Establishing the Role of DC-SIGN and Glycoprotein H for KSHV Entry in B Lymphocytes

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A Thesis by

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Submitted in partial fulfillment of the requirements for the degree of
Master of Science in Pharmaceutical Sciences
August 2021

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June 2021
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ACKNOWLEDGEMENTS

First and foremost, I would like to acknowledge my advisor Dr. Jennifer Totonchy for her support and guidance throughout this whole two year journey. Her knowledge, enthusiasm and passion for Immunology and Virology have deeply inspired me for my future endeavors. I would also like to acknowledge Dr. Romina Nabiee for being an amazing mentor in the beginning of this process. Her technical and conceptual support was immense and I am forever thankful. I would like to acknowledge my lab-mates Feryal Aalam, Nedaa Alomari, Emily Romero, and Jesus Ramirez for their continuous support in the laboratory setting and life support in general. I would like to acknowledge my thesis committee Dr. Surya Nauli and Rennolds Ostrom, Ph.D. for their insightful comments in my thesis draft and during our annual meeting. Overall, I would like to acknowledge Chapman University School of Pharmacy for allowing me to further my career as a Scientist.
Establishing the role of DC-SIGN and glycoprotein H for KSHV entry in B lymphocytes

by Nancy Palmerin

Kaposi sarcoma-associated herpesvirus, also known as KSHV or HHV-8, is an emerging pathogen and the causative agent of multiple cancers in immunocompromised patients. KSHV is known to cause four types of malignancies: endothelial-based Kaposi Sarcoma (KS), two rare B cell-based lymphomas; Primary effusion lymphoma, Multicentric Castleman’s disease and a recently characterized inflammatory disorder called KSHV-associated inflammatory cytokine syndrome (KICS). Unfortunately, all of the diseases associated with KSHV infection are fatal, and to this day there is no known cure. The purpose of this study is to understand the viral entry process of KSHV in tonsil-derived B lymphocytes. This study will particularly explore the role of KSHV glycoprotein gH and cell surface receptor DC-SIGN.

The first aim of this project will investigate whether the cell surface receptor, DC-SIGN, is required for viral entry of KSHV on tonsillar B lymphocytes. Various approaches will be performed. First, characterization of tonsillar B lymphocytes subsets that express DC-SIGN will take place via flow cytometry followed by analysis on whether KSHV can infect DC-SIGN+ or DC-SIGN- B cells. Second, manipulation of DC-SIGN will be performed utilizing a Neutralizing antibody (anti-DC-SIGN), to observe the change in KSHV infection on tonsillar B lymphocytes. Lastly, a DC-SIGN depletion strategy will be performed to vigorously answer the question of whether DC-SIGN is required for KSHV entry. The second aim that will be addressed is whether the KSHV glycoprotein H (gH) is required for entry into tonsil derived B lymphocytes. In vitro infection of tonsil derived B lymphocytes with a mutant KSHV virus that lacks gH, but retains all the other essential KSHV glycoproteins (KSHV-ΔgH) will be performed and analyzed via flow cytometry. We will also combine these approaches with the KSHV-ΔgH mutant virus in order to determine...
whether manipulation of DC-SIGN affects entry of this mutant into B lymphocytes. This study will help understand the initial modes of KSHV transmission in tonsillar B lymphocytes which is relatively understudied in the field and pave the way for the development of future therapeutics to prevent KSHV transmission.
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<td>AIDS</td>
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<td>ORF</td>
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<td>xCT</td>
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<td>HAART</td>
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CHAPTER I - INTRODUCTION

1.1 Herpesviruses

Herpesviruses are a family of DNA viruses known to infect both animals and humans [4]. There are a total of eight herpes viruses known to infect humans: Herpes Simplex Virus 1 (HSV-1), Herpes Simplex Virus 2 (HSV-2), Varicella-Zoster Virus (VZV), Epstein-Barr virus (EBV), cytomegalovirus (CMV), human herpesvirus 6 (HHV-6), human herpesvirus 7 (HHV-7), and Kaposi Sarcoma Herpes Virus (KSHV)[4]. These eight human herpesviruses belong to the Herpesviridae family of DNA viruses [4], and are divided into 3 subfamilies: Alphaherpesvirinae, Betaherpesvirinae, and Gammaherpesvirinae based on their biological properties (Figure 1) [5]. Members of Alphaherpesvirinae are Herpes Simplex Virus 1 (HSV-1), Herpes Simplex Virus 2 (HSV-2), and Varicella-zoster virus (VZV) [6]. These particular viruses are known to infect a broad host of cell types and are known to be neuroinvasive. Alphaherpesvirinae infection and replication predominantly occurs in epithelial cells. Subsequently transmission of viral infection occurs in the neurons, where the virus is known to establish persistent infection in the nerves [7]. Herpes simplex virus 1 and Herpes simplex virus 2 can be classified as sexually transmitted diseases and are the major causes of cold sores and genital herpes [6]. Varicella-zoster virus is responsible for shingles (herpes zoster) and chickenpox (varicella) [8].

Members of Betaherpesvirinae are: Cytomegalovirus, Human Herpesvirus 6, and Human Herpesvirus 7 [9]. These viruses have a stringent cellular tropism towards: epithelial cells, fibroblasts, monocytes, lymphocytes, and T cells but are also lymphotropic where they have extensive reproductive cycles [10]. Cytomegalovirus is linked to infections in the nervous system and is known to cause congenital infections and mononucleosis [11]. Human Herpesvirus 6 is the causative agent of Roseola infantum, a nonmalignant rash found predominantly in infants [12].
Human Herpesvirus 7 is associated with various muscular rashes in children [13]. Both Human Herpesvirus 6 & Human Herpesvirus 7 bring clinical manifestations such as seizures, fevers, and rashes [14]. Members of *Gammaherpesvirinae* are Epstein-Barr virus (EBV), and Kaposi Sarcoma Herpes Virus (KSHV). EBV and KSHV are known to have the most limited host range [15]. This particular class of herpesviruses are the only members known to infect lymphocytes, but also infect a variety of non-lymphocyte cell types, and are associated with human cancers [15]. Overall, all herpes viruses share similar characteristics but the most common are: structure of the virion, latent and lytic life cycles, cascade system of gene expression, assembly, and imperceptible infection [1].
1.2 Kaposi Sarcoma Human Herpesvirus

Kaposi Sarcoma-associated herpesvirus (KSHV, or HHV-8) is an oncogenic virus which is widely known for its association with the Acquired Immunodeficiency Syndrome/ Human Immunodeficiency Virus (AIDS/HIV) epidemic [16]. Kaposi Sarcoma (KS) was first discovered in 1872 by physician, Dr. Moritz Kaposi, where he characterized a Kaposi Sarcoma tumor as an aggressive tumor with multi pigmented skin lesions [17]. Before the AIDS epidemic, Kaposi Sarcoma was a rare neoplasm that primarily infected elderly men in Eastern Europe and the Mediterranean [5]. In 1994, Chang et al. revealed that Kaposi Sarcoma Herpes Virus was the etiological agent of Kaposi Sarcoma. KSHV was identified through polymerase chain reaction-based subtractive analysis where a KS lesion of an AIDS patient was removed via biopsy and compared to the patient’s unaffected skin [18].

KSHV is now known to cause several types of human malignancies that are primarily present in people that are immunosuppressed or have acquired immunodeficiency syndrome (AIDS). The human malignancies are: Kaposi Sarcoma (KS), Primary effusion lymphoma (PEL), Multicentric Castleman’s disease (MCD) and a recently characterized inflammatory disorder called KSHV-associated inflammatory cytokine syndrome (KICS) [19]. Kaposi Sarcoma is the most common KSHV-associated tumor. Kaposi Sarcoma can be described as an endothelial based cancer with distinctive multicentric angioproliferative spindle cell lesions [20]. There are four distinct variants of Kaposi Sarcoma: Classical Kaposi Sarcoma, Endemic Kaposi Sarcoma, Iatrogenic Kaposi Sarcoma and AIDS associated Kaposi Sarcoma. These four variants differ in the severity of infection and the extent on which the patient has immunosuppression [20]. Classical KS is described as a rare form of Kaposi Sarcoma that is prevalent in HIV negative elderly patients in Eastern European regions, the Mediterranean, and Middle Eastern origin [5]. Endemic Kaposi Sarcoma is predominantly in Africa, infecting HIV negative children, men, and women [21]. Iatrogenic Kaposi Sarcoma, also known as transplant KS, develops in patients that undergo immunosuppressive therapy after organ transplantation. AIDS associated Kaposi Sarcoma commonly develops in HIV infected individuals and is a major cause of death in these patients [22].
PEL and MCD can be described as two B-cell based lymphoproliferative disorders associated with KSHV [16]. PEL is a true clonal lymphoma and develops in the body as peritoneal, pleural, and pericardial effusions [23]. While MCD is a polyclonal reactive lymphoproliferative disease that manifests as follicular and vascular alterations in the lymph nodes. Besides these two disorders caused by KSHV there is a newly characterized condition known as KICS. KICS and MCD have similar presentations with overproduction of certain inflammatory cytokines i.e Interleukin 6 and Interleukin 10 (IL-6 and IL-10) but KICS lacks pathologic evidence associated with MCD [20]. Distribution of KSHV infection varies geographically [24]. Prevalence of KSHV parallels the prevalence of Kaposi Sarcoma [24]. In the adult population, KSHV infection is found to be present in sub-Saharan Africa at >80% seroprevalence, 30% in the Mediterranean, at 10% in Western and 8% Northern Europe [25, 26]. It should be noted that the seroprevalence of KS increases drastically in KS patients that are infected with HIV [27]. Studies have shown KS patients that are HIV positive may display seropositive results for infection during initial examination but upon retesting, KS individuals display seronegative results. These results reveal that negative serology is not always indicative of lack of infection [28].

1.3 Kaposi Sarcoma Herpesvirus Transmission

Despite nearly three decades of KSHV research the exact mechanism of KSHV transmission is exceedingly unclear [29]. Modes of transmission differ depending on the background prevalence in the population [30]. Transmission may occur during childhood and/or adulthood and is spread via nonsexual and sexual routes [31]. KSHV was initially considered to only be transmitted via sexual routes during the AIDS pandemic [16]. Now, sexual routes are known to occur in non-endemic areas such as the United States and Western Europe where KSHV transmission is prevalent among men who have sex with men [25]. Studies have shown that men who have sex with men have roughly 40% higher rate of infectivity from that compared to heterosexual encounters [25]. Nonsexual routes of KSHV transmission occur in endemic areas such as Sub Saharan Africa where much of the transmission is thought to occur during childhood [32]. Researchers have hypothesized
that KSHV may be transmitted in the early stages of childhood via mother to child transmission [33]. There are a variety of different instances where mother to child transmission has been shown to occur. Mother to child transmission may occur during gestation where the child attains the KSHV virus prior to being born. This transmission tends to occur when the mother contains KSHV viral load in the female genital tract [31]. In other instances, the mother may transmit the virus to the child via breastfeeding [34]. Studies have revealed that breast milk can contain KSHV DNA[34]. One last and major instance of mother to child transmission is via salivary transmission. Studies have identified instances where the mother premasticates the food of the infant to feed it and the child later displays KSHV infection [35, 36].

Overall, KSHV can be detected in peripheral blood mononuclear cells, saliva, semen, breast milk and prostate glands, but salivary shedding has been deemed the major reservoir of infectious KSHV transmission[37, 38] . KSHV viral DNA is more frequently present and at higher levels in saliva than any other fluid from KSHV patients undergoing treatment.[39]. Studies have also demonstrated that the oral cavity plays a significant role in KSHV infections [40]. The oral cavity contains cells such as: epithelial cells, B lymphocytes, lymphatic endothelial cells and fibroblasts that KSHV is able to infect [41]. With that in mind, numerous labs, as well as our group, have implemented the use of tonsils as a primary model to study the early stages of KSHV infections [29, 53].

1.4 Kaposi Sarcoma Herpesvirus Life Cycle

KSHV has two life cycles following infection of host cells; latent and lytic replication. Both of these phases are characterized by distinctive patterns of gene expression and both are essential for the development of KSHV associated diseases [42]. KSHV is known to be predominantly in the latent state with only 1-3% of cells associated with lytic replication at a time [43] . Establishment of latent infection occurs initially during acute infection in immunosuppressed individuals [40]. In latent infection, the KSHV DNA genome persists as a circular episome in the nucleus where it is in a quiescent state involving no production of virions. The purpose of latency is to allow the
virus to establish a persistent infection, and, in some cases, latency can be maintained via immune-mediated inhibition of lytic replication[44, 45]. There are a few latent associated genes expressed in this phase including LANA (latency-associated nuclear antigen, vCyclin (viral cyclin), v-FLIP (viral Fas like inhibitory protein), and various viral microRNAs [22].

LANA, a nuclear antigen that is 230 kDa and roughly 1162 amino acids long, is thought to be a highly versatile protein during latency [44]. LANA is essential for maintaining, replicating and segregating the viral genome throughout each generation of the host cell. LANA has the capability of binding and interacting with various proteins, such as p53, to suppress transcriptional activity and achieve inhibition of apoptosis and cell proliferation. These interactions allow LANA to mediate viral persistence for KSHV [22]. v-Cyclin’s main duty is to function as a modulator during latency to sustain cell proliferation and the cell cycle. v-Cyclin modulates phosphorylation and inhibition of: CDK inhibitors, H1, histones, pRb protein, and p27 to form v-Cyclin-CDk6 complex. v-Cyclin-CDk6 mediates phosphorylation of nucleophosmin and facilitating interactions between CDK6, LANA, and nucleophosmin to promote KSHV latency [22, 46]. Homologous to v-Cylin is another viral protein called v-FLIP. v-FLIP is an activator for both classical and alternative NF-κB pathway which induce cell survival signals [47]. v-FLIP has the capability of blocking apoptosis, viral reactivation and lytic replication in different cases [48]. For example, blockage of apoptosis was performed by inhibiting procaspase 8 cleavage which blockage of viral reactivation and lytic replication was performed by suppressing the AP-1 pathway [48].

During lytic infection, also referred to as the productive phase, KSHV replicates its genome and produces infectious virions that are assembled and released from the cell via cellular lysis [40]. The majority of KSHV infected cells in humans are latently infected and contribute to persistent infection [49]. But the presence of lytic genes is still critical for the pathogenesis of KSHV-associated diseases. The lytic replication genes that are present in infected cells are divided into three classifications: immediate early, early, and late genes [44]. Lytic gene functions include, but are not limited to, regulation of cell cycle, survival, and immune evasion [50]. After all lytic genes are expressed, the commencement of virus assembly occurs in the nucleus which subsequently
leads to the viral progeny to be released from the host cell [44]. It should be noted that latency is reversible and has the potential of reactivating due to environmental conditions to enter lytic replication [22]. There is a balance between these two life cycles to give KSHV the capability of achieving persistent infection and achieving efficient spread of the virus to new hosts.

1.5 Kaposi Sarcoma Herpesvirus Cellular Tropism

KSHV exhibits an extensive cellular tropism as it infects a diversity of target cells in vitro and in vivo [52]. This broad cellular tropism is due to KSHV having distinct routes of entry depending on the cell type [52]. In vivo, KSHV DNA has been detected in: human B-cells, peripheral blood B-cells, epithelial cells, monocytes, fibroblast, keratinocytes, endothelial cells, and para endothelial spindle cells [3]. In vitro, KSHV infects endothelial cells, monocytes, fibroblasts, B-cells, and epithelial cells lines of nonhuman and human origin [53].

We can also obtain clues as to the in vivo tropism of KSHV by observing the cell types in which it causes disease. For example, KSHV DNA has been detected in lymphoproliferative diseases such as MCD and PEL [54]. The immunological features of these diseases have led many to speculate that MCD arises in naïve B-cells while PEL arises in post germinal center memory subsets [55, 56]. However, this may not always be the case, studies have revealed that KSHV infection can cause MCD and PEL via defects in germinal center reactions and or reduction of memory cells [56, 57]. Overall, the initial cell types to be infected in both diseases is still unknown. In individuals with KS, KSHV DNA has been found in a B-cell fraction of peripheral blood and endothelial cells in malignant tumors [58, 59].

Altogether, KSHV has high susceptibility towards B lymphocytes in vivo, since it provides KSHV with an environment where it is capable of establishing latency and persisting during the whole life of their host [60]. Unfortunately, B lymphocytes have been notoriously difficult to infect with KSHV in vitro, due to lack of appropriate receptor expression among other factors [61]. Our lab, along with several other groups, have successfully performed in vitro infections on tonsil derived B lymphocytes [62].
1.6 Kaposi Sarcoma Herpesvirus Molecular Machinery

The structure of KSHV includes an electron-dense nucleocapsid with a tightly packaged double stranded DNA virus (genome of 165kb and 90 open reading fragments) [63]. The capsid is surrounded by a layer of tegument that contains several capsid associated proteins such as Open Reading Fragments (ORFs): 45, 52, 11, 21, 33, 63, 64, and 75, several loosely associated proteins and microRNAs. [2]. The last layer that surrounds the whole particle is the viral envelope which consists of a lipid bilayer containing various viral glycoproteins. These viral envelope glycoproteins are: gB (ORF8), gH (ORF22), gL (ORF47), gM (ORF39), gN (ORF53), ORF4, and gpK8.1. The conserved glycoproteins that are found in all herpesviruses are gB, gH, gL, gM, and gN [63]. The conserved glycoproteins are thought to play an important role for virus entry and KSHV infection since they mediate initial virus and host cell interactions (Figure 2) [2].

1.6.1 Kaposi Sarcoma Herpesvirus Viral Envelope Glycoproteins

As mentioned above, the glycoproteins that surround the viral envelope of KSHV are: gB (ORF8), gH (ORF22), gL (ORF47), gM (ORF39), gN (ORF53), ORF4, and gpK8.1. The KSHV glycoprotein B is a 110 kDa protein that is thought to be a key player in the initial interaction for KSHV entry into the cell and is thought to be the perpetrator of membrane fusion [2]. Glycoprotein B (gB) undergoes cleavage and yields two linked polypeptides at 75kDa and 54kDa with complex sugars and high mannose [64]. These conformational changes cause a shift in the target cells and brings them to close proximity making fusion more likely to occur [64]. KSHV glycoprotein H (gH) and glycoprotein L (gL) form a non-covalent linked hetero-dimer complex where gH is 120 kDa and gL is 42 kDa. This hetero-dimer complex is found to be a vital epitope for KSHV specific neutralizing antibodies in KS patients [65]. Previous studies have shown that gH reaches the cell surface independently from gL [66]. The role of gH and its presence being essential in KSHV entry remains poorly understood. KSHV glycoprotein M (gM) and glycoprotein N (gN) form a heterodimer [67]. gN is utilized for the processing of gM. Both, gN and gM are overall known to be involved in virus penetration [2]. Glycoprotein K8.1 (gpK8.1A) gives rise to two open reading frames: gpK 8.1A and gp K8.1B. gp K8.1A consists of 228 amino acid protein while gp K8.1B
encodes a 167 AA glycoprotein sequence [68]. There are a variety of glycoproteins that are either still unknown or poorly understood in the KSHV infection process.

1.7 Kaposi Sarcoma Herpesvirus Viral Entry and Infection

Understanding how KSHV enters cells is of critical importance for the pathogenesis in the host and for treatment strategies[2]. The current entry process of KSHV is poorly understood due to the fact that KSHV possesses diverse modes of entry depending on the cell type. What is currently known in the field is that KSHV has the capability of infecting cells by binding and entering the host target cells through a sequential, multistep process that includes interactions among various host cell receptors and various viral envelope glycoproteins. The interactions between glycoproteins and host cell receptors facilitate attachment, fusion, entry, replication and assembly.[53]. The mechanism of KSHV viral entry can be divided into a variety of different steps (Figure 3). The initial step in the KSHV viral entry process consists of the binding of host cell receptors to the KSHV viral glycoproteins [3]. Interactions of host cell receptors and KSHV viral glycoproteins ultimately triggers signal transduction via various pathways that assist with viral entry and trafficking[52]. This process may involve activation of tyrosine kinases that lead to recruitment of signaling complexes that induce internalization [52]. For example, binding of KSHV to integrins leads to phosphorylation of focal adhesion kinase (FAK), which leads to the activation of cell processes such as adhesion, proliferation, endocytosis and cell death [3]. Induction of FAK is
also known to activate other kinases such as the family of Src kinases, PI3-K, Rho GTPases, and Diaphanous 2 to regulate cell trafficking [3].

Following signal induction is viral entry into the target cells [3]. It should be noted that KSHV employs different routes of entry depending on the cell type. For example, in fibroblasts, endothelial cells, epithelial cells and monocytes, KSHV is shown to enter via endocytosis [3]. Endocytosis within itself comprises different sub-routes of entry such as clathrin-mediated endocytosis, phagocytosis, caveolae mediated endocytosis, and macropinocytosis [52]. In endothelial cells, the route of endocytosis KSHV partakes in is macropinocytosis while in fibroblasts KSHV enters via clathrin mediated endocytosis [69].

Once the virus is fully internalized into the endosomes, the KSHV viral envelope fuses with endosomal membranes allowing release of the capsid into the cytoplasm [3]. The capsid is then transported within the cytoplasm via dynein motors and microtubules to ultimately reach the nucleus [70]. The virus moves to the nucleus where the viral capsid is released and delivers the viral DNA [3]. This then leads to viral gene expression and host cell gene reprogramming. [3].

1.8 Kaposi Sarcoma Herpesvirus Host Cell Surface Receptors

As mentioned above, the initial step in the KSHV viral entry process consists of the binding of KSHV viral glycoproteins to KSHV host cell receptors. It should be noted that KSHV host cell receptors fall into two main categories: Cellular Binding Receptors and Cellular Entry Receptors [71]. Cellular Binding Receptors are used to promote binding and concentration of the virus onto the target cell. A known binding receptor for KSHV is Heparan Sulfate (HS) [72]. Heparan Sulfate is a sulfated polysaccharide of the sulfate proteoglycan family that serves as an attachment factor for a variety of viruses such as: KSHV [73], HIV [74], Hepatitis E [75], Dengue [76], and RSV [77].

HS is present on various cell types: fibroblasts, endothelial cells, epithelial cells and monocytes [2]. Within these cell types, the binding of HS with glycoproteins gB, gHgL, and K8.1 have been shown to occur during the KSHV entry process [78]. To identify if HS was essential for KSHV
entry, individual studies pre-treated the target cells with soluble HS (a neutralizing agent against HS) [2, 60, 64, 78, 79]. Results displayed that HS was dispensable for viral entry but that in the presence of HS, interactions were enhanced for entry into cells [2].

Other studies have also demonstrated that heparan sulfates are dispensable in tonsillar derived B lymphocytes [62]. This particular study removed HS via a heparinase treatment from the tonsillar derived B lymphocytes and overall displayed that KSHV still had the capability of infecting these cells. The combination of all these studies overall reveal that HS can be utilized as an attachment factor to enhance entry but it is not essential for entry in all cell types [2].
The second type of host cellular receptor are known as cellular entry receptors [71]. There are various cellular entry receptors that KSHV is known to engage with depending on cell type. The cellular entry receptors for KSHV include, but are not limited to, Ephrin receptors (Eph), integrin receptors, cystine/glutamate antiporter (xCT), and Dendritic Cell-specific Intercellular adhesion molecule-3-grabbing non-integrins (DC-SIGN) [52]. Ephrin receptors (Eph) are type-I transmembrane proteins that are part of the receptor tyrosine kinase family [71]. The Eph receptors consist of various forms and are vital for signaling pathways in cancer proliferation and differentiation[80]. Eph receptors can be found on endothelial cells, epithelial cells, fibroblasts cells, and B-cells [71]. One form of Eph receptor known as EphA2 can be used in the KSHV entry process. Studies have shown that EphA2 is essential for entry in endothelial cells by knocking down Eph2A and displaying no presence of infection after [81]. Studies have also indicated that gHgL can bind to the EphA2 receptor and induce EphA2 phosphorylation, which ultimately leads to internalization [82].

Integrins are cell surface receptors responsible for interactions between cells and the extracellular matrix [71]. Studies have indicated that alpha and beta dimers of integrins serve as receptors for KSHV infection in fibroblasts, monocytes, epithelial cells and endothelial cells [71]. Studies have shown that KSHV gB interacts with different types of integrins such as: α3β1, αVβ3, and αVβ5 for entry into these cells [2, 68, 72, 83]. gB possesses a binding motif (Arginine-Glycine-Aspartate, also referenced to as: RGD) that is the potential binding site for these intergin host cell receptors [78]. The interaction of gB with these integrins activates a cascade of signaling pathways that, in turn, result in internalization of the virus. Besides the gB glycoprotein interaction, studies suggest that integrins alone are indispensable in KSHV infection while others studies state otherwise. One study suggests that integrins are critical for mediating entry, signal transduction, and viral-gene expression for KSHV in endothelial cells, and fibroblasts[78]. But other studies reveal data that KSHV utilizes heparan sulfates and Ephrin receptor routes, independent of integrins, to infect endothelial cell lines[84]. Thus, the importance of integrins for KSHV entry in these specific cell types remains largely unknown.
Cystine/glutamate antiporter (xCT), is a 12-transmembrane, 501 amino acid glutamate/cysteine exchange transporter protein that is 125kDa [85]. The primary function of xCT is cystine uptake for intracellular glutathione synthesis to maintain intracellular redox balances during oxidative stress[86]. xCT is highly expressed on malignant tumors including lymphomas, breast carcinoma, glioma and prostate cancer [87]. Reports have shown that xCT is an integral component of a complex that mediates cellular entry and fusion of KSHV [86]. xCT forms a complex with CD98, integrins and other signaling molecules to promote KSHV entry and fusion in various cell types such as: endothelial cells, fibroblasts and B-cells[87]. Studies have also shown that xCT has a multi-functional role in KSHV infection to ultimately promote cell survival and tumorigenesis[87].

DC-SIGN also known as, Dendritic Cell-Specific ICAM-3-Grabbing Non-integrin, CD209, is a type II C-type (calcium-dependent) lectin receptor [71]. DC-SIGN is highly expressed in cells of the immune system such as dendritic cells (DCs), in dermal and mucosal tissues, monocytes, macrophages, B lymphocytes from peripheral blood, and tonsillar B lymphocytes [88, 89]. In monocytes, macrophages, dendritic cells, and B lymphocytes, glycoprotein gB has the ability to bind to host cell receptor DC-SIGN [2, 88]. Studies have shown that gB binds to DC-SIGN in a dose dependent manner, but this interaction has yet to be deemed essential for KSHV entry in any cell type [90]. DC-SIGN is utilized as an entry receptor by many viruses such as Ebola virus, HIV-1, Hepatitis C, Ebola Virus, KSHV, SARS coronavirus, and Dengue virus [91]. Overall, DC-SIGN is deemed important as it plays a critical role in the innate and adaptive immune response by facilitating dendritic cell signaling, adhesion and migration [92].

1.9 B-cell Immunology

B-cells, also known as B-lymphocytes, are a class of white blood cells that are an essential component in adaptive immune system [93]. B-cell development commences in the bone marrow where B-cells acquire their antigen specific receptor and are released into the peripheral lymphoid tissues for final maturation (Figure 4) [94]. In the peripheral lymphoid tissues, B-cell differentiation
is subdivided into two distinctive pathways: T-cell dependent pathway or T-cell independent pathway [95].

T-cell dependent pathways require the interaction between B-cells and T-cells for B-cell differentiation [96]. T-cell dependent pathways occur in specialized micro-environments within lymphoid tissues called germinal centers. The germinal center environment is subdivided into two major compartments: Dark zone compartment and the light zone compartment. Within the dark zone compartment, B-cells undergo proliferation, class-switch recombination and somatic hypermutation; a process in which the function and affinity of their antigen receptors is refined via recombination, random mutation, and selection. B-cells proliferate into centroblasts and centrocytes but ultimately leave the dark zone as centrocytes [96]. In the light zone, the transformed centrocyte acquires an antigen on their B-cell receptor (BCR), which they internalize, process and present to helper T-cells via a Major Histocompatibility Complex Class II molecule (MHC II). Helper T-cells recognize and interact with the MHC II molecule via their T-cell receptor and CD4+ molecule [97]. Subsequently, T-cells up-regulate CD40L surface proteins and bind to the CD40 protein on the centrocytes leading to the secretion of cytokines. Secretion of cytokines promote centrocytes to undergo further rounds of proliferation, somatic hypermutation and exit the germinal center reactions as plasmablast, memory cells, or plasma cells [97].

In T-cell-independent pathways, B-cells do not interact with T-cells nor undergo germinal center reactions for B-cell differentiation [98]. T-cell independent pathways occur in the marginal zone of the spleen which is located at the border of the red and white pulp adjacent to the marginal sinus [99]. In this particular pathway, naive B-cells bind to T-cell independent antigens via their BCR. T-cell independent antigens such as lipopolysaccharides (LPS) and polysaccharides (PS), also referred to as Pathogen Associated Molecular Patterns (PAMPs), elicit repetitive epitope units within their structure that ultimately provide the first signal of activation [100]. Pattern recognition receptors known as Toll-like receptors (TLR), recognize this initial signal and interact with PAMPs to elicit B cell proliferation and differentiation into plasmablast and plasma cells. In the T-cell independent pathway, memory cells can not be induced due to the absence of T cell help [100].
Overall, the goal of each pathway is to elicit antigen-induced activation in B-cells during their process of development for them to produce plasmablast, memory (T-cell dependent pathway) and plasma cells [100]. It should be noted that memory cells are critical cell types for the humoral immune response as their duty is to recognize foreign antigens that have previously entered the cell and elicit rapid immune responses [101]. While plasmablasts are a transient population of cells that are in the process of maturing into plasma cells. Lastly, plasma cells are long lived cells that primarily reside in the bone marrow and secrete high-affinity antibodies [102].

As a lymphotropic herpes virus, KSHV has affinity for B lymphocytes leading to development of PEL and MCD [54]. So far, there have been a few in vitro KSHV infection models of B lymphocytes focusing on the CD19+ population of B-cells [54, 103, 104]. Within these studies, data has suggested that KSHV infection may be driving B-cell differentiation in infected samples, i.e. KSHV infected B-cells lead to plasmablast differentiation [103]. Our lab has pioneered the detailed examination of B lymphocyte lineages in KSHV infection, and has characterized B lymphocyte tropism of KSHV in human tonsil lymphocytes [62]. We demonstrated that a wide variety of B-cell lineages are targeted by KSHV infection, and that CD138+ plasma cells (both the CD20+ and CD20-) are highly targeted populations by KSHV. This susceptibility of plasma cells is not due to CD138 (heparan sulfate) and overall B-cell infection is independent of HSPS. How diverse populations of the B-cells are targeted by KSHV, and what means of cellular entry is used by KSHV remains to be answered.

1.10 Justification for and Significance of the Study

Despite extensive KSHV research, there is still no effective treatment to fully eradicate KSHV [105]. Additionally there is no standard guideline to manage the current KSHV associated diseases [105]. KSHV treatments are dependent on the type of KSHV associated disease, the tumor location, the tumor progression, severity of the symptoms and immune system tolerance [105]. KSHV treatments that are in use to combat this virus are highly variable depending on the geographic location [105]. U.S treatment strategies include but are not limited to antiviral therapy, antiretroviral therapy, excisional biopsy, radiation therapy, chemotherapy, immunotherapy, and
protease inhibitors [105]. While other countries such as Sub Saharan Africa, where KS is the predominant cancer in those living with HIV/AIDS, do not have U.S treatments readily accessible [106, 107].

The most common treatment for prevention of KSHV associated diseases in the U.S is antiviral therapy [105]. In KSHV diseases associated with PEL and MCD, antiviral drugs have been shown to reduce KSHV viral load. This outcome is due to these diseases expressing highly lytic phase genes [108]. In KSHV-MCD several toxic chemotherapy treatments have also been utilized to combat this disease [108]. Studies have overall shown that KSHV-KS does not respond well to antiviral treatments due to the small quantity of lytic KSHV genes in the infected cells [105].

![Figure 4](image.png)

**Figure 4.** Scheme of the stages of B cell differentiation in human tonsil.

Highly active antiretroviral therapy (HAART) or HAART in combination with chemotherapy have been implemented as treatment for people with KS and HIV/AIDS [109]. Whereas, the incidence of KSHV has not declined in these individuals. Studies have demonstrated that some individuals fully recover with these treatments while others display resistance to the medication [110]. This resistance is indicative that people living with KS and HIV/AIDS need more than immune reconstitution to prevent the disease [62].

KSHV associated diseases consist of many challenges for treatment strategies due to its persistent lifelong latent infection with recurring activation for active replication [49]. Exploring new insights in the early stages of KSHV transmission is needed to offer new effective and less toxic therapeutics for KSHV associated diseases [105]. Although KSHV glycoprotein and host cell
receptor interactions have been well established on numerous cell types as described in the KSHV host cell receptor section of this thesis, there is limited data available characterizing how KSHV enters primary B lymphocytes.

Studies have shown that DC-SIGN, can be utilized as an entry receptor for KSHV infection in human myeloid dendritic cells, macrophages, and activated peripheral blood B-cells [88]. To evaluate if DC-SIGN is required for KSHV infection in human activated peripheral blood B-cells, a previous study blocked DC-SIGN expression on target cells with a DC-SIGN neutralizing antibody [anti-DC-SIGN MAb] prior to infection. 24 hours post infection, the distribution of KSHV infection was assessed. Results displayed that pretreatment of target cells with anti-DC-SIGN blocked ~90% of KSHV infection. Study concluded that expression of DC-SIGN is essential for KSHV infection in B-cells [89]. However, this conclusion is not valid for tonsil-derived B-cells, or any other B-cell due to the fact that this study was strictly performed on peripheral blood B-cells and additionally analysis of specific B-cell subsets in the blood was not performed. Thus, the role of DC-SIGN in tonsil-derived B-cells for KSHV entry and in specific B-cell lineages remains unknown.

A previous study from our lab and our collaborators at the City of Hope, generated a mutant KSHV virus lacking gH, but retaining all of the other KSHV viral envelope glycoproteins (KSHV-ΔgH) in order to characterize the requirement of glycoprotein H in KSHV biology [111] . This study assessed the infectivity of KSHV-ΔgH in numerous cell types in vitro such as epithelial cells, endothelial cells, a B-cell line (MC116) and fibroblasts. Results revealed that glycoprotein H is required for infection in epithelial cells, endothelial cells, and fibroblast, but not required in MC116 B-cell lymphoma cells.

These previous studies have led us to take several approaches to elucidate the essential glycoproteins and host cells receptors in KSHV entry and infection of tonsil-derived B lymphocytes. We will test whether the cell surface receptor, DC-SIGN, is required for viral entry and infection in tonsil derived B-cell lineages and whether the glycoprotein H is required for entry on tonsil derived B-cell lineages. We will also combine these approaches with the KSHV-ΔgH mutant virus in order to determine whether manipulation of DC-SIGN affects entry of this mutant into B lymphocytes.
Determining these roles will help provide novel targets for therapeutic intervention in KSHV diseases and the prevention of KSHV transmission.

CHAPTER II-METHODOLOGY

2.1 Materials

Dulbecco’s Modified Eagle Medium (DMEM) with high-glucose and L-glutamine and no sodium pyruvate was purchased from Caisson Labs (Smithfield, UT, US). Fetal Bovine Serum was purchased from R&D Systems (Centennial, CO). Trypsin-EDTA, 0.25%, sterile-filtered, BioReagent, suitable for cell culture, 2.5 g porcine trypsin and 0.2 g EDTA. 4Na per liter of Hanks’ Balanced Salt Solution with phenol red were obtained from Sigma Aldrich (St.Louis, MO). Penicillin Streptomycin L-glutamine (PSG) was purchased from Corning (Corning, NY). RBC lysing solution was created in-house with 0.15M ammonium Chloride (Sigma Aldrich), 10mM potassium bicarbonate (Bio Basic), 0.1 EDTA (Sigma Aldrich). Dimethyl Sulfoxide (DMSO) was purchased from Bio Basic (Ontario, Canada). Roswell Park Memorial Institute Medium (RPMI) was purchased from Cytiva (Marlborough, MA). DNase I (deoxyribonuclease I), Grade II, from bovine pancreas, 100 mg was purchased from Sigma-Aldrich (St.Louis, MO). Primocin 500 mg was obtained from Invivogen (San Diego, CA). Bovine Serum Albumin (BSA) was purchased from Cytiva (Marlborough, MA). Sodium Azide was purchased from Sigma-Aldrich (St.Louis, MO). Antibodies for flow cytometry were acquired from BD Biosciences (San Diego, CA) and Biolegend (San Diego, CA): anti-human IgD-BUV395 (BD 563823), anti-mouse CD77-BV510 (BD563630), anti-mouse CD138-BV650 (BD 555462), anti mouse CD27-BV750 (BD 563328), anti-mouse CD19-PerCPCy5.5 (BD 561295), anti-human CD38-APC (BD 560158), anti-mouse CD20-APC7 (BL 302313), and anti-mouse DC-SIGN-PE-Cy7 (2µl/test BD 330114), Zombie Violet™ Fixable Viability (BL 423113). Mojosort™ Human Pan B-cell isolation kit was purchased
from Biolegend San Diego, CA (BL 480082). Miltenyi CD209 (DC-SIGN) Microbeads Kit were purchased from Miltenyi Biotec Inc Auburn, CA (Miltenyi 130-092-868).

2.2 Cell lines

Cell free iSLK-derived BAC16-rKSHV-ΔgH-eGFP was generated in the Ogembo laboratory at City of Hope, Duarte, CA [111]. Cell free iSLK-derived BAC16-rKSHV-WT-eGFP were generated as described in [112]. CDw32 L cell lines were obtained from American Type Culture Collection (ATCC), and underwent irradiation protocol as discussed in [29]. De-identified human tonsil specimens were obtained from the National Disease Research Interchange (NDRI) Note: The use of deidentified tonsil specimens was not subject to IRB review as human subjects research.

2.3 Laboratory Equipment

Miltenyi Biotec QuadroMACs Magnetic Cell separator and MojoSort™ Magnet were utilized to perform cell separation. Flow cytometry reading on all experiments was performed using BD LSRFortessa Flow Cytometer. FlowJo Software & RStudio was utilized to analyze data and perform statistical analysis.

2.4 Methods

2.4.1 Preparation of cell-free recombinant KSHV virions

iSLK cell lines harboring BAC16-KSHV-WT-eGFP [112] and BAC16-KSHV-ΔgH-eGFP [111] were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% Cosmic Calf Serum (CCS), PSG, puromycin (1μL/mL), G418 (1μL/mL), and hygromycin (8μL/mL) at 37°C in 5% CO₂. For virus preparations, 12x T185 flasks at 80-90% confluence were stimulated for 72 hours with 3mM sodium Butyrate and 2μM Doxycycline hyclate. At 3 days post induction, supernatants were clarified by centrifugation at 1700rpm at 12min 4°C and filtered with a 0.45um vacuum filter. Virions were pelleted out of clarified supernatant over 25% sucrose in TNE (50mM Tris[pH7.4], 100mM NaCl, 0.1mM EDTA, pH7.4) by centrifugation at 22,000 rpm for 2 hours.
Virus pellets were resuspended in 2ml TNE and stored at -80 °C. Infectious titer doses were determined for iSLK-BAC16-KSHV-WT-eGFP by serial dilution infection on human fibroblasts and quantified at 3 days post infection via flow cytometry. Infectious titer doses were determined for iSLK-BAC16-KSHV-∆gH-eGFP by calculating equal genome copy number to that of iSLK-BAC16-KSHV-WT-eGFP via quantitative PCR (qPCR).

2.4.2 Isolation of primary lymphocytes from Human Tonsil

De-identified human tonsil specimens were obtained from the National Disease Research Interchange (NDRI) following routine tonsillectomies. Less than 24 hours post-surgery, tonsil specimens were shipped and delivered in DMEM+PSG to the laboratory. Primary lymphocytes were extracted via dissection and maceration of tonsil tissues in RPMI media. Lymphocyte-containing media were passed through a 40µm filter, and pelleted at 1500rpm for 5 minutes. Red blood cell lysing solution (0.15M ammonium chloride, 10mM potassium bicarbonate, 0.1M EDTA) was utilized to lyse red blood cells present in the lymphocyte preparation. Following 3 minutes of RBC lysis, lymphocytes were diluted in 50ml of PBS, manually counted and pelleted at 1500rpm for 5 minutes. Aliquots of 1e8 cells were resuspended in 1ml of freezing media (90% FBS, 10% DMSO) and cryopreserved.

2.4.3 Magnetic Cell Separation of Total B-Lymphocytes from Tonsillar Primary lymphocytes

Tonsil primary lymphocyte suspensions were thawed at 37°, slowly diluted to 5ml with RPMI. and pelleted at 1500rpm for 5 minutes. Pellets were resuspended in 1ml RPMI with 20%FBS,and 100µg/ml DNase I, and 100µg/ml Primocin. Cells were maintained in a low-binding 24 well plate at 37 °C and 5% CO₂ incubator for two hours. After recovery, total lymphocytes were counted and Total human B cells were isolated using Mojosort™ Human Pan B-cell isolation kit (Biolegend 480082) according to manufacturer’s instructions. Bound cells that were non B-cells were retained and maintained in 1ml RPMI, 20% FBS and 100ug/ml at 37 °C and 5% CO₂ incubator.
2.4.4 Infection of Total B lymphocytes with KSHV

Recombinant KSHV virion preparations were used at the dose needed to infect 20% of human fibroblast cultures at 3 days post-infection (ID20) and were diluted in serum free media RPMI in a total of 400µl for each 1 million total B lymphocytes. For experiments including KSHV-∆gH, the WT virus was used at ID20 doses and KSHV-∆gH was used at an equivalent genome dose. In all experiments Mock-infected cultures were included as an internal reference for the GFP positive signal and to allow analysis of culture-specific effects. Cells in infection media were spinoculated at 1000rpm for 30 minutes at 4˚C in 12x75mm round bottom tubes. After spinoculation, tubes were incubated at 37˚C for an additional 30 minutes. After incubation, infected cultures were transferred to X-ray irradiated CDW32 L cells in a 48 well plate and reconstituted with 20% fetal bovine serum, 100ug/ml of Primocin and 1 million cells from the bound fraction of the B cell isolation. Reconstituted lymphocyte cultures were incubated at 37˚C, 5% CO₂ for the duration of the experiment. At 3 days post-infection, cells were harvested for analysis by flow cytometry for B-cells lineages and KSHV infection (See section 2.4.7).

2.4.5 DC-SIGN Neutralization

Total B lymphocytes were isolated as in 2.4.3 and were incubated with Human DC-SIGN/CD209 Antibody [anti-DC-SIGN mAb] (R&D system MAB161-100) at varying concentrations (0ug/ml, 2.5ug/ml, 5ug/ml) for 30 minutes on ice prior to infection, culture and analysis as described in 2.4.4.

2.4.6 DC-SIGN Depletion

Total B lymphocytes were isolated as described in 2.4.3 and were further separated into DC-SIGN+ (bound) and DC-SIGN- (unbound) fractions using CD209 (DC-SIGN) MicroBeads (Miltenyi 130-092-868) according to manufacturer’s instructions. Following separation, half of the DC-SIGN-B cells were reconstituted with DC-SIGN+ B cell fraction and the remaining cultures remained depleted. These depleted and reconstituted samples were Mock-infected or infected with KSHV-WT and KSHV-∆gH, cultured and analyzed as described in 2.4.4.
2.4.7 Flow cytometry

At day 0 (baseline) or at 3 days post-infection (3 dpi), 5e5 lymphocytes were aliquoted into a 96-well round bottom plate and pelleted at 1500 rpm for 5 minutes. Resuspension of the pellet was performed with 100µl PBS containing zombie violet fixable viability stain (BL Cat# 423113) and incubated on ice for 15 minutes. After incubation 100ul PBS, containing the following: 2% FBS and 0.5% BSA (FACS Block) was added to the wells. Cells were pelleted at 1500rpm 5 minutes and resuspended in 200ul FACS Block followed by a 10 minute incubation on ice. Cells were pelleted at 1500rpm for 5 minutes and resuspended in 50µl of PBS containing the following 0.5% BSA and 0.1% Sodium Azide (FACS Wash), 10µl BD Brilliant Stain Buffer Plus and antibodies as follows: IgD-BUV395 (2.5µl/test BD 563823), CD77-BV510 (2.0 µl/ test BD 563630), CD138-BV650 (2µl/test BD 555462), CD27-BV750 (2µ/test BD 563328), CD19-PerCPCy5.5 (2.0µl/test BD 561295), CD38-APC (10µl/test BD 560158), CD20-APCH7 (2ul/test BL 302313), and DC-SIGN-PE-Cy7 (2µl/test BD 330114) and incubated on ice for 15 minutes. After incubation, 150µl FACS Wash was added. Cells were pelleted at 1500rpm for 5 minutes followed by two washes with FACS Wash. Cells were collected in 200µl FACS Wash for flow cytometry analysis. Sample data and appropriate compensation controls were acquired on a BD Fortessa X20 flow cytometer and analyzed using FlowJo Software (Table 1, Supplemental Figure 1).

2.4.8 RT-PCR

At 3 days post infection, 2e6 lymphocytes were harvested into Trizol and an equal volume of DNA/RNA shield (Zymo Research R110-250) was added. RNA extraction was performed using Zymo Directzol Microprep (Zymo Research R2060) according to manufacturer instructions. RNA was eluted in 10µl H2O containing 2U RNase inhibitors and a second DNase step was performed for 30 minutes using the Turbo DNA-Free kit (Invitrogen AM1907M) according to manufacturer instructions. One-step RT-PCR cDNA synthesis and preamplification of GAPDH, LANA and K8.1 transcripts was performed on 15ng of total RNA using the Superscript III One-step RT-PCR kit (ThermoFisher 12574026) and 2µM outer primers for
each target gene as follows: GAPDH outer forward (5’-TCGGAGTCAACGGGATTTGGT-3’),
GAPDH outer reverse (5’- GGGTCTTACT CCTTGGAGGC-3’), LANA outer forward (5’- AATGGGAGCCACC GGTAAG-3’), LANA outer reverse (5’- CGCCCTTAAAC GAGAGGAAGTG-3’), K8.1 outer forward (5’-ACCGTCGGTTGTTGAGGATAA-3’), K8.1 outer reverse (5’-
TCGTGGAACGCACAGGTAAA-3’). Duplicate no RT (NRT) control reactions were assembled for each lineage/sample containing only Platinum Taq DNA polymerase (Thermofisher 15966005) instead of the Superscript III RT/Taq DNA polymerase mix. After cDNA synthesis and 20 cycles of target pre-amplification, 2µl of pre-amplified cDNA or NRT control reaction was used as template for multiplexed real-time PCR reactions using TaqProbe 5x qPCR MasterMix-Multiplex (ABM MasterMix-5PM), 5% DMSO, primers at 900nM and probes at 250nM against target genes as follows: GAPDH forward (5’-TCGGAGTCAACGGGATTTGGT-3’), GAPDH reverse (5’- GGGTCTTACT CCTTGGAGGC-3’), GAPDH probe (5’[HEX]-ACGCCACAGTTT CCCGAGG- [BHQ1]3’), LANA forward (5’-AATGGGAGCCACC GGTAAG-3’), LANA reverse (5’- CGCCCTTAAAC GAGAGGAAGTG-3’), LANA probe (5’ [6FAM]-ACACAAATGCTGGCCAGCCC[G-BHQ1]3’), K8.1 forward (5’-
ACCGTCGGGT GGTGAGGATA-3’), K8.1 reverse (5’- TCGTGGAAACGCACAGGTAAA-3’), K8.1 probe (5’[FAM]-TGCGCGTCTCTTCTCTTAGTGTTG-[TAMRA]3’) and analyzed using a 40 cycle program on a Biorad real time thermocycler. Data is represented as quantitation cycle (Cq) and assays in which there was no detectable Cq value were set numerically as Cq=41 for analysis and data visualization.

2.4.9 Statistical Analysis

Data plots and statistical analysis were performed in Rstudio software (version 7.0) using ggplot , reshape, dplyr, and tidyverse packages. Statistical analysis was performed using R package: rstatix. Specific statistical methods such as independent t-test and Pearson correlations were implemented.
Specific statistical tests and the resulting values are described in detail in the corresponding figure legends.

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<tr>
<th>Table 1: Lineage definitions for lymphocyte subsets used in the study</th>
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CHAPTER III-RESULTS

3.1 Most DC-SIGN+ B-cells are Germinal Centers, Naives, and Transitional Lineages at Baseline

We first wanted to establish the lineage-specific expression of DC-SIGN in tonsil-derived B-cells prior to infection. This analysis allows us to determine if DC-SIGN expression correlates with lineage-specific susceptibility to KSHV infection and also provides a baseline for comparison of infected cultures to determine whether our lymphocyte culture system changes DC-SIGN expression over time. These results reveal that for most tonsil lymphocyte preparations 10-15% of tonsil-derived B-cells were positive for DC-SIGN (Figure 5A). When we performed lineage analysis (Table 1, Supplemental Figure 1) on DC-SIGN positive B-cells, we determined that most B-cell lineages, with the exception of MZ-like, were present within the DC-SIGN+ population and germinal center, naive, and transitional lineages were highly represented (Figure 5B).

3.2 KSHV infection does not alter DC-SIGN expression on B-cells and expression of DC-SIGN on KSHV-infected cells is highly variable.

To determine whether our culture system and/or KSHV infection alters the distribution of DC-SIGN within B cell lineages, we performed KSHV infection and analysis at 3 dpi for 10 tonsil lymphocyte preparations quantitating DC-SIGN+ B cells and the distribution of B cell lineages within the DC-SIGN+ population for both Mock and KSHV-infected cultures. These results reveal that there was no statistically significant change in overall DC-SIGN expression on B lymphocytes in Mock vs. KSHV-infected cultures (Figure 5C, left panel). Moreover, we found that although significant numbers of DC-SIGN+ B cells were present in KSHV-infected cultures, few of these DC-SIGN+ cells were KSHV infected (as shown by the expression of the GFP reporter that is constitutively expressed from the recombinant KSHV genome), indicating that infection is not particularly enriched in the DC-SIGN+ fraction (Figure 5C, right panel). However, since overall
KSHV infection is low in these cultures (~1% of B-cells) we analyzed the data to examine the frequency of DC-SIGN expression on KSHV-infected (GFP+) cells specifically (Figure 5D). This data shows that the presence of DC-SIGN in KSHV-infected lymphocytes is highly variable. Finally, we analyzed the B cell lineage distribution for DC-SIGN+ B cells in Mock and KSHV-infected conditions and found no significant influence of infection on DC-SIGN distribution (Figure 5E). Taken together, these data show minimal impact of infection on DC-SIGN expression and distribution indicate that expression of DC-SIGN within infected cells varies substantially based on donor. Thus these results do not strongly support or refute the hypothesis that DC-SIGN is used as an attachment receptor for KSHV infection in B lymphocytes.

3.3 Neutralization of DC-SIGN increases KSHV Infection of B lymphocytes

In order to more directly determine whether DC-SIGN is required for KSHV entry into B lymphocytes, we performed experiments in which we blocked DC-SIGN using increasing concentrations of a DC-SIGN neutralizing antibody prior to infection and assessed the magnitude and distribution of KSHV infection at 3 dpi. Surprisingly, these results revealed increased KSHV infection at 3 dpi in most samples (Figure 6A). In order to account for the variable susceptibility of tonsil samples to KSHV infection in our model [62], we analyzed this data as a change in GFP using controls with no antibody treatment as a normalization factor. In this analysis we can clearly see that the majority of tonsils included in this analysis show increased infection in response to DC-SIGN neutralization, and the difference was statistically significant (p=0.02) at the 5µg/ml dose (Figure 6B).

Next, we wanted to determine whether neutralization of DC-SIGN influences the distribution of KSHV infection within B-cell lineages. In these experiments, if a particular lineage shows decreased targeting by KSHV in the presence of DC-SIGN neutralization, we can conclude that DC-SIGN is important for viral entry in that cell type. Our results show no statistically significant increase or decrease in KSHV infection of any specific B-cell lineage. However, infection of memory, MZ-
Figure 5: DC-Sign expression in human tonsil with and without KSHV infection. (A) Flow cytometry analysis of DC-SIGN expression on the surface of viable B cells in 11 unique tonsil lymphocyte specimens at day 0 (baseline). (B) Analysis as in (A) showing the distribution of B cell subsets within DC-SIGN+ B cells. Red diamonds indicate the mean of 11 tonsil samples. (C) DC-SIGN expression at 3 days post-infection (dpi) in Mock-infected and KSHV-infected cultures (left panel, n.s.) or in KSHV-infected GFP+ B cells (right panel). (D) Frequency of DC-SIGN expression on GFP+ B lymphocytes in KSHV-infected cultures at 3 dpi. (E) Distribution of B cell lineages within DC-SIGN+ B-cells as in (B) for Mock and KSHV-infected cultures at 3 dpi (n.s.). Where indicated, data point color scheme indicates unique tonsil specimens and can be compared between sub-panel within each portion of the figure.
like and naive B-cells was increased in most of the samples (Figure 6C). These results, therefore, do not identify any B-cell subset in which DC-SIGN is critical for entry.

We next wanted to determine whether increased the overall infection we observed with DC-SIGN neutralization (Figure 6B) was correlated with changes in infection of any B-cell subsets. In order to examine this, we plotted the change in frequency of GFP within B cell subsets against overall change in GFP frequency at the 5μg/ml neutralizing antibody dose and calculated correlation coefficients via the Pearson method (Figure 6D). Although power analysis indicates that these correlations are not strong enough to be statistically significant with this sample number, we observe that samples with increased GFP also displayed increased proportions of infected plasma, MZ-like and memory B-cell lineages. These observations indicate that these particular lineages do not depend upon DC-SIGN for KSHV entry.

3.4 Neutralization of DC-SIGN increases Plasma Cell frequency in KSHV-infected cultures

In order to determine whether the increase in GFP+ B-cells with DC-SIGN neutralization was due to increased frequency or targeting of a specific B-cell subtype, we performed lineage analysis on these samples. Analysis of overall lineage frequencies (infected and uninfected) in these cultures at 3 dpi revealed that most lineages did not change with DC-SIGN neutralization in either the Mock or KSHV-infected conditions (Figure 7A and Figure 7B). However, we did observe a statistically significant increase in the plasma cell population at the 5μg/ml dose only in the KSHV-infected conditions (Figure 7A). This observation is particularly interesting given our recent publication showing that plasma cells are highly targeted by KSHV early in infection [62]. We hypothesized that DC-SIGN neutralization was increasing overall infection by increasing targeting of B-cell lineages that undergo lytic replication, thereby acting as a point of expansion for the virus. To test this hypothesis, we performed RT-PCR analysis for LANA (a latent gene) and K8.1 (a lytic gene) on 8 unique tonsil specimens with and without DC-SIGN neutralization at 5μg/ml. These results show that there is no difference in lytic gene expression between untreated cultures and
Figure 6: Effect of DC-SIGN neutralization on KSHV infection in tonsil B lymphocytes. (A) Flow cytometry analysis of KSHV infection of 10 unique tonsil specimens based on GFP+ B lymphocytes at 3 dpi with indicated doses of DC-SIGN neutralizing antibody (n.s.) (B) analysis as in (A) normalized within samples to the 0µg/ml dose in order to remove between-sample variability. p=0.02 comparing 0µg/ml to 5µg/ml using student’s T-test with Holm correction for multiple comparisons). (C) Analysis of frequency of plasma cells within viable B cells in Mock or KSHV-infected cultures at 3 dpi with indicated doses of DC-SIGN neutralizing antibody. p=0.05 comparing 0µg/ml to 5µg/ml in KSHV-infected conditions using student’s T-test with Holm correction for multiple comparisons. (D) Flow cytometry analysis based on the distribution of GFP+ B lymphocytes within each B-cell lineage at 3dpi with indicated doses of DC-SIGN neutralizing antibody (n.s) (E) Normalized correlation analysis showing linear relationships (Pearson’s correlation coefficient) comparing the change in frequency of GFP within B cell lineages and the change in overall GFP frequency at 5µg/ml neutralizing antibody dose (F) Taqman RT-PCR analysis on 8 unique tonsil specimens with duplicate technical replicates for each reaction condition showing expression of LANA (latent) and K8.1 (late lytic) genes normalized within-reaction to GAPDH housekeeping gene expression (n.s.). Where indicated, data point color scheme indicates unique tonsil specimens and can be compared between subpanels within each portion of the figure.
cultures treated with DC-SIGN neutralizing antibodies (Figure 7C). These results suggest that the mechanism for increased infection with DC-SIGN neutralization is not increased viral spread within the cultures.

3.5 KSHV gH is not required for KSHV infection of tonsil derived B lymphocytes

In a recent study from our laboratory and our collaborators at the City of Hope, a mutant KSHV virus lacking gH, but retaining all of the other KSHV viral envelope glycoproteins (KSHV-ΔgH) was generated to characterize the requirement of gH in KSHV entry for adherent cells such as epithelial cells, endothelial cells, a B-cell line (MC116) and fibroblasts. Results revealed that gH is required for entry in the adherent cells, but was not required for entry into the MC116 lymphoma cell line[111]. These results suggest that gH may be dispensable for entry into B-cells. In order to test this hypothesis in our primary tonsil lymphocyte system, we utilized the same mutant virus that was constructed for this previous study, which lacks gH but retains all the other KSHV glycoproteins (KSHV-ΔgH). We performed infections with 7 unique tonsil lymphocyte samples and performed infections with KSHV-WT, KSHV-ΔgH and Mock infection as described above, and assessed the distribution of KSHV infection at 3 dpi using flow cytometry. It should be noted that because KSHV-ΔgH cannot infect fibroblasts we cannot use our normal system for functional titration of the virus stock to normalize infection. Thus, for these experiments we performed titration of the KSHV-WT stock as usual on fibroblasts to determine the optimal infectious dose, and then used genome quantitation of both KSHV-WT and KSHV-ΔgH virus stocks in order to calculate an equivalent genome dose for KSHV-ΔgH. These results reveal that, overall in the data set, KSHV-ΔgH infects tonsil-derived B cells at levels comparable to those seen with KSHV-WT. However, within tonsil samples, 4 of 7 samples showed higher levels of GFP+ B lymphocytes in KSHV-ΔgH compared to WT (Figure 8A). We wanted to determine whether the difference in infection efficiency with KSHV-WT vs. KSHV-ΔgH was related to the levels of B-cell subsets in the tonsil samples at the time of infection. To do this analysis, we calculated the difference between the percent of GFP+ B cells with each virus on a per-sample
Figure 7: Neutralization of DC-SIGN increases Plasma Cell frequency in KSHV-infected cultures. Analysis of frequency of lineages within viable B cells in Mock (A) or KSHV-infected (B) cultures at 3 dpi with indicated doses of DC-SIGN neutralizing antibody at PC has p=0.05 comparing 0µg/ml to 5µg/ml in KSHV-infected conditions using student’s T-test with Holm correction for multiple comparisons. (C) RT-PCR analysis of KSHV transcripts at 3dpi with indicated doses of DC-SIGN neutralizing antibody (n.s.).
basis (GFP in KSHV-ΔgH - GFP in KSHV-WT) and compared this value to the sample-specific baseline frequency of each B-cell lineage. This data reveals that KSHV-ΔgH infectivity is highly correlated with baseline levels of double negative B cells (an atypical memory B cell subtype), but that KSHV-ΔgH had lower infectivity in samples with high levels of transitional B cells (Figure 8B). These results may suggest that KSHV infection of transitional B-cells relies more heavily upon gH-dependent entry mechanisms compared to entry into double negative B-cells. When we examined the distribution of GFP within B-cell lineages at 3 dpi for these experiments, we found that there was no significant difference in B-cell targeting between KSHV-WT and KSHV-ΔgH (Figure 8C). Finally, we wanted to determine whether the distribution of B-cell lineages within the infected (GFP+) fraction was different between KSHV-WT and KSHV-ΔgH. This analysis showed that there was also no significant difference between KSHV-WT and KSHV-ΔgH infection (Figure 8D). Thus, neither the amount of infection within any B-cell subset nor the proportion of B-cell subsets within the infected fraction differed between KSHV-WT and KSHV-ΔgH. Taken together, these results indicate that KSHV-ΔgH (a virus which is globally defective in non-B cell types) can infect primary B cells, and displays similar infectivity and B cell lineage tropism when compared to KSHV-WT. We would conclude from this data that overall susceptibility of individual tonsil specimens to infection with KSHV-ΔgH is dependent upon the B-cell composition of the specimen, suggesting that gH does play a role in entry into B lymphocytes. However, gH is not strictly required for KSHV entry into any B-cell lineage, suggesting that this glycoprotein does not play a pivotal role in KSHV infection of B lymphocytes in human tonsil.

3.6 Depletion of DC-SIGN in KSHV-WT and KSHV-ΔgH infections reveals multiple, entry mechanisms for KSHV in tonsil B lymphocytes

In order to examine whether KSHV-ΔgH uses DC-SIGN to enter into B lymphocytes and to validate our earlier DC-SIGN neutralization data (Figure 6) via another method, we performed experiments with 6 unique tonsil specimens where we isolated total B lymphocytes and performed a second step where we separated DC-SIGN+ and DC-SIGN- B-cells. Because DC-SIGN+ cells
Figure 8: KSHV-∆gH infection in tonsil B lymphocytes. (A) Flow cytometry analysis of 7 unique tonsil specimens infected with KSHV-WT and KSHV-∆gH based on GFP+ B lymphocytes at 3 dpi (B) Correlation analysis showing linear relationships (Pearson’s correlation coefficient) between baseline frequency of B-cell lineages and the change in GFP between KSHV-∆gH and KSHV-WT on a per-sample basis. Power analysis indicates with this sample number correlations with r≤0.86 are statistically significant. (C) Distribution of KSHV-WT and KSHV-∆gH infected cells (GFP+) within B cell lineages at 3 dpi (n.s.) (D) Distribution of B-cell lineages within infected (GFP+) fraction for KSHV-WT and KSHV-∆gH. Red diamonds indicate mean for each condition (n.s.). Where indicated, data point color scheme indicates unique tonsil specimens and can be compared between sub-panels within each portion of the figure.
represent a minor population in tonsil B-cells (Figure 5A) we did not have sufficient cell numbers
to infect the populations separately. Therefore we adopted a strategy where we either infected
DC-SIGN-depleted populations or reconstituted the depleted population with DC-SIGN+ B cells
prior to infection. Analysis of KSHV infection in these cultures revealed that KSHV-WT infection
is uniformly higher in DC-SIGN reconstituted cultures compared to DC-SIGN depleted cultures.
This trend was similar in KSHV-ΔgH infected cultures with the exception of one tonsil sample
in which infection was increased in reconstituted culture for KSHV-WT and decreased in the
same condition with KSHV-ΔgH (Figure 9A). Next, we examined the distribution of GFP within
B-cell lineages for DC-SIGN depleted vs. reconstituted cultures in order to determine whether
DC-SIGN is required for entry into any particular B-cell subset. Consistent with our neutralization
data, depletion of DC-SIGN did not abolish infection of any B-cell lineages with KSHV-WT.
Consistent with our previous analysis (Figure 8) there was no statistically significant difference
in infection comparing WT to ΔgH for either depleted or reconstituted fractions. For KSHV-WT,
although infection of most lineages tended to be higher in the reconstituted fractions, there was
no significant difference between DC-SIGN depleted and reconstituted cultures for any lineage
with KSHV-WT. Results were similar for KSHV-ΔgH with the exception of centrocytes (the
CD77 negative sub-population of germinal center B cells) where infection was absent in the DC-
SIGN depleted culture vs. the DC-SIGN reconstituted culture (p=0.03) (Figure 9B). These results
support our previous conclusions that DC-SIGN plays a minor role as a receptor for KSHV entry
into tonsil-derived B cells, and that the KSHV glycoprotein gH is not required for entry into any
B-cell lineages. However, our data reveals that in the absence of both the DC-SIGN receptor
and the gH glycoprotein, KSHV cannot infect centrocytes. This implies that KSHV has only two
entry mechanisms for centrocytes: (1) a gH-dependent mechanism that uses a receptor other than
DC-SIGN, and (2) a gH-independent mechanism that uses DC-SIGN as the receptor. In KSHV-
WT, both entry mechanisms are functional and no difference is seen when DC-SIGN is depleted
because the gH-dependent entry mechanism is intact. When gH is absent but DC-SIGN is present
(KSHV-ΔgH reconstituted) entry into centrocytes is mediated by the interaction of another KSHV-
gp and DC-SIGN. When gH is absent and DC-SIGN is absent, KSHV has no entry mechanism for centrocytes (Figure 9C).

Taken together, our examination of the roles of KSHV-gH and cellular DC-SIGN reveals that:

1. KSHV-gH is not required for entry into tonsil-derived B lymphocytes.
2. KSHV-gH can participate in KSHV entry into centrocytes via a DC-SIGN independent entry mechanism.
3. DC-SIGN is not necessary for KSHV entry into any B-cell lineage.
(4) DC-SIGN does play a role in KSHV entry into tonsil-derived B-cells, but in all B-cell subtypes alternative entry mechanisms exist.

Our results presented in this study provide a first glimpse into the complexity of KSHV entry in the lymphocyte compartment and highlight that multiple lineage-dependent entry mechanisms are employed by KSHV which depend upon multiple cellular receptors and multiple KSHV glycoproteins. Further studies are needed to establish the roles of additional receptor-gp interactions in these important cell types in order to rationally design therapies and vaccine strategies that will effectively limit KSHV entry into, and spread within, the human immune system.
CHAPTER IV-DISCUSSION & FUTURE DIRECTIONS

DC-SIGN is deemed important for the body’s innate and adaptive immune system for many reasons. DC-SIGN has the responsibility of recognizing and capturing pathogens, facilitating dendritic cell signaling, adhesion and migration, and overall initiating T cell responses [92]. DC-SIGN is highly expressed in cells such as dendritic cells (DCs), dermal and mucosal tissues, monocytes, macrophages, B lymphocytes from peripheral blood, and tonsillar B lymphocytes [88, 89]. The role of DC-SIGN as a receptor for various viruses such as HIV-1, HCV, Ebola virus, CMV, dengue virus, and the SARS coronavirus has been well characterized. DC-SIGN is known to facilitate viral entry for KSHV, in human myeloid dendritic cells, macrophages, and activated peripheral blood B-cells.

However, the existing literature on how DC-SIGN facilitates KSHV entry in tonsil derived B lymphocytes is highly unknown. The only known study to perform an in-depth analysis on the role of DC-SIGN as an entry receptor for KSHV infection was performed by Rappocciolo et. al. in 2008 [89]. This particular study utilized peripheral blood B-cells that were activated with CD40 ligand (CD40L) and interleukin 4 (IL-4) prior to infection to manipulate the proliferation of cells in vitro. Wild-type KSHV virons derived from BCBL-1 PEL cells were used to infect activated peripheral blood B-cells, and RT PCR was utilized to quantitate the copies of KSHV DNA present in the cells after KSHV infection. In contrast, our studies utilized iSLK derived BAC16 recombinant KSHV virus and resting tonsil derived B lymphocytes with no additive stimulant due to our recent publication[62] showing that tonsil derived B lymphocytes do not need to be activated for infectivity within our culture model. Importantly, we utilized multi-color flow cytometry for our analysis method which allows us to see KSHV infection using the constitutively-
expressed GFP reporter present in the BAC16 genome alongside surface antigen markers for B cell lineages, allowing us to examine not only the total amount of infection in all B-cell, as was done in Rappocciolo et. al., but also infection of specific B-cell subtypes on a per-cell basis. Thus, our studies, for the first time ever, provide an in-depth report into the role of DC-SIGN for KSHV entry in tonsil derived B lymphocytes that provides insight into whether DC-SIGN influences the B-cell lineage-specific tropism of KSHV. Moreover, although DC-SIGN is known to be expressed on B cell in both peripheral blood and tonsil, this study establishes the B-cell lineage distribution of DC-SIGN in tonsil for the first time and shows that DC-SIGN expression is variable based on donor, and most DC-SIGN+ B-cells in tonsil are germinal center, naive, and transitional phenotypes. When we employed a neutralizing antibody to disrupt any interaction between DC-SIGN and KSHV glycoproteins, we observed significantly increased KSHV infection at the highest dose (5µg/ml), which was also the optimal dose for inhibition of DC-SIGN - ICAM3 interaction during characterization of the neutralizing antibody by the manufacturer. These results differ from those reported in Rappocciolo et. al., where use of the same anti-DC-SIGN neutralizing antibody clone but at much higher concentrations (20µg/ml) effectively blocked KSHV infection of activated peripheral B-cells[89]. Our analysis of the distribution of KSHV infection within B-cell lineages did not reveal any change in KSHV targeting of B-cells with DC-SIGN neutralization, and RT-PCR results show no increase in lytic replication in culture where the neutralizing antibody was used. Thus our data provides no evidence that the increase in overall infection with DC-SIGN neutralization is a result of altered KSHV targeting or spread. We did observe an increase in total plasma cell numbers in DC-SIGN neutralized, KSHV-infected lymphocyte cultures. Our previous work has shown that our lymphocyte culture system does not favor the survival of plasma cells but KSHV infection increases overall plasma cell numbers at 3 dpi. Recently, activation of DC-SIGN signaling via antibody binding was shown to promote survival of B lymphoma cells [113]. Thus, the combination of DC-SIGN neutralization and KSHV infection may synergistically promote the survival of plasma cells in our culture system and we speculate that the presence of plasma cells promotes KSHV infection via unknown mechanisms that are possibly related to the cytokine
milieu. Overall our results collectively show that DC-SIGN is not required for KSHV entry into any B-cell lineage.

The KSHV viral envelope is known to consist of conserved viral glycoproteins: gB, gH, K8.1 and gL found in all herpes viruses. These specific glycoproteins are thought to mediate KSHV attachment, fusion and entry into host target cells. To date, it is unknown which viral glycoproteins are essential for KSHV entry in tonsil derived B lymphocytes. We recently showed that gH is essential for KSHV entry into epithelial cells, endothelial cells and fibroblasts but is not required for entry into the MC116 B-cell lymphoma cell line. The current study extends these findings and shows that gH is also not required for entry into primary tonsil lymphocytes. Interestingly, our results revealed tonsil donor-specific differences in the infectivity of KSHV-ΔgH compared to KSHV-WT, and that these differences are correlated to the frequencies of transitional and double negative B-cells in the original tonsil sample. Moreover, our experiments in which we employed depletion of DC-SIGN+ B-cells show that there is a gH-dependent mechanism for KSHV entry into centrocytes. These data collectively demonstrate that, although gH is not strictly required for entry into any B-cell type, there are gH-dependent entry mechanisms in tonsil-derived B-cells.

Our work presented here highlights that gH and DC-SIGN are not critical for entry in tonsil derived B cells, but both factors can play a role in KSHV entry of specific B-cell lineages. Our results clearly indicate that KSHV has multiple diverse entry mechanisms in tonsil lymphocytes which remain to be established.

Our study does have some limitations that require further research. In particular, for the neutralization experiment we chose to utilize a neutralizing antibody that may not be optimal for the inhibition of DC-SIGN and KSHV glycoprotein interactions. The anti-DC-SIGN antibody we utilized is optimized to neutralize the interaction of DC-SIGN with ICAM-3/CD50. It should be noted that the DC-SIGN receptor is a tetramer protein composed of four individual domains: the C-terminal carbohydrate recognition domain (CRD), the neck-repeat region, the transmembrane domain, and the N terminal cytoplasmic tail [114]. Due to DC-SIGN’s role in the process of viral infection and its structural complexity, many groups believe that blocking the sugar binding site, I-CAM-3
epitope in the carbohydrate recognition domain (CRD) will overall inhibit DC-SIGN and not allow the virus to enter [114]. This assumption is believed to be true because the ICAM-3 epitope is located in the center of the carbohydrate recognition domain where most molecular processes are mediated in the DC-SIGN receptor [114]. Importantly, the biochemistry of any interactions between DC-SIGN and KSHV glycoproteins has not been established. The fact that our results show increased infection with DC-SIGN neutralization could imply that the neutralizing antibody may be binding to a different epitope on DC-SIGN and that the antibody is not neutralizing the actual interaction of the viral glycoproteins to DC-SIGN. More research is needed to decipher the real interactions between DC-SIGN and KSHV glycoproteins to be able to fully neutralize those specific interactions. It will be interesting in future studies to explore the actual interactions of the virus with the host cell receptor by utilizing a Fluorescence Resonance Energy Transfer (FRET) immunoassay to detect and track the specific interactions and subsequently develop accurate neutralizing antibodies against the neutralizing epitopes on DC-SIGN. Moreover, our results with DC-SIGN neutralization highlight the need to better establish the role of DC-SIGN signaling in our tonsil lymphocyte cultures and its impact on KSHV infection.

Our study is also limited on the fact that only one conserved glycoprotein for KSHV was studied, glycoprotein gH. Future studies are needed to determine if the other conserved glycoproteins: gB, gM, gN, and gpK8.1 are essential for KSHV entry. In order to study this, mutant viruses for each individual glycoprotein need to be produced. With the production of these mutant viruses we will have the opportunity to unravel different entry mechanisms for KSHV in tonsil derived B-cells and to determine what viral glycoproteins are essential for entry. Furthermore, studies are needed to establish the roles of additional receptor-gp interactions in these important cell types in order to rationally design therapies and vaccine strategies that will effectively limit KSHV entry into, and spread within, the human immune system.
Supplemental Figure 1: Gating scheme for B cell lineages by flow cytometry

Defined B cell lineages
(1) All CD19+
(2) CD19+CD20-
(3) CD19+CD20+
(4) All plasma cells
(5) CD20- plasma cells
(6) CD20+ plasma cells
(8) Transitional
(9) Plasmablast
(10) Germinal Center
(11) Naive
(12) MZ-like
(13) Memory
(14) Double negative
REFERENCES

56. Totonchy, J., Extrafollicular activities: perspectives on HIV infection, germinal center-


73. Garrigues, H.J., et al., KSHV attachment and entry are dependent on αVβ3 integrin


