COMPARISON OF HHV-8 VIRAL TITERS AND CYTOKINE PROFILES BETWEEN MALES AND FEMALES AT RISK OF KAPOSI'S SARCOMA DEVELOPMENT

by

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ABSTRACT

Human herpesvirus 8 (HHV-8) is the causative agent of Kaposi's sarcoma- a cancerous tumor of endothelial origin. Development of KS among men who have sex with men (MSM) is preceded by viral reactivation as determined by increasing viral antibody levels and viral load in circulating immune cells. Thus, prevention of viral reactivation should result in decreased KS incidence.

HHV-8 infection rates are not significantly different between men and women, yet the occurrence of KS is dramatically different between genders. The prevalence of KS in MSM (15-60%) is more frequent than that of heterosexual men (0-9%), and occurs even less frequently (<1%) in the female population. Males are approximately around 13 times more likely to develop KS than women. This strong sexual bias brings up several possible factors that may explain the disparity between KS development and HHV-8 infection rates between men and women. Two of these factors are: First, a more robust natural immune response occurs in women following a primary infection, resulting in higher viral antibody titers. The presence of higher viral antibody titers may control viral reactivation, thus reducing the risk of KS development. Second, there may be a hormonal regulation of viral reactivation. Our laboratory has found the presence of functional estrogen response elements in the HHV-8 genome and preliminary results suggest that in the presence of estrogen, spontaneous reactivation of HHV-8 in B cells is reduced. Prevention of viral

reactivation would decrease KS development and in this hypothesis, viral antibody titers would be decreased among women due to the lack of viral reactivation (compared to men). In addition, increased viral reactivation among men may result in increased levels of proinflammatory cytokines, which would further modulate their immune response.

To test the hypotheses that men and women mount differing immune responses to HHV-8 infection, HHV-8 antibody titers were determined in cohorts of men and women at risk of developing KS. Circulating proinflammatory cytokine profiles were also analyzed between HHV-8 infected males and females to determine if levels of proinflammatory cytokines differ between genders. It is hypothesized that women will have a more robust immune response resulting in fewer viral reactivation events while men, due to a lessened immune response will reactivate more frequently, and as a result will have higher levels of proinflammatory cytokines compared to women. The public health significance of this thesis is in regards to more effective treatments to combat or suppress KS development in males.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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1.0 INTRODUCTION

1.1 HUMAN HERPESVIRUS-8

Herpesviruses are a family of double stranded DNA viruses that are prevalent in most animal species (1, 2) Over 130 herpesviruses have been identified, eight of which infect humans (1). One of these viruses is human herpesvirus 8 (HHV-8). HHV-8 is part of the gamma herpesvirus family of DNA tumor viruses (3). HHV-8 was discovered as the etiologic agent of Kaposi's sarcoma in 1994 by Chang and coworkers (4).

Its genome is 160 kb and contains a diverse set of genes that regulate signaling, transformation, and extensive immune evasion (5). Like all herpesviruses, HHV-8 establishes lifelong latency in its host, with intermittent periods of lytic replication (3). This herpesvirus has the ability to induce neoplasia in its hosts, and is also associated with different types of malignancies and lymphoproliferative diseases of B cells (3). Specifically, HHV-8 is associated with several diseases, such as primary effusion lymphoma (PEL), some forms of multicentric Castleman's disease (MCD), and Kaposi's sarcoma (KS) (6, 7, 8, 9).

1.2 HUMAN HERPESVIRUS-8 GENOME AND REPLICATION

The HHV-8 genome is 165kb, and contains over 90 ORF's, at least 11 of which are homologs to cellular proteins involved in signal transduction, cell cycle regulation, inhibition of apoptosis, and immune modulation (5, 10, 11). HHV-8 genes contain an upstream promoter, regulatory sequence, and initiation site, which is followed by a 5' nontranslated sequence, the ORF itself, followed by 3' nontranslated regions, and finally a polyadenylation signal (1). The coding region of the HHV-8 genome is flanked by high GC multiple terminal repeat units of 801 base pairs (12, 13). The coding region is a long unique region (LUR) that encodes the approximate 90 genes (14). HHV-8 dedicates large amounts of coding region in order to produce proteins that are able to evade and downregulate the host's immune system (15). These mechanisms interfere with both innate and adaptive immune responses. Some of these genes include viral IL-6, macrophage inflammatory proteins 1-3 (MIP), and various chemokines (1, 13, 14). HHV-8 can alter the immune environment of the host by blocking apoptosis, decreasing MHC class I surface expression, and altering interferon pathways (1, 16). HHV-8 encodes several glycoproteins expressed in the virion, including gB, gH, gL, gM, and gN (17).

1.2.1 Latency

During latency, HHV-8 infected cells are non-permissive for replication (6, 18). Only some viral genes are expressed, encoding proteins only necessary for maintaining the virus within the host where it exists as a covalently closed circular episome (19, 20). Viral structural proteins are not expressed, and no progeny virus are produced (3). HHV-8 has incorporated several cellular genes, most likely during the evolutionary development of the virus. These homologous genes are

able to facilitate virus replication, transformation, and survival (14, 21). Latency is essential to the pathogenesis of HHV-8, and the majority of cancerous KS cells are latently infected with the virus (1).

1.2.2 Lytic Replication

During lytic replication, all ORF's of the viral genome are expressed, which leads to successful viral replication, cell lysis, and subsequent cell death (1). Both latent and lytic HHV-8 genes are felt to contribute to viral oncogenesis (6). This process is facilitated via transformation, which has the ability to cause normal cells to proliferate in an uncontrolled manner (21). The infection of B-cells is a necessary step in KS development, as HHV-8 infection develops from latently infected B-cells (22). Rappocciolo et al. described this by demonstrating that activated B-cells cannot only be infected with HHV-8, but can produce infectious virus (23).

1.3 HHV-8 BINDING AND ENTRY RECEPTORS

HHV-8 requires two separate receptors: (1) binding receptors and (2) entry receptors, in order to infected cells. HHV-8 subunits must first attach to the binding receptor, which then may interact with the entry receptor, allowing penetration of the virion into the cell.

In order for HHV-8 to enter a host cell, the viral envelope glycoproteins and other cell membrane components must interact according to the following steps: First, the virus must bind and attach to the cell surface, interact with a specific entry receptor, and enter the host cell via mechanisms such as endocytosis or membrane fusion (1, 76). HHV-8 is then able to release its capsid into the cytoplasm of the host cell, post cell fusion (76). Binding receptors are also required for proper entry into the host cells by HHV-8, but are not sufficient for entry (2). The interaction between glycoproteins and binding receptors tethers the virion to the host cell surface, and then the virus is able to interact with entry receptors (2, 76).

1.3.1 Heparin Sulfate

Heparin sulfate (HS) is “ubiquitous cell surface” molecule, which can bind to a variety of ligands and is important for many cellular activities (24). HS is a binding receptor for HHV-8 on target cells, and has been shown to bind both glycoproteins (gp)K8.1 and gB (24). K8.1 is present in two forms- K8.1A or K8.1B, and both are an integral part of the HHV-8 viral envelope (25).

Nelpel et al. demonstrated that K8.1 is capable of binding to heparin sulfate on the surface of cells, but is not capable of entering cells through this mechanism. It is believed that K8.1 is one of the proteins involved in this complex process.

1.3.2 Integrin α3β1–like (CD 49c/29)

Integrins are heterodimeric receptors that contain α and β glycoproteins (24). Chandran et al. demonstrated that RGD (Arg-Gly-Asp) amino acids are capable of binding to integrin α3β1–like (CD 49c/29). The group analyzed the amino acid sequence of HHV-8 glycoproteins, and determined that gB contains the RGD motif, and is also capable of entering target cells via this mechanism. This group also demonstrated the ability of gB to interact with α3β1. It has been established that α3β1 integrin is a cellular receptor for HHV-8 infection.

1.3.3 xCT

xCT is an amino acid transporter and HHV-8 entry receptor (26). It is one of four known HHV-8 entry receptors, and is present on macrophages, and endothelial cells. Parsons et al. determined that expression of the entry receptor xCT significantly increases as an individual develops KS. This suggests that increased xCT expression increases cell permissiveness for HHV-8 to enter more cells, and the specificity of xCT expression on varying cell types (26). This may make xCT an ideal target for treatment or prevention of developing KS (27).

1.3.4 EPHRIN

Recently, Boshoff et al. reported that the ephrin receptor A2 (EphA2), which is a tyrosine kinase, is able to bind to HHV-8 glycoproteins (specifically gH and gL), which allows the virus to enter target cells effectively, particularly endothelial cells (28). Upon phosphorylation, EphA2 is capable of promoting endocytosis of the host cell, giving this receptor an important role in the infection of cells. Like xCT, possible therapies for HHV-8 infection could be blocking this receptor as a therapeutic strategy to decrease ongoing lytic infection of cells, which has been demonstrated to lead to angiogenesis, and increasing inflammation in the host (28).

1.3.5 DC-SIGN

DC-SIGN, or Dendritic Cell-Specific Intracellular adhesion molecule 3-Grabbing Non-integrin, is a C-type lectin receptor that is present on DCs as well as activated macrophages (29). The DC-SIGN receptor present on macrophages can bind mannose-type carbohydrates (PAMPS) that are found on viruses and other pathogens. DC-SIGN on DCs mediates interactions with endothelium, activation of CD4 T-cells, etc. Rappocciolo and colleagues have demonstrated that DC-SIGN is a cellular entry receptor for HHV-8 on DC's, macrophages, and activated B-cells (29, 30). In vivo, these are the typical cell types that are "natural targets" for HHV-8 entry (17). Hensler et al. demonstrated that soluble gB is able to bind DC-SIGN (17).

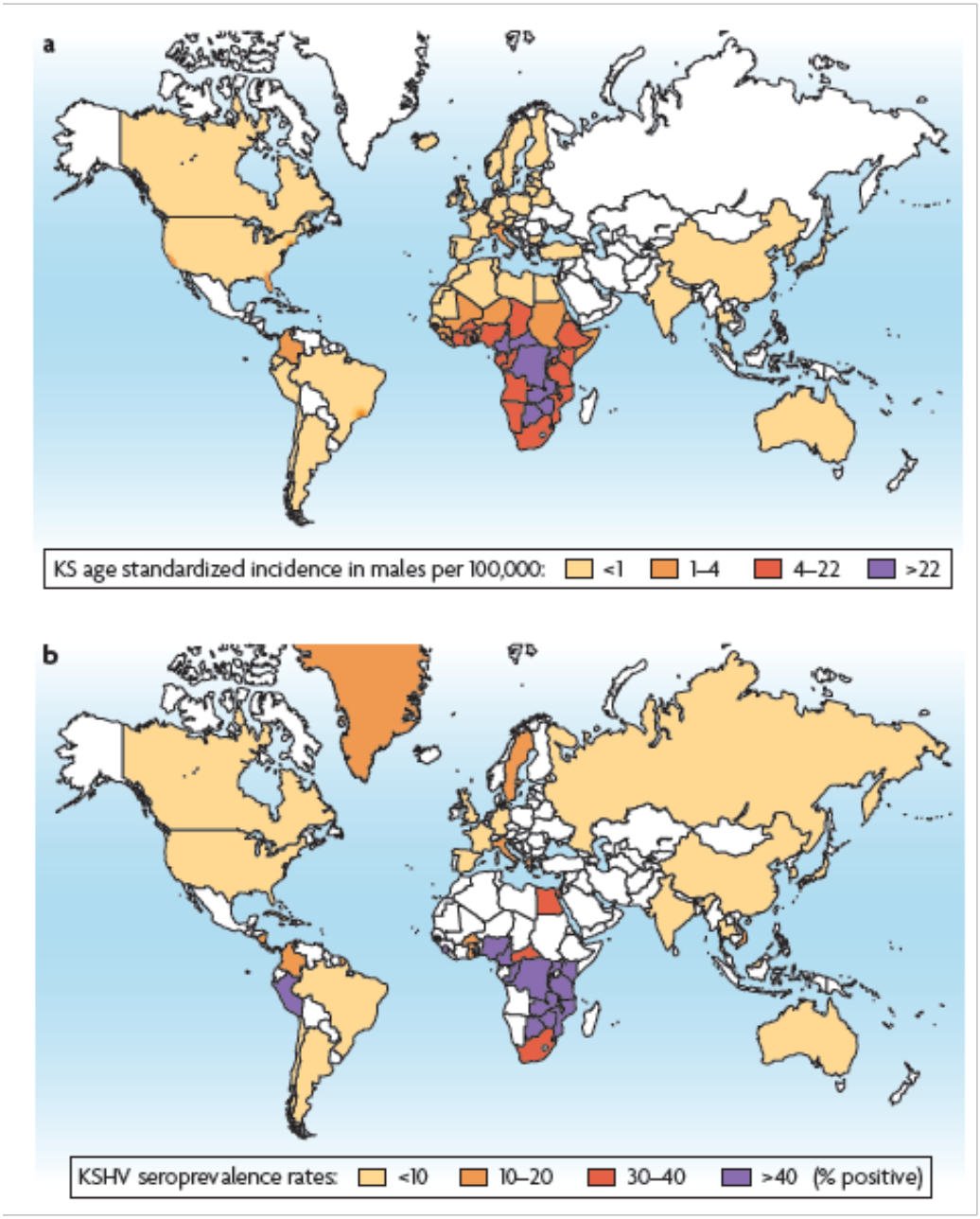
1.4 KAPOSI'S SARCOMA

KS is a cancerous tumor of endothelial origin (14) and is composed of a mixture of irregularly shaped, round capillaries, and spindle-shaped cells often with infiltrating mononuclear cells (1, 14). KS usually presents with pigmented skin lesions, which can eventually lead to more serious ulcerating tumors (14). Risk factors for the development of KS in HHV-8 infected individuals include increased HHV-8 viral load, HIV seropositivity, and immunosuppression (31). In order for KS to develop, the virus needs to efficiently establish a persistent infection in the host (15).

The detection of HHV-8 infection in an individual is most often based on the presence of antibodies against either lytic or latent viral antigens (14, 31). DNA sequences of HHV-8 have been detected in all forms of KS tumors, but can also be detected via PCR from PBMCs (peripheral blood mononuclear cells) and BCBLs (body cavity based lymphoma) of both KS subjects, and otherwise healthy individuals (32, 33, 34). However, PCR methods can be time consuming, expensive, and not as sensitive as serological assays (1). For this reason, testing for specific antibodies to HHV-8 is a less expensive and simpler way to determine infection. Current methods for detection of HHV-8 antibodies include enzyme-linked immunosorbant assay (ELISA), western blot, and immunofluorescent assay (IFA) (1, 35).

The outbreak of KS in the early 1980's led in part to the discovery of AIDS. KS is one of the most common AIDS-associated malignancies in the population (31) (Figure 1). Though HHV-8 infection is evenly distributed throughout the population, prevalence of KS is not evenly distributed (36, 37). The prevalence of KS in homosexual men (15-60%) is much more frequent than that of heterosexual men (0-9%), and occurs even less frequently in the female population

(38). Males are around 13 times more likely to develop KS versus women (2, 3). There are 4 forms of KS (3).



(A), Incidence of Kaposi's sarcoma (KS) in the male population, age-standardized. (B) Seroprevalence of HHV-8, compiled from multiple studies. (Figure reprinted with permission from Mesri, E. A., E. Cesarman, et al. (2010). "Kaposi's sarcoma and its associated herpesvirus." Nat Rev Cancer 10(10): 707-719).

Figure 1 Seroprevalence of HHV-8 versus Seroprevalence of KS.

1.4.1 Classical Kaposi's sarcoma

Classical KS is mainly associated with elderly men in Mediterranean countries, and is also known as sporadic KS (2, 3). The most common countries associated with this form of KS are Italy and Israel. Classical KS is usually nonaggressive, and is often associated with lesions on the lower extremities (40). Generally, the older the subjects are, the faster the disease progression (14, 3).

1.4.2 Endemic Kaposi's sarcoma

Endemic KS was discovered in Africa prior to the onset of the AIDS epidemic (42). It is found in both men and women, and most notably, young children. This form of KS tends to be more aggressive than classical KS, and can affect parts of the body like the lymph nodes. Endemic KS is the leading cause of cancer in children in sub-Saharan Africa (40).

1.4.3 Iatrogenic Kaposi's sarcoma

Iatrogenic KS is transplant-associated, due to treatment with immunosuppressants (3). It normally occurs in subjects after solid-organ transplants that were subsequently treated with immunosuppressive drugs to combat organ rejection (2, 7). Withdrawal of therapy can sometimes

lead to KS regression. Iatrogenic KS often has a most rapid disease progression compared to classical KS, but it is also able to present as a chronic infection as well (14)

1.4.4 AIDS-Associated Kaposi's sarcoma

AIDS-associated KS is the most aggressive form of KS (43). The disease is often associated with a high tumor burden and rapid disease course, which is very different from the timecourse of infection seen in non-AIDS subjects (6, 44,). AIDS-associated KS involves not only the lower extremities and skin, but also the upper body, lymph nodes, and head (7). It may also disseminate to other organs such as the lungs, liver, spleen, and GI tract (14). In the 1980's, individuals with AIDS have a 50% lifetime rate of developing KS, however this percentage has declined since antiretroviral drugs were introduced (14, 6).

1.4.5 Development and Distribution of Kaposi's sarcoma

The distribution of KS is not evenly distributed throughout the population. KS development only occurs in a small proportion of the HHV-8 population, and only after a long viral latency period (5, 47). The development of KS is dependent on several factors, including the host's underlying immune status, HHV-8 viral titers, HIV coinfection, and immunosuppression (5, 48, 49). The prevalence of KS does not match with HHV-8 seroprevalence (which is much more evenly distributed amongst sexes). This leads us to believe that women are somehow protected against developing the cancer.

The higher prevalence of KS in men versus women begs the question as to why there is such a strong sex bias between sexes. This study has suggested two possible factors that could be influencing this bias:

(1) A more robust humoral response in women (As measured by a higher viral titer compared to men):

- Could decrease the chances of reactivation and developing KS versus men due to a stronger immune response to HHV-8.
- May correlate with higher proinflammatory cytokines in women.

1.5 ANTIBODIES

Antibodies and neutralizing antibodies are an integral part of the humoral immune response against viral infection. The immune system uses antibodies to identify and neutralize pathogens, such as HHV-8 by recognizing antigens, and targeting a specific epitope for each pathogen. They have been implicated in controlling the progression of herpesvirus-associated diseases (57, 58). If women are capable of eliciting a stronger initial immune response to HHV-8 versus men, this would result in higher antibody titers. Higher antibody titers in females may be able to effectively combat and suppress reactivation of HHV-8 over time, while males are at a higher risk of reactivation, due to a less robust immune response. The role of neutralizing antibodies in controlling KS infection is not yet clear. Previous studies have detected the presence of nAb in HHV-8 infected individuals, and examined the ability of HHV-8 to inhibit the nAb response against infection. Wood et al. found that the total level of antibodies and neutralizing antibodies was significantly higher in KS subjects. Kimball et al. examined inhibition of HHV-8 viremia at

1:10 or 1:50 dilutions of seropositive subject samples, which was not seen in the seronegative subjects (59). Several other research groups such as Ledgerwood et al. and Moss et al. have examined virus neutralization based on flow cytometry methods, however no group to date has explored this technique with HHV-8 (60). In this study, a GFP expressing virus, BAC16, was used to examine the level of neutralizing antibodies in males versus females.

1.6 THE BAC16 VIRUS

BAC16 was generated by the J. Jung laboratory, and is a KSHV bacterial artificial chromosome (BAC) clone that is derived from the rKSHV.219 virus. It contains the BAC16 bacmid, which consists of a full-length KSHV genome and GFP under the control of the EF1α promoter. This bacmid was transfected into the KS cell line, iSLK. SLK cells were originally isolated in Israel in 1990 from a tumor biopsy of the oral mucosa of an immunosuppressed HIV-1 subject (62). iSLK stands for inducible SLK cells. They contain the KSHV RTA (replication and transcription activator) gene (ORF50), under the control of doxycycline. The RTA protein is necessary and sufficient to reactivate the switch from latency to lytic replication (61, 62). The BAC16 virus can be reactivated to productive replication by treating iSLK cells with doxycycline for three days.

(2) Sex hormone (estrogen) that limits viral reactivation in women (Resulting in lower viral titers compared to men):

- Estrogen may be decreasing HHV-8 replication, which may suppress KS reactivation in women.
- May correlate with lower proinflammatory cytokines in women.

1.7 ESTROGEN

Estrogen plays an important role in many biological functions, and is mediated by the estrogen receptor (ER) (53). ER's are ligand-dependent transcription factors that are able to bind to specific DNA sequences within the promoter of a gene (estrogen response elements) to activate gene expression. ER's bind with high affinity to ERE's and are widely expressed in different tissue types (54). There are 2 subtypes of estrogen receptors- ERα and ERβ, both of which are expressed in vascular endothelial cells, smooth muscle cells, and myocardial cells (54, 55). However both receptors are functionally distinct and differ in some tissue distribution, DNA-binding domains, and activation of estrogen promoters (52). Estrogen is known to participate in the regulation of several genes that are involved in proliferative vascular diseases, specifically their pathogenesis. This may be due to cell-cell communications, regulation of inflammatory genes, etc. (52, 56). It is hypothesized that estrogen may exhibit a protective effect on certain neovascular disorders, such as KS due to altering vascular effects such as vascular relaxation and vasodialation, which may reduce progression of the lesions (52).

Previous research in the Jenkins' laboratory has shown that estrogen response elements (ERE's) can be found in front of several HHV-8 genes, which are able to bind estrogen receptor proteins, and that these ERE's appear to be functional in modulating HHV-8 transcription (Patrick Shea). Also, that estrogen can induce transcription of some HHV-8 genes, and in the presence of estrogen, there is an increase in transcription of HHV-8 genes with ERE's in proximity to their

promoters (Rebeca Compaore- a previous MS student in the Jenkins' lab). Preliminary research has also shown that in the presence of estrogen, HHV-8 does not reactivate *in vitro.*

1.8 PROINFLAMMATORY CYTOKINES

Cytokines are hormonal, intra- and inter-cellular messengers that are responsible for many biological effects in the immune system including immune cell attraction, activation of humoral and cell-mediated immunity and modulation of specific immune responses (64). They are small nonstructural proteins with molecular weights ranging from 8 – 40,000 d (64). Most nucleated cells are capable of synthesizing cytokines, and they are also capable of responding to specific cytokines, depending on whether or not they express the appropriate receptor (64). Some of the major producers of cytokines include B and T-cells, DCs, macrophages, epithelial cells and endothelial cells. Cytokines are a very diverse group of messengers, and can contribute to stimulating the production of cells, growth and differentiation of cells (developmental processes), regulating the immune system, etc. (65). Cytokines can be divided into proinflammatory and anti-inflammatory cytokines. The primary role of anti-inflammatory cytokines is to reduce inflammation and promote healing of the host. Alternatively, proinflammatory cytokines essentially promote disease progression by increasing inflammation in the host (64). For this study, proinflammatory cytokines will be the main focus of analysis.

KS is an "inflammatory cytokine-mediated angioproliferative disease" (2). Specifically, KSHV has an ORF (K14) that is homologous to cellular OX2- which stimulates primary

monocytes, macrophages, and dendritic cells to produce excessive amounts of proinflammatory cytokines, such as IL-1β, 7, and TNF- α (66). This means that KS has a strong ability to induce inflammation, and promote cytokine-mediated angiogenic proliferation of infected cells (67, 66). Excessive levels of proinflammatory cytokines can lead to uncontrolled tissue damage, and progression of diseases (68). Previous research has demonstrated that HHV-8 infected endothelial cells are driven to proliferate by proinflammatory factors, and that there is an abnormal excess of cytokines and chemokines produced by B-cells infected with HHV-8 (65).

HHV-8 infection is able to trigger the release of proinflammatory cytokines that can alter the maturation of human cells. Specifically, IL-6 and TNF-α were increased after HHV-8 infection of DCs (69). However, this has not been examined in regards to reactivation. HHV-8 binding and entering into cells can cause the release of cytokines and chemokines (69). Cytokines also have the ability to prevent differentiation of cells in to DCs, decrease antigen uptake and presentation to combat infection, and even alter cell surface marker phenotypes (70). Chung et al. reported that KS appears to be a hyperplastic disorder that is caused in part by the local production of inflammatory cytokines, specifically IL-1, IL-6, and TNF-α. The researchers supported this conclusion by demonstrating that the infiltration of inflammatory cells in KS lesions preceded the transformation of spindle-shaped endothelial cells. They also reported that the production of these inflammatory cytokines are responsible for the activation of vessels and endothelial cells, which has the ability to increase adhesiveness, and the recruitment of more immune cells to the site, thus increasing inflammation. (66). Finally, cytokines have the ability to polarize DCs to promote a Th2 vs. Th1 immune response. Specifically, certain cytokines have the ability to polarize DC's towards the induction of a Th2 response, which can contribute to the persistence of HHV-8 (69).

Th1 cells are an essential component of the immune system, specifically the adaptive immune response. Th1 cells activate macrophages, and are important for cell-mediated immune response to pathogens. They are also important for the activation of CTLs. Th2 cells produce IL-4, 5, 10, and 13, which are important in antibody responses and production. Th2 cells also activate granulocytes (70).

Because KS has the ability to stimulate cells to produce large amounts of proinflammatory cytokines, altering the maturation of different cell types, examining cytokine profiles of males versus females would also be an important step in understanding more about the variances in immune system regulation between sexes. This study examined 10 different proinflammatory cytokines between males and females to determine if there were differences in the subject's sera samples. Differences could indicate disease progression in males if proinflammatory cytokines are increased in this population. The 10 cytokines examined were:

1.8.1 IFN-γ

Interferon gamma is the main Th1 cytokine, and it is a cytokine that produces a proinflammatory response that is responsible for killing intracellular pathogens (68). Specifically, IFN-γ activates macrophages, has the ability to inhibit viral replication, and can increase CD8+ activity (64, 66) Other functions of IFN-γ include increasing antigen presentation, inducing production of IgG from B-cells, and suppresses Th2 differentiation (67). This cytokine is produced by lymphocytes, and is involved in numerous pathways for a number of medical disorders (64).

1.8.2 IL-1β

Interleukin-1β is a proinflammatory cytokine that was originally referred to as a lymphocyte-activating factor (LAF), is produced by activated macrophages, and is an important component in the inflammatory response (64, 66). IL-1β is involved in several activities such as cell differentiation, proliferation, and cell death of B and T-cells. This cytokine is also involved in the induction of prostaglandins, which is a mediator of many biological conditions such as regulating hormones, controlling growth of immune cells, and causing vasodialation (68).

1.8.3 IL-10

Interleukin 10 is an anti-inflammatory cytokine that downregulates Th1 cytokines, and co-stimulatory molecules present on macrophages (66). IL-10 can also increase B-cell survival and growth, and may be capable of inhibiting other proinflammatory cytokines, such as those mentioned above (68). This cytokine is also capable of inhibiting the synthesis of certain cytokines, such as interferons, I2, and 3, which suppress Th1 proinflammatory responses (67)

1.8.4 IL-12p70

Interleukin 12 production favors the generation of Th1 cells, and is produced by DC's, B-cells, and macrophages (67). Specifically, IL-12 is an important component in differentiating naïve

T-cells into Th1 cells, and to promote NK cells to stimulate IFN-γ (68). It also has the ability to stimulate other cytokines like IFN-γ and TNF-α. IL-12 is able to mediate cytotoxic activity of other cell types, and unlike IL-8, is anti-angiogenic (64). IL-12 is encoded by two separate genes- IL-12A (p35) and IL-12B (p40). IL-12p70 is the homodimer of these two submits (64). Recently, there has been discussion as to if IL-12 may be a useful component in some anti-cancer therapies.

1.8.5 IL-13

Interleukin 13 behaves similarly to IL-4- favoring the generation of Th2 cell stimulation and growth. IL-13 has been determined to induce changes in hematopoetic cells, B-cell maturation, and differentiation. IL-13 triggers macrophage activity, increasing proinflammatory activity (68).

1.8.6 IL-2

Interleukin 2 is also known as T-cell growth factor, and stimulates the proliferation of T-cells. IL-2 is necessary for the differentiation, and growth of T-cells (69). When an antigen binds to a TCR, IL-2 secretion is stimulated. IL-2 production levels are associated with diseases such as HIV infection, which is an important confounding factor in our study that will be explored later (69).

1.8.7 IL-4

Interleukin 4 is also known as B-cell stimulatory factor, and lymphocyte stimulatory factor. This cytokine is produced by Th2 cells, and can help activate B-cells, stimulate DNA synthesis, and is associated with the promotion of IgE and eosinophilic responses (68). IL-4 production also favors the generation of Th2 cells, including their stimulation and growth. Some of the biological functions of IL-4 include T-cell proliferation, plasma cell development, and decreased production of Th1 cells (68).

1.8.8 IL-6

Interleukin 6 is another B-cell stimulatory factor, as well as a cytotoxic T-lymphocyte (CTL) differentiation factor. IL-6 is very important in inducing inflammation and apoptosis in humans (63). It is released from many cell types, and plays an important role in differentiating B-cells into Ig secreting cells (64, 66)

1.8.9 IL-8

Interleukin 8 is a chemokine that is produced by macrophages, epithelial, and endothelial cells. IL-8 not only induces chemotaxis in target cells, but also causes other cells to migrate towards the site of infection (66). It can also induce phagocytosis, and angiogenesis. IL-8 is highly associated with inflammation, and is increased by oxidative stress (63, 64).

1.8.10 TNF-α

Tumor necrosis factor alpha is a cytokine that is highly involved in inflammation, and mainly produced by activated macrophages, CD4 cells, and NK cells (68). TNF-α is an immune regulator, and is able to induce cell death and inflammation in some tumor cell lines (66). TNF-α is capable of stimulating IL-1, IL-12, and can induce sepsis and inflammation (67, 68).

2.0 SPECIFIC AIMS

This study examined two possible reasons why women are protected against KS development, and men are at a much higher risk of developing the disease, regardless of HHV-8 seroprevalence. The first hypothesis is that there is a more robust humoral response in women, which we suspect would result in higher antibody titers versus males. This would allow women to mount a stronger immune response to HHV-8, and become more protected against reactivation of the virus and development of KS. The second hypothesis involves hormonal control of HHV-8, which may be interfering with viral reactivation and therefore suppressing the development of KS in women. If this hypothesis is correct, we would expect male antibody titers would be increased due to increased reactivation events resulting in their predisposition to develop KS much more frequently compared to females. It is also hypothesized that males will have increased levels of proinflammatory cytokines versus females, due to increased viral reactivation. Two specific aims will be completed to examine each of these hypotheses:

AIM 1: Determine HHV-8 antibody titers in a cross-sectional study between MSM in the Multicenter AIDS Cohort study (MACS) and women in the Women's Interagency HIV Study (WIHS).

I. Perform a cross-sectional study using matched samples between males and female serum samples to explore differences in HHV-8 titers and infection:

1. Perform IFA's on serum samples to determine HHV-8 antibody titers of MACS and WIHS study participants.

2. Examine differences in titer levels based on HIV status, gender, and race.

II. Examine differences in levels of neutralizing antibodies between matched men and women.

1. Quantify BAC16 virus stock via a TCID50 assay.
2. Perform neutralization assays and determine the level of infection by the number of cells expressing GFP via flow cytometry 48 hpi.

AIM 2: Compare the levels of proinflammatory cytokines between men and women.

1. Utilize a Meso Scale Discovery (MSD) Proinflammatory Pane V-PLEX kit that analyzes serum samples for 10 different human proinflammatory cytokines.
2. Compare the levels of cytokines between males and females.

3.0 MATERIALS AND METHODS

3.1 CELL LINES

BCBL-1 cells are a HHV-8 positive, EBV negative B-cell line that originated from a pleural effusion lymphoma and were obtained from the NIH AIDS repository (71). BCBL-1's were cultured in RPMI supplemented with 10% fetal bovine serum (FBS), 0.1% Gentamycin, and grown at 37C and 5% CO_2.

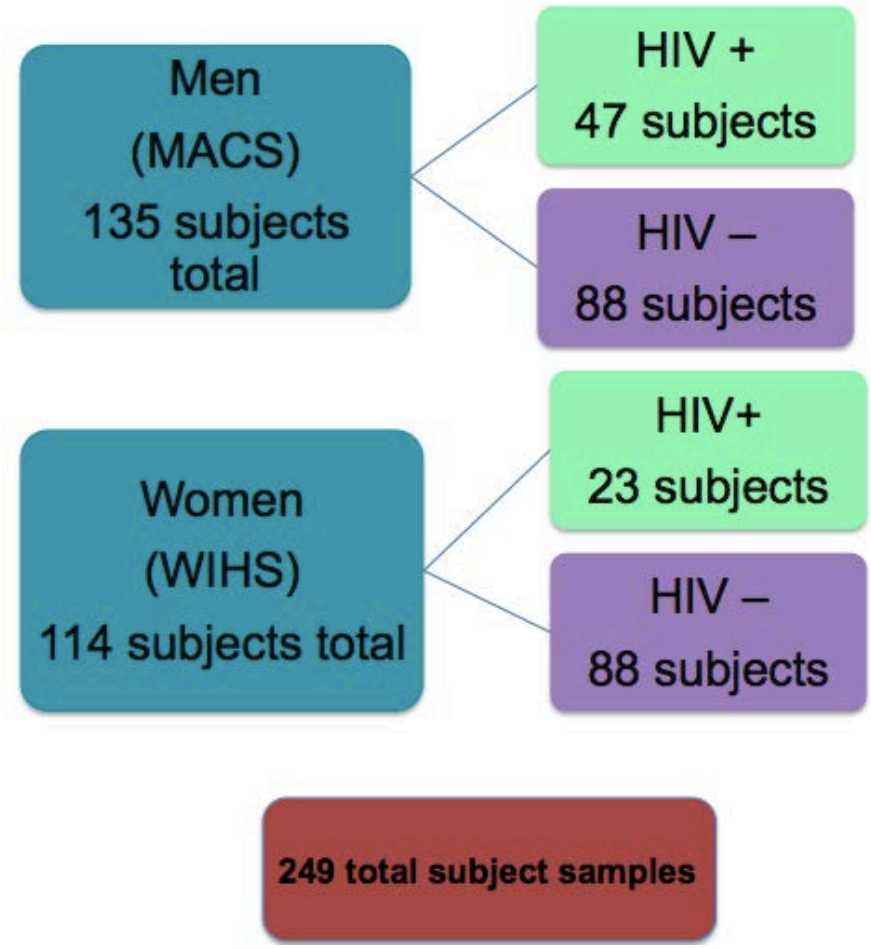
K562 cells originated from a chronic myelogenous erythroleukemia (72). K562-DC-SIGN cells were generated by transfecting K562 cells with a plasmid that expresses the DC-SIGN protein under the control of the immediate-early CMV promoter (obtained from Ben-Hur Lee- UCLA). Transformed cells were selected in media containing 400 μg/mL G418. K562-DC-SIGN cells were constructed by Dr. Heather Hensler in Dr. Jenkins' laboratory. Expression of DC-SIGN was confirmed by flow cytometry. K562-DC-SIGN cells were cultured in RPMI supplemented with 10% fetal bovine serum (FBS), 0.1% Gentamycin, and grown at 37C and 5% CO_2.

SLK cells were originally isolated in Israel in 1990 from a tumor biopsy of the oral mucosa of an immunosuppressed HIV-1 subject (61). iSLK stands for inducible SLK cells, which are induced by doxycycline via the RTA (replication and transcription activator) gene- which controls the switch from latency to lytic replication (61, 62). iSLK cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 1μg/ml puromycin, 250 μg/ml G418, 1,200 μg/ml hygromycin B and grown at 37C and 5% CO_2.

3.2 SUBJECT SAMPLES

A total of 249 total subject serum samples were used for this study. 135 male and 114 female samples. Within the male cohort, there were 47 HIV infected and 88 HIV uninfected subjects while within the female cohort, there were 23 HIV infected and 88 HIV uninfected subjects the WIHS (Figure 2).

Male samples were obtained from the MACS study, which is an ongoing study of HIV-1 and AIDS infection in MSM in the United States beginning in 1984. Participants include HIV seropositive and high-risk HIV seronegative subjects. Female samples were obtained from WIHS (The Women's Interagency HIV Study). The WIHS study is an ongoing study of HIV-1 and AIDS infection in women, beginning in 1994 and also consists of HIV seropositive and high-risk seronegative subjects. Both MACS and WIHS serum samples were matched as closely as possible to HIV status, CD4 counts, age, HAART treatment, and race (Figure 3). For the cytokine analysis of MACS and WIHS serum samples, only HIV seronegative samples were utilized. This was to remove the potential confound of HIV coinfection on proinflammatory cytokine levels in the subject groups. Due to the WIHS group only having 23 HIV+ subjects, and the MACS having 47 HIV+ subjects, there were not enough matching samples, so multiple WIHS samples had to be matched to single MACS samples. The MACS and WIHS study protocols were approved by the institutional review boards of each of the participating centers, and the University of Pittsburgh. Informed consent was obtained from all the men and women.



135 subject from the Multicenter Aids Cohort Study (MACS) and 144 subjects from the Women's Interagency HIV Study (WIHS) were used for this study. Out of these male group 47 subjects were HIV+, and 88 subjects were HIV-, for the female group, 23 subjects were HIV+ and 88 subjects were HIV-, for a total of 249 total subject samples. WIHS subject samples were tested via IFA for HHV-8 seroprevalence by Rebeca Compaore.

Figure 2 Subject samples from the MACS and WIHS studies.

14216	10101496	Hispanic	Hispanic	SP	SP	365	2003.362	9000	46.513	49.29863	2.78563014	587	457	ELISA	130	Not on HAART	At Jenkins Lab
14011	30203446	Black	Black	SP	SP	380	2003.209	2004.052	51.543	48.65753	2.88546575	99	81	ELISA	18	Not on HAART	At Jenkins Lab
14078	30100551	Black	Black	SP	SP	365	2003.017	9000	33.626	36.6274	3.00139726	1135	935	ELISA	200	Not on HAART	At Jenkins Lab
24341	10310982	Black	Black	SP	SP	490	2008.274	9000	30.0787	25.31781	4.76089528	640	639	ELISA	1	Not on HAART	Newly Selected
23032	20101804	Black	Black	SP	SP	170	1992.55	9000	35.14096	35.73973	0.59876488	156	150	IFA	6	Not on HAART	Newly Selected
23028	10311491	Black	Black	SP	SP	160	1991.918	9000	40.39726	36.00548	4.39178082	105	99	IFA	6	Not on HAART	Newly Selected
23191	20101018	Black	Black	SP	SP	75	1990.735	1996.715	36.36438	36.30411	0.06027397	395	404	ELISA	9	Not on HAART	Newly Selected
23076	10310514	Black	Black	SP	SP	75	1988.602	9000	35.42849	33.87671	1.55177783	136	131	ELISA	5	Not on HAART	Newly Selected
23031	10311439	Black	Black	SP	SP	200	1993.799	9000	35.74521	36.11781	0.37260274	378	376	IFA	2	Not on HAART	Newly Selected
23005	10100569	Black	Black	SP	SP	230	1995.636	1997.06	36.65753	38.18556	1.52802605	146	145	IFA	1	Not on HAART	Newly Selected
23014	10102955	Black	Black	SP	SP	80	1988.085	1997.443	39.70492	36.09863	3.6062879	444	446	ELISA	2	Not on HAART	Newly Selected
23086	30409151	Black	Black	SP	SP	230	1995.661	1998.034	36	38.18308	2.18308257	294	286	Both	8	Not on HAART	Newly Selected
23139	10100331	Black	Black	SP	SP	160	1991.844	9000	31.92603	35.94795	4.02191781	97	85	IFA	12	Not on HAART	Newly Selected
13093	30306608	White	White	SP	SP	140	1991.009	9000	29.11781	26.21918	2.89863014	242	222	IFA	20	Not on HAART	Newly Selected
24023	10103250	Black	Black	SP	SP	470	2007.272	2011.548	33.88219	37.09589	3.21369863	543	534	ELISA	9	Not on HAART	Newly Selected
34064	10205408	White	White	SP	SP	365	2002.346	2006.376	40.50137	45.19726	4.69589041	748	719	ELISA	29	Not on HAART	Newly Selected
23030	30203612	Black	Black	SP	SP	75	1987.515	9000	37.06849	39.45497	2.38648102	309	311	ELISA	2	Not on HAART	Newly Selected
23034	10310691	Black	Black	SP	SP	75	1987.548	9000	38.41918	40.1863	1.76712329	474	467	ELISA	7	Not on HAART	Newly Selected
33071	20102046	White	White	SP	SP	80	1987.934	9000	41.48767	45.20274	3.71506849	280	246	ELISA	34	Not on HAART	Newly Selected
13146	10311566	Hispanic	Hispanic	SP	SP	120	1990.023	9000	42.24658	44.47397	2.22739726	125	174	IFA	49	Not on HAART	Newly Selected
23244	20100612	Black	Black	SP	SP	75	1991.622	9000	35.84658	36.87397	1.02739726	83	35	ELISA	48	Not on HAART	Newly Selected
44049	20205450	Black	Black	SP	SP	365	2002.713	2003.732	26.6249	26.88915	0.26424882	620	625	ELISA	5	Not on HAART	Newly Selected
13022	10101460	Hispanic	Hispanic	SP	SP	150	1991.373	9000	38.48462	39.02497	0.54034733	14	34	IFA	20	Not on HAART	Newly Selected
23044	10102880	Black	Black	SP	SP	140	1990.987	9000	46.72401	43.80822	2.91578711	12	18	IFA	6	Not on HAART	Newly Selected
23207	10100266	Hispanic	Hispanic	SP	SP	160	1991.923	9000	34.73973	31.43288	3.30684932	420	408	ELISA	12	Not on HAART	Newly Selected
23157	20205119	Black	Black	SP	SP	75	1990.166	9000	37.19171	39.92329	2.73158171	3	22	ELISA	19	Not on HAART	Newly Selected
33028	10205232	White	White	SP	SP	120	1990.103	1996.928	25.18334	29.40548	4.22214238	365	367	ELISA	2	Not on HAART	Newly Selected
23080	20100561	Black	Black	SP	SP	100	1989.164	9000	40.01096	37.74247	2.26849315	105	141	IFA	36	Not on HAART	Newly Selected
24219	10102258	Hispanic	Hispanic	SP	SP	390	2003.444	2009.802	32.72603	31.73699	0.9890411	509	465	ELISA	44	Not on HAART	Newly Selected
13217	10310780	White	White	SP	SP	230	1995.639	9000	34.02466	38.69184	4.66718317	669	653	Both	16	Not on HAART	Newly Selected
13226	10102967	Black	Black	SP	SP	240	1996.337	1997.276	44.22951	44.70411	0.47460139	217	202	Both	15	Not on HAART	Newly Selected

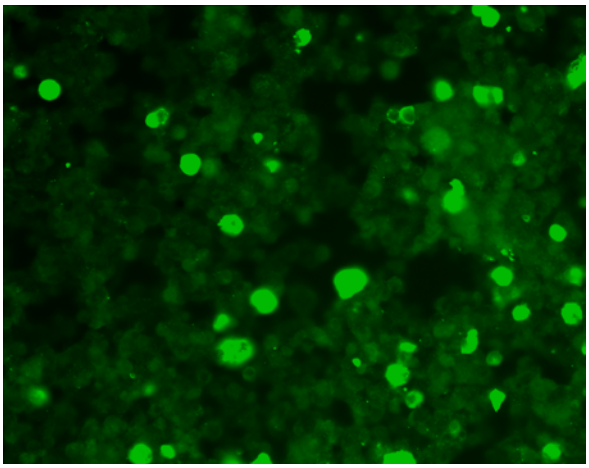
MACS and WIHS serum samples were matched as closely as possible on factors such as age, race, CD4 counts, HIV seropositivity, and HAART status.

Figure 3 Matched MACS and WIHS subject samples.

3.3 ENHANCED LYTIC IMMUNOFLUORESCENT ASSAY

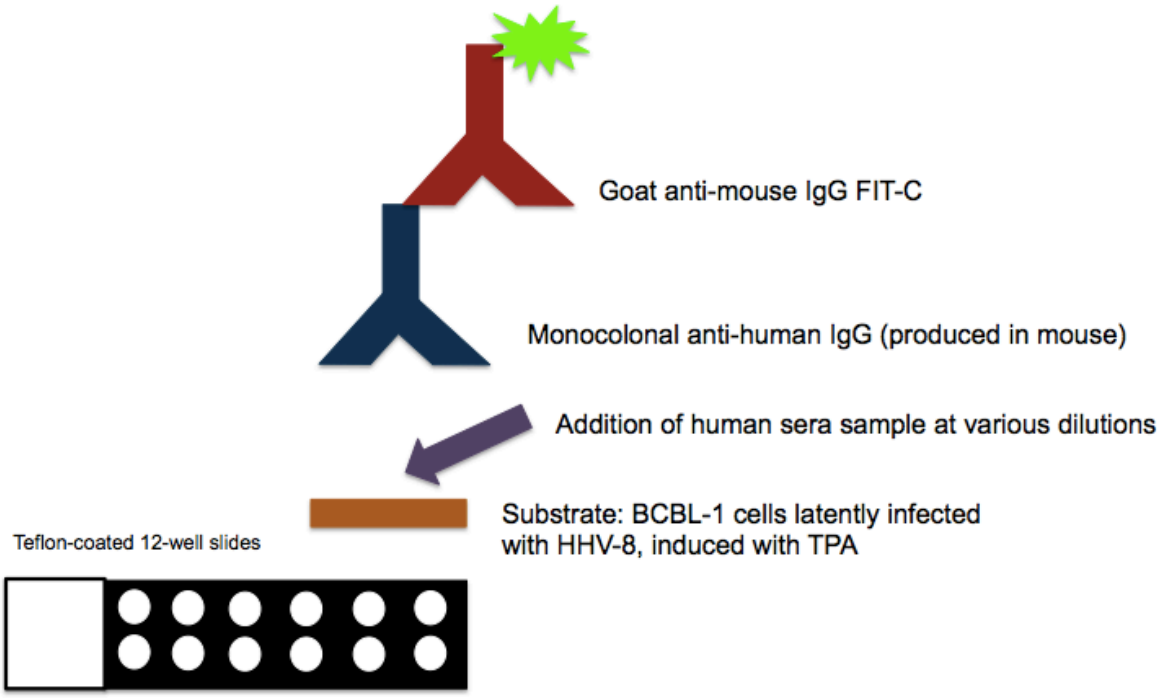
HHV-8 antibody titers were determined using an enhanced lytic immunofluorescence assay. The IFA procedure was performed as previously described (Hoffman et al). Briefly, BCBL-1 cells (at a concentration of $2x10^5$ cells/mL) were induced with 20ng per mL TPA (12-O-tetradecanoylphorbol-13-acetate) for 5 days. This results in reactivation of latent HHV-8 to a productive infection in approximately 15-20% of the cells. The cells were collected, washed with 1x PBS and fixed in in 4%-paraformaldehyde. Following fixation, the cells were washed 3 times with PBS and permeabilized with 0.05% Triton-X. The cells were washed twice with PBS, resuspended to a concentration of 2×10^7 cells/ml and $2x10^5$ cells (10µL) added to each well on a 12-well Teflon coated slide (Fisher Scientific). The cells were allowed to dry at room temperature and the slides stored at -20C until use.

For the IFA assays, duplicate wells from the 12-well slides were blocked with 20µL 10% goat serum at 37C for 1 hour and then treated with the human serum samples for 1 hour at 37C. Controls consisted of known positive and negative controls. Subject serum samples were added in dilutions ranging from 1:50 – 1:25600. Slides were then washed two times in 1X PBS for 5 minutes at room temperature. The secondary antibody (mouse anti-human IgG monoclonal antibody at a dilution of 1:200) was added to each of the samples and allowed to incubate at 37C for 1 hour. Slides were once again washed two times. Finally, a tertiary FITC-labeled antibody (goat anti-mouse IGG at a dilution of 1:100) was added to each of the cells, and incubated at 37C for 30 minutes. Slides were then washed again for 10 minutes in the dark, and stored in the dark until visualized via fluorescence microscopy (Figure 4 and 5).



Induced BCBL-1 slides were loaded onto 12-well Teflon-coated slides. Human sera samples were added to the slides, along with a monoclonal antibody and FITC-labeled tertiary antibody to detect HHV-8 lytic proteins. The presence of HHV-8 antibodies is indicated by the cells emitting green fluorescence. Photo provided by Dr. Frank Jenkins.

Figure 4 Positive IFA using BCBL-1 cells and human serum.



A more in-depth look at the IFA experimental design, including BCBL-1 cells induced with TPA, dilutions of human sera, monoclonoal anti-human IgG antibody, and a tertiary goat anti-mouse IgG FIT-C for detection via fluorescence microscopy.

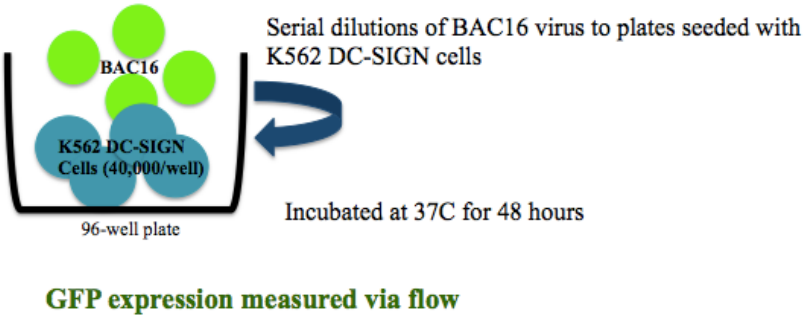
Figure 5 Enhanced Lytic Immunofluorescent Assay Experimental Design.

3.4 BAC16 VIRUS

The BAC16 GFP virus was introduced into iSLK cells by the J. Jung laboratory, and the cells are maintained in 1µg/ml puromycin, 250 µg/ml G418, and 1,200 µg/ml hygromycin B (62). The BAC16 virus was isolated from this cell line by inducing the cells with doxycycline (1µg/ml) and sodium butyrate (1mM). 4 days after induction, the supernatant was collected and centrifuged at 950 x g for 10 minutes at 4C to remove cells and debris. The supernatant was the spun at 25,000 g for 3 hours in an ultracentrifuge to pellet the virus, and the virus was resuspended in a small volume of PBS.

3.5 TCID50

Levels of infectious BAC16 virus in each viral stock prepared, was determined by a TCID50 assay. To perform the TCID50 assay, $4x10^4$ K562-DC-SIGN cells were seeded in a 96 well U-bottom plate, and dilutions of the virus (10^{-1} to 10^{-5}) were added to each of the wells (15μL of each dilution). Controls consisted of uninfected wells. Each dilution was placed in 6 replicate wells. The plates were incubated at 37C for 48 hours, and then the cells were fixed with 2% paraformaldehyde. Detection of GFP expression (due to the BAC156 virus infecting the K562-DC-SIGN cells) was measured by flow cytometry on an BD Accuri C6 flow cytometer. The mean fluorescence intensity (MFI) of the uninfected cells was averaged and this level + 2 S.D. used as the cutoff for positive infection. An infected well whose MFI was above the cutoff value was scored as positive for infection. The infectious titer was ten calculated using the Reed Muench method (75) (Figure 6).

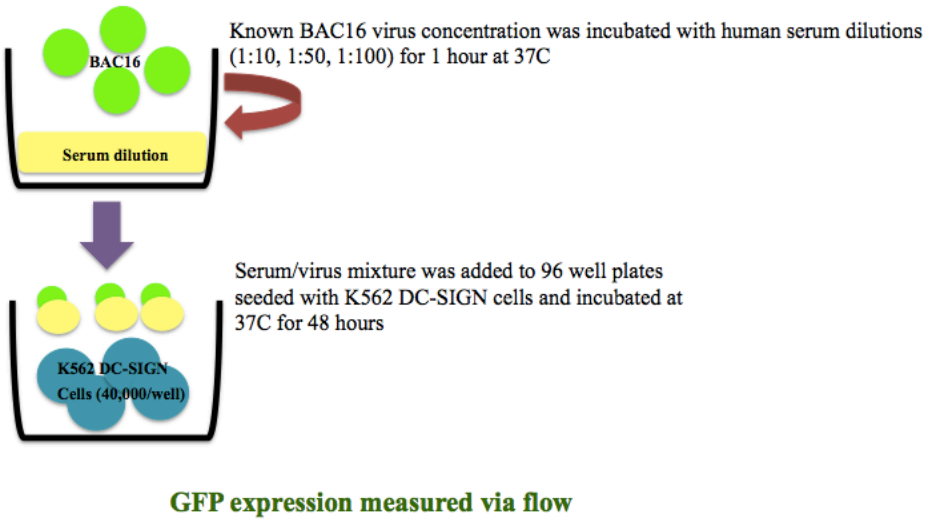


The TCID50 with the harvested BAC16 virus was performed by seeding a 96-well plate with 40,000 cells/well of K562 DC-SIGN cells. Serial dilutions of the BAC16 virus were added to the plates (6 replicates) in concentrations of 10^{-1} to 10^{-5}, along with an uninfected control as a baseline. The plate was incubated at 37C for 48 hours, and GFP expression was measured via flow cytometry.

Figure 6 TCID50 experimental design.

3.6 NEUTRALIZATION ASSAY

For the neutralization assays, human serum samples at dilutions of 1:10, 1:50, and 1:100 were added to a known concentration of the virus based on the TCID50 results. This serum/virus mixture was incubated for 1 hour at 37C, and each dilution diluted further to concentrations of 10^{-1} and 10^{-2}. The virus/serum samples were added to K562-DC-SIGN cells in 96 well plates(40,000 cells/well) and incubated at 37C for 48 hours. The cells were fixed with 2% paraformaldehyde and analyzed by flow cytometry. The amount of infectious virus in each sample (following neutralization) was measured by TCID50 assay (Figure 7). Neutralization outcome is reported as the virus dilution resulting in at least 50% neutralization.



The neutralization assay was completed by adding a known concentration of virus (via the TCID50 assay) to various dilutions of human serum samples (1:10, 1:50, and 1:100) along with an uninfected control, and a control with no serum added. The virus/serum mixture was incubated at 37C for 1 hour, and then added to a 96-well plate seeded with K562 DC-SIGN cells at 40,000 cells/well. The plate was incubated at 37C for 48 hours, and the GFP expression was measured via flow.

Figure 7 Neutralization assay experimental design.

3.7 CYTOKINE ANALYSIS

To examine levels of proinflammatory cytokines in male and female serum samples, a state-of-the-art multiplex ELISA kit was used (Meso Scale Discovery; MSD). Cytokine levels were measured using the MSD 96-well Proinflammatory Panel 1 (human) V-PLEX Kit (Figure 8). This assay measures the following cytokines and chemokines: IFN-γ, IL-1β, IL-10, IL-12 p70, IL-13, IL-2, IL-4, IL-6, IL-8, TNF-α. Each of these cytokines are important in the development of an inflammatory response, in immune regulation such as activating adaptive immunity and directing a Th1 vs Th2 T-cell response. The MSD assay was performed following the manufacturer's

instructions. The plate was read using an MSD plate reader and levels of the different cytokines determined using MSD software. In total, 47 MACS seronegative samples and 25 WIHS seronegative samples were tested, in duplicate. Cytokine levels that were below the standard curve for that cytokine were given the value of 0 while samples whose levels were above the standard curve were given the value of the highest concentration in the standard curve.

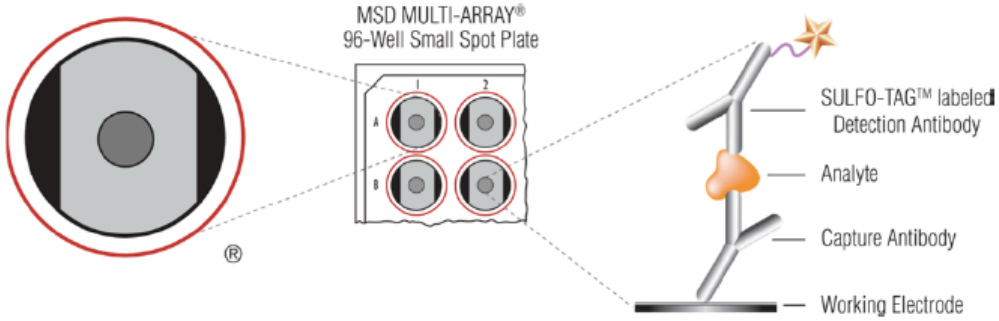


Diagram of the MSD Proinflammatory cytokine kit. This assay measures levels of 10 of the most common proinflammatory cytokines: IFN-γ, IL-1β, IL-10, IL-12 p70, IL-13, IL-2, IL-4, IL-6, IL-8, TNF-α. This is done via a sandwich immunoassay, where an electrode is below each well on the 96-well plate. A capture antibody is coated on the bottom of the MSD plate, serum samples from MCS and WIHS subjects were added, and then the plate was read utilizing a plate reader. *Obtained from the MSD Proinflammatory cytokine (Human) V-PLEX Kit manual.*
Figure 8 MSD 96-well proinflammatory panel 1 (human) V-PLEX kit.

3.8 DATA ANALYSIS

SPSS was used to analyze differences between gender, HIV status, and race for the IFA antibody titers using non-parametric testing (Independent samples: Mann-Whitney U). SPSS was also used to analyze these differences with the MSD proinflammatory cytokine measurements also using non-parametric testing (Independent samples: Mann-Whitney U). The distributions

examined in the male and female population based on race were also completed using nonparametric testing, using the Independent samples- Kruskal-Wallis test. For the correlation analysis, a non-parametric Spearman's rank correlation coefficient was utilized.

4.0 RESULTS

4.1 AIM 1

4.1.1 Differences in HHV-8 antibody titers between men and women

HHV-8 antibody titers were determined using the enhanced Lytic IFA assay. This assay measures antibodies directed against lytic viral proteins, and an end-point dilution was used to determine the antibody titer of each subject: 135 male and 114 female samples (Figure 2). Each sample was tested in duplicate and the identity of the samples were blinded to the IFA reader.

As seen in Table 1, the male group had a mean +/- SEM. HHV-8 antibody titer of 1,464 +/- 4963 and the female group had a mean HHV-8 antibody titer of 1,071 +/- 3811. This represents a significant increase in HHV-8 antibody titers among men (n = 135) compared to women (n = 114; p=0.000). Also seen in Table 1, there was a significant increase in male antibody titers in the HIV seropositive groups compared to the women (p = 0.036). However, there was a significant increase in female antibody titers in the HIV seronegative group compared to men (p = 0.009). There were no significant differences between males or females between HIV+ and HIV+ subjects (p = 0.215 and p = 0.217) (Table 1).

Next, differences in antibody titers were examined between races (Caucasians, African Americans, and Hispanics) for both male and female subject groups. As seen in Table 2, there were no significant differences between antibody titers in any of the three race groups (p = 0.644)

in the male study. As seen in Table 3, there were also no significant differences between antibody titers in any of the three race groups in the female study.

4.1.2 Neutralization Assay

The BAC16 virus stock was tested by Dr. Paolo Piazza to determine if the virus was successfully able to infect three different cell lines expressing DC-SIGN. This was completed in order to determine the most appropriate cell line to complete the neutralization assay experiments with. Cells were infected with BAC16 in 100µl total volume in a 96-well plate. The virus was removed after 3 hours, and dead cells were excluded by the use of amine binding dies (Aqua from Invitrogen). GFP expression was then measured via flow cytometry using a BD LSR Fortessa™ Cell Analyzer.

Figures 9, 10, and 11, show the infection of three different cell lines expressing DC-SIGN with the BAC16 virus at concentrations of 1:0, 1:10, and 1:100: 721.221 DC-SIGN, which is an "HLA null" EBV positive B-cell lineage; CHO DC-SIGN, which are Chinese hamster ovary cells; and K562 DC-SIGN, which is an erythroleukemia cell line. The results demonstrate that all three cell lines were successfully able to be infected by the BAC16 virus. However, the highest levels of infection were seen in the K562 DC-SIGN cells (Figure 11). Infection with the BAC16 virus at 48 hours showed 42.2% of the K562 DC-SIGN cells expressing GFP from the virus.

Figure 12 displays the growth curves of the three cell lines expressing DC-SIGN infected with BAC16 from 0 to 72 hpi. The most prolonged level of GFP expression over 48 hours was

seen in the K562 DC-SIGN cell line, so this line was chosen for all future experiments. Next, the level of DC-SIGN expression on the cells were analyzed via flow using an anti-DC-SIGN antibody. This was completed with help by Dr. Paolo Piazza. The percentage of cells expressing DC-SIGN, and thus able to be successfully infected by the BAC16 virus, was 81.5% (Figure 13). Upon enrichment using an EasyStep anti APC magnetic separation kit (which reduces non-specific binding and purifies the cells), the percentage of DC-SIGN positive cells was 98% (Data not shown). In order to keep the amount of BAC16 virus used in each neutralization assay consistent, a TCID50 was used to quantify each BAC16 virus harvest. This was completed due to the variability in virus concentration for each individual harvest. Typically, the virus concentration ranged from $2.5 \times 10^5 – 3.5 \times 10^5$ infectious particles/mL.

4.1.3 Examining differences in levels of neutralizing antibodies between matched MACS and WIHS samples

Upon flow cytometry measurement of the percentage of GFP (thus presence of virus) in the neutralization assay samples, the results were not what was expected. First, known HHV-8 seropositive and HHV-8 seronegative serum were used to test the effectiveness of the assay, along with uninfected controls containing no BAC16 virus, and another control containing no serum. The average percentage of GFP in the uninfected control wells was 0.1%. The average percentage of GFP in the wells containing no serum (only BAC16 virus and K562 DC-SIGN cells) was 28.5%. The known HHV-8 seropositive serum samples had an average of 0.2% GFP expression. However, the known HHV-8 seronegative serum samples had an average of 0.15% GFP expression. Upon discovering this discrepancy, the serum samples were heat inactivated at 56C for 30 minutes to

remove any confounding complement, and the neutralization assay was performed again. Unfortunately, the results remained the same- extremely low levels of GFP in each sample containing serum, regardless of if the serum was HHV-8 seropositive or negative (Table 4).

4.1.4 Summary of Aim 1

Aim 1 was designed to investigate differences in antibody titers between HIV+ and HIV- subject groups in males and females. Upon testing, these results demonstrated that antibody titers were significantly higher in the males versus the female population overall ($p = 0.00$). Male titers were also significantly higher in the HIV seropositive group ($p = 0.036$). Oppositely, female titers were significantly higher in the HIV seronegative group versus males ($p = 0.009$). It was also shown that there were no significant differences between males or females between HIV+ and HIV+ subjects ($p = 0.215$ and $p = 0.217$). Next, xAim 1 examined any possible differences between races (Caucasian, African American, and Hispanics) amongst male and female groups. No significant differences were found between antibody titers in any races, both in the male and the female groups.

The second part of Aim 1 involved investigating differences in neutralizing antibodies using the BAC16 virus. The virus was successfully able to infect three different cell lines expressing DC-SIGN (721.221 DC-SIGN, CHO DC-SIGN, and K562 DC-SIGN). K562 DC-SIGN cells showed the most prolonged and consistent level of infection over 48 hours, and were utilized for future studies. DC-SIGN expression of this cell line was examined, which showed an 81.5% expression of DC-SIGN, and then a 98% expression of DC-SIGN upon cell enrichment.

The TCID50 to titer the BAC16 virus stock was successful, however due to issues with the neutralization assay itself, no further assays were performed, and future work will be conducted in our laboratory to determine why these results are occurring.

In Aim 2, we measured the levels of 10 different proinflammatory cytokines in males versus females, to determine if males will have an increased level of proinflammatory cytokines that coincides with their increased antibody titers overall versus the female group. This may lead to their increased rates of reactivation and may also explain why men are at a much greater risk of developing KS.

The results in Aim 1 showed that antibody titers in males were significantly higher than females overall. In line with these results, levels of proinflammatory cytokines were analyzed between male and female populations. It is hypothesized that males, who reactivate more frequently than females, will have a higher level of proinflammatory cytokines versus women, which has been proven by previous studies to lead to further KS disease progression and dissemination of virus throughout the body.

4.2 AIM 2

4.2.1 Analyzing Differences in Proinflammatory Cytokines Between Genders

For this aim, 47 MACS samples and 25 WIHS samples were examined for their levels of proinflammatory cytokines. All of these subject samples were HIV negative, in order to reduce the confounding factor of HIV infection on cytokine levels in the serum samples. Upon analysis of the MSD cytokine panel, it was discovered that 7 of the 10 cytokines tested were significantly elevated in males versus females. These cytokines were: IL-10, IL12p70, IL-1β, IL-2, IL-6, IL-8, and TNFα. The average values for each cytokine in the male and female groups, along with standard deviations, can be seen in Table 5.

4.2.2 Analyzing Differences in Proinflammatory Cytokines Between Races

Upon determining that there were significantly higher levels of proinflammatory cytokines in males versus females, this study next examined any possible differences between races and cytokine levels between three different races: (1) Caucasian, (2) African American, and (3) Hispanic.

Proinflammatory cytokine levels were first examined in the male and female Caucasian population only. It was determined that there was a significant difference between male and female Caucasians in their levels of IL-6 ($p = 0.024$) (Table 6). The average values for each cytokine in the male and female groups, along with standard deviations for Caucasians only, can also be seen in Table 6.

Proinflammatory cytokine levels next were examined in the male and female African American population only. It was determined that there was a significant difference between male and female African Americans in their levels of IL12p70 ($p = 0.030$), IL-1β ($p = 0.007$), IL-2 ($p = < 0.005$), IL-6 ($p = < 0.005$), IL-8 ($p = < 0.005$), and TNF-α ($p = 0.005$) (Table 7). The average values for each cytokine in the male and female groups, along with standard deviations for African Americans only, can be also seen in Table 7.

Significant differences in cytokine levels were examined in the Hispanic population only. It was determine that there was a significant difference between male and female Hispanics in their levels of IL-2 ($p = 0.002$), IL-6 ($p = 0.007$), and IL-8 ($p = 0.001$) (Table 8). The average values for each cytokine in the male and female groups, along with standard deviations for Hispanics only, can also be seen in Table 8.

4.2.3 Analysis of correlation between IFA antibody titers and proinflammatory cytokine levels

Finally, correlations were examined between IFA titers and the 10 proinflammatory cytokines studied in Aim 2. This analysis was used to determine if antibody titers had any effect on proinflammatory cytokine production, or if any proinflammatory cytokines had a positive or negative correlation with one another. The analyses were broken down into 3 groups (1) HIV SN MACS + WIHS cohorts together (Table 9), (2) HIV SN MACS (Table 10), and (3) HIV SN WIHS (Table 11). In the first group, including both men and women, there were no significant differences

between any of the cytokines. There were also no differences in the second group (MACS HIV SN), although IL-2 had a p value of 0.05. In the final group, HIV SN WIHS, IL-12p70 was significantly different- meaning that there was a positive correlation between IFA titer and IL12-p70 in the women.

4.2.4 Summary of Aim 2

Interestingly, 7 out of the 10 proinflammatory cytokines examined in the MSD panel were significantly elevated in total males versus females (IL-10, IL12p70, IL-1β, IL-2, IL-6, IL-8, and TNFα). In the Caucasian population, 1 proinflammatory cytokine (IL-6) was significantly higher in males versus females. In the African American population, 6 proinflammatory cytokines were significantly higher in the male population (IL12p70, IL-1β, IL-2, IL-6, IL-8, and TNF-α) Finally, in the Hispanic group, 3 proinflammatory cytokines (IL-2, IL-6, and IL-8) were significantly higher in the male population versus the female. These results overall and between Caucasians, African Americans, and Hispanics show that males have significantly higher levels of proinflammatory cytokines versus women in the HIV seronegative population. No strong correlations were seen when examining IFA titers and proinflammatory cytokines, except in the HIV SN WIHS group, where IL-12p70 was seen to be positively correlated to an increase in antibody titer.

Table 1 MACS and WIHS antibody titers +/- SEM for HIV seropositive and HIV seronegative groups.

Group (n)	Mean Titer +/- SEM	p-value
MACS (135) **WIHS** (114)	1464 +/- 328 1071 +/- 465	**0**
MACS HIV SP (47) MACS HIV SN (88)	2435 +/- 223 946 +/- 832	0.215
WIHS HIV SP (23) WIHS HIV SN (88)	427 +/- 94 1261 +/- 601	0.217
MACS HIV SP (47) **WIHS** HIV SP (26)	2435 +/- 223 427 +/- 94	**0.036**
MACS HIV SN (88) **WIHS** HIV SN (88)	946 +/- 832 1261 +/- 601	**0.009**

Antibody titers were measured using an enhanced lytic immunofluorescent assay and end-point dilution.

Table 2 MACS antibody titers +/- SEM by race.

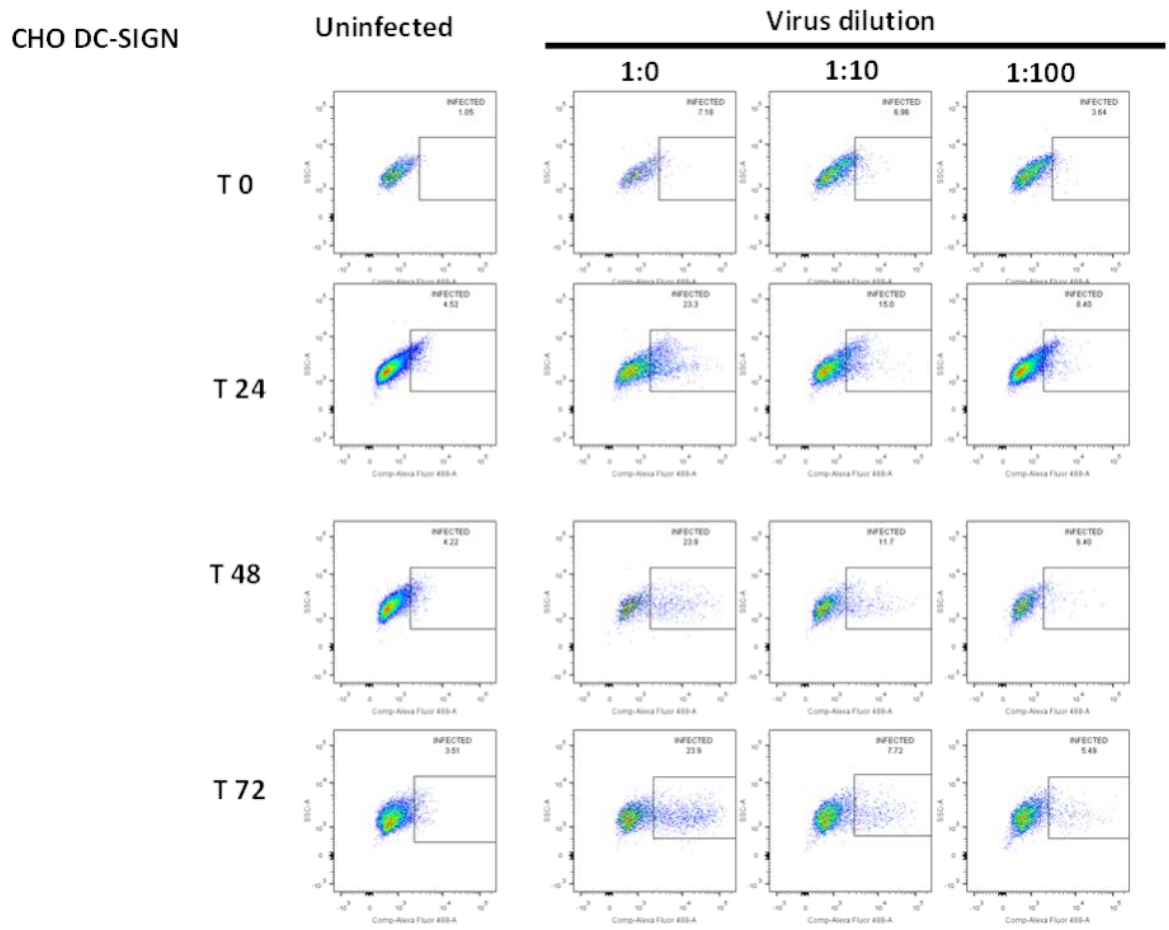
MACS by RACE (n)	Mean Titer +/- SEM	p-value
Caucasian (15)	833 +/- 407	0.644
African American (95)	1755 +/- 458	
Hispanic (25)	740 +/- 149	

Antibody titers were measured using an enhanced lytic immunofluorescent assay and an end-point dilution on Caucasian, African American, and Hispanic HIV seronegative men.

Table 3 WIHS antibody titers +/- SEM by race.

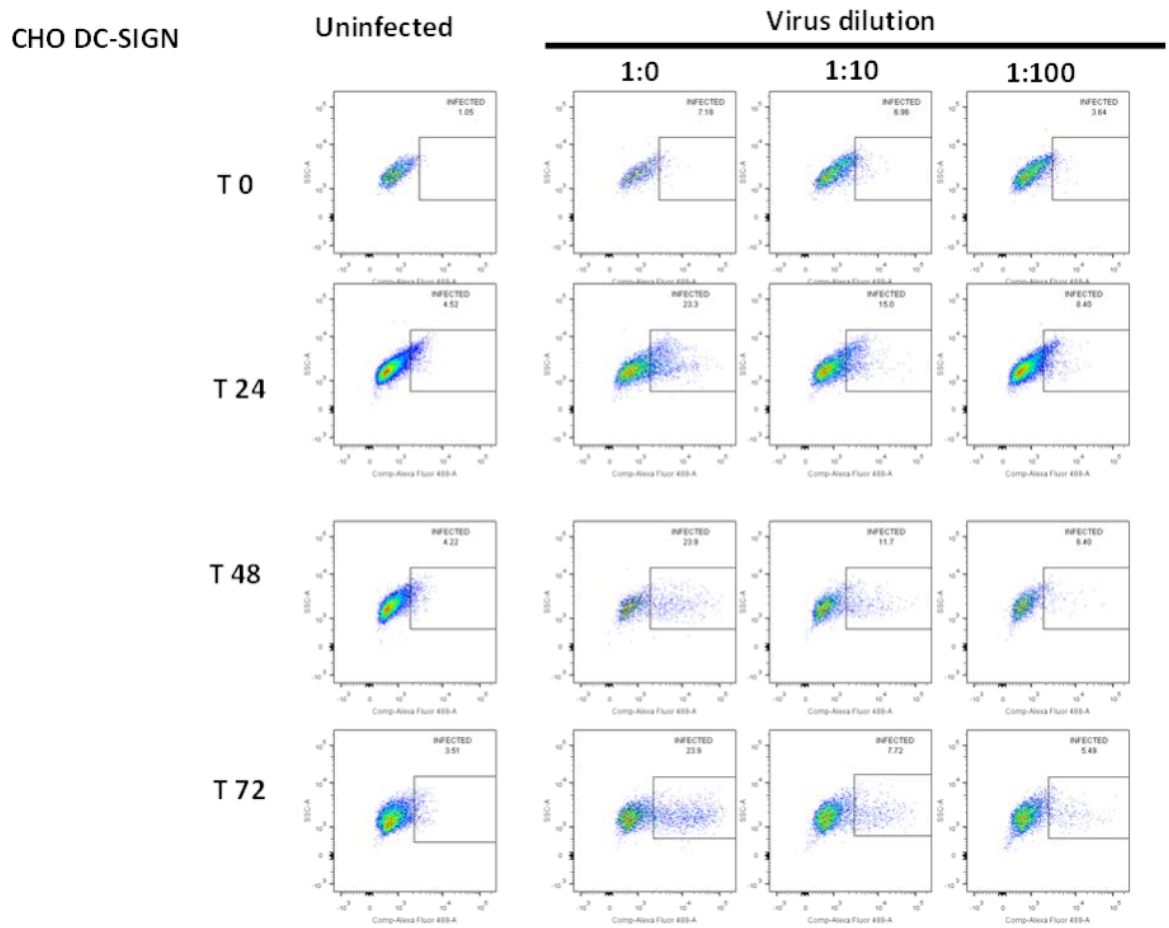
WIHS by RACE (n)	Mean Titer +/- SD	p-value
Caucasian (12)	1217 +/- 538	0.168
African American (81)	1201 +/- 648	
Hispanic (21)	486 +/- 169	

Antibody titers were measured using an enhanced lytic immunofluorescent assay and an end-point dilution on Caucasian, African American, and Hispanic HIV seronegative women.



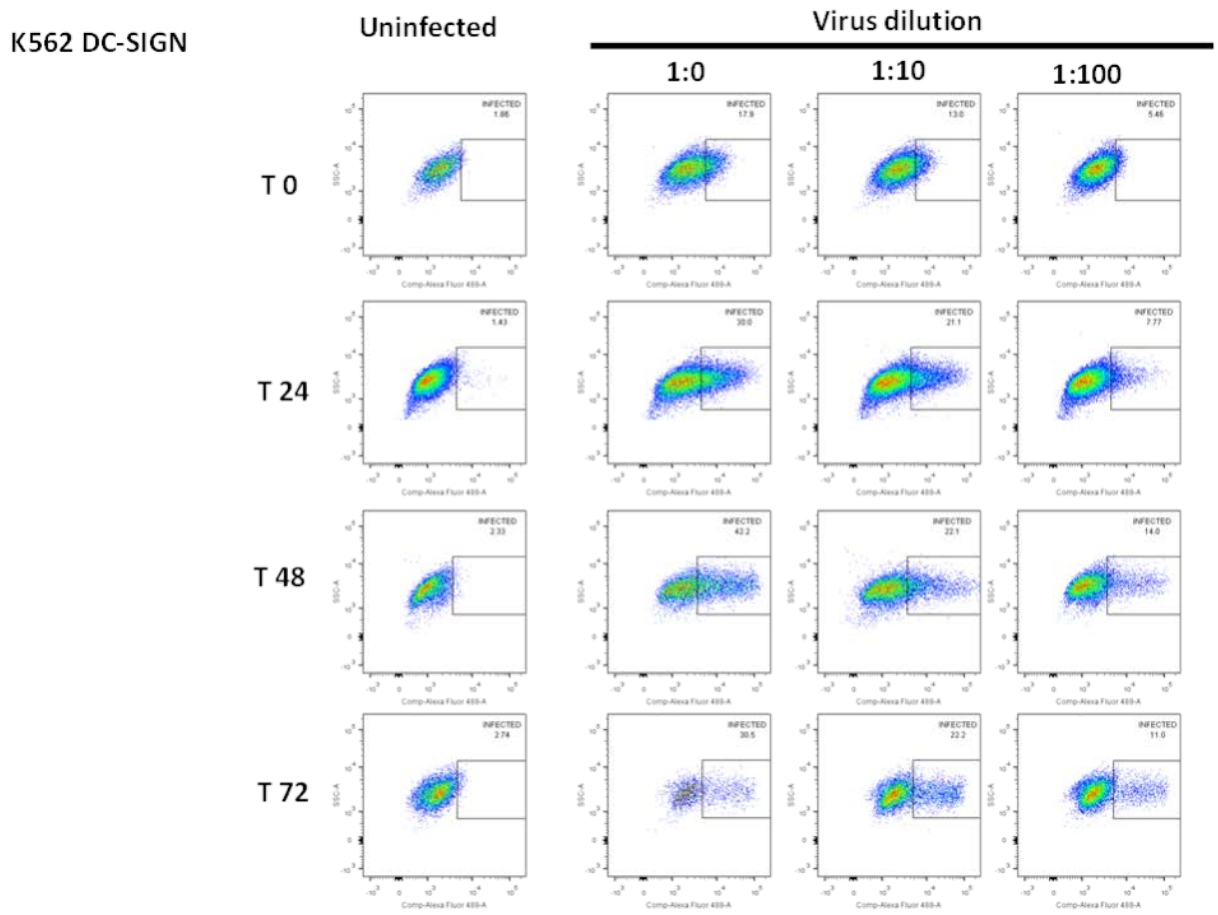
Infection of 721.221 DC-SIGN cells with BAC16 virus. 721.221 DC-SIGN cells are an HLA null EBV positive B-cell lineage. This line was able to be infected by the BAC16 virus at concentrations of 1:0, 1:10, and 1:100. Provided by Dr. Paolo Piazza.

Figure 9 BAC16 virus infection of 721.221 DC-SIGN cells.



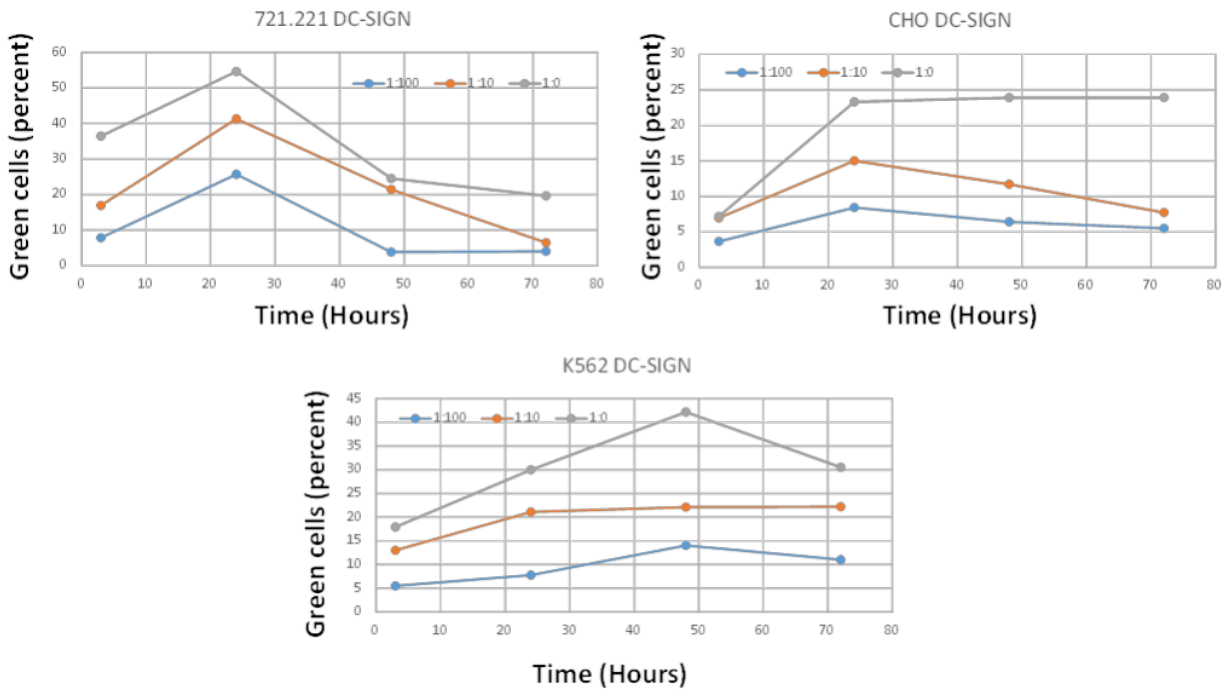
Infection of CHO DC-SIGN cells with the BAC16 virus. CHO DC-SIGN cells are Chinese hamster ovary cells. This line was able to be infected by the BAC16 virus at concentrations of 1:0, 1:10, and 1:100. Provided by Dr. Paolo Piazza.

Figure 10 BAC16 virus infection of CHO DC-SIGN cells.



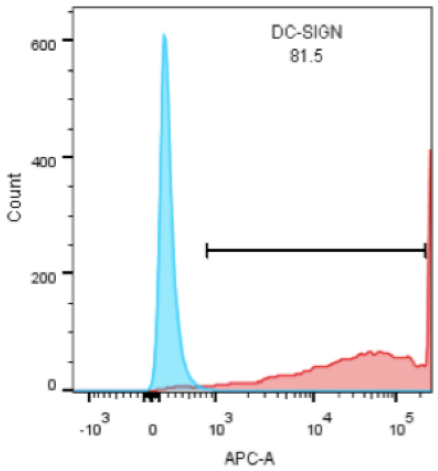
Infection of K562 DC-SIGN cells with BAC16 virus. K562 DC-SIGN cells are an erythroleukemia cell line. This line was able to be infected by the BAC16 virus at concentrations of 1:0, 1:10, and 1:100, and showed the highest level of infection at 48 hours. This was the cell line chosen to perform the TCID50 and neutralization assays using the BAC16 virus. Provided by Dr. Paolo Piazza.

Figure 11 BAC16 virus infection of K562 DC-SIGN cells.



72-hour growth curve with 721.221 DC-SIGN cells, CHO DC-SIGN cells, and K562 DC-SIGN cells, measuring the percentage of GFP expressing cells (BAC16 infected cells). 721.221 and CHO DC-SIGN cells peak at 24 hours, while K562 DC-SIGN cells peak at 48 hours, and have the highest and most consistent level of infect. Provided by Dr. Paolo Piazza.

Figure 12 Growth curve of cell lines expressing DC-SIGN infected with BAC16.



Expression of DC-SIGN was measured in K562 DC-SIGN cells using an anti DC-SIGN antibody. 81.5% of the K562 DC-SIGN cells were expressing DC-SIGN at this time, before enrichment. Post-enrichment, the percentage of DC-SIGN positive cells was 98% (Data not shown). Provided by Dr. Paolo Piazza.

Figure 13 DC-SIGN expression of K562 DC-SIGN cells.

Table 4 Neutralization Assay Results.

MACS SERUM							
HHV-8 seropositive	6 replicates each						
1:10	0.2	0.4	0.3	0.3	0.1	0.2	0.3
1:50	0.2	0.3	0.2	0.2	0.1	0.3	0.1
1:100	0.1	0.1	0.1	0.1	0.2	0.1	0.2
HHV-8 seronegative							
1:10	0.2	0.3	0.1	0.2	0.2	0.1	0.1
1:50	0.1	0.3	0.3	0.1	0.2	0.2	0.3
1:100	0	0.1	0	0	0.2	0.2	0.1

Dilutions of HHV-8 seropositive and HHV-8 seronegative serum samples added to an exact concentration of the BAC16 virus, and incubated with K562 DC-SIGN cells at 37C for 48 hours. The plates were then subsequently measured for GFP via fluorescence microscopy.

Table 5 MSD proinflammatory cytokine V-PLEX panel (MACS vs. WIHS total).

WIHS VS MACS (all)	WIHS (n=25) Mean +/- SD	MACS (n=47) Mean +/- SD	p-value
IFN-γ	24 +/- 55	45 +/- 111	0.284
IL-10	1 +/- 2	3 +/- 12	**0.048**
IL-12p70	0.5 +/- 1	57 +/- 249	**0.008**
IL-13	20 +/- 24	198 +/- 621	0.855
IL-1β	19.73 +/- 24	23 +/- 41	**0.012**
IL-2	1 +/- 3	18 +/- 55	**< 0.005**
IL-4	2 +/- 6	9 +/- 36	p = 0.863
IL-6	0.1 +/- 0.2	126 +/- 424	**< 0.005**
IL-8	3 +/- 9	256 +/- 766	**< 0.005**
TNF-α	5 +/- 7	12 +/- 15	**< 0.005**

Results of the MSD panel examining 10 proinflammatory cytokines between all HIV seronegative males and females. Significant differences demonstrate an significant increase in proinflammatory cytokines in males versus females.

Table 6 MSD proinflammatory cytokine V-PLEX panel (MACS vs. WIHS Caucasian).

WIHS VS MACS (Caucasian)	WIHS (n=3) Mean +/- SD	MACS (n=6) Mean +/- SD	p-value
IFN-γ	34 +/- 55	115.46 +/- 254.46	1
IL-10	6 +/- 2	4.07 +/- 5.43	0.714
IL-12p70	2 +/- 1	157 +/- 383	1
IL-13	68 +/- 25	482 +/- 1069	0.548
IL-1β	68 +/- 25	40 +/- 68	0.381
IL-2	9 +/- 3	34 +/- 46	0.262
IL-4	18 +/- 6	21 +/- 52	0.167
IL-6	0.2 +/- 0.2	276 +/- 623	**0.024**
IL-8	26 +/- 9	434 +/- 845	p = 0.262
TNF-α	19 +/- 7	25 +/- 32	p = 0.548

Results of the MSD panel examining 10 proinflammatory cytokines between Caucasian HIV seronegative males and females. Significant differences demonstrate an significant increase in proinflammatory cytokines in males versus females.

Table 7 MSD proinflammatory cytokine V-PLEX panel (MACS vs. WIHS African American).

WIHS VS MACS (African American)	WIHS (n=16) Mean +/- SD	MACS (n=31) Mean +/- SD	p-value
IFN-γ	26 +/- 67	34 +/- 77	0.323
IL-10	0.6 +/- 0.8	1 +/- 3	0.074
IL-12p70	0.4 +/- 0.6	55 +/- 260	**0.03**
IL-13	16 +/- 12	178 +/- 610	0.964
IL-1β	16 +/- 12	18 +/- 36	**0.007**
IL-2	0.4 +/- 0.7	5 +/- 12	**<0.005**
IL-4	0.6 +/- 1	8 +/- 38	0.937
IL-6	0.2 +/- 0.2	121 +/- 449	**<0.005**
IL-8	1 +/- 1	193 +/- 827	**<0.005**
TNF-α	3 +/- 2	8 +/- 7	**0.005**

Results of the MSD panel examining 10 proinflammatory cytokines between African American HIV seronegative males and females. Significant differences demonstrate an significant increase in proinflammatory cytokines in males versus females.

Table 8 MSD proinflammatory cytokine V-PLEX panel (MACS vs. WIHS Hispanic).

WIHS VS MACS (Hispanic)	WIHS (n=6) Mean +/- SD	MACS (n=10) Mean +/- SD	p-value
IFN-γ	15 +/- 20	34 +/- 58	0.635
IL-10	0.3 +/- 0.2	9 +/- 25	0.958
IL-12p70	0.2 +/- 0.3	4 +/- 9	0.093
IL-13	16 +/- 5	84 +/- 161	0.713
IL-1β	16 +/- 5	30 +/- 45	0.875
IL-2	0.1 +/- 0.3	47 +/- 109	**0.002**
IL-4	0.3 +/- 0.6	2 +/- 4	0.263
IL-6	0.7 +/- 0.1	59 +/- 108	**0.007**
IL-8	0.7 +/- 1	348 +/- 528	**0.001**
TNF-α	3 +/- 2	18 +/- 20	0.073

Results of the MSD panel examining 10 proinflammatory cytokines between Hispanic HIV seronegative males and females. Significant differences demonstrate an significant increase in proinflammatory cytokines in males versus females.

Table 9 Total HIV SN MACS and WIHS HHV-8 antibody titer and proinflammatory cytokine correlation.

MACS (n = 135) WIHS (n = 114)		HHV-8 IFA Titer	IFN gamma	IL-10	IL12p70	IL-13	IL-1 beta	IL-2	IL-4	IL-6	IL-8	TNF alpha
HHV-8 IFA Titer	Correlation Coefficient	1	0.075	0.074	0.225	-0.074	-0.172	0.061	0.049	0.078	0.062	0.136
	Sig. (2-tailed)	.	0.532	0.535	0.058	0.537	0.15	0.611	0.682	0.513	0.603	0.255
IFN gamma	Correlation Coefficient	0.075	1	.466**	.422**	0.449	0.457	.441**	.299**	0.363	0.452	.426**
	Sig. (2-tailed)	0.532	.	0	0	0	0	0	0.011	0.002	0	0
IL-10	Correlation Coefficient	0.074	.466**	1	.450**	0.343	.249**	0.595	.312**	0.493	.544**	0.565
	Sig. (2-tailed)	0.535	0	.	0	0.003	0.035	0	0.008	0	0	0
IL12p70	Correlation Coefficient	0.225	.422**	.450**	1	0.473	.180**	.512**	0.471	0.487	.547**	.427**
	Sig. (2-tailed)	0.058	0	0	.	0	0.131	0	0	0	0	0
IL-13	Correlation Coefficient	-0.074	.449**	.343**	.473**	1	.712**	.391**	.329**	0.28	.486**	.592**
	Sig. (2-tailed)	0.537	0	0.003	0	.	0	0.001	0.005	0.017	0	0
IL-1 beta	Correlation Coefficient	-0.172	.457**	.249*	0.18	0.712	1.000**	.171*	0.298	-0.066	.197**	.321*
	Sig. (2-tailed)	0.15	0	0.035	0.131	0	.	0.15	0.011	0.582	0.097	0.006
IL-2	Correlation Coefficient	0.061	.441**	.595**	.512**	0.391	.171**	1.000**	.394**	0.749	.858**	.652**
	Sig. (2-tailed)	0.611	0	0	0	0.001	0.15	.	0.001	0	0	0
IL-4	Correlation Coefficient	0.049	.299*	.312**	.471**	0.329	.298*	.394**	1.000**	0.25	.295*	.353**
	Sig. (2-tailed)	0.682	0.011	0.008	0	0.005	0.011	0.001	.	0.034	0.012	0.002
IL-6	Correlation Coefficient	0.078	.363**	.493**	.487**	0.28	-.066**	.749**	.250**	1	.816**	.481**
	Sig. (2-tailed)	0.513	0.002	0	0	0.017	0.582	0	0.034	.	0	0
IL-8	Correlation Coefficient	0.062	.452**	.544**	.547**	0.486	.197**	.858**	.295**	0.816	1.000**	.693**
	Sig. (2-tailed)	0.603	0	0	0	0	0.097	0	0.012	0	.	0
TNF alpha	Correlation Coefficient	0.136	.426**	.565**	.427**	0.592	.321**	.652**	.353**	0.481	.693**	1.000**
	Sig. (2-tailed)	0.255	0	0	0	0	0.006	0	0.002	0	0	.

The total number of MACS and WIHS HIV seronegative serum samples were compared using both their HHV-8 viral titers from the IFA results in Aim 1, to the proinflammatory cytokine analysis in Aim 2 using a Pearson's correlation coefficient to determine possible positive and negative associations between titers and cytokine levels.

Table 10 HIV SN MACS HHV-8 antibody titer and proinflammatory cytokine correlation.

MACS (n = 47)			HHV-8 IFA Titer	IFN gamma	IL-10	IL12p70	IL-13	IL-1 beta	IL-2	IL-4	IL-6	IL-8	TNF alpha
Spearman's rho	HHV-8 IFA Titer	Correlation Coefficient	1	-0.118	-0.108	-0.031	-0.134	-0.186	-0.287	0.027	-0.226	-0.25	-0.04
		Sig. (2-tailed)	.	0.431	0.469	0.835	0.369	0.211	0.05	0.855	0.126	0.09	0.788
	IFN gamma	Correlation Coefficient	-0.118	1	.456**	.548**	0.57	0.627	.488**	.378**	0.524	0.518	.309*
		Sig. (2-tailed)	0.431	.	0.001	0	0	0	0.001	0.009	0	0	0.034
	IL-10	Correlation Coefficient	-0.108	.456**	1	.423**	0.404	.365*	0.57	.285*	0.574	.564**	0.455
		Sig. (2-tailed)	0.469	0.001	.	0.003	0.005	0.012	0	0.052	0	0	0.001
	IL12p70	Correlation Coefficient	-0.031	.548**	.423**	1	0.61	.307*	.430**	0.563	0.52	.474**	.430**
		Sig. (2-tailed)	0.835	0	0.003	.	0	0.036	0.003	0	0	0.001	0.003
	IL-13	Correlation Coefficient	-0.134	.570**	.404**	.610**	1	.717**	.519**	.392**	0.637	.716**	.722**
		Sig. (2-tailed)	0.369	0	0.005	0	.	0	0	0.006	0	0	0
	IL-1 beta	Correlation Coefficient	-0.186	.627**	.365*	.307*	0.717	1.000**	.535*	.339*	0.486	.643**	.558*
		Sig. (2-tailed)	0.211	0	0.012	0.036	0	.	0	0.02	0.001	0	0
	IL-2	Correlation Coefficient	-0.287	.488**	.570**	.430**	0.519	.535**	1.000**	.433**	0.759	.789**	.517**
		Sig. (2-tailed)	0.05	0.001	0	0.003	0	0	.	0.002	0	0	0
	IL-4	Correlation Coefficient	0.027	.378**	0.285	.563**	0.392	.339**	0.433	1.000**	0.439	.363**	0.372
		Sig. (2-tailed)	0.855	0.009	0.052	0	0.006	0.02	0.002	.	0.002	0.012	0.01
	IL-6	Correlation Coefficient	-0.226	.524**	.574**	.520**	0.637	.486**	.759**	.439**	1	.845**	.471**
		Sig. (2-tailed)	0.126	0	0	0	0	0.001	0	0.002	.	0	0.001
	IL-8	Correlation Coefficient	-0.25	.518**	.564**	.474**	0.716	.643**	.789**	.363**	0.845	1.000**	.662**
		Sig. (2-tailed)	0.09	0	0	0.001	0	0	0	0.012	0	.	0
	TNF alpha	Correlation Coefficient	-0.04	.309*	.455**	.430**	0.722	.558*	.517**	.372**	0.471	.662*	1.000**
		Sig. (2-tailed)	0.788	0.034	0.001	0.003	0	0	0	0.01	0.001	0	.

The total number of WIHS HIV seronegative serum samples were compared using both their HHV-8 viral titers from the IFA results in Aim 1, to the proinflammatory cytokine analysis in Aim 2 using a Pearson's correlation coefficient to determine possible positive and negative associations between titers and cytokine levels.

Table 11 HIV SN WIHS HHV-8 antibody titer and proinflammatory cytokine correlation.

WIHS (n = 25)			HHV-8 IFA Titer	IFN gamma	IL-10	IL12p70	IL-13	IL-1 beta	IL-2	IL-4	IL-6	IL-8	TNF alpha
Spearman's rho	HHV-8 IFA Titer	Correlation Coefficient	1	0.371	0.259	.411*	0.009	0.009	0.163	.177*	0.131	0.163	0.108
		Sig. (2-tailed)	.	0.068	0.211	0.041	0.964	0.964	0.435	0.396	0.532	0.437	0.608
	IFN gamma	Correlation Coefficient	0.371	1	.429*	0.055	0.15	0.15	.340*	0.224	0.217	0.464	.670*
		Sig. (2-tailed)	0.068	.	0.033	0.794	0.475	0.475	0.096	0.282	0.297	0.019	0
	IL-10	Correlation Coefficient	0.259	.429*	1	0.293	0.303	.303*	0.518	0.449	0.261	.324*	0.745
		Sig. (2-tailed)	0.211	0.033	.	0.155	0.141	0.141	0.008	0.024	0.208	0.114	0
	IL12p70	Correlation Coefficient	.411*	0.055	0.293	1	.287*	0.287	0.371	0.435	.270*	0.517	0.128
		Sig. (2-tailed)	0.041	0.794	0.155	.	0.165	0.165	0.068	0.03	0.193	0.008	0.54
	IL-13	Correlation Coefficient	0.009	0.15	0.303	0.287	1	1	0.404	0.145	-0.197	0.437	0.382
		Sig. (2-tailed)	0.964	0.475	0.141	0.165	.	.	0.045	0.49	0.345	0.029	0.06
	IL-1 beta	Correlation Coefficient	0.009	0.15	0.303	0.287	1	1	0.404	0.145	-0.197	0.437	0.382
		Sig. (2-tailed)	0.964	0.475	0.141	0.165	.	.	0.045	0.49	0.345	0.029	0.06
	IL-2	Correlation Coefficient	0.163	0.34	.518**	0.371	0.404	0.404	1.000**	0.72	0.102	0.575	.580**
		Sig. (2-tailed)	0.435	0.096	0.008	0.068	0.045	0.045	.	0	0.628	0.003	0.002
	IL-4	Correlation Coefficient	0.177	0.224	.449*	.435*	0.145	0.145	.720*	1.000*	0.281	0.435	.361*
		Sig. (2-tailed)	0.396	0.282	0.024	0.03	0.49	0.49	0	.	0.174	0.03	0.076
	IL-6	Correlation Coefficient	0.131	0.217	0.261	0.27	-0.197	-0.197	0.102	0.281	1	0.253	0.157
		Sig. (2-tailed)	0.532	0.297	0.208	0.193	0.345	0.345	0.628	0.174	.	0.222	0.454
	IL-8	Correlation Coefficient	0.163	.464*	0.324	.517**	0.437	.437*	0.575	.435**	0.253	1.000*	0.47
		Sig. (2-tailed)	0.437	0.019	0.114	0.008	0.029	0.029	0.003	0.03	0.222	.	0.018
	TNF alpha	Correlation Coefficient	0.108	.670**	.745**	0.128	0.382	.382**	.580**	0.361	0.157	.470**	1.000**
		Sig. (2-tailed)	0.608	0	0	0.54	0.06	0.06	0.002	0.076	0.454	0.018	.

The total number of MACS HIV seronegative serum samples were compared using both their HHV-8 viral titers from the IFA results in Aim 1, to the proinflammatory cytokine analysis in Aim 2 using a Pearson's correlation coefficient to determine possible positive and negative associations between titers and cytokine levels.

5.0 DISCUSSION

KS was first described in 1872 as an “indolent tumor” in elderly Mediterranean men (6). Over 100 years later, HHV-8 was discovered as the etiologic agent of KS (4, 12). Since the initial description of AIDS in the early 1980’s, KS is the most common AIDS-associated malignancy (31). Though much has been discovered about the virus in the past several decades (presentation of the virus, transmission, immune evasion strategies, etc.) there is still much to be learned about the development of KS in different populations, particularly men versus women (6, 12, 13, 74). The prevalence of KS is not evenly distributed throughout the population (13, 20). Though both females and males both are infected with HHV-8, KS is seen mainly in the male population, at a rate of roughly 13:1 versus females.

In this study, two hypotheses were generated to explain why there is such a strong sexual bias in KS development. The first possibility as to why men develop KS more often than women, is a more robust humoral response in women, which would result in a reduced incidence of KS. This suggests that women may have higher antibody titers compared to their male counterparts, leading to a more robust humoral immune response. This may possibly suppress KS reactivation in females.

The second hypothesis is hormonal control, which may limit viral transcription replication of HHV-8, resulting in lower antibody titers in females, and a reduced incidence of KS. Specifically, estrogen may be controlling or preventing reactivation in women. Previous research in our laboratory has established compelling evidence that estrogen plays an important role in

HHV-8 infection by showing that ERE's in the HHV-8 genome can modulate gene transcription, and that estrogen can induce the transcription of HHV-8 genes. These data emphasizes the importance of estrogen in controlling varying aspects of HHV-8 pathogenesis and gene transcription. For this reason, males would have higher antibody titers compared to women, who may have decreased or suppressed viral transcription due to estrogen. Along with this hypothesis, we predicted that due to males developing KS more frequently than females, they would have increased levels of proinflammatory cytokines versus women, as many proinflammatory cytokines can actually assist in HHV-8 infection and disease progression.

The present study was completed to analyze these two hypotheses between males versus females who are infected with HHV-8, and to determine which is the most logical to explain the KS gender bias. This study provides an important step in determining why women do not develop KS as often as men do, and understanding this gender bias could possibly lead to more effective treatments or preventative therapies for the development of KS.

First, this study analyzed differences in antibody titers between males and females. This was competed via an enhanced lytic immunofluorescent assay (IFA) on 135 male and 114 female samples (Figure 2). These samples were matched as closely as possible on factors such as HIV status, CD4+ T-cell counts, race, age, etc. (Figure 3). The IFA's were completing using the BCBL-1 cell line, which is latently infected with HHV-8 (Figures 4 and 5). It was found that the male group had a significantly higher mean antibody titer versus women (1,464 vs. 1,071).

However, antibody titers between HIV+ and HIV- men and women significantly differed. In the HIV seropositive group, male titers were significantly higher than the female group. In the HIV seronegative group, female titers were significantly higher than the male group (Table 1). These results suggest that there may be another factor which is causing females to mount a stronger immune response to HHV-8 in the HIV seronegative group. It is also noted that there is no significant difference in antibody titers in males or females between genders (Tables 2 and 3).

There are several possible hypotheses as to why antibody titers in females are higher in the HIV- group, and male antibody titers are higher in the HIV+ group. Some of these factors include the timeline of infection- whether HIV infection or HHV-8 infection occurred first. For example, if a female would become infected with HIV first, and subsequently infected with HHV-8, the female would likely have a decreased T-cell count that may attribute to a decreased immune response to HHV-8 (31). This may explain why women have a significantly lower HHV-8 titer in the HIV seropositive group, and a significantly higher titer in the HIV seronegative group. It could also be suggested that the women in the WIHS study had become infected with HIV before becoming infected with HHV-8. Alternatively, the MACS study group contains many men with extremely "high risk" sexual lifestyles. This behavior may also suggest increased exposures to HHV-8 over time, meaning multiple exposures to the virus, possibly increasing a male's chance of reactivation over time. Overall, these serological results demonstrate that males are mounting a stronger humoral response compared to women, which leads us to believe that the second hypothesis, hormonal control, may be responsible for the gender bias associated with KS development.

This study next examined neutralizing antibodies between males versus females. Neutralizing antibodies are an important component of the immune response against viral infection, but their role in KS infection has not been studied in depth (57). They have been implicated in the control of many diseases, included several herpesvirus-associated diseases. There have been several previous studies that have detected the presence of neutralizing antibodies in HHV-8 infected subjects. First, Wood et al. found that KS subjects had a higher level of nAb versus those who had not yet developed KS. Second, Kimball et al. demonstrated that HHV-8 infection was able to be inhibited by nAb in seropositive subject samples, and this effect was not seen in seronegative subjects. To date, no group has utilized flow cytometry to examine neutralizing antibody levels within HHV-8 infected subjects.

The neutralization assay was completed using a GFP-expressing BAC16 virus, and was subsequently infected by Dr. Paolo Piazza into three different cell lines that express DC-SIGN. The K562 DC-SIGN cell line was chosen as the optimal line for our neutralization assays (Figures 9, 10, 11, and 12). These cells were enriched to increase the percentage of expression of DC-SIGN, thus increasing the chances of viral infection. The virus was tittered via a TCID50 assay, and then the neutralization assay was completed on known HHV-8 + and known HHV-8 – samples. The samples were analyzed via flow cytometry for the level of GFP remaining in each well. A lower level of GFP corresponded to a lower concentration of virus in the sample, thus a higher level of neutralizing antibodies in the subject serum.

Unfortunately, the results from the neutralization assays were inconclusive. Upon flow cytometry measurement of GFP expression, the average percentage of GFP in the uninfected

control wells was 0.1%. The average percentage of GFP in the wells containing no serum (only BAC16 virus and K562 DC-SIGN cells) was 28.5%. The known HHV-8 seropositive serum samples had an average of 0.2% GFP expression. All three of these experimental groups were as to be expected, but unfortunately the GFP expression percentage in the HHV-8 seronegative sample was 0.15%. Because this subject was not infected with HHV-8, they should have no antibodies specific for HHV-8. For this reason, the negative sample should not be neutralizing any of the BAC16 virus in culture. This leads us to believe that there is some component of the serum itself and the BAC16 virus that is either killing the harvested virus, or neutralizing the virus via an alternative mechanism. Originally, we suspected it might be due to the complement cascade killing the virus, but even after heat inactivating the serum, almost no virus was present in the HHV-8 seronegative samples again.

To further examine our second hypothesis, this study then examined the levels of 10 proinflammatory cytokines to determine if we would observe differences between males and females. It is hypothesized that men, who reactivate more frequently and have been shown to have overall higher antibody titers, will have higher levels of proinflammatory cytokines in their serum. It has been demonstrated in previous studies that proinflammatory cytokines can increase disease progression of HHV-8, and development of KS. This may explain why men are at a greater risk of developing KS versus women. Because KS is an inflammatory cytokine mediated disease, the induction of inflammation is an essential component of disease development and progression (68). Previous research by Jenkins et al. demonstrated that HHV-8 infection triggers the release of proinflammatory cytokines, specifically IL-6, IL-8, and TNF-α (66). For our study, only HIV

seronegative subjects were examined for proinflammatory cytokines, as HIV is a potential confound when examining inflammation in HHV-8 infected subjects.

Upon MSD analysis, it was determined that 7 of the 10 cytokines tested were significantly elevated in the male population: IL-10, IL12p70, IL-1β, IL-2, IL-6, IL-8, and TNFα (Tables 4). More specifically, in the Caucasian male population, IL-6 was significantly higher in males (Tables 5) In the African American population, IL-1β, IL-2, IL-6, IL-8, and TNF-α were all significantly higher in males (Table 7). Finally, in the Hispanic population, IL-2, IL-6, and IL-8 were all significantly higher in males (Table 8).

There were three cytokines that were not significantly different between males and females in any of the groups. These are IFN-γ, IL-4, and IL-13. IFN-γ. IFN-γ is crucial and necessary for innate and adaptive immunity against many types of pathogens- viral, bacterial, etc. Thus, it is possible to suggest that males and females will not differ significantly in their levels of IFN-γ, as it is such an important component of our immune system as a whole. The other two cytokines which did not differ between genders are IL-4 and IL-13, both which behave similarly in that they drive the immune system towards a Th2 response versus a Th1 response. Th1 cells produce proinflammatory cytokines, which would kill viruses like HHV-8 (68). So, it could be concluded that both males and females appear to be both favoring a Th1 response, in an attempt to combat HHV-8 infection.

The proinflammatory cytokines significantly upregulated in males: IL-10, IL12p70, IL-1β, IL-2, IL-6, IL-8, and TNFα, each have their own inflammatory effects on the immune system.

First, IL-10 is an anti-inflammatory cytokine that actually downregulates Th1 immune responses (68). Downregulation of the Th1 immune response could have implications towards HHV-8 infection, both positively and negatively. Positively, it could be the body's attempt to reduce inflammation, which is being brought on via HHV-8 infection. Negatively, it could be a mechanism of HHV-8 to downregulate cytokines like IFN-γ, which are critical to a healthy immune system, and would aid in combatting infection. IL-10 is the only cytokine out of the 7 mentioned above that has anti-inflammatory effects on the body (68).

The next cytokine that is upregulated in males is IL-12p70, which favors the generation of Th1 cells, and promotes cytolytic activity. IL-1β promotes inflammation by activating lymphocytes, and produces prostaglandins, which has the ability to induce fevers. IL-2 stimulates the proliferation and growth of T-cells. IL-6 is a B-cell stimulatory factor, and a CTL differentiation factor, which is extremely important in inducing inflammation and apoptosis in humans (63). IL-8 induces chemotaxis, and causes cells to migrate towards sites of infection, thus highly associated with inflammation and angiogenesis (64). Finally, TNFα is also highly involved in regulating other immune cells, and can induce apoptosis, inflammation, and also has the ability to inhibit tumor growth and viral replication (67). It also stimulates IL-1 production (66).

All of these results demonstrate several possible conclusions. First, it is clear that men are eliciting a stronger humoral response to HHV-8 versus women- both overall and in the HIV+ group. However, women's titers were significantly higher in the HIV- group. This may have to do with the timecourse of infection, multiple exposures to HHV-8, etc. But this will need to be examined in more depth in future studies. In general, male antibody titers were significantly higher

than women's titers, which coincides with our second hypothesis, that men will have increased HHV-8 antibody titers, coinciding with an increase in proinflammatory cytokines. Along with this, women will have decreased antibody titers due to a possible role of estrogen in suppressing reactivation and development of KS.

Because of this, we examined whether men would have an increased proinflammatory cytokine response to HHV-8 versus women, which can cause damage to the body and actually enhance KS disease progression. From our data, it is clear that men are mounting a stronger proinflammatory response to HHV-8 infection versus women- both generally, and also between races. Jenkins et al. previously reported that IL-6, IL-8, and TNF-α were increased after HHV-8 infection, and were able to alter the maturation of immune cells (66). These cytokines were also shown to be associated with lytic viral replication. Many of these cytokines favor cytolytic activity, apoptosis, and chemotaxis (64, 67). Others are B-cell stimulators that may ultimately aid in disseminating infection within the infected host (66). This is due to the fact that HHV-8 targets B-cells for infection and replication, to produce more infectious virus. However, both the males and females are infected with HHV-8 in this cytokine analysis, and both are mounting an immune response to the virus. These results do not mean that women are not producing these cytokines, simply that males are producing these proinflammatory cytokines in greater concentrations, which appears to have a positive effect on disease progression, which may be why men develop KS more frequently than women.

Finally, we examined the correlation between HHV-8 IFA titers and proinflammatory cytokine levels in both the male and female groups (Figures 9, 10, and 11). Using only the HIV

seronegative samples for comparison, 3 groups were tested: (1) Both MACS and WIHS HIV SN cohorts together, (2) MACS HIV SN, and (3) WIHS HIV SN. In the first group analyzing both cohorts, there were no significant differences in cytokine levels based on HHV-8 antibody titer. This was also observed in the MACS group as well. In the WIHS group, however, IL-12p70 was significantly different ($p = 0.041$) and a correlation coefficient of 0.411, meaning there is a positive correlation between a higher HHV-8 antibody titer in women, and an increase in IL-12p70. This data demonstrates that the higher HHV-8 antibody titers in the male group does not correlate with higher proinflammatory cytokine levels. In women, a positive correlation was seen with 1 out of the 10 cytokines tested. This leads us to believe that the HHV-8 antibody titer is not what is driving the proinflammatory cytokine response. This means that the higher the titer a patient has does not mean they will have higher levels of proinflammatory cytokines.

5.2 PUBLIC HEALTH SIGNIFICANCE

Identifying the differences in antibody titers between different subject groups has not been investigated in the context of males versus females at this level. The results of this study play an important role in understanding the result of HHV-8 infection and possible KS development. These results will hopefully aid those in the future by examining other mechanisms that may be important in KS development- whether it may be disease progression, or disease suppression. Identifying that our first hypothesis, that women will have a more robust overall humoral response, was rejected. However, our second hypothesis, that men will generally have higher antibody titers and proinflammatory cytokines, which will ultimately lead to KS, was supported. This is an important step in understanding KS development and disease progression. Particularly due to the fact that

men are producing much higher amounts of several cytokines versus females, though both are infected with HHV-8. In the future, this information could help researchers and physicians understand mechanisms behind disease development, and may be able to use this information to suppress or decrease the chances of reactivation in subject groups.

6.0 FUTURE DIRECTIONS

Upcoming studies should be focused on two particular components:

(1) Examining the neutralization assay further. It is clear that there is some component in the human serum samples we have obtained, or possibly the viral harvest, that is interfering with the quantification of BAC16 virus, even in the HHV-8 negative samples. I also believe it would be important to perform a longitudinal study on the serological samples, with much more information about times of infection, reactivation events, etc. by having multiple blood samples. This would allow us to examine the relationship between HIV and HHV-8 more closely as well.

(2) Examining hormonal control, specifically estrogen. The completed aims from our second hypothesis were supported: That men will elicit a stronger immune response to HHV-8, resulting in higher viral antibody titers and an increased level of proinflammatory cytokines. The next logical step would be to examine sex hormones and estrogen in more depth, by determining their methods of possibly limiting viral transcription and replication, or exactly how it may be suppressing disease progression in females. Previous research in our laboratory has shown that ERE's are able to control HHV-8 transcription, but the exact mechanisms and effects of this control should be examined in more depth. It would be essential to examine exactly how estrogen can be controlling or suppressing KS reactivation in women.

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