protein expression can be detected in the prostates of men from Tobago. Viral protein expression is not present in the cancer cells, but in the surrounding tissues (section 4.3.3, Figure 13). Among the Tobago biopsy subgroup, no viral protein expression was

seen in the seronegative subjects. Viral protein expression, in the seropositive subjects, was similar to what we saw in the MACS cohort (chapter 2). In the Tobago biopsy subgroup we saw expression of at least one viral protein in 75% of the seropositive subjects.

The Tobago prostatectomy subgroup allowed us to compare areas with cancer and areas without cancer in the same subject. Within the Tobago prostatectomy subgroup, all but three subjects expressed viral protein. In five of the six seronegative subjects that expressed viral protein, both K8.1 and LANA-1 was seen. All of the seronegative patients underwent re-testing to reconfirm that they were seronegative. Upon retesting, the subjects remained negative by the serological test. Since the majority of the seronegative subjects expressed both viral proteins, we believe them to be HHV-8 positive by protein expression, but with low HHV-8 antibody titers. Recent studies in our laboratory have shown that individuals can sero-revert (unpublished data, MacDonald, Bunker and Jenkins). Presumably sero-reversion is a result of the antibody titers falling below the sensitivity of the serological assay.

We were able to determine the percentage of positive cells for each marker in the Tobago cohort. This allowed us to determine that there was a significant difference in viral protein expression within the same patient between cancerous and non-cancerous areas of the prostate. Viral protein expression, both LANA-1 and K8.1, was higher in the Tobago cancerous sections versus the non-cancerous sections. This suggests that the virus is influencing the microenvironment in which the tumor develops. To lend further support, we predominately saw LANA-1 in the endothelial cells, where cancer

develops. We are suggesting that the virus aids in tumor development by influencing the microenvironment in which the tumor develops.

The PUCG cohort allowed us to examine a subset of men from the United States. Similar to what we saw in the Tobago cohort, among the HHV-8 seropositive subjects, viral protein expression was detected in multiple regions of each section, but not in the cancer. Some of the subjects showed both lytic and latent protein expression in the stained regions, while other regions contained only latent protein expression and only lytic protein expression. Six of the seronegative subjects showed protein expression (section 5.3, Table 14) with K8.1 expression the more predominate protein expressed. We were able to quantify the number of cells expressing viral proteins, but found no difference between the seronegative and seropositive subjects by Mann-Whitney U.

As discussed previously, we found that viral gene expression was in the prostatic glandular epithelium as well as the infiltrating macrophages in all cohorts. LANA-1 expression appeared to be expressed predominantly in the epithelial cells of the prostate. K8.1 appeared to be expressed predominately in what looked like macrophages. Viral IL-6 was stained for only in the MACS cohort, and appeared only in what looked like the macrophages. This suggests that HHV-8 establishes a latent infection in prostatic cells and that infiltrating macrophages do not enter the prostate latently infected. The expression of viral lytic genes in the macrophages may indicate that the macrophages have either become infected with HHV-8 in the prostate or have engulfed cells expressing viral proteins.

Next we sought to demonstrate that the presence of the virus in the prostate was associated with an increased inflammation. We used the CD68 macrophage marker to determine infiltrates as well as looking at CD20 for B-cell infiltrates. For the Tobago and PUGC cohorts we were able to quantify the number of cells present.

When we stained the MACS cohort for CD68, we found three distinct patterns of macrophage clustering. The lowest level of CD68 staining had an occasional macrophage present in the tissue and was labeled no inflammation (section 2.6, Figure 7B). There were two different CD68 staining patterns reflecting increased inflammation. Sections that exhibited pronounced areas of staining consisting of several cells in each area were labeled as focal staining (section 2.6, Figure 7 D,E). Sections that exhibited many individual or small groups of macrophages located throughout the tissue were labeled diffuse (section 2.6, Figure 7 G,H). Among the seropositive MACS samples, 20% (1/5) of the samples that did not express viral proteins exhibited inflammation compared to 54.5% (6/11) of the samples that expressed at least one viral protein. Thus expression of at least one viral protein was associated with increased inflammation.

We found no significant difference inflammatory infiltrates of the Tobago biopsy subgroup, based on CD68 expression, between the seropositive and the seronegative subjects. This could be a product of our sample size being so low. We only had 7 seronegative and 12 seropositive patients; perhaps if we were able to increase the sample size, we would find significance. It also may be explained by the presence of prostate cancer in these biopsies. It is well documented that prostate cancer is associated with increased infiltration, including macrophage infiltrates. The presence of

cancer-related infiltrate in these needle biopsies may mask any effects of viral protein expression. Another possible explanation is that these men were undergoing a biopsy of the prostate. That is a traumatic injury to the organ, injury is a possible cause of inflammation. For these reasons, it is not unreasonable that we have not found a difference in inflammation, as marked by CD68 staining, in the seropositive and seronegative prostates of the Tobago biopsy subgroup.

With the Tobago Prostatectomy subgroup we were able to analyze areas with and without cancer. We used a Wilcoxon-Signed Ranks test to determine that viral antigen expression resulted in an increased macrophage infiltrate. This suggests that in areas of cancer there is a higher amount of viral replication, due to K8.1 presence, and an increased immune infiltrate.

We also showed that within the same patient, the areas with cancer exhibited an increased immune infiltrate, both CD20 and CD68, over the areas that were cancer-free. The increased immune infiltrate could be a result of just the cancer or a combination of the cancer and the virus. Our earlier results with the cancer-free prostates of the MACS cohort suggest that at least some of the increased in immune infiltrate is due to the presence of the virus.

We were also able to determine total number of positive cells for each protein in the PUCG cohort samples. These results showed no differences between the inflammatory markers, CD68 and CD20, with respect to subjects that were antigen positive, subjects that were LANA-1 positive, and subjects that were K8.1 positive.

Taken together, these data suggest that the virus is present in the prostates of seropositive men, but it is not in the cancer. From the Tobago prostatectomy subgroup we can surmise that presence of HHV-8 in the prostate can result in an increased macrophage infiltrate. The difference in antigen expression seen in the cancer vs. non-cancer supports our hypothesis that the virus infects cells in the prostate, establishes a chronic inflammation and sets up a microenvironment that aids in tumor development.

For the Tobago prostatectomy and PUCc cohorts we collected data on the gp130 R148G polymorphism. When these data are taken into consideration we find that the gp130 high risk allele exhibited an increased macrophage infiltrate in the non-cancerous areas when compared to non-cancerous areas from individuals homozygous for the gp130 low risk allele in the Tobago subgroup. This suggests that the gp130 high risk allele is associated with an increased inflammation.

For the PUCc cohort, no difference was seen in immune infiltrate based on gp130 allele status. The gp130 genotype is not as prevalent in the U.S. as it is in Tobago (section 5.3, table 15). We know from the Tobago data that HHV-8 combined with the high risk G/G gp130 polymorphism produces an odds ratio of 3.1 for prostate cancer. The high risk polymorphism is found only in 3.7% of the U.S. cohort compared to the 23.4% seen in the Tobago cohort. Also, in the Tobago cohort, the gp130 high risk polymorphism was associated with an increased inflammation in the non-cancerous areas (section 4.3.4, table 10). In fact, among the PUCc cohort, there were no homozygous high risk (G/G) allele genotypes seen. This suggests that the lack of association between the high risk gp130 allele and inflammation in the U.S. population is due to the low prevalence of this allele in the population.

Following the observation that the gp130 R148G polymorphism was associated with increased inflammation in the Tobago prostatectomy subgroup, we decided to look to see if this polymorphism was functional by assessing LCLs developed from men in Tobago. We assessed the effect of rhIL-6, rhIL-11, and OSM on LCL lines representing all three genotypes. All of the Tobago LCLs showed a proliferative dose response to rhIL-6 with the homozygous high risk genotype showing the greatest response followed by the heterozygous genotype and then the homozygous low risk genotype. These results demonstrate that the R148G polymorphism in the gp130 signaling receptor is functional; in that it exhibits an increased proliferative response on LCLs from men with the G/G genotype. In testing the LCL's proliferative response to 25ng of rhIL-6, rhIL-11, and rhOSM, we saw that the response was specific to rhIL-6. This suggests that the polymorphism found in gp130 is affecting the binding region of the gp80/IL-6 complex. The R148G mutation is located in the D2 region of the gp130 protein which is known to be involved in the binding of gp80/IL-6 and is not believed to be involved with IL-11 and OSM binding. This could explain why IL-11 and OSM do not affect the growth of the LCLs in the same manner that IL-6 does.

We have also shown that each genotype shows a distinctly different STAT3 activation response to rhIL-6 at 25ng. The low risk allele shows a double peak pattern with increases in activation at 15 and 45 minutes. The heterozygous allele shows a drop early followed by strong activation after 15 minutes. While the high risk allele shows immediate strong activation that remains for 30 minutes.

Together the growth curve and STAT3 activation data suggest that the high risk G/G allele infers a growth advantage to B cells over the other the alleles. The continuous

activation of STAT-3 beginning at 15 minutes and continuing until 45 minutes suggests that STAT-3 in the high risk allele is constitutively active. Constant activation of STAT3 is known to infer a growth advantage in the activated B-cell subtype of diffuse large B-cell lymphomas.

The observation that IL-11, a cytokine that shares the gp130 homodimer as its signaling receptor with IL-6, did not infer a growth advantage, suggests that this effect is IL-6 specific. We believe that IL-6, through an elevated STAT-3 activation, infers a growth advantage, which can lead to aberrant cellular growth.

These data presented here taken all together show that HHV-8 can be found in the prostate. Expression of viral antigen is associated with an increased macrophage infiltrate. The cancer cells do not contain the virus; the virus is in the surrounding cells. The gp130 polymorphism is functional and is associated with viral protein expression and increased macrophage infiltrate. Taken together these data support the hypothesis that HHV-8 can serve as a co-factor for the development of prostate cancer by establishing a chronic infection which results in chronic inflammation increasing the risk of cancer development. In addition, the data also suggests that genetic factors may be important in the overall immune response.

7.2 Future Directions

Recent studies focusing on monocyte chemoattractant protein-1, MCP-1, found that IL-6 induces MCP-1 expression in myeloma cells ¹²¹. Nesbit and colleagues have shown

that MCP-1 stimulation of monocytes led to tumor formation in nontumorigenic melanoma cells ¹²¹. MCP-1 is a member of the CC chemokine family and is known to recruit monocytes, NK cells, and certain subpopulations of T-cells. It is classified as an inflammatory chemokine ¹²². If IL-6 can induce MCP-1 expression in melanoma, it may induce expression in prostate cancer. We propose that future work look at the expression of MCP-1 both in the tissue and in the supernatant from IL-6 induced LCLs from Tobago.

Further experiments on the R148G polymorphism effect on the inflammatory response are needed as well. We propose looking at immune cell migration experiments, in order to see if B-cells stimulated with IL-6 can induce migration of immune cells.

We propose testing the hypothesis that Tobago men with the high risk gp130 polymorphism have a heightened immune response. We suggest testing the immune response of Tobago men to a vaccine challenge. It is possible to measure the immune response to challenge with an influenza vaccine. Inoculate the men with the influenza vaccine, draw blood every week and measure the immune response. This will show if the high risk men respond faster than the low risk.

Another option is to look at to mouse for an animal model to study the gp130 polymorphism. Mouse gp130 has a proline at the site of the polymorphism, but if a mutation that is similar to the one seen in humans is introduced, it may be possible to study the effects in a mouse model.

It is also necessary to increase our genotyping and sample number in subjects from the United States. We believe R148G gp130 polymorphism is involved in the development

of prostate cancer in Tobago. Since the polymorphism has been found at only 3.7% in those tested so far in the U.S., it is understandable why we don't see the association in the United States.

The next step for the gp130 R148G polymorphism is to discern the crystal structure of it and IL-6. If the polymorphism is affecting the D2 binding region, the crystal structure will show us that.

7.3 Public Health Significance

Prostate cancer is the most common cancer among men in the United States and is the leading site of new cancer cases, at 25% of all cancers found in men ¹. It is second only to lung cancer as a cause of cancer death in the U.S. The annual incidence of prostate cancer has increased by more than 30% over the last 25 years while the mortality rate has increased approximately 14% over the last 60 years ². It has been said that if a man lives to be 80 he will die with prostate cancer and not from it. Supporting this idea is the statistics that one in 30 men will develop prostate cancer during their life time; and one in six of those men will develop invasive prostate cancer ¹.

We have shown a direct relationship between prostate cancer and HHV-8. We found that the presence of the virus results in an increase macrophage infiltrate, which is associated with poorer outcomes in prostate cancer ¹⁸. Our data strongly support the hypothesis that HHV-8, a sexually transmitted agent, is involved in the development of prostate cancer. With this new data, it is now possible to begin educational campaigns for the association of this sexually transmitted agent and prostate cancer. We also

believe that anti-inflammatory agents may reduce the risk of prostate cancer. The results of this dissertation lend support to applying anti-inflammatory agents to the regime of prevention of prostate cancer.

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