HUMAN HERPESVIRUS-8 INTERACTIONS WITH DENDRITIC CELLS

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Human herpesvirus-8 (HHV-8, also known as Kaposi's sarcoma associated herpesvirus, KSHV) is a gamma-2 herpesvirus and is the etiological agent of Kaposi's sarcoma, primary effusion lymphoma and a subset of Multicentric Castleman's disease. We have previously shown that HHV-8 uses DC-SIGN (CD209) for entry into susceptible cell types, including immature dendritic cells. In the present study, we demonstrate that DC-SIGN expression renders previously non-permissive cells permissive to HHV-8 infection. Also, we have demonstrated that HHV-8 infection of dendritic cells and endothelial cells results in the expression of some viral lytic proteins initially but subsequently switches to only latent protein expression. However, infection appears to be non-productive as the infected cells maintain viral DNA copies at a low level but this level does not increase over time, nor is encapsidated viral DNA found in the supernatant. Secondly, we demonstrate that the glycoprotein B homologue of HHV-8 binds to DC-SIGN in a dose-responsive manner and that DC-SIGN binds HHV-8 in a region of the carbohydrate recognition domain that is unique, though overlapping, with the HIV-1 gp120 and ICAM-2/3 binding sites. Lastly, we demonstrate that infection of immature DC results in the expression of IL-6, TNF-α, MIP-1α, MIP-1β, and IL-12p40, but not bioactive IL-12p70. This cytokine release occurs quickly after infection and is maintained for up to 72 hours post-infection, suggesting that virus binding is sufficient for at least some of the cytokine release and that the virus may be active in skewing infected cells to illicit a T_H2 response. The significance of these findings from a public health standpoint centers on the fact that while HHV-8-related cancers have decreased in incidence in the United States, they still represent a serious global health concern in other countries. Our findings give insight into the initial interactions of HHV-8 and its target cells and

as a result, can be used for the design of targeted therapies to prevent viral infection and spread.

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Preface

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1.0 Chapter 1: Introduction

1.1 Human Herpesvirus 8

Human herpesvirus 8 (HHV-8, also known as Kaposi's sarcoma-associated herpesvirus, KSHV) is a gamma herpesvirus and is the causative agent of Kaposi's sarcoma (KS), primary effusion lymphoma (PEL) and a subset of Multicentric Castleman's disease (MCD). The virus was first discovered in 1994 by Chang and Moore who used a representational difference assay to show the presence of unique herpesviral DNA sequences in the tissues of patients with KS, which they then named KSHV[35]. While KS had been identified in 1872 by Moritz Kaposi, it was considered a rare cancer, affecting mostly elderly men of Mediterranean decent (termed Classical KS) [106]. Rare cases were also observed in transplant recipients (termed Iatrogenic or post-transplant KS) and it has also been shown to be widespread in sub-Saharan Africa (Endemic KS)[82,205]. In the early to mid-1980s, however, new cases of KS were observed in young men who showed none of the previous risk factors for KS[67,86,99]. It was later found that these young men were the first AIDS cases, and, while at the time it was not understood whether HIV was involved in the development of KS, the cancer was listed as one of the defining illness for AIDS. It was only with Chang and Moore's discovery that it was understood that KS resulted from a co-infection in these patients with both HIV and HHV-8.

HHV-8 is most closely related to Rhesus Rhadinovirus (RRV), while its nearest human herpesvirus relative is Epstein-Barr virus (EBV, HHV-4).[6] While all human herpesviruses share considerable homology, HHV-8 has higher homology to gamma-herpesviruses, but is distinct as it contains several genes unique to itself.

Like other herpesviruses, HHV-8 is an enveloped virus with a large DNA genome, specifically encoding for over 80 proteins[178]. The genome consists of a 140.5kb region with GC-rich terminal repeats on each side[178]. The virus contains many genes which are conserved among herpesviruses and have been named accordingly, but also contains at least 15 genes unique to HHV-8 among the herpesviruses which have been designated as such with the prefix "K". Most of these unique genes show homology to human cellular genes and act in subversion of cellular responses to HHV-8. For example, the K3 and K5 gene products are ubiquitin ligases which assist in immune evasion by causing the degradation of MHC and other immune-related molecules, and the K13 gene product is a virally encoded FLIP (FLICE (FAS-associated death-domain-like IL-1beta-converting enzyme) -inhibitory protein) which functions in apoptosis avoidance.[15,45,100,128] The majority of viral proteins are expressed only during active viral replication and a few in the early stages of non-productive infections.[115,172] Only five genes have been shown to be associated with the persistent, or latent, stage of the viral life cycle: LANA-1 (Latency-associated nuclear antigen-1, ORF 73), viral cyclin D (ORF72), kaposin (ORF K12), vIRF-2 (viral Interferon Regulatory Factor-2, LANA-2, ORFK11.1) and vFLIP (ORF K13).[26,56,108,115,149,170]

Like other herpesviruses, HHV-8 encodes a variety of glycoproteins that are expressed on the virion. There are 6-8 HHV-8 virion-associated glycoproteins known to date: the herpesvirus conserved glycoproteins gB, gH, gL, gM, gN, the HHV-8 unique glycoprotein K8.1A and the gene products of ORFs 28 and 68, which are predicted to be glycoproteins (Table 1).[233] Both ORF28 and ORF68 gene products do not currently have demonstrated functions in HHV-8, but have been predicted to be glycoproteins based on their homology to the EBV proteins BDFL3 (gp150) and BFLF1 (UL32 family glycoprotein), respectively. Prior studies on HHV-8 and other herpesviruses have demonstrated that the gH and gL glycoproteins form a complex, as do gM and gN. Expression of gH on the cell surface requires co-expression of gL and

Table 1: HHV-8 glycoproteins, function and homologues.

Glycoprotein	ORF	Conservation	Homologues	Function
gB	8	Alpha, beta, gamma	All gB	Attachment, infection
K8.1	K8.1	Unique	None	Attachment
gH	22	Alpha, beta, gamma	All gH	Fusion?
gL	47	Alpha, beta, gamma	All gL	Fusion?
gM	39	Alpha, beta, gamma	All gM	Assembly, Egress?
gN	53	Alpha, beta, gamma	All gN	Assembly, Egress?
?	28	gamma	MHV-68 ORF 28, EBV gp150	Unknown
?	68	?	EBV BFLF1	Unknown

antibodies to both glycoproteins can neutralize virus infectivity, but do not inhibit binding of virions to the cell surface.[151] In other herpesviruses, gL is a soluble protein that requires expression of gH for membrane anchoring.[58] gH on the other hand, is a single membrane spanning protein with several hydrophobic domains shown to be involved in fusion activities.[85] The gM and gN glycoproteins also form a complex in the infected cell. Koyano and colleagues have reported that the gM/gN complex may play a role in virus entry and egress through the modulation of viral glycoprotein trafficking and/or membrane fusion.[114] Herpesviral homologues of gM appear to be multi-membrane spanning, are mostly non-essential components of the virion and have low or no glycosylation.[27,55] Homologues of gN appear to be type I glycoproteins, but have very small extracellular domains and appear to have functions in viral

assembly and egress.[137] The K8.1A glycoprotein has been reported to bind to heparan sulfate in the early stages of viral attachment. It has been shown to contain a complex glycan structure and a recent study reported that K8.1A was not essential for virus replication[135]. gB has been the most studied HHV-8 glycoprotein and has been shown to have multiple roles. Firstly, gB has been shown to bind heparan sulfate for attachment similar to K8.1A[3]. Additionally, gB is essential for egress from cells[117], and antibodies directed against gB can neutralize virus infectivity.[3] Furthermore, gB activates Focal Adhesion Kinase (FAK) signaling and cytoskeletal rearrangements[188]. Specifically, gB binds integrins through its RGD sequence which phosphorylates FAK and induces downstream actin rearrangements via RhoA and cdc42 Rho GTPases in a Src-dependent manner, which appears to activate transport of viral DNA to the nucleus[153,188,216]. These events appear to be required for entry of HHV-8 into target cells and requires MEK and ERK cascade induction[116,152,187]. Additionally, gB is a type I glycoprotein with a large extracellular domain and is highly mannosylated[11].

1.1.1 Viral Entry

In vivo, HHV-8 appears to have a limited cell tropism. In the context of the HHV-8-related cancers, viral DNA can be found in many tissues throughout the body, but represents the presence of the cancer in those compartments rather than infection of tissue specific cell types. Thus far, HHV-8 has been found in B cells, endothelial cells, CD34+, and monocytic cells *in vivo*[21,22,94,174]. *In vitro* infections of various primary and transformed endothelial cells are well documented in the literature[20,43,120,147,162]. On the other hand, *in vitro* infection of primary or cultured B cells had been unachievable until the discovery that CD40L and IL-4 activation of primary B cells causes the cells to become susceptible to infection through the upregulation of the viral receptor, DC-SIGN[20,171]. Our lab and others have also shown that HHV-8 can infect cells of monocyte lineage, including monocyte-derived dendritic cells (MDDC) and IL-13 differentiated macrophages *in vitro*[20,172]. Recent studies have also shown that human hematopoietic progenitor CD34+ cells can also be infected by HHV-8 *in vitro* and a SCID mouse

model corroborates the *in vivo* tropism observed in KS patients. [94,228] Studies have shown that keratinocytes, primary and immortalized epithelial cells, and human foreskin fibroblasts (HFF) can also be infected by HHV-8 *in vitro* [2,5,34,59] Others have also shown that the limited tropism of HHV-8 can be expanded to include many other laboratory adapted cell lines through the use of polybrene[14]. Polybrene is a cationic polymer which neutralizes membrane charge and causes virus aggregation, resulting in an entry mechanism that is independent of interaction of the virus with the surface receptor[48,49]. Therefore, those cell lines that have been shown to be infected with HHV-8 with the addition of polybrene do not necessarily reflect natural targets for the virus either *in vitro* or *in vivo*.

Initial interactions of a virus and the cell surface can vary in complexity from binding of a single viral protein to a single cell surface protein, to an intricate process in which several viral proteins interact with one or more cell surface molecules in a coordinated and precise, albeit sometimes redundant, sequence. Herpesviruses utilize complex multi-step binding and entry processes involving binding, entry and in some cases, separate fusion receptors[30,92,196]. Binding receptors serve to concentrate the virus on the surface of the cell, providing better access to entry receptors. This virus-binding receptor interaction is reversible and not essential for virus entry. Most herpesviruses use surface glycosaminoglycans, typically heparan sulfate, as their initial binding receptor[92,196]. In contrast, entry receptors are involved in viral entry by either triggering fusion between the viral envelope and cell membrane or by inducing endocytosis with subsequent membrane/envelope fusion inside of the endosome. Endosomal fusion in many cases requires a pH change and a possibly a separate fusion receptor. Binding to entry receptors is often irreversible, is required for virus entry, and can involve more than one viral glycoprotein. Three major criteria for defining an entry receptor are: 1) The receptor must be naturally present on cells that can be entered by the virus, and not present on those that cannot

(unless the virus uses multiple entry receptors); 2) Entry can be blocked by interference with the receptor (antibody, siRNA, etc.); and 3) Artificial addition of the receptor onto a non-permissive cell type renders that cell line permissive for entry and binding[190].

HSV-1 entry is the most characterized of all of the herpesviruses. HSV-1 glycoprotein C (gC) initially attaches to heparan sulfate, though gC is non-essential and can be replaced by glycoprotein B (gB) in proteoglycan attachment.[95] Subsequently, glycoprotein D (gD), which is unique to alphaherpesviruses, binds to one of three entry receptors: Nectin-1; herpesvirus entry mediator (HVEM); or 3-O-Sulfotransferase modified Heparan Sulfate (3-O-HS).[118,189,226] It is then thought that the binding of gD triggers the fusion-related glycoprotein complex, gB, gH and gL, to induce fusion with the cellular membrane.[92] It is undetermined whether gB interacts directly with a cellular protein, but association with lipid rafts has been observed.[16] Glycoprotein H has also been shown to bind to $\alpha v\beta 3$ integrin, though the function of this binding is unknown.[164] The minimum requirements for HSV entry are gD, gB, gH and gL, and at least one of the cellular entry receptors.

In the case of HHV-8, both the viral glycoprotein B homologue (gB, ORF 8) and the unique K8.1A glycoprotein can interact with heparan sulfate, a binding receptor.[3,19,219] In each case, binding is reversible and not necessary for infection, similar to the heparan sulfate interaction with other herpesviruses.

Akula et al. have shown that HHV-8 gB binds to integrin $\alpha 3\beta 1$ (CD49c/29) via an internal RGD motif and uses this integrin protein as a post-binding replication factor for *in vitro* infection of endothelial cells and human foreskin fibroblasts (HFF).[4] HHV-8 infectivity of HFF cells was inhibited with peptides containing the gB RGD motif. However, the study also suggests that the $\alpha 3\beta 1$ integrin is not solely responsible for viral entry into target cells as virus binding and infectivity could only be partially blocked by anti-integrin antibodies. Possibly more importantly, this study demonstrated that binding of gB to the integrins through the RGD motif resulted in

activation of the focal adhesion kinase (FAK) pathway.[4,220] Thus, attachment of gB to integrins appears to be a post-binding event that may be important in cell signaling, but its role in viral entry is unclear. Because of their ubiquitous expression, integrins do not seem to be likely candidates for the singular entry receptor of HHV-8, which has been shown to have a limited host cell range. In fact, the cellular distribution of $\alpha 3\beta 1$ integrin does not correspond with HHV-8 permissive cell types.[172] Similarly, Garrigues et al. has shown that gB binds the $\alpha v\beta 3$ integrin via its RGD motif and that this integrin may also function in the entry of HHV-8 into some cell types in a comparable manner.[74]

Another lab has recently suggested that xCT, a cystine transport molecule, serves as the entry and fusion receptor for HHV-8. Kaleeba et al.[104,105] have reported that using an *in vitro* fusion assay, cells expressing HHV-8 glycoproteins can fuse with cells expressing xCT. This study also shows that over-expression of xCT in cell lines can cause an increase in virus infection of these cells and that antibody against xCT could partially block virus infection. However, levels of fusion seen in particular cell types only partially correlate with the amount of xCT mRNA expression in those cells. More importantly, cell types that can be readily infected *in vivo* and *in vitro*, like human microvascular endothelial cells (HMVEC), express large amounts of xCT mRNA, but have little fusion activity using the assay, while those cell types that cannot be infected without the use of polybrene (which bypasses receptor usage), such as Mel-1700, express high amounts of xCT mRNA and show high rates of fusion, suggesting that HHV-8 susceptibility, xCT expression and fusion activity do not correlate well[104,105]. In addition, the work of Kaleeba et al. indicates that fusion between the HHV-8 viral envelope and cell membranes must occur at the level of the cellular membrane, at a neutral pH. This is contrast to work from the Chandran laboratory clearly demonstrating endocytosis of HHV-8 during infection of fibroblast cells.[2] This same group has also shown that xCT and CD98, a molecule known to co-localize with xCT, forms complexes with integrins $\alpha 3\beta 1$, $\alpha v\beta 3$, and $\alpha v\beta 5$ and that

xCT/CD98 is not required for entry of HHV-8, but as antibodies against these molecules suppressed viral gene expression, may serve a function post-entry[215]. Lastly, syncitia formation has not been described in HHV-8 infection or reactivation, as has been demonstrated with other viruses that fuse at the cell surface under neutral pH, disputing the hypothesis that HHV-8 fuses at the surface of the cell as predicted in the xCT model. It is for these reasons that we believe that neither $\alpha 3\beta 1$, $\alpha v\beta 3$, nor xCT satisfy the aforementioned criteria for entry receptors[190] of HHV-8, but may, however, serve other important purposes in viral infection or have overlapping function in binding.

We have recently determined that HHV-8 binds to the c-type lectin, DC-SIGN (CD209).[172] DC-SIGN and the related molecule, DC-SIGNR (also called L-SIGN), are pathogen pattern recognition receptors shown to initiate the uptake of several bacteria, fungi, and viruses[79,90,191], by binding to high mannose-containing glycoproteins. We have shown that HHV-8 binding and infectivity can be significantly blocked using anti-DC-SIGN antibodies or mannan (a natural ligand for DC-SIGN). Furthermore, cell lines not previously permissive to HHV-8 infection become permissive upon transfection of DC-SIGN-expressing plasmids. Most strikingly, however, DC-SIGN is expressed *in vitro* on a subpopulation of all cell types shown to be infected *in vivo*, specifically, interleukin 4 (IL-4)/CD40 ligand (CD40L) activated B cells, IL-13 activated macrophages and endothelial cells.[171,172] Also, DC-SIGN is expressed on a significant subset of cell types that can be infected under *in vitro* conditions: keratinocytes, mesenchymal stem cells, and most recently, MDDCs ([171,172] and data not shown). Thus, DC-SIGN is the only currently described HHV-8 receptor that satisfies all the previously mentioned criteria required for it to be a viral entry receptor for HHV-8[190].

The HHV-8 viral ligand for DC-SIGN is yet unknown. Strong candidates for a viral ligand of a cellular receptor (also termed the viral attachment protein, VAP) typically have large extracellular domains, are generally type I glycoproteins, and absolutely must be components of the virion.

1.1.2 Viral Replication

HHV-8 infection of most cell types *in vitro* results in the production of a limited number of lytic transcripts produced, without virus progeny production[115,172]. As a method for *in vitro* productive infection of any cell line has only recently been demonstrated[171], the temporal pattern of viral gene expression is unknown as yet. Various studies have investigated the genes involved in phorbol ester-induced reactivation of primary effusion lymphoma (PEL) lines, giving a preliminary outline to alpha, beta and gamma-designated genes[103,115,131], however it has yet to be determined whether this outline will match up with the gene cascade of natural infection. It is known that the major replicative switch protein of HHV-8 is the Rta (Regulator of Transcription Activation) homologue (ORF50) and has been shown to activate a large number of viral genes[225]. Various studies have shown that expression of Rta alone is sufficient for reactivation of latently infected cell lines[87,133,134].

As previously mentioned, HHV-8 latency is characterized by the expression of the five latency associated proteins (LANA-1, vCyclin, kaposin, vIRF-2 and vFLIP) and a lack of progeny virus production. LANA-1 appears to function in tethering the viral episome to the host chromatin, maintenance of latency, regulation of host cellular pathways, and immune evasion[217]. vCyclin is a viral homologue of the cellular cyclin-D protein, and functions as a surrogate in cellular cyclin cascades for cell cycle regulation[123]. Kaposin is thought to function in transformation[149] and has recently been shown to have a role in the stabilization of cellular cytokine mRNAs[141]. vIRF-2, also known as LANA-2, functions to regulate early immuno-responsive genes via suppression of interferon induced PKR[26,69]. Lastly, vFLIP, is a viral FLICE (FAS-associated death-domain-like IL-1beta-converting enzyme) inhibitory protein which acts to inhibit apoptosis through activation of I_KB kinase[127]. As described, it is possible to see how these proteins work together to create a cellular environment in which it is easy for the virus to persist by acting in immune evasion and anti-apoptosis.

1.1.3 Pathogenesis

Like other herpesviruses, HHV-8 has the ability to persist in the host through the establishment of a lifelong non-productive latent state, characterized by the expression of the latency associated proteins and the lack of progeny virion production. Presumably, in the case of a primary infection with HHV-8, the virus goes through some level of lytic replication to set up a sufficient reservoir of virus in the host and then switches to a latency program for maintenance. The difficulty in determining this lies in the fact that primary infections with HHV-8 are largely asymptomatic, with only a small proportion of persons citing a rash[181], lymphoadenophathy, or flu-like symptoms[222]. It appears that infected persons may experience a period reactivation of the virus but that this phenomenon is likely infrequent[222].

The seroprevalence of HHV-8 appears to be much lower than other human herpesviruses, some of which are near ubiquitous. Reports of HHV-8 seroprevalence in the general population vary in the US between 5-10%.[1,102] However, infection rates are higher in other geographic areas, such as Africa (over 40%[60,122,192]), the Mediterranean[32,33,39], and South America[52,143,195], or in specific groups, such as homosexual men (HIV negative population 20-38%, 30-48% total homosexual population[140]).

As already stated, HHV-8 was first discovered as the causative agent of Kaposi's sarcoma. KS occurs in four types: Classical which occurs in elderly men of Mediterranean decent, AIDS-related, transplant-related, and endemic which occurs in otherwise healthy persons in Africa[111]. In recent years, the incidence of AIDS-related KS has decreased due to the successful use of HAART therapy which allows HIV infected persons to maintain a more competent immune system which in turn, keeps HHV-8 in a latent state[75]. KS typically appears as blue-red or purple lesions on the skin and is thought to arise from endothelial cells. The cancer itself is a neoplasm arising from vascular origins and is characterized by angiogenic spindle-shaped cells associated with an immune infiltrate. Treatment for the cancer generally

involves augmenting the immune system (for example, reduction of HIV-related immunosuppression or cessation of post-transplant related chemically induced immunosuppression).

Primary Effusion Lymphoma (PEL) is a rare non-Hodgkin's lymphoma which is associated with a lack of solid tumor mass and an indeterminate phenotype and is often associated with an Epstein-Barr virus (EBV) co-infection.[63] Again, the highest frequency of PEL occurs in AIDS patients, but can also arise in the context of post-transplant immunosuppression and even more rarely in HHV-8-infected persons with no other risk factors.

Multicentric Castleman's Disease (MCD) is a lymphoproliferative disease associated with expansion of lymph node germinal centers, hypergammaglobulinemia, and increased B cell expansion. It occurs in two forms (a vascular hyline variant and a plasma cell variant), though HHV-8 is only associated with the plasma cell variant[160].

1.2 DC-SIGN

DC-SIGN (CD209) was discovered as a dendritic cell-specific c-type lectin receptor[81]. It is a type II transmembrane protein composed of an intracellular tail, variable neck region and carbohydrate recognition domain[64,77]. Initial studies showed that DC-SIGN binds ICAM-3 in the process of cellular cross-talk between DC and T cells[80] and proposed its function as a pathogen recognition receptor[79] (Figure 1). Reports have also shown that DC-SIGN interactions with ICAMs can aid in dendritic cell movement in tissues[72,78], and acts as a receptor for the internalization of antigen for processing and presentation[61,146]. DC-SIGNR (DC-SIGN-related, also known as L-SIGN) is a related molecule with similar function originally shown to be expressed on liver sinusoidal endothelial cells[13]. DC-SIGN has also been shown to be present at low levels on endothelial cells[148]. Macrophages have also been shown to express DC-SIGN[37,193] and we have shown that DC-SIGN is expressed on activated B cells both *in vitro* and

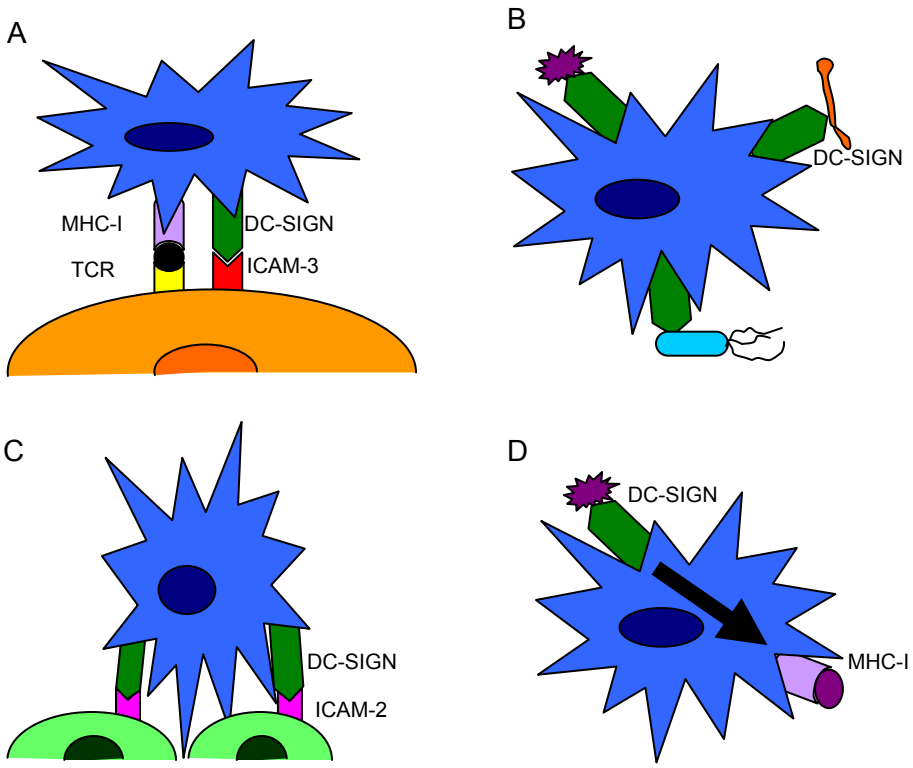

Figure 1. DC-SIGN is multi-functional. A. DC-SIGN acts in T cell priming by interacting with ICAM-3 to assist in the formation of the immune synapse. This interaction acts as the initial contact between DC and T cells permitting subsequent interaction between signaling molecules on each cell. B. DC-SIGN acts as a pathogen recognition receptor for viruses, bacteria and fungi on DC and results in signaling to activate DC. C. DC-SIGN binds ICAM-2 on endothelial cells to allow DC migration from the blood to tissues. D. DC-SIGN acts as an antigen uptake receptor by binding pathogens and targeting them for antigen processing and MHC-I presentation.

in vivo[171,173]. Most recently, studies suggest that DC-SIGN appears to localize to lipid rafts on the cell surface [28] and forms multimers, of which the ability is based on the length of the DC-SIGN neck region[64,185].

DC-SIGN has been shown to bind to several pathogens in a specific manner. It was first shown to bind gp120 of HIV-1, internalize the virus, and aid in trans-infection of T lymphocytes[79], a function that was later shown to be shared with DC-SIGNR[167]. Some studies have also suggested that DC-SIGN may be used for the infection of dendritic cells (DCs),

adding more controversy to debate on whether DCs are infected with HIV-1 or are simply carriers for the virus[25]. DC-SIGN was then also shown to bind Ebola virus through its envelope glycoprotein GP1 and enhancing *cis*- and *trans*-infection of susceptible cells[7,191]. *Mycobacterium tuberculosis* was also shown to use DC-SIGN as a receptor and further work has shown this interaction also to have immunosuppressive effects[83,206]. Both DC-SIGN and DC-SIGNR bind to hepatitis C virus glycoprotein E2[73,129,130,168], while hepatitis B virus binds neither[158]. Severe Acute Respiratory Syndrome coronavirus uses DC-SIGN for entry and trans-infection, though DC do not become infected with the virus[229]. Dengue virus uses DC-SIGN as an entry receptor[154,208], while the related West Nile virus uses DC-SIGNR instead[47]. Alphaviruses, such as Sindbis virus also use DC-SIGN as a receptor[110]. Avian H5N1 influenza appears to use DC-SIGN as a portal for both *cis*- and *trans*-infections[223]. Measles virus does not require DC-SIGN exclusively for infection, but attachment and entry into dendritic cells is blocked in the presence of anti-DC-SIGN antibodies[53]. HCMV is the only herpesvirus besides HHV-8 to use DC-SIGN as an entry receptor by binding via its gB homologue[90]. Moreover, this study has shown that HSV-1 gB or gD and VZV gB or gE did not bind DC-SIGN. However, a recent report has shown that HSV-1 and HSV-2 both bind DC-SIGN through gB and gC but that DC-SIGN does not act as an entry receptor for HSV, but rather enhances *cis*-infection of DC[51]. It has also been shown to be a binding partner for *Leishmania amastigotes*[44], *Helicobacter pylori*, *Leishmania mexicana*, *Schistosoma mansoni*[8], *Candida albicans*[29], *Aspergillus fumigatus*[183,184], *Leptospira interrogans*[76], *Escherichia coli*[109,230], *Streptococcus pneumoniae* serotypes 3 and 14[112], *Yersinia pestis*[231], and specific strains of *Neisseria meningitidis*[199] and *Neisseria gonorrhoeae*[230]. Therefore, while many pathogens bind DC-SIGN, suggesting its promiscuity as a receptor, these interactions are still specific and have different consequences depending on the pathogen.

It has been demonstrated that DC-SIGN/DC-SIGNR binds to high mannose glycan structures.[65] Recently, Lin and coworkers reported that DC-SIGN/DC-SIGNR binds with a

higher affinity to high mannose glycoproteins than to glycoproteins with complex structures.[124] Thus, the high mannose HIV-1 gp120 and the glycoprotein of Ebola virus bind to DC-SIGN. In both cases, this was related to the cell of origin of the virus, as gp120 produced in PBMCs bound to DC-SIGN while gp120 produced in macrophages did not. The gp120 produced in PBMCs has a predominately high mannose glycan structure, while the protein produced in macrophages is primarily a complex structure. Studies have shown that DC-SIGN exhibits some variation in the glycans it recognizes, preferring Galβ1-4(Fucα1-3)GlcNAc (Le^x) trisaccharides which are expressed multivalently. DC-SIGN also binds with intermediate affinity to oligomannose-type N-glycans, diantennary N-glycans expressing Le^x and GalNAcβ1-4(Fucα1-3)GlcNAc (LacdiNAc-fucose), while DC-SIGN did not bind to N-glycans expressing core-fucose with either α1-6 or α1-3 linkage to the Asn-linked GlcNAc[213]. Additionally, it has been shown that DC-SIGN and DC-SIGNR may have slight differences in their preferences for specific sugars. For instance, DC-SIGNR does not bind $Lewis^x$ antigens, while DC-SIGN does[214].

HIV-1 gp120 has been shown to bind to a region of DC-SIGN that is separate, but overlaps with that of the ICAM-2/3 binding site.[204] For the most part, this is in accordance with another published report showing that the conserved Ca^{+2} binding residues of DC-SIGN were important to both ICAM and gp120 binding. However, the reports conflict on the V351 residue of DC-SIGN, which one group found to be non-essential to either binding, but the other found to be important in ICAM binding but not to gp120 binding.[82]

As previously mentioned, binding to DC-SIGN may result in a functional or phenotypic change in the host cell, though this process is unclear as the signaling properties of DC-SIGN are poorly understood. The cytoplasmic tail of DC-SIGN contains several potential signaling motifs, including a di-leucine motif (LL), a tri-acidic cluster (EEE) and a tyrosine-based motif (YXXL), all of which are somewhat conserved in c-type lectins. For example, DC-SIGNR shares

the di-leucine motif and tri-acidic cluster, but not the tyrosine-based motif. Both the di-leucine motifs and the tri-acidic cluster typically function in internalization. In fact, it has been shown that truncations of the final 20 or 35 amino acids of the cytoplasmic tail of DC-SIGN or targeted mutations in the di-leucine motif render DC-SIGN unable to be internalized.[61,119,229] Also, to this end, it has been postulated that the structure of the ligand may be responsible for determining the intracellular fate of the DC-SIGN-bound molecule. For example, Hepatitis C virus has been specifically shown to be targeted to non-lysosomal compartments (from which it escapes) as opposed to Lewis-X antigen, another molecule which binds DC-SIGN, is directed to lysosomes.[132]

However, current studies suggest that the YXXL motif may not be functional as it has been found that DC-SIGN is not phosphorylated and mutation of the tyrosine residue does not affect DC-SIGN signaling.[31,70] Nonetheless, several groups have determined that ligation of DC-SIGN does result in signaling, suggesting that the functions of its cytoplasmic tail is currently poorly understood. Specifically, it has been demonstrated that ligation of DC-SIGN on MDDC and transfected cell lines using an agonist antibody results in phosphorylation of ERK1/2 and Akt, but not p38MAPK.[31] Furthermore, DC-SIGN was determined to be located in lipid rafts, could be co-precipitated with Lck and Syk, and also triggered PLC-γ phosphorylation and calcium mobilization. Most significantly, this antibody ligation and subsequent cellular signaling was functional, in that it resulted in the production of IL-10, which was increased in the presence of TNF-α, an inflammatory cytokine, or LPS, a ligand for TLR4, but not a synthetic TLR2 ligand. The importance of this finding lies in the knowledge that several organisms known to bind DC-SIGN have been shown to preferentially elicit a T_H2 response, which can lead to impaired pathogen recognition and is often associated with chronic or latent infection. To this end, it has been shown that the *Mycobacterium tuberculosis* cell wall component, ManLAM, which interacts with DC-SIGN, results in IL-10 production, inhibition of IL-12 production, and impaired DC

maturation.[71,83] Furthermore, this study also showed that ManLAM interaction with DC-SIGN disrupted the signaling that took place due to *M. tuberculosis* interaction with TLR4. More recently, this group has also determined that binding of several different pathogens to DC-SIGN results in activation of Raf-1 and subsequent acetylation of NF-κB p65 which lead to extended transcriptional activity of NF-κB and specifically increased IL-10 transcription.[88] Similarly, it has recently been shown that HIV gp120 induces the production of IL-10 via an ERK-dependent pathway, impairs the function of immature MDDC, and these effects are dependent on gp120 mannosylation and interaction with mannose-binding c-type lectins.[186] Therefore, it appears that interaction of certain pathogens with DC-SIGN results in signaling that overrides signaling that occurs as a result of concurrent TLR signaling and that the consequences of this interaction may result in creating an environment in which the pathogen is able to persist. To this end, it has also been shown that HIV-1 or anti-DC-SIGN monoclonal antibody binding to DC-SIGN results in specific triggering of Rho-GTPase via the guanidine nuclear exchange factor, LARG (leukemia-associated RhoGEF) and this activation retains DCs in an immature state allowing for synapse formation between DC and T cells and results in MIP-1α, RANTES and TNF-α production[97].

It has long been thought that IL-12 production leads to a T_H1 induction in which the consequences are expression of IFN-γ and cytotoxic T cell responses.[17,155,209] The classical T_H2 response, characterized by the induction of humoral responses, arises from the production of IL-10 or IL-4[176,177,197]. For this reason, it has been proposed that specific pathogens may be able to skew the immune response they induce to their own advantage[161]. Specifically, since a T_H1 response is thought to be preferred to clear viral infections, and a T_H2 response by itself can lead to persistence, it would be plausible to suggest that pathogens may have developed tactics to induce IL-10 or IL-4 and prevent IL-12 production. Thus, the determination that binding of DC-SIGN by antibody or pathogen leads to a T_H2 response has far-reaching

consequences.[31,186] More recently, this field has expanded to include a new set of T_H responses, the T_H17 subset[84,113,202]. This subset is induced in human cells through TNF-α, IL-21 and IL-23 (a member of the IL-12 family which uses the IL-12p40 subunit and a unique p19 subunit) and is negatively regulated by IL-2, IL-27, IL-4 and IFN-γ.[38,84,101,121,203] This subset was originally described based on its role in allergy and autoimmunity, but also appears to have a role in response to infection, although this role is less clear[10,201,212]. For example, T_H17 responses appear to be protective against *Klebesiella pneumoniae*, but responsible for the immune-mediated tissue damage seen in *Citrobacter rodentium* infections.[200] Little has been studied as far as the effects of this subset in viral infection, although one recent study showed increased IL-17 production by these cells in the context of HIV infection, but the outcome of this induction was unclear.[138]

As other studies have shown that interaction of other pathogens with DC-SIGN can lead to skewing of the immune response towards an environment that promotes persistence of that pathogen, it is important to determine whether binding of HHV-8 by DC-SIGN also supports these effects. Also, since persistence of latent virus is a necessity for the development of HHV-8-related cancers, it is feasible to consider that therapies targeted towards balancing the immune response to the virus would be an ideal proactive treatment for cancer prevention.

In summary, DC-SIGN is c-type lectin found largely on cells of lymphocyte lineage whose function involves both attachment and pathogen recognition. The mechanisms and consequences of DC-SIGN ligation are only partially defined, but some studies suggest that pathogen interactions with this molecule may have immunosuppressive effects. Further work is required to determine the effects of HHV-8 binding to DC-SIGN.

2.0 Chapter 2: Specific Aims and Hypothesis

2.1 Statement of Problem and Hypothesis

HHV-8 entry is poorly understood. Previously, two molecules, xCT and integrins, have been suggested to be involved in HHV-8 entry, but appear to be act in post-entry stages of infection.[215] Recent work in our lab has shown that HHV-8 uses DC-SIGN as an entry receptor, as DC-SIGN is present on all in vivo targets for HHV-8 and blocking of DC-SIGN on these cells can inhibit infection. A requirement for viral entry receptors is that expression of the protein in a non-permissive cell renders the cell permissive. We propose to test DC-SIGN's ability to act in this manner in this study. Furthermore, no studies on the mechanism of interaction between DC-SIGN and HHV-8 have been performed thus far. Therefore, we propose to determine which HHV-8 glycoprotein binds DC-SIGN and to determine if the binding region is shared with binding regions for other DC-SIGN ligands ICAM-2 and the HIV glycoprotein gp120. These findings are essential to designing targeted therapies that prevent HHV-8 infection but do not interfere with normal immune processes.

Additionally, ligation of DC-SIGN has been shown to have immunosuppressive effects.[31,83,97] We and others have shown that HHV-8 infection *in vivo* induces a non-robust CTL response[23,89,126,222] and that DCs infected *in vitro* are poor antigen presenting cells[172]. Specifically, these DC did not upregulate maturation markers, downregulated endocytosis functions, and were unable to present antigen to T cells. Furthermore, these detrimental effects were also observed in uninfected DC in co-culture with infected DC, suggesting that infected DC give off a soluble mediator or interact with uninfected DC directly. Since HHV-8 can interact with target cells through DC-SIGN, it follows that DC-SIGN ligation may contribute to these

observed effects. To this end, we will infect DC to determine whether they release any cytokines or chemokines that may act in suppressing antigen presentation capabilities of DC. These findings will be important to delineating the mechanisms HHV-8 uses to evade the immune response and therefore, useful in designing therapeutic treatments.

Hypothesis: We believe that DC-SIGN acts as an entry receptor for HHV-8 through interaction with the viral glycoprotein gB in a manner similar to ICAM-2 and gp120. We also hypothesize that the binding of DC-SIGN results in the initiation of cytokine and chemokine signaling which contributes to the ability of the virus to evade the immune system.

2.2 Specific Aims

2.2.1 Specific Aim 1

Test the hypothesis that DC-SIGN expression is required for HHV-8 infection and that infection of dendritic cells and endothelial cells results in a productive infection.

K562 cells expressing DC-SIGN will be constructed to determine if expression of DC-SIGN makes previously non-susceptible cell lines susceptible. Cells will be assessed for expression of DC-SIGN by IFA and western blot. K562 and K562-DC-SIGN cells will be infected with HHV-8 and stained for viral proteins by immunofluorescence, in order to demonstrate that K562 cells can only be infected with HHV-8 when DC-SIGN is expressed on the cell surface.

Secondly, we will determine if HHV-8 replicates in DC and HMVEC-d by analysis of kinetics of viral DNA replication upon infection with HHV-8. Real time PCR will be used to determine HHV-8 DNA levels in cell pellets and supernatants of infected MDDC and HMVEC-d.

2.2.2 Specific Aim 2

To test the hypothesis that the HHV-8 glycoprotein gB binds to DC-SIGN.

Soluble forms of HHV-8 glycoproteins K8.1A and gB and soluble DC-SIGN will be assessed for interaction by ELISA and Far Western blot. These assays are expected to show that gB, but not

K8.1A, binds DC-SIGN in a dose-responsive manner. We will also determine whether specific mutations in the DC-SIGN CRD affected the ability of HHV-8 to infect cell lines expressing these altered forms.

2.2.3 Specific Aim 3

To determine temporal expression of cytokines induced upon infection of MDDC with HHV-8.

As ligation of DC-SIGN can be immunosuppressive and we have observed that DC infected with HHV-8 are defective in their antigen presentation ability due to a paracrine factor, we will determine whether HHV-8 infection of MDDC resulted in the production of an altered pattern of cytokines.

3.0 Chapter 3: Requirement for DC-SIGN in HHV-8 infection and kinetics of viral infection of MDDC and endothelial cells

3.1 Preface

We have demonstrated that DC-SIGN acts as an entry receptor for HHV-8. In this chapter, we will test the hypothesis that DC-SIGN expression is required for HHV-8 infection and that infection of dendritic cells and endothelial cells results in a productive infection.

All experiments were preformed by Heather Hensler with the exception of the cell culture and infections shown in Figure 4, which were done in conjunction with Mariel Jais. Results shown here have been published directly or referred to in two recent publications by our lab[171,172].

3.2 Summary

We have previously shown that DC-SIGN serves as a receptor for HHV-8 on dendritic cells, macrophages and activated B cells. Thus, we hypothesized that expression of DC-SIGN on a non-permissive cell type could confer permissiveness to that cell type. We have created K562-DC-SIGN cells that express DC-SIGN and can be infected with HHV-8. Additionally, as our finding that HHV-8 infects MDDC is a unique observation for these cells, we examined kinetics of viral replication in these cells. We found that HHV-8 infection results in some viral protein expression, but shows no viral DNA replication, similar to that observed for HHV-8 infection of endothelial cells. These results clarify the initial events in HHV-8 infection, which is important for the design of targeted preventative therapies.

3.3 Introduction

We have previously shown that DC-SIGN acts as a viral entry receptor for HHV-8 on monocyte-derived dendritic cells (MDDC), macrophages and activated B cells[171,172]. In each case, infection of these cell types could be inhibited by antibodies directed against DC-SIGN or pretreatment of the cells using a DC-SIGN natural ligand, such as mannan. As DC-SIGN is present on each of the major cell types that can be infected *in vivo*, and blocking of the receptor can prevent infection, DC-SIGN satisfies two of the criteria for defining an entry receptor[190]. As part of these studies, we sought to determine whether DC-SIGN could satisfy the third criterion, which states that expression of the receptor protein should enable infection of previously non-permissive cell lines.

Additionally, as infection of MDDC is a new finding, it is important to characterize the fate of this infection. Thus far, HHV-8 infections of cell types *in vitro* have been only partially characterized. Viral DNA can be found and viral proteins are expressed upon HHV-8 infection of keratinocytes[34]. It has been shown that HHV-8 infects human foreskin fibroblasts (HFF) via endocytosis, and results in the expression of some viral RNA transcripts[2,115]. Infection of endothelial cells has also been shown to result in the expression of viral RNA transcripts and protein[115]. However, studies on endothelial cells have gone further and showed that infection of these cells *in vitro* results in a mixed population in which the majority of cells are in a latent state with a very small amount in a lytic replicative state from which a low level of infectious virus is produced[43]. Though in each of these cases, viral DNA is present in the infected cells, a quantitative look at levels of viral DNA in the context of *in vitro* infection has not yet been performed.

For these reasons, we wished to further characterize the infection of DC-SIGN expressing cells. To this end, we created a cell line that could be rendered permissive for HHV-8 infection by expression of DC-SIGN. Furthermore, we demonstrated that infection of MDDC results in the presence of a steady level of viral DNA in cells over time, but no active replication

of virus, as shown by a lack of increasing copy number in either the cell pellets or the supernatants and this is comparable to the kinetics of HHV-8 infection of endothelial cells.

3.4 Materials and Methods

3.4.1 Cell lines

K562 cells, a human erythrocytic leukemia cell line, were obtained from ATCC, and were chosen for their inability to be infected by HHV-8. Stable K562 transfectants of DC-SIGN were made by transfecting a plasmid vector expressing DC-SIGN (pcDNA3-DC-SIGN[166], NIH AIDS Repository) into K562 cells using Lipofectamine 2000 (Invitrogen) and selecting single cell derived, G418-resistant clones. Clones were screened for surface expression of DC-SIGN by immunofluorescence using an anti-DC-SIGN polyclonal antibody (Calbiochem). K562 and K562-DC-SIGN cells were maintained in RPMI-1640 (Cellgro) supplemented with 10% fetal calf serum (Atlanta Biologicals). Trex BCBL-1-RTA cells, PEL cells engineered to express the Rta protein in a tetracycline inducible manner (obtained from Jae Jung, Harvard University),were maintained in RPMI-1640 (Cellgro) supplemented with 10% fetal calf serum (Atlanta Biologicals)[150]. Monocyte-derived dendritic cells were cultured by positively selecting CD14+ cells from peripheral blood from HHV-8 seronegative donors using MACS beads (Miltenyi). Cells were differentiated by six day incubation at 1×10^6 cells/mL in AIM-V medium (Invitrogen) supplemented with 1000 U/mL of recombinant human interleukin-4 (IL-4) (R&D Systems) and human granulocyte-monocyte colony-stimulating factor (GM-CSF)[221]. Primary human adult dermal microvascular endothelial cells (HMVEC-d) were purchased from Lonza and maintained in EGM-2 supplemented with the corresponding Bullet Kit (Lonza) according to the manufacturer's instructions.

3.4.2 Immunofluorescence

Infection of target cells was visualized by immunofluorescence (IFA) using antibodies against the HHV-8 proteins ORF59, K8.1 and LANA-1 (ABI). Briefly, cells were fixed in 1%

paraformaldehyde, permeabilized in 0.1% Triton-X100 in PBS and dried to poly-L-lysine coated slides. Cells were blocked in 10% goat serum in PBS for 30 min at 37˚C and subsequently incubated with the primary antibody at 37˚C for 1 hr (ORF59 and K8.1 at 1:1000, LANA-1 at 1:500 in PBS). Cells were washed in PBS and incubated with goat anti-mouse-IgG-FITC (ORF59 and K8.1, Santa Cruz) or goat anti-rat-IgG-FITC (LANA-1, Dako) at 1:100 in PBS at 37˚C for 1 hr. K562-DC-SIGN were confirmed for DC-SIGN expression by IFA using a polyclonal anti-DC-SIGN antibody (1:100, Calbiochem) and goat-anti-rabbit IgG-FITC (1:100, Santa Cruz) in a similar fashion. Images were captured using a Nikon Eclipse E800 microscope with Nikon camera model U-III and processed on Q Capture software (Quantitative Imaging Corp, Copyright 2007).

3.4.3 Western blots

Proteins or cell lysates were subjected to SDS-PAGE and transferred to Immobilon PVDF membrane (Millipore). Membranes were blocked in 3% milk/PBS/0.05% Tween-20 and incubated with anti-DC-SIGN polyclonal antibody at (1:5000, Calbiochem). Membranes were washed 3 times in PBS/0.05% Tween-20 and then incubated with goat anti-rabbit IgG-HRP (1:70,000, Pierce). Membranes were washed in a similar fashion and subsequently developed using the West Pico Chemiluminescence kit (Pierce).

3.4.4 HHV-8 virus production and infections

TRex BCBL-1 RTA cells[150] were induced using 1 μg/ml Doxycycline and incubated for 3 days. Supernatant was collected by centrifugation and concentrated to approximately 1/100 of the original volume using 100,000 MWCO (molecular weight cut off) or 1,000,000 MWCO Centricons (Millipore & VivaSpin). Virus was stored at -80˚C until use. Infections were performed by infecting 10^6 cells in the smallest possible volume at 37˚C for 1-4 hrs, followed by removal of the virus. Infected cells were incubated at 37˚C for up to 72 hours. As viral MOI cannot be easily assayed for HHV-8, we quantified viral DNA copy input as described below and infected cells with 5-50 encapsidated viral DNA copy equivalents/cell as shown.

3.4.5 Quantitative PCR for HHV-8

HHV-8 DNA levels were measured using a real-time TaqMan PCR assay with primers and probe against the HHV-8 alkaline exonuclease gene (ORF37) (ORF37F, 5'-TCGGTGGCGATGCTTTAGAC-3', ORF37R, 5'-TGAAGCAGACGATGCTTTGC-3', ORF37PROBE, 5'-6-FAM-TGCTAACCCCCGTCTACTTTCCCCG-TAMRA-3')[198]. Standard curves of known quantities (between $5x10^4$ and $5x10^2$) of commercially available HHV-8 DNA (ABI) were prepared for comparison of unknown samples. To determine encapsidated viral DNA copy number in virus preparations, 50μl of concentrated supernatant was treated with DNase I at 10U/ml (Invitrogen) according to the manufacturer's specifications, followed by phenol chloroform extraction. To determine viral DNA levels in infected cells, cellular DNA was extracted using the QIAamp Blood DNA Miniprep kit (Qiagen). To determine viral DNA levels from encapsidated virus in cell culture supernatants, the experimental samples were pelleted by ultracentrifugation and treated with DNase I at 10U/ml (Invitrogen) according to the manufacturer's specifications, followed by phenol chloroform extraction. DNA samples were stored at -20˚C until use. PCR was performed in duplicate for each sample using the Taqman PCR Core Reagent kit (Applied Biosystems) and analyzed using the following conditions: 95˚C for 10 min, followed by 45 cycles of 95˚C for 15 sec and 60˚C for 1 min. To verify that this assay detected lytic virus replication, we analyzed DNA samples from TRex BCBL-1 RTA cells[150]. We induced viral replication by the addition of 20 ng/ml Doxycycline and collected samples at 24, 48, and 72 h for isolation of DNA and analysis of HHV-8 viral load by our real-time TaqMan PCR assay. Our results demonstrated a significant increase in viral DNA in the induced cells relative to the uninduced cells (data not shown).

3.5 Results

As part of our study demonstrating that DC-SIGN serves as a receptor for HHV-8 on DCs, macrophages and activated B cells[171,172], we sought to determine if the expression of DC-SIGN

in a non-permissive cell line could render that cell line permissive to HHV-8 infection. To this end, we constructed DC-SIGN expressing K562 cells by transfecting a DC-SIGN expressing plasmid (pcDNA3-DC-SIGN[166]) into K562 cells and selecting a G418-resistant single cell derived clone. Multiple clones were tested and single clone with high surface expression, referred to as K562-DC-SIGN, was used in all experiments. K562 cells were chosen for expression due to their ease of use, lack of endogenous DC-SIGN expression, and inability to be infected with HHV-8. Both K562 (Figure 2, A) and K562-DC-SIGN (Figure 2, B) were then tested for expression of DC-SIGN by immunofluorescence and western blot (previously demonstrated to be 44kDa[80], Figure 2, C). In both of these figures, a polyclonal antibody was used for testing, but cells were also positive using a monoclonal antibody against DC-SIGN (data not shown). Cells were also shown to be positive for protein expression by FACS and mRNA expression by real-time RT-PCR[171,172].

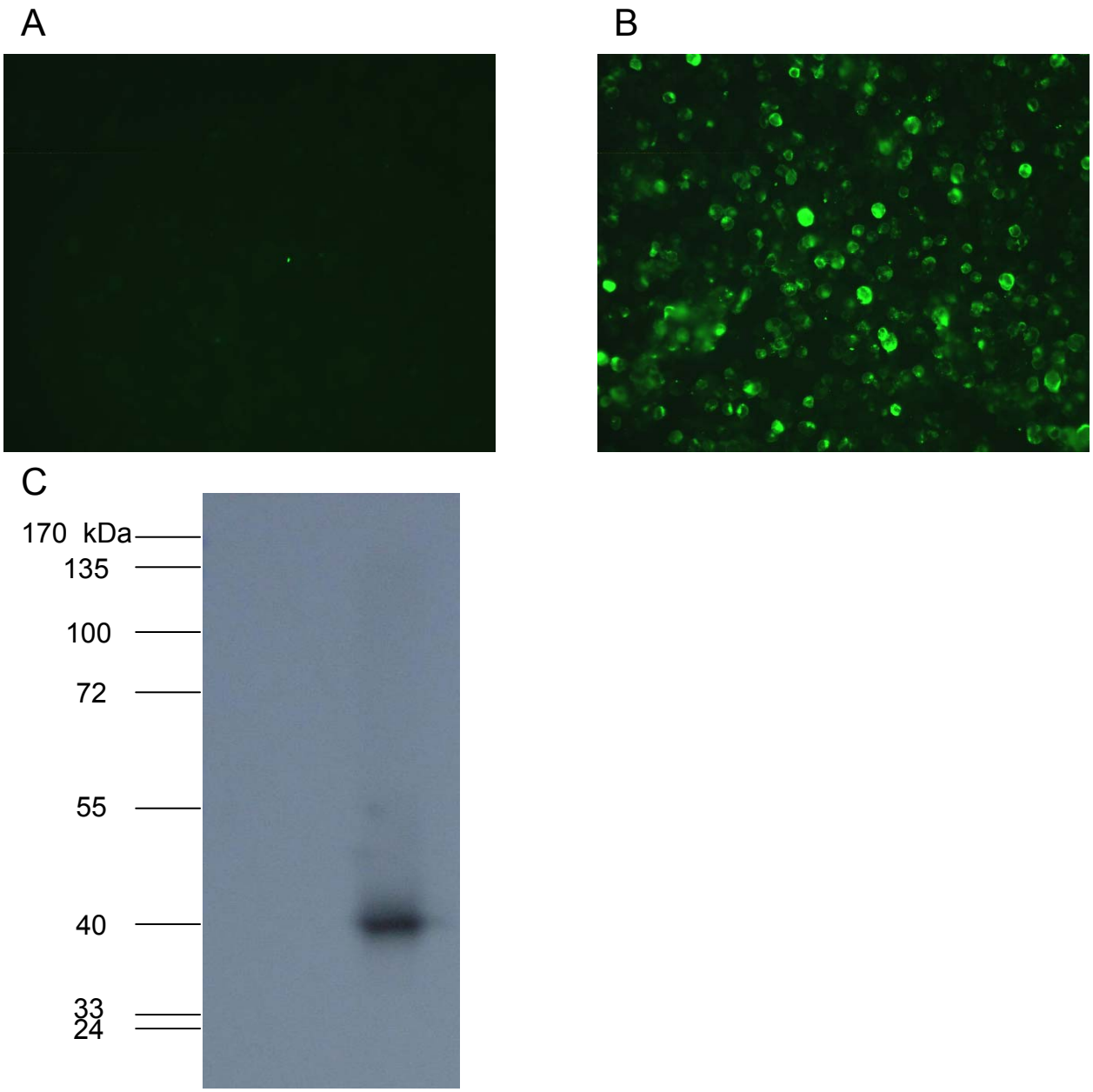

Figure 2. K562-DC-SIGN cells express DC-SIGN protein. A & B. K562 (A) or K562-DC-SIGN cells were stained with anti-DC-SIGN polyclonal antibody and visualized for expression of DC-SIGN. C. Lysates of K562 (lane 1) or K562-DC-SIGN (lane 2) were examined by western blot for DC-SIGN expression using anti-DC-SIGN polyclonal antibody.

Next, K562 and K562-DC-SIGN cells were tested for their permissiveness to HHV-8 infection. Cells were infected for 24 hours and viral infection monitored by immunofluorescence using antibodies directed against the viral proteins ORF59 (Figure 3, A and B) and K8.1 (C, D, and E). As shown in Figure 3, K562-DC-SIGN cells (B & D) could be infected by HHV-8 as shown by expression of two viral proteins (ORF59, cytoplasmic and perinuclear, and K8.1, cytoplasmic and cell surface), while K562 cells (E) were resistant to infection as evidenced by the lack of viral protein expression. Cells were also stained for a third viral protein, LANA-1, at 0, 48 and 72 hours post-infection with similar results (data not shown). Cells stained with only the secondary antibodies were negative (data not shown).

As our finding that MDDC could be infected with HHV-8 was the first observation of its kind, we were interested in determining whether HHV-8 replicated in these cells or if infection was abortive as has been shown for other cell types[115]. To this end, we employed a method for isolation of viral DNA from infected cell pellets and supernatants. This DNA was then used in a quantitative PCR assay against the viral ORF37 gene. MDDC were infected with either 5 or 50 encapsidated DNA copies/cell, washed and cell pellets and supernatants harvested at 0, 24, 48, and 72 hours post-infection. As shown in Figure 4, A, viral DNA was present in the cell pellets at higher levels initially and then maintained at less than 1 copy/cell throughout the 72 hours. Encapsidated DNA was found in the supernatant only at extremely low levels, implying that infectious virus was not released from these cells. These results suggest HHV-8 does not replicate in MDDC, despite the fact that some viral proteins are expressed in infected cells[172]. This is similar to the pattern observed in endothelial cells (Figure 4, B). In this case, however, the endothelial cells were not washed after infection, and as a result DNA was observed in the supernatant, but did not increase over time, similar to levels found in the cell pellets.

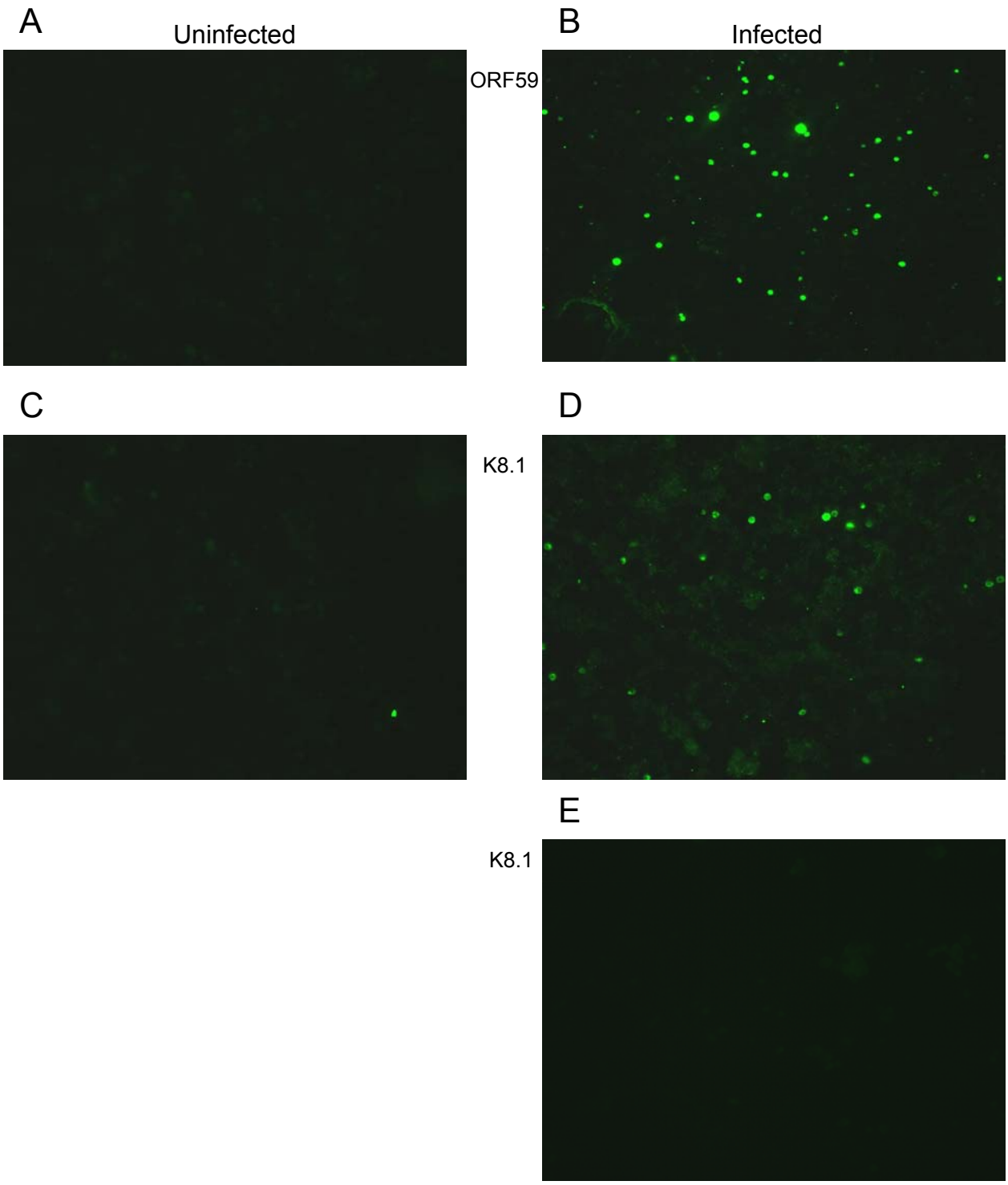

Figure 3. HHV-8 infects K562-DC-SIGN cells. K562 (E) or K562-DC-SIGN (A-D) were infected (B, D, E) or left uninfected (A, C) and stained with either anti-ORF59 (A, B) or anti-K8.1 (C, D, E) at 24hrs post-infection. A. K562-DC-SIGN, uninfected, anti-ORF59. B. K562-DC-SIGN, infected, anti-ORF59. C. K562-DC-SIGN, uninfected, anti-K8.1. D. K562-DC-SIGN, infected, anti-K8.1. E. K562, infected, anti-K8.1

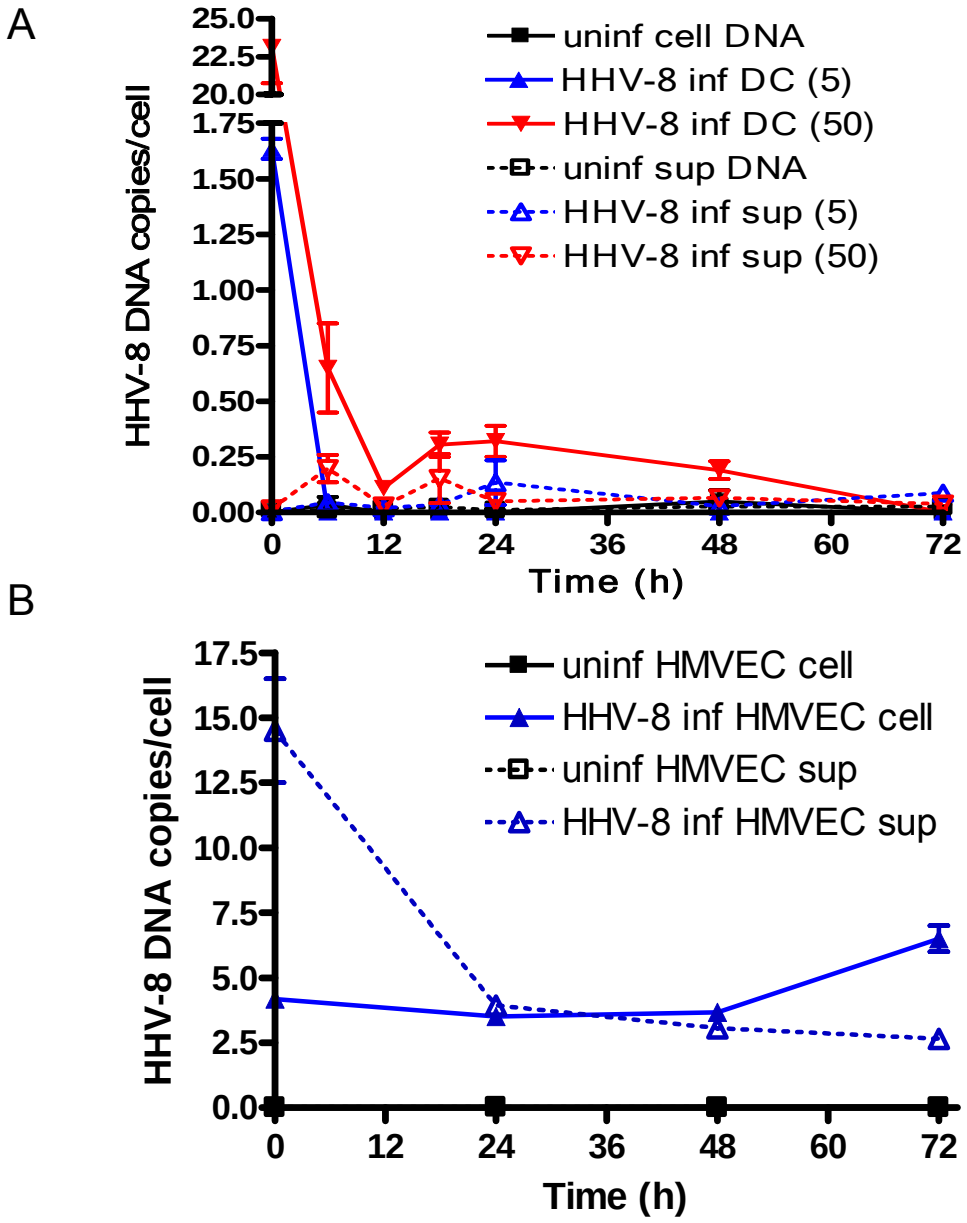

Figure 4. HHV-8 infection of MDDC and HMVEC is abortive. A. MDDC were infected with HHV-8 at the equivalent of 5 (▲, △) or 50 (▼, ▽) viral DNA copies/cell or left uninfected (■,□) and both supernatant and cell pellets were subject to quantitative real-time PCR. B. HMVEC-d were infected with the equivalent of 10 viral DNA copies/cell (▲, △) or left uninfected (■,□) and both supernatant and cell pellets were subject to quantitative real-time PCR. Representative of greater than 5 experiments each, with separate donors for each experiment. Error bars represent duplicate samples. In the case of supernatant samples, copies/cell refers to the DNA copies calculated to be derived from one cell.

3.6 Discussion

We have shown that DC-SIGN expression confers susceptibility to K562 cells which are normally non-susceptible to HHV-8 infection. We created the stably-transfected K562-DC-SIGN cell line from a single cell-derived clone and characterized its DC-SIGN expression by western blot, immunofluorescence, FACS and RT-PCR[171,172]. We have also shown that this cell line can be infected with HHV-8 *in vitro* as evidenced by expression of viral proteins ORF59, K8.1 and LANA by immunofluorescence. This finding is of importance as it gives further credence to the evidence that DC-SIGN serves as an entry receptor for HHV-8. This is supported by the fact that the major cell types that can be infected with HHV-8 express DC-SIGN and infection of these cells can be blocked by anti-DC-SIGN antibodies or natural ligands for DC-SIGN[171,172]. As a result of these findings, DC-SIGN is the only HHV-8 receptor to satisfy all of the accepted criteria for defining a viral entry receptor[190]. Subsequent work in our lab has shown that Raji cells that artificially express DC-SIGN also become susceptible to HHV-8 infection[171]. Interestingly, infection of both K562-DC-SIGN and Raji-DC-SIGN cells appears to be productive as both show increases in viral DNA over time.

This is in contrast to our findings shown here that HHV-8 does not replicate in MDDC or HMVEC. In each of these cases, viral proteins are expressed, but viral DNA does not replicate[172]. Studies on DC infection by HHV-8 *in vivo* have had conflicting results[36,156,175], but in each of these cases, subjects were not screened for HHV-8 seropositivity prior to assessment of DC infection. As HHV-8 seropositivity is low in the general public, inability to demonstrate infected DCs may have simply been due to the use of sample populations who were not even infected with HHV-8. However, dendritic cells have been found in KS lesions[62], and future studies are planned to investigate whether DCs are infected *in vivo*. For this reason, we cannot say that *in vivo* infection of either DCs or endothelial cells does not result in viral replication. It is possible that lack of viral replication *in vitro* is a result of the inability to accurately reproduce *in*

vivo conditions in an *in vitro* culture system. Future studies will be needed to assess the rationale for why some cell types (activated B cells, K562-DC-SIGN and Raji-DC-SIGN) replicate virus *in vitro*, while others (dendritic cells, endothelial cells) do not.

4.0 Chapter 4: HHV-8 Glycoprotein B binds to the cellular receptor DC-SIGN

4.1 Preface

While we have shown that DC-SIGN binds HHV-8, the viral glycoprotein responsible for that binding is unknown. In this chapter, we will test the hypothesis that the HHV-8 glycoprotein gB binds to DC-SIGN.

All experiments were preformed by Heather Hensler.

4.2 Summary

We have recently shown that HHV-8 uses DC-SIGN as an entry receptor for dendritic cells, macrophages and activated B cells. The viral attachment protein for DC-SIGN is unknown. HHV-8 virions contain 5 conserved herpesviral glycoproteins, a single unique glycoprotein, and 2 predicted glycoproteins. Previous studies have shown that DC-SIGN binds highly mannosylated glycoproteins. We have confirmed earlier reports showing that the HHV-8 glycoprotein B is highly mannosylated, while the glycoprotein K8.1 has complex glycosylation. In this study, we show that HHV-8 gB but not K8.1 binds DC-SIGN in a dose-dependent manner. We also show that HHV-8 binds DC-SIGN in its carbohydrate recognition domain in a position that is overlapping, though slightly unique, to the published binding regions for ICAM-2/3 and HIV-1 gp120. These results clarify some of the initial events in HHV-8 entry and can be used for the design of targeted preventive therapies.

4.3 Introduction

We have demonstrated that DC-SIGN is a cellular receptor for HHV-8 [171,172]. In addition to its expression on monocyte-derived dendritic cells (MDDCs), DC-SIGN is also expressed on activated macrophages and B-cells and its isomer, DC-SIGNR is expressed on endothelial cells[171,172,194]. These cell types represent natural targets for HHV-8 *in vivo*. Studies on the interactions between DC-SIGN and other viruses known to use DC-SIGN as an entry receptor, such as HIV, Ebola, hepatitis C, CMV and others, have demonstrated that viral glycoproteins are the viral attachment proteins (VAPs) responsible for binding to DC-SIGN, or its endothelial cell-expressed homologue, DC-SIGNR.[46 73,79,90,124,168,191]. Specifically, the majority of these studies have demonstrated that viral glycoproteins with a high mannose glycan structure bind to DC-SIGN/DC-SIGNR[65]. Like other herpesviruses, HHV-8 encodes a variety of glycoproteins that are expressed on the virion. There are 6-8 HHV-8 virion-associated glycoproteins known to date: the herpesvirus conserved glycoproteins gB, gH, gL, gM, gN, the HHV-8 unique glycoprotein K8.1A, and the gene products of ORFs 28 and 68, which are predicted to be glycoproteins[233]. Studies in our lab as well as others, have determined that the HHV-8 gB glycoprotein produced in B cells has a high mannose glycan structure while other glycoproteins such as K8.1A and gN have a predominately complex structure[11,114,227]. The glycan structure of the remaining 5 HHV-8 glycoproteins is not known. As gB has a high mannose glycan structure, it is a prime candidate for the viral attachment protein that binds DC-SIGN.

Studies investigating the binding site of DC-SIGN's natural ligands, ICAM-2/3 and the HIV gp120 protein have reported that the binding site of gp120 is separate, but overlapping with that of ICAM-2/3, and that the conserved Ca^{+2} binding residues of DC-SIGN were important to binding of both gp120 and ICAM-2/3 (see Figure 10).[82,204] DC-SIGN contains two predicted Ca^{+2} binding sites, one formed by E347, N349, E354, N365, and D366 and another by D320, Q323, N350, and D355[82]. For the most part, these studies agreed on important residues for binding of both molecules, with the exception of V351, an amino acid located in the "binding

pocket" of DC-SIGN and which was suggested to be important in binding by Geitenbeek, et al.[82], but not by Su, et al[204]. Additionally, the crystal structure of DC-SIGN suggests that the "binding pocket" is formed with N311 on one end[65]. This mutation was suggested to confer preferential ligand binding, but was surprisingly shown to not be involved in gp120 or ICAM binding[65,204]. Interestingly, a mutation at the other end of the pocket in D367, was shown to be important to ICAM binding, but not gp120 binding[204]. As we have shown that HHV-8 binds DC-SIGN[171,172], we wished to determine whether HHV-8 binds in a similar or distinct manner to the other two ligands. To this end, we have made cell lines expressing of a panel of 11 DC-SIGN point mutations in which individual amino acids have been mutated to an alanine, along with the corresponding wild-type DC-SIGN DNA to determine the binding region of HHV-8.

In the present study, we demonstrate that gB but not K8.1 binds DC-SIGN in a dose-dependent manner. Infections of mutant DC-SIGN-expressing cell lines with HHV-8 demonstrated that HHV-8 binds DC-SIGN in a region that overlaps, but is slightly unique compared to the ICAM and gp120 binding sites.

4.4 Materials and Methods

4.4.1 Soluble proteins

Baculovirus-expressed soluble DC-SIGN was obtained in purified form from Dr. Sal Butera[169] (Centers for Disease Control, Atlanta, GA). Baculoviruses expressing the soluble HHV-8 glycoproteins K8.1 and gB [219,220] to which 6X Histidine tags have been introduced, were obtained from Dr. Bala Chandran (Rosalind Franklin University, North Chicago, IL). Baculoviruses were used to infect SF9 cells which were incubated for 6 days at 28˚C. Cell pellets were lysed using I-PER insect cell lysis solution (ThermoFisher/Pierce) in the presence of TPCK (0.01mM), TLCK (0.01mM) and aprotinin (1:100), and proteins were purified using a His-binding Cobalt Column (ThermoFisher/Pierce) according to manufacturer's instructions. Proteins were dialyzed overnight against PBS to remove imidizole using a 10,000 MWCO Slide-

a-lyzer (ThermoFisher/Pierce). Proteins were stored at -80˚C until use. Protein concentration was determined using the Bio-Rad Protein Assay kit per the manufacturer's protocol (Bio-Rad, Hercules, CA) and relative amounts confirmed by western blot and Coomassie staining.

4.4.2 Endoglycosidase digests

Soluble gB and K8.1 were digested with Endoglycosidase H or PNGaseF (NEB) at 37˚C for 1 hr according to the manufacturer's instructions. Lysates were subjected to SDS-PAGE and western blots were performed as described.

4.4.3 Western blots

Proteins or cell lysates were subjected to SDS-PAGE and transferred to Immobilon PVDF membrane (Millipore). Membranes were blocked in 3% Carnation dry milk/PBS/0.05% Tween-20 and incubated with anti-K8.1 monoclonal antibody (1:3000, ABI), anti-gB-N1 rabbit antisera (1:2500), or anti-DC-SIGN polyclonal antibody (1:5000, Calbiochem) in PBS/0.05% Tween-20. The anti-gB-N1 rabbit polyclonal antisera was produced by Sigma-Genosys against the HHV-8 gB peptide TFQTSSSPTPPGSSS (aa 30-46) as described[220]. Membranes were then incubated with secondary antibodies goat anti-mouse IgG-HRP (K8.1, 1:60,000) or goat anti-rabbit IgG-HRP (gB and DC-SIGN, 1:70,000) (Pierce) in PBS/0.05% Tween-20 and subsequently developed using the West Pico Chemiluminescence kit (Pierce).

4.4.4 Far Western Blot

Soluble proteins were subjected to SDS-PAGE (12% for gB and Sf9 lysate, 15% for K8.1) under denaturing conditions and transferred to Immobilon PVDF membrane (Millipore). Soluble DC-SIGN protein was labeled in vitro with EZ-Link NHS-LC-LC-Biotin according to the manufacturer's instructions at a molar coupling ratio of 5:1 for 30 minutes at room temperature (ThermoFisher/Pierce). Membranes were blocked for 7.5 minutes in Superblock T20 (ThermoFisher/Pierce) at room temperature and washed twice in PBS/0.05% Tween-20. This step likely allowed for protein renaturation as length of blocking had an effect on binding ability (data not shown), although this was not confirmed. Membranes were then incubated with DC-

SIGN-biotin in probe dilution buffer (0.3% BSA/1% normal goat serum/PBS) at 10mg/ml at 37˚C for 1 hr. Five washes were performed using PBS/0.05% Tween-20. The membranes were then incubated with avidin-HRP substrate diluted into 7.5ml PBS/0.05% Tween-20 according to the manufacturer's instructions (ABC Elite kit, Vector labs) for 1 hour at 37˚C. Five washes were performed using PBS/0.05% Tween-20. Blots were developed using Vector Red diluted into 14ml of deionized water according to the manufacturer's instructions (Vector labs) and rinsed in tap water to stop the reaction when sufficient color change had developed.

4.4.5 ELISA

Polysorp 96 well plates (Nunc) were coated with the soluble HHV-8 glycoproteins in 1X bicarbonate buffer (ThermoFisher/Pierce) overnight at 4˚C. Plates were washed with PBS/0.05% Tween-20, air-dried and stored at -20˚C. Plates were blocked using 1% bovine serum albumin in PBS/0.05% Tween-20 for 30 minutes at room temperature and subsequently incubated with DC-SIGN that had been conjugated with biotin (ThermoFisher/Pierce) for 1 hour at room temperature. Plates were washed thoroughly and incubated with streptavidin-HRP (R&D Systems) for 1 hour at room temperature. Plates were developed using color-change substrate (R&D Systems) and the reaction was stopped using 2N H_2SO_4. Plates were read at 450nm/540nm. ELISAs to demonstrate protein presence were performed by blocking using 1% bovine serum albumin in PBS/0.05% Tween-20 for 30 minutes at room temperature. Wells were then incubated with an anti-gB antisera (Sigma Genosys) or anti-K8.1 monoclonal antibody (Advanced Biologics Inc) at 1:500 in PBS/0.05% Tween-20 for 1hr at room temperature and followed with goat anti-rabbit or goat anti-mouse peroxidase conjugated IgG antibodies (Pierce) at 1:350 in PBS/0.05% Tween-20 for 1hr at room temperature. Amounts of proteins were Plates were developed using color-change substrate (R&D Systems) and the reaction was stopped using 2N H_2SO_4. Plates were read at 450nm/540nm (data not shown).

4.4.6 Cell lines

SF9 cells were purchased from Invitrogen. K562 cells were obtained from ATCC. Stable K562 transfectants of both wild type and point mutants of DC-SIGN were made by transfecting plasmids containing individual point mutations in DC-SIGN (backbone for mutations was wild-type DC-SIGN in pcDNA3 (Invitrogen) obtained from Benhur Lee, UCLA, Los Angeles, CA)[204] into K562 cells using Lipofectamine 2000 (Invitrogen) and selecting single cell derived G418 resistant clones. Clones were screened for surface expression of DC-SIGN using an anti-DC-SIGN polyclonal antibody (Calbiochem) recognizing an epitope outside of the mutated regions by immunofluorescence (data not shown). DNA was purified from the cell lines and sequenced to confirm the identity of each of the clones (data not shown). Trex BCBL-1-RTA cells were obtained from Jae Jung (Harvard University, Boston, MA)[150].

4.4.7 HHV-8 Infection

TRex BCBL-1 RTA cells[150] were induced using 1 μg/ml Doxycycline and incubated for 3 days. Supernatant (containing infectious HHV-8) was collected and concentrated to approximately 1/100 of the original volume using 100,000 MWCO or 1,000,000 MWCO Centricons (Millipore). Virus was stored at -80˚C until use. Infectivity was tested via infection of K562-DC-SIGN cells and visualization of viral protein production by immunofluorescence. Infections were performed by infecting 10^5 cells in the smallest possible volume at 37˚C for 1-4 hrs, followed by removal of the virus. Infected cells were incubated at 37˚C for 48 hrs.

4.4.8 Immunofluorescence

Infection of target cells was visualized by immunofluorescence (IFA) using antibodies against the HHV-8 proteins ORF59, K8.1 and LANA-1 (ABI). Initially, infected cells were stained with antibodies against all three proteins. These results demonstrated that ORF59 expression was always present in infected cells and therefore in later experiments we only used antibodies against ORF59 to demonstrate viral infection. In order for cells to be scored as positive for infection, at least 20% of the cells had to be positive by IFA. Cells scored as negative for

infection did not demonstrate viral protein expression. For immunofluorescence, cells were fixed in 1% paraformaldehyde, permeabilized in 0.1% Triton-X100 in PBS and dried to poly-L-lysine coated slides. Wells were blocked in 10% goat serum in PBS for 30 min at 37˚C and subsequently incubated with the primary antibody at 37˚C for 1 hr. Wells were washed in PBS and incubated with goat anti-mouse-IgG-FITC (ORF59 and K8.1, Santa Cruz) or goat anti-rat-IgG-FITC (LANA-1, Dako) at 37˚C for 1 hr. K562 cells expressing DC-SIGN mutants were confirmed for DC-SIGN expression by IFA using a polyclonal anti-DC-SIGN antibody (Calbiochem) and goat-anti-rabbit IgG-FITC (Santa Cruz) in a similar fashion. Images were captured using a Nikon Eclipse E800 microscope with Nikon camera model U-III and processed on Q Capture software (Quantitative Imaging Corp, Copyright 2007).

4.5 Results

As it had been previously shown that HHV-8 gB was highly mannosylated, while K8.1 had a complex glycosylation pattern[11,227], we sought to confirm that the baculovirus constructs we obtained were consistent with this observation under our expression conditions. To this end, purified soluble proteins made in Sf9 cells were digested with Endoglycosidase H (EndoH), which cleaves N-linked glycoproteins at the chitobiose core of high mannose and some hybrid oligosaccharides, or PNGaseF, which cleaves N-linked glycoproteins between the innermost GlcNAc and asparagine residues of high mannose, hybrid and complex oligosaccharides. As shown in Figure 5, gB was cleaved to a similar extent by both PNGaseF and Endoglycosidase H, as demonstrated by the migration of a lower molecular weight band (black vs. dotted arrows), suggesting that gB glycosylation is predominantly high mannose. Glycoprotein K8.1, on the other hand was only fully cleaved with PNGaseF, suggesting a complex glycosylation pattern.

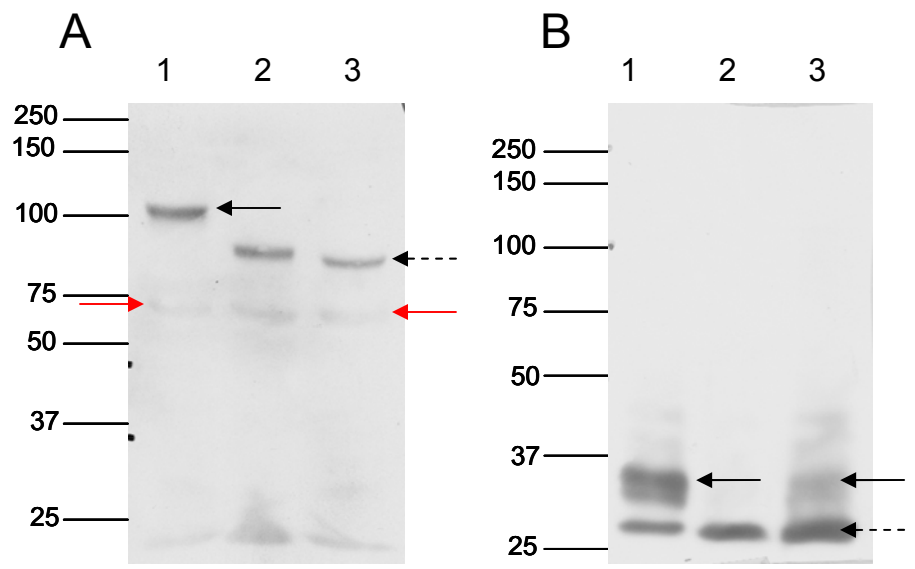

Figure 5. gB is highly mannosylated and K8.1 has complex glycosylation. A-B. Soluble gB (panel A) or K8.1 (panel B) was left untreated (lane 1) or digested with PNGaseF (lane 2) or EndoH (lane 3) and western blotted with the corresponding antibody. Arrows show full length bands, dotted arrows show digested full length proteins, and red arrows show undigested and digested sizes of gB ~68kDa cleaved portion.

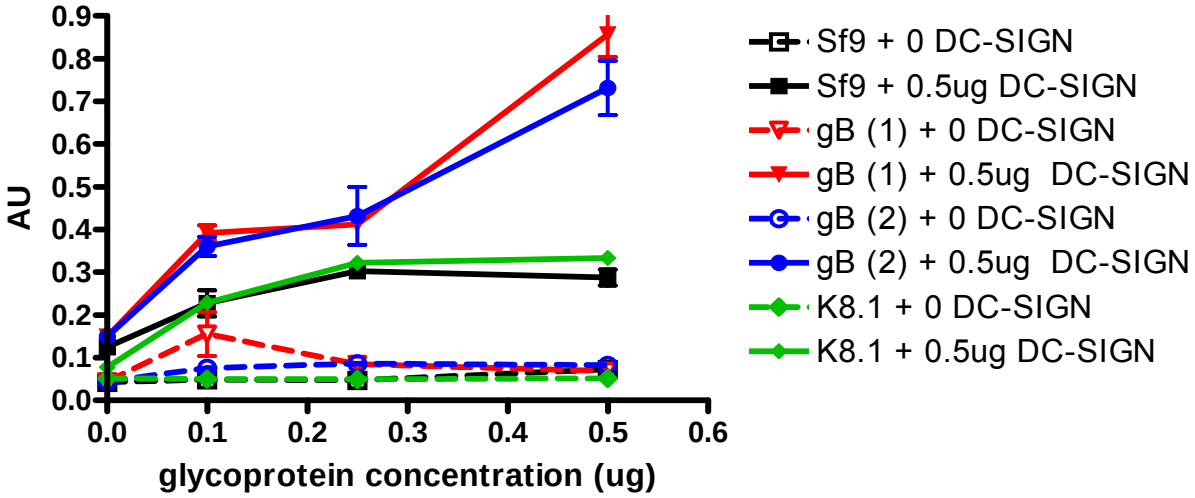

Figure 6. gB binds DC-SIGN in a dose-dependent manner. ELISAs were performed using plates bound with uninfected Sf9 lysates (black lines), 2 independent preparations of gB (1 & 2, red and blue lines) or K8.1 (green lines) at varying amounts. Plates were incubated with 0μg (dotted lines) or 0.5μg (solid lines) of soluble DC-SIGN, and probed for DC-SIGN. Error bars represent results of duplicate wells. Representative of multiple experiments.

Next, we sought to determine if either K8.1 or gB was capable of binding DC-SIGN. To this end, we constructed an ELISA in which soluble glycoproteins were bound to a plate and allowed to interact with biotin labeled soluble DC-SIGN. Binding of the soluble DC-SIGN was detected using Streptavidin-HRP as described in Materials and Methods. Two negative controls were used; uninfected Sf9 lysate was His-purified in the same manner as the soluble proteins and bound to the plate in place of the purified glycoproteins (black lines), and wells containing bound glycoproteins or the uninfected Sf9 lysate were exposed to blocking buffer rather than DC-SIGN (dotted lines). As shown in Figure 6, gB from two separate preparations (denoted 1 and 2, blue and red) bound to DC-SIGN in a dose-dependent manner, while K8.1 (green) bound to similar levels as the uninfected Sf9 lysate (black).

As a second method to demonstrate gB binding to DC-SIGN, we performed Far Western blot analyses. Briefly, varying amounts of soluble gB, K8.1 or uninfected Sf9 lysate were subjected to SDS-PAGE, transferred to membrane and subsequently incubated with biotinylated soluble DC-SIGN. As seen in Figure 7, DC-SIGN bound only to the immobilized gB and this binding was dose-responsive as shown by bands of increasing density. There was no binding detected of soluble DC-SIGN to the blots containing K8.1 (Figure 7, A) or the uninfected Sf9 lysate (Figure 7, B) at similar protein levels. The full length gB protein is approximately 112 KDa. When expressed on virions, it is often cleaved into two smaller proteins with molecular weights of approximately 75 and 59kDa.[11]. The soluble version we have used has a full length of about 100kDa and cleaved forms of 68 and 36 KDa[220]. An advantage of the Far Western blot analyses is that it allows us to determine which of the gB bands bind DC-SIGN. The soluble DC-SIGN bound two of the three gB bands; the full length undigested band of ~100 KDa and the smaller cleaved band of ~36 KDa (Figure 7, C). A western blot of the soluble gB proteins analyzed in Figure 7, C is shown in Figure 7, D. The gB proteins were detected using the gB-N1 antisera which is directed against a peptide located in the ~68kDa cleaved portion. As a result, the only gB bands detected by this sera are the full length and 68K bands as shown

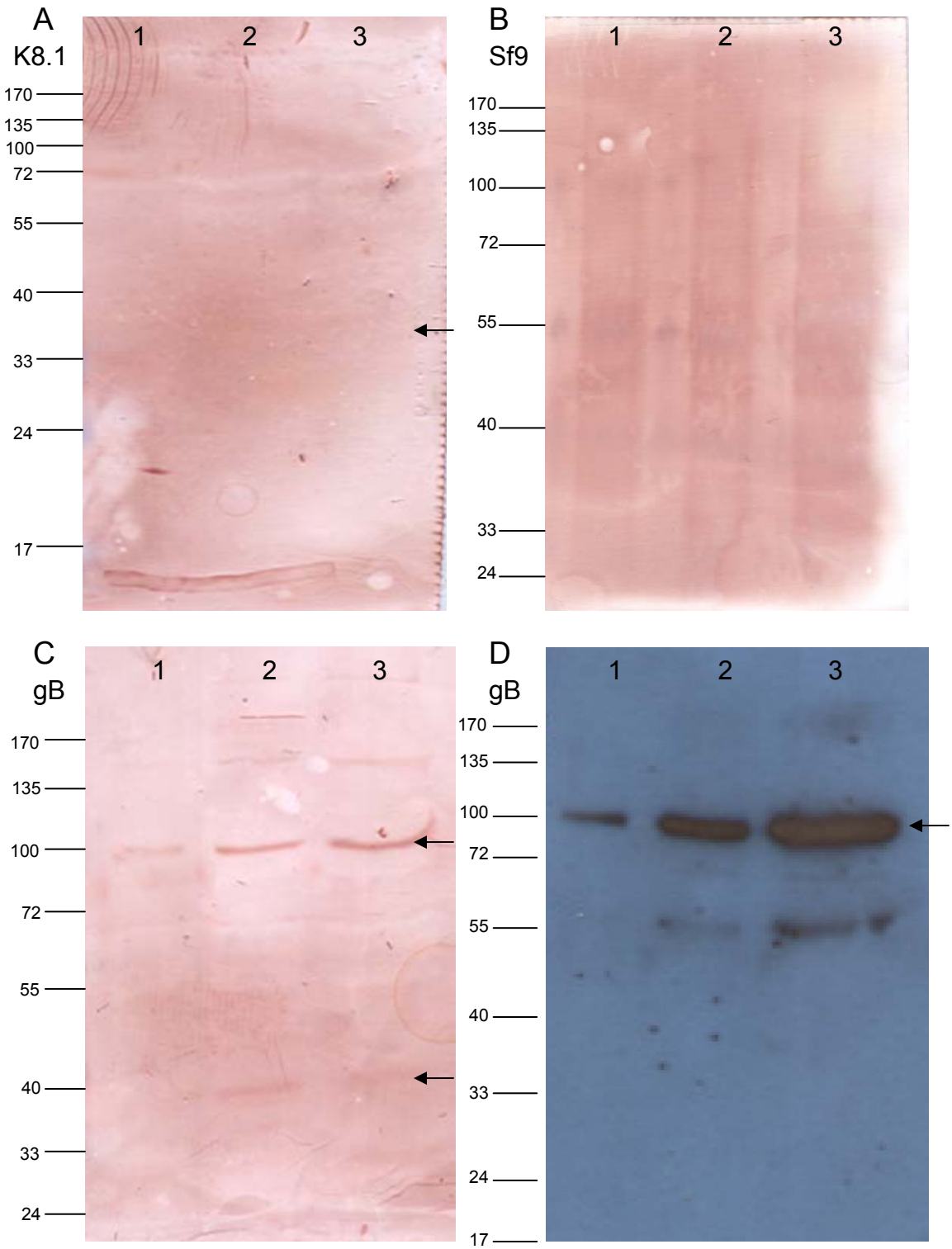

Figure 7. gB binds DC-SIGN in a Far Western blot. A-C. Lysates (Lane 1, 1.4μg, 2, 3.5μg and 3, 6.9μg) of soluble K8.1 (A), uninfected Sf9 (B), or soluble gB (C) were treated with soluble DC-SIGN in a Far Western blot. D. Corresponding western blot of soluble gB at the same amounts. Arrow in A shows the predicted size of soluble K8.1 (~37kDa) and arrows in C & D show the full length gB band (~100kDa) and a low molecular weight cleaved band (~36kDa). Representative of multiple experiments.

Figure 7, D. It is interesting to note that in Figure 5, A, while the full length soluble gB band (black arrows) is reduced in size upon endoglycosidase digestion, the ~68kDa band (arrowheads) does not appear to be reduced in size. This is surprising, as the predicted glycosylation sites are predominantly located in this region[165]. Therefore, these results suggest that gB can bind DC-SIGN through a region between aa. 440-702.

Previous studies have shown that DC-SIGN binds host (ICAM-2/3) and pathogen ligands (HIV gp120) in separate but overlapping sites[82,204] Therefore, we sought to determine whether HHV-8 binding of DC-SIGN matched either of these two binding sites. To this end, K562 cell lines expressing one of 11 different mutations in the DC-SIGN carbohydrate recognition domain were infected with HHV-8. As described in Materials and Methods, the expression and genotype of each mutant cell line was confirmed by IFA and DNA sequencing (data not shown). It follows, that if the particular cell line is permissive for HHV-8 infection, the DC-SIGN mutation expressed in that cell line is not in a position which affects HHV-8 entry. Conversely, if the cell line is not permissive, it suggests that the mutation does affect HHV-8 entry, likely due to its inability to bind DC-SIGN. Parental K562 cells and K562-DC-SIGN expressing cells served as negative and positive controls, respectively. (see Figure 3 for an example). These cells served as the basis for the determination of infection of the mutant cell lines. As shown in Table 2, we found that mutations in amino acids in positions 345, 346, 347, 349, 351, 355, and 367 prevented infection of HHV-8, while 311, 360, 361 and 366 had no effect on infection.

Table 2. HHV-8 infectivity of K562-DC-SIGN point mutants.

K562 cell line	HHV-8 Infection
K562 (parental)	Negative
K562-DC-SIGN (wild type)	Positive
K562-DC-SIGN-N311A	Positive
K562-DC-SIGN-R345A	Negative
K562-DC-SIGN-G346A	Negative
K562-DC-SIGN-E347A	Negative
K562-DC-SIGN-N349A	Negative
K562-DC-SIGN-V351A	Negative
K562-DC-SIGN-D355A	Negative
K562-DC-SIGN-S360A	Positive
K562-DC-SIGN-G361A	Positive
K562-DC-SIGN-D366A	Positive
K562-DC-SIGN-D367A	Negative

4.6 Discussion

In this study, we have identified an HHV-8 glycoprotein that serves as a viral attachment protein responsible for binding to DC-SIGN. Specifically, we have shown that gB is a binding partner for DC-SIGN, while K8.1 is not. This interaction was shown to be dose-responsive both by ELISA and Far Western blot. These results agree with previously published results showing that DC-SIGN binds high mannose glycoproteins (gB) preferentially over complex sugars (K8.1)[65]. Additionally, two recent studies have shown that DC-SIGN binds to the CMV and HSV-1 and -2 gB homologues[51,90]. Currently, glycosylation patterns of the six other HHV-8 glycoproteins have not been determined, save for gN, which has been shown to have complex glycosylation[114]. We cannot discount that other glycoproteins can bind DC-SIGN, as we have only investigated gB and K8.1 binding. Future studies will address whether other HHV-8

glycoproteins can be used in place of gB for entry of HHV-8. However, this finding suggests that gB binds to DC-SIGN to allow the virus to be endocytosed into the cell, where it escapes from the endosome in a pH-dependent manner (Hensler and Jenkins, unpublished results,[171])

Interestingly, it appears that the lower molecular weight (~36kDa) cleaved portion of gB (corresponding to aa. 440-702 of the soluble protein) binds to DC-SIGN based on our Far Western results. This is somewhat unexpected as this region is nearest to the transmembrane region of the protein in the sequence[220], and is predicted to have less glycosylation sites than the larger molecular weight portion. However, as the structure of HHV-8 gB has not been resolved, we cannot determine the precise location of this region once the protein has folded. Alignment with the sequence of HSV-1 gB, whose structure is known, suggests that this region would correspond to the Domains III-V (core, crown, and arm) which are located on the main stalk and top surface of the protein, rather than the cleaved flexible base regions (Domains I & II) which contain the fusion loops[91,93]. Therefore, based on these results we propose that gB binds DC-SIGN at its tip, which would avoid hindering the flexibility of the arm regions and subsequent insertion of the fusion loops into the cell membrane. Future studies are required to prove this hypothesis, as well as, whether gB binds DC-SIGN in a glycosylation dependent manner.

We have also shown that HHV-8 infects cells by binding to the DC-SIGN CRD in regions that overlap both ICAM and gp120 binding sites. Negative (parental K562) and positive controls also behaved appropriately. Interestingly, however, the pattern of binding in the CRD does not overlap perfectly with either of the published binding regions for ICAMs and gp120, but rather appears to have its own slightly different pattern (Figure 8). Previously published studies assessed comparative binding levels of ICAMs and gp120 and found that both of these ligands have low to mid-level binding to most of the mutants[82,204]. It is therefore possible that some of the mutants bound HHV-8 at a lower level and still resulted in infection. Therefore, our characterization likely reflects only positions that are essential for infection of HHV-8 via DC-

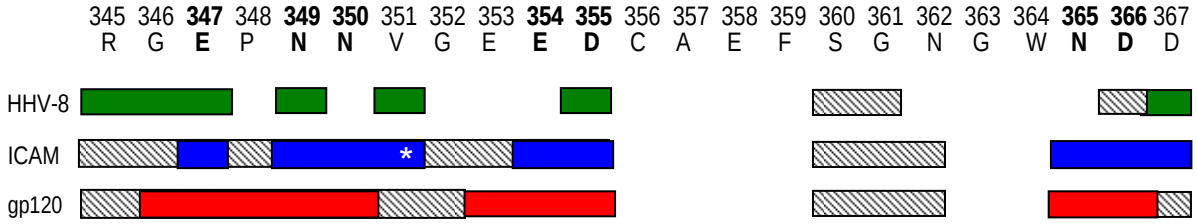

Figure 8. HHV-8, ICAM and gp120 binding sites on DC-SIGN CRD[82,204]. The amino acid sequence of the DC-SIGN CRD is shown. Amino acids shown to be involved in ICAM (blue) or gp120 (red) binding by the other studies[204] are highlighted below the single-letter amino acid code. White asterisk refers to disputed mutation between the two studies. Letters in bold refer to c-type lectin conserved Ca^{+2} binding sites. Green bars show mutations shown to affect HHV-8 infection. Hatched bars show mutations tested in each study that did not affect binding or infection. Mutation N311A not shown.

SIGN. Also, as these studies measured binding of ICAMs or gp120 to the DC-SIGN mutants and ours measured infectivity of the DC-SIGN mutant-expressing cells, it is possible that some of the DC-SIGN mutants could yet be found to bind HHV-8 without permitting infection.

This study has identified key components of the initial protein-carbohydrate interactions required for initiation of HHV-8 infection. Identification of the kinetics of early infection can lead to the development of specific anti-viral therapies designed to prevent infection of susceptible cells which may, in turn, also prevent the development of HHV-8-related cancers.

5.0 Chapter 5:

Cytokine production by human herpesvirus 8-infected dendritic cells

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Running title: “Cytokine production by HHV-8 infected DC”

5.1 Preface

DC-SIGN ligation has been shown to result in the production of cytokines and the consequences of that ligation appear to have effects in immune deregulation[31,83,97,186]. For this reason, we proposed that DCs infected with HHV-8 would produce similar cytokines upon infection. We found that infected DC produced T_H2-skewing cytokines, suggesting that DC-SIGN ligation by HHV-8 could contribute to immune evasion and an ability to persist in the host.

The following manuscript has been accepted for publication in the Journal of General Virology and all experiments were performed by Heather Hensler. The chapter is presented as a direct copy of the manuscript published. An addendum follows the manuscript addressing additional points not made in the manuscript.

5.2 Summary

We have previously shown that human herpesvirus-8 (HHV-8) infected dendritic cells (DC) undergo incomplete maturation and have defective antigen presenting function. Here we examined the effects of HHV-8 infection on cytokine production which is critical to the function of DCs. We detected expression of IL-6, TNF-α, MIP-1α, MIP-1β, RANTES and IL-12p40, 2-6 h post-infection and which peaked by 15-24 h. The expression decreased 24-48 h post-infection, with the exception of TNF-α, which remained high throughout the 72 h. Interestingly, while IL-12p40 expression increased post-infection, bioactive IL-12p70 was not detected in the supernatants. These results suggest an intentional skewing of cytokine production in HHV-8 infected DCs towards induction of a T_H2 response.

5.3 Results and Discussion

Human herpesvirus-8 (HHV-8, also known as Kaposi's sarcoma-associated herpesvirus, KSHV) is a gamma-herpesvirus and is the causative agent of Kaposi's sarcoma, primary effusion lymphoma and a subset of Multicentric Castleman's disease [144]. We have previously reported that primary infection with HHV-8 in HIV-negative adults does not result in pronounced clinical symptoms despite the presence of cellular and humoral immune responses and a detectable viremia [222]. Analysis of cytotoxic T lymphocyte (CTL) responses to viral proteins expressed during a primary infection revealed a distinct but relatively non-robust immune response [222]. The reason for this diminished immune response in normal individuals is not well understood but could reflect an effect of HHV-8 infection on antigen presenting cells. In support of this hypothesis, we have shown that HHV-8 can infect monocyte-derived dendritic cells (MDDC) through the C-type lectin dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN; CD209). While this infection is non-productive, it results in an altered pattern of dendritic cell (DC) function including loss of endocytotic ability, altered surface marker expression, and loss of antigen presentation ability, suggesting a partial rather than full maturation of these cells [172]. These effects may directly result in the dampened immune response during a primary HHV-8 infection (as described by a weak T cell response and low antibody titers) that we and others have observed [23,89,126,222]. We therefore sought to determine if HHV-8 infection of DCs results in an altered pattern of cytokine production that could be related to the loss of DC function.

DCs were generated from enriched CD14+ monocytes grown for 6 days in AIM-V media and supplemented with IL-4 (1000U/ml) and GM-CSF (1000U/ml) and infectious HHV-8 was purified from supernatants of TPA-induced BCBL-1 cells as previously described[172]. Cells were infected with gradient-purified virus equivalent to 50 viral DNA copies per cell as previously described[172]. Cells were not washed following absorption, to avoid loss of cytokines released

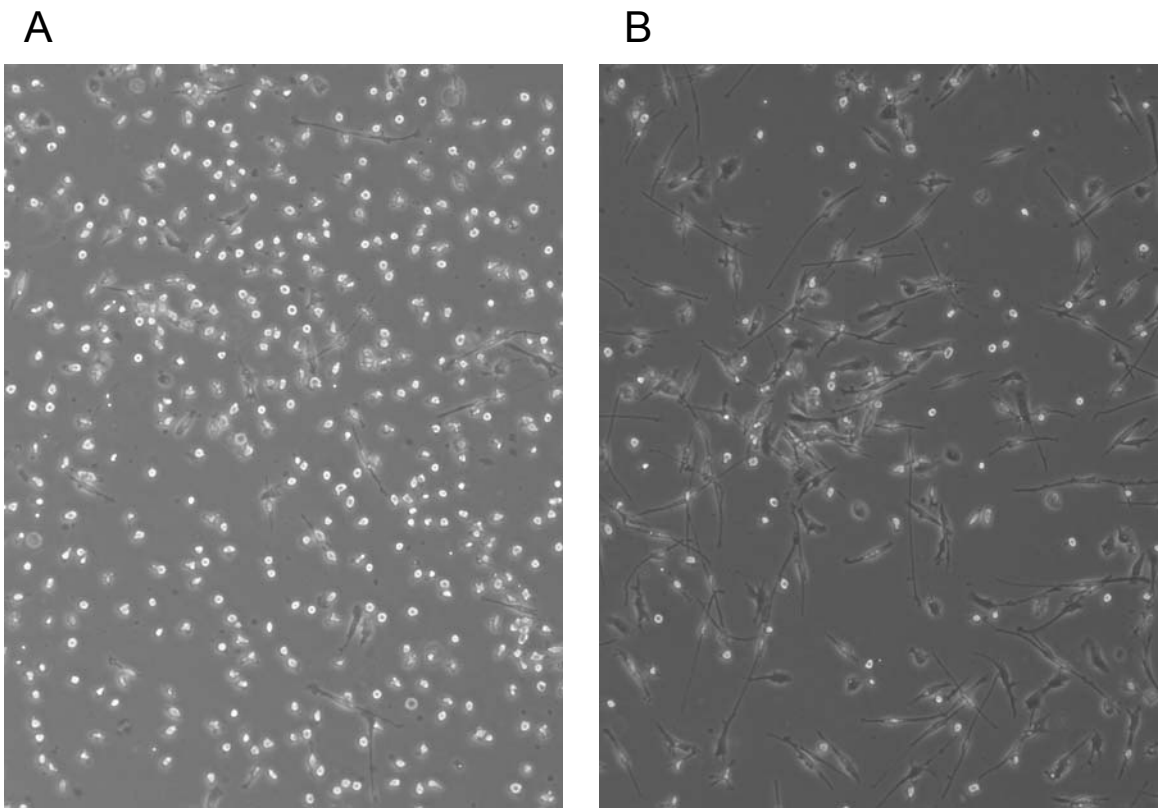


Figure 9. DC infected with HHV-8 develop a distinct morphology similar to matured DC. DC from an HHV-8-negative donor were left uninfected (A) or infected with HHV-8 (B) for 24h and photographed under brightfield microscopy.

upon initial interaction with the virion. Immature DCs infected with purified HHV-8 for 24hs appear morphologically distinct from uninfected DC as shown in Figure 9. Viral infection resulted in the DCs exhibiting an elongated appearance similar to *in vitro*-matured DC.

We have previously demonstrated that HHV-8 infection of immature DCs resulted in a loss of endocytosis and a uniform decrease in cell surface receptors even though less than 100% of the cells are infected [172]. Thus the effects of viral infection appear to be global in the culture rather than isolated to infected cells. Therefore, we wanted to determine whether HHV-8 infection could trigger the release of cytokines or chemokines that could be responsible for altering the maturation profile of the cells. To this end, supernatants from DC cultures that had been infected with purified HHV-8 or left uninfected for 48 h were subjected to a comparative screening for cytokine secretion using kits obtained from Biosource and assayed by Luminex

technology (Figure 10). As interleukin-4 (IL-4) and granulocyte macrophage colony stimulating factor (GM-CSF) were added exogenously to induce differentiation of monocytes to DC, comparisons of these cytokines between uninfected and infected cultures could not be determined as they exceeded the detection limit. Interferon-γ (IFN-γ), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (FGF-b), granulocyte colony stimulating factor (G-CSF), hepatocyte growth factor (HGF), IL-13, and IL-17 were not detected in either set of cultures (data not shown). IL-1β, IL-2, IL-5, monocyte chemoattractant protein-1 (MCP-1), IL-10, and IL-15 were detectable at low levels in both uninfected and infected cultures (Figure 10). IL-6 showed the greatest difference between uninfected and infected cultures (approximately 18-fold), while tumor necrosis factor-α (TNF-α) (approximately 3-fold), Eotaxin (approximately 3-fold), macrophage inflammatory protein-1α

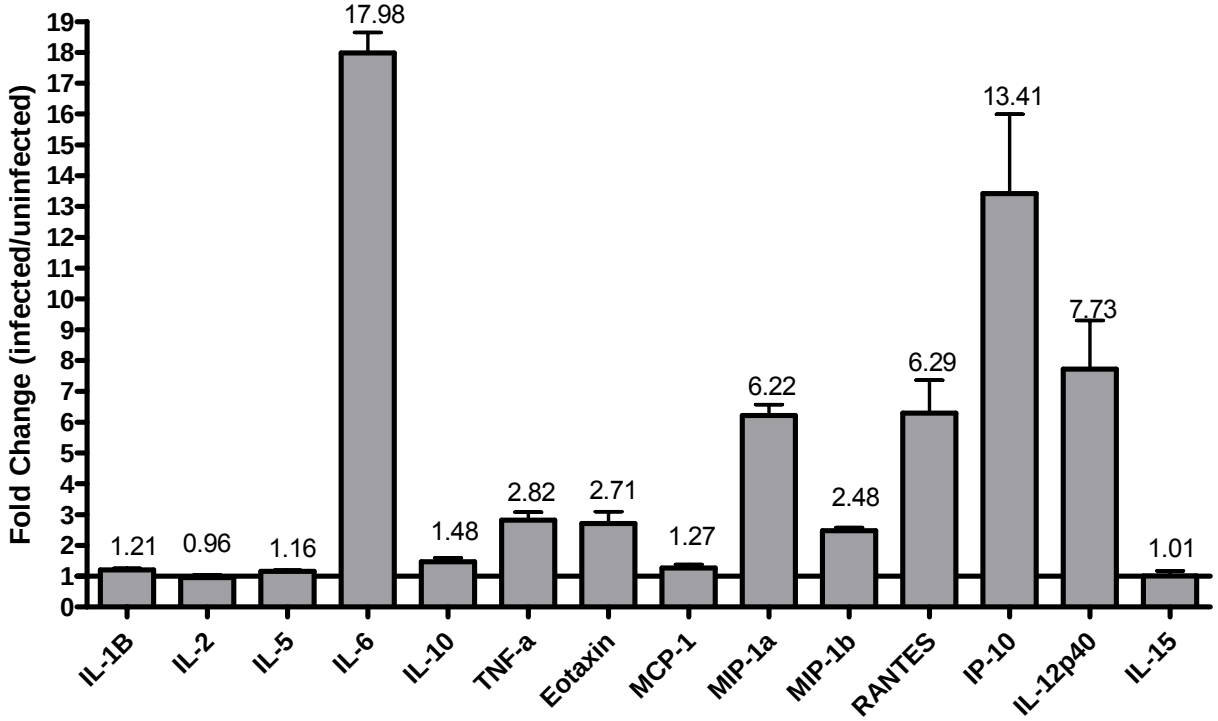


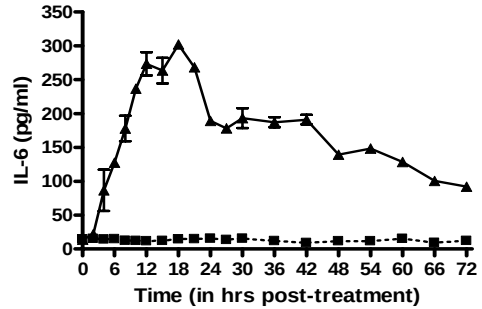
Figure 10. Changes in cytokine expression in HHV-8 infected DC versus uninfected DC. Supernatant samples from DC from an HHV-8-negative donor were infected or left uninfected, harvested 48h after infection and relative cytokine amounts were determined by Luminex. Representative of duplicate samples for each of two sample populations.

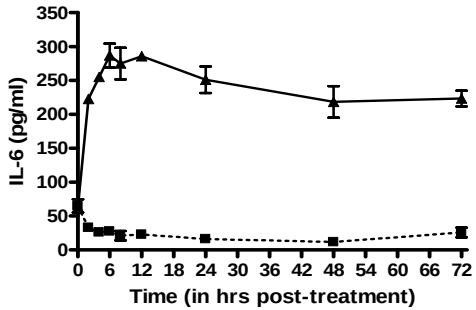
(MIP-1α) (approximately 6-fold), macrophage inflammatory protein-1β (MIP-1β) (approximately 2.5-fold), RANTES (approximately 6-fold), IFN-γ inducible protein-10 (IP-10) (approximately 13.5-fold), and IL-12p40 (approximately 8-fold) all showed greater than 2-fold increases in infected cultures (Figure10).

Since morphological changes to HHV-8 infected DCs could be observed as early as 6h post-infection (data not shown), we wanted to determine how quickly these cytokines were released into the media after infection. To this end, we performed ELISA (R&D Systems) for TNF-α, IL-6, MIP-1α, MIP-1β, RANTES and IL-12p40, all which showed a minimum of 2-fold increases in the HHV-8 infected cultures in the previous Luminex screening. We also tested for IFN-α and IL-12p70 by ELISA, neither of which were included in the Luminex screening. For each cytokine, DC from 2 HHV-8-negative donors were infected with HHV-8 or remained uninfected, and supernatant samples were removed from the cells at various times.

Additionally, as a positive control to mimic cytokine release from immature DCs stimulated to become mature DCs, the DCs from a third HHV-8-negative donor were treated with LPS (250ng/ml, Calbiochem) or recombinant trimeric CD40L (1 μg/ml, Amgen), and supernatant samples were harvested at corresponding times. In this case, a third donor was used as sufficient cells could not be purified from a single donor to perform the necessary experiments to include infection and protein treatment at one time. At each timepoint, supernatant was removed from a well containing 10^6 cells and thus represents the total accumulated amount of each cytokine in the supernatant between the time of infection and the time of harvest. As shown in Figure 11, each of the cytokines that showed viral infection-related changes by Luminex also had noticeable differences as determined by ELISA. IFN-α was not detected in uninfected or infected DC (data not shown). IL-6 was induced between 2-6 h post-infection, peaked between 6-18 h post-infection and decreased after 18 h. TNF-α production

A


IL-6 (pg/ml)
Time (in hrs post-treatment)


IL-6 (pg/ml)
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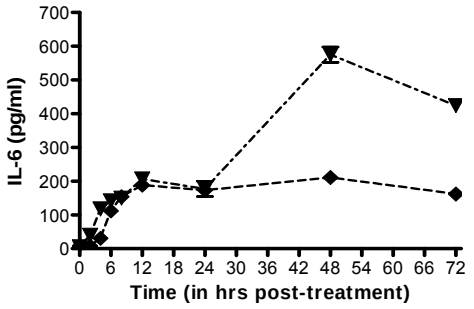
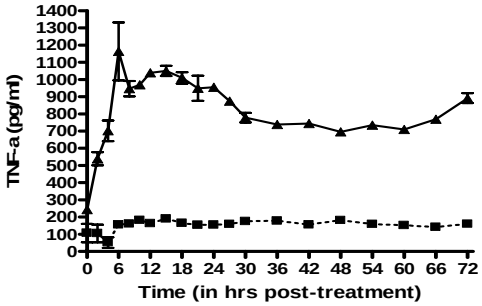
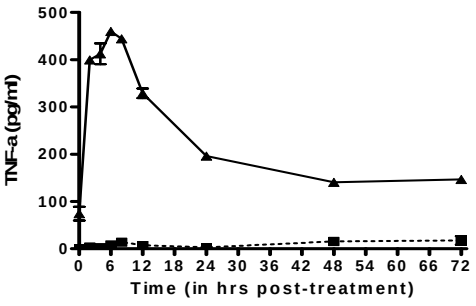
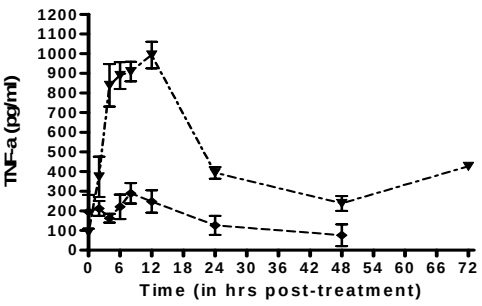
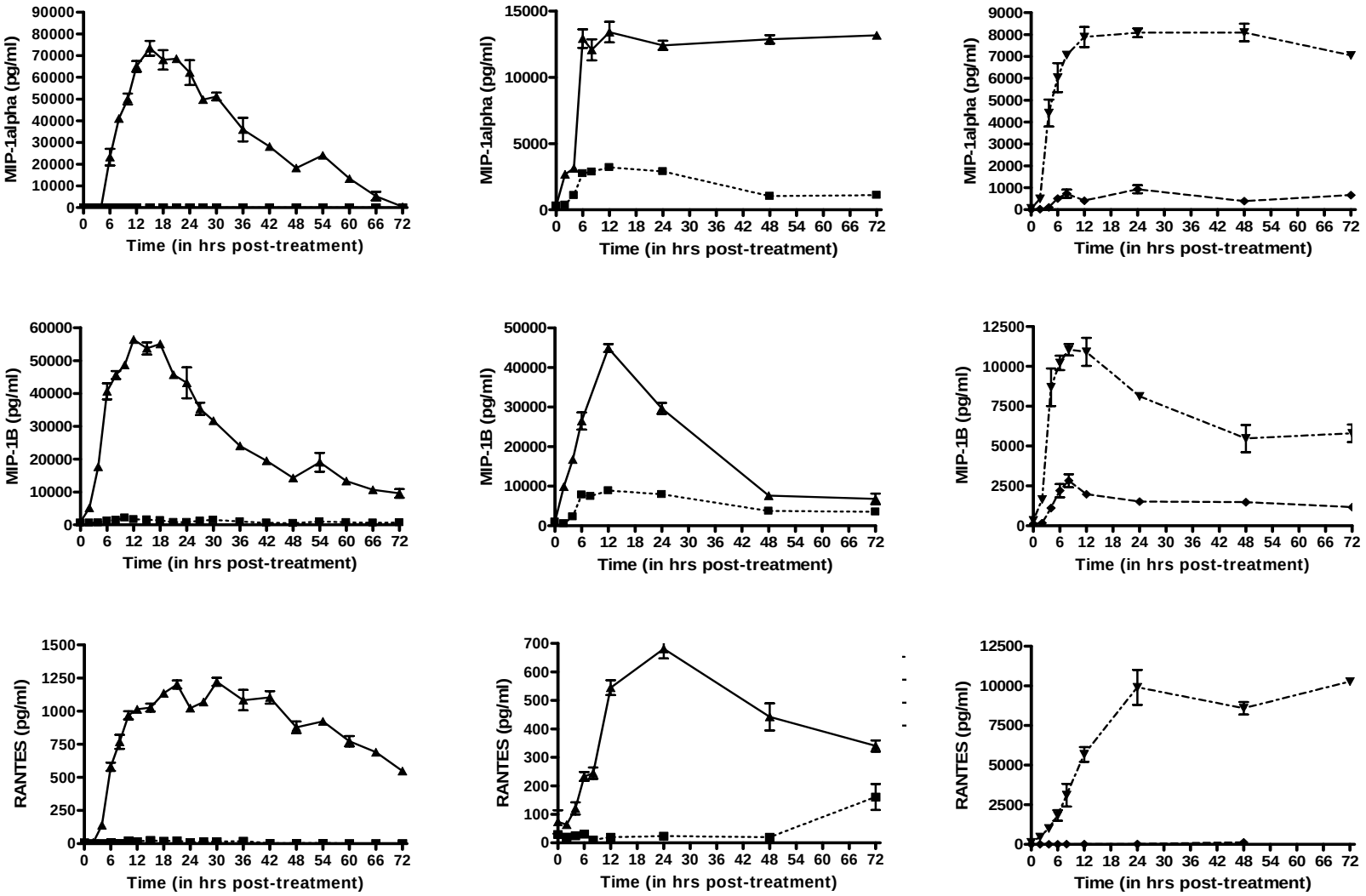

IL-6 (pg/ml)
Time (in hrs post-treatment)


TNF-a (pg/ml)
Time (in hrs post-treatment)


TNF-a (pg/ml)
Time (in hrs post-treatment)


TNF-a (pg/ml)
Time (in hrs post-treatment)

B


MIP-1alpha (pg/ml)
MIP-1B (pg/ml)
RANTES (pg/ml)
Time (in hrs post-treatment)

C

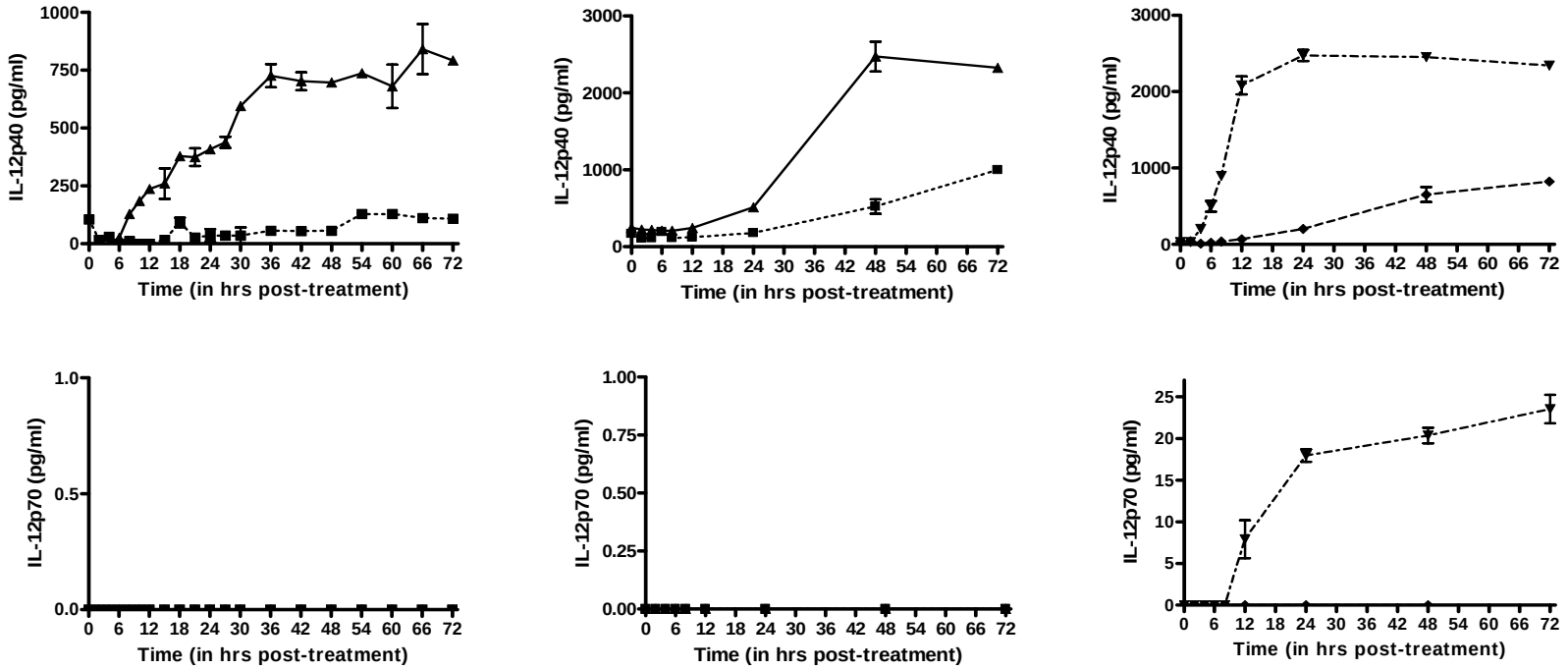


Figure 11. Temporal expression of selected cytokine expression in HHV-8 infected DC. A, B, & C. DC were infected with HHV-8 (▲, solid line), untreated (■, dotted line), treated with LPS (♦, dashed line) or trimeric CD40L (▼,dot/dash line), supernatant samples were harvested at selected times post-treatment, and analyzed by ELISA for IL-6 and TNF-α (A), MIP-1α, MIP-1β, and RANTES (B) or IL-12p40 and IL-12p70 (C). Each column represents the results from separate donors. Error bars represent duplicate samples.

began within 2 h post-infection, peaked at 6 h and decreased thereafter. The chemokines MIP-1α, MIP-1β, and RANTES had a slower induction with peaks 12-24 h post-infection and decreased to low levels thereafter. Lastly, as shown in the Luminex screening, IL-12p40 was induced to high levels beginning between 12-18 h post-infection and peaking 48-72 h later. However, bioactive IL-12p70 was not detected in either the uninfected or infected cultures at any time point, but was detected when immature DC were treated with trimeric CD40L.

As previously mentioned, we have shown that HHV-8-infected DCs exhibit an intermediate phenotype that is neither immature nor fully mature ($CD83^{low}$, $HLA\text{-}DR^{+}$, $HLA\text{-}ABC^{low}$, $DC\text{-}SIGN^{low/-}$). As these effects were not limited to infected cells but also included uninfected cells in the same culture [172] (data not shown), we suspected a paracrine effect and sought to determine which cytokines were being produced by infected cells. We found that pro-inflammatory cytokines (IL-6 and TNF-α), chemokines (MIP-1α, MIP-1β, RANTES), and potentially T_H2-skewing cytokines (IL-10, IL-12p40 but not IL-12p70) were increased after HHV-8 infection. This is consistent with reports of IL-6, IL-10, TNF-α, MIP-1α, MIP-1β, and RANTES expression in cultured PEL and KS cells, and in KS biopsies [9,24,57,211]. Lack of IL-12 production in response to maturation stimuli has also been observed in PEL cell lines [9] and purified peripheral blood myeloid DC from KS patients [54]. Indeed, exposure to certain stimuli can polarize DC to promote a T_H2 rather than a T_H1 phenotype [50], and lack of IL-12 expression contributes to this skewing [96]. As it is known that DC polarized towards T_H2 induction can contribute to persistence of viruses, [161], lack of IL-12 production by HHV-8 infected DC suggests this mechanism may be at work in this case.

These findings are of interest in the context of our previous observations that in vitro infected DC show limited functionality after infection with HHV-8. Interestingly, recent studies have shown that both infection of monocytes by HHV-8 or exposure to PEL-derived cytokines results in the inability of these cells to differentiate into DCs, decreases antigen uptake and

presentation, and alters their surface marker phenotype [41,42]. Moreover, others have shown that peripheral blood myeloid DC from classical KS patients have similar defects that are attributable to a soluble factor [54]. For instance, TNF-α stimulus in the absence of an accessory signal (i.e., LPS or CD40L) has been implicated in the production of partially or semi-mature DC, which could lead to peripheral tolerance [136,179]. Moreover, efficient IL-12 p70 production is necessary for generating robust CTL responses [210]. Given that HHV-8-infected persons have a dampened CTL response to the virus [23,89,126,222], the lack of IL-12p70 observed in our experiments suggests that HHV-8 could have developed a mechanism to prevent the production of IL-12p70 either through transcriptional repression of the p35 subunit or over-production of potentially antagonistic p40 homodimers [218]. However, a quantitative assay for determination of p40 homodimers in humans is not available.

We postulate that at least some of these cytokine and chemokine responses are caused by viral binding and entry into the cell rather than production of viral proteins, as our lab has shown that HHV-8 infection of DCs is non-productive[172]. Moreover, the cytokine and chemokine responses were induced as early as 2 h post-infection of the DCs using gradient-purified virus, and preliminary experiments using gradient-purified UV-irradiated virus were also able to induce cytokine responses (data not shown). Indeed, the HHV-8 viral OX2 homolog, which has been shown to be incorporated into the virion, is capable of inducing IL-1β, IL-6, MCP-1, and TNF-α in circulating monocytes, macrophages, and DCs[40]. Recent studies have shown that activating-antibody or HIV gp120 binding to DC-SIGN results in the production of TNF-α, MIP-1α, RANTES, and IL-10 [31,97,186]. Furthermore, these same studies indicate that DC-SIGN ligation on DCs can result in lack of maturation, lessened T cell stimulatory capacity, and lack of surface receptor upregulation. These responses appear to be dependent on mannosylation of viral glycoproteins. As HHV-8 encodes at least one highly mannosylated glycoprotein (gB) [11]

and the virus is known to bind to DC-SIGN [171,172], it is plausible that DC-SIGN binding to virus is at least partly responsible for the dampened immune responses previously observed in HHV-8 infected DC.

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5.4 Addendum

Virus used in this study was purified on Histodenz density gradients in an effort to remove contamination such as cellular debris, viral proteins, DNA and RNA. We cannot be certain all debris was removed and we acknowledge that there is a possibility some of the observed effects might be due to contamination of the virus prep.

The impetus for the initiation of these experiments was based on findings that all DC in a culture of both uninfected and HHV-8 infected DC showed morphological and functional changes. Our interest in determining cytokine patterns by infected cells was based on the idea that cytokines produced by infected cells would have effects not only on that cell, but on others in the culture. However, another option not discussed in this paper is that infected DC might form tunneling nanotubules connecting them to uninfected cells[157,180]. In this way, the infected cells might also be able to have an effect on uninfected cells through the transfer of calcium flux, vesicles, viral cell surface proteins, or other small molecules induced by viral infection which can be passed along nanotubes.

Additionally, experiments in which DC-SIGN was blocked by monoclonal antibody were also performed, but not shown. The results of these experiments were of limited use as

antibody treatment resulted in the production of some cytokines. This observation was expected as it is in accordance to prior studies showing that DC-SIGN antibody ligation can induce DC-SIGN signaling.[31]

Nomenclature cross-reference: MIP-1α/CCL3, MIP-1β/CCL4, IP-10/CXCL-10, RANTES/CCL5, Eotaxin/CCL11, MCP-1/CCL2

6.0 Chapter 6: Discussion

6.1 Summary

Previously, our lab has shown that HHV-8 enters DCs, macrophages and activated B cells via DC-SIGN[171,172]. These findings are of importance for several reasons. Firstly, this is the first description of *in vitro* infection by HHV-8 of both DCs and B lymphocytes of any type. Secondly, infection of activated B cells is productive, which is also the first observation of such an infection *in vitro*. Finally, DC-SIGN serves as an entry receptor for HHV-8 in both of these cell types.

To extend these findings, we have shown that DC-SIGN expression confers permissiveness to a cell line, K562, which is normally non-permissive to HHV-8 infection (Figure 3). Taken with the fact that all of the major cell types that can be infected with HHV-8 *in vivo* also express DC-SIGN and infection of these cells can be blocked by anti-DC-SIGN antibodies or natural ligands for DC-SIGN[171,172], DC-SIGN satisfies all of the accepted criteria for defining a viral entry receptor[190]. Thus far, DC-SIGN is the only proposed HHV-8 receptor to fulfill all of these criteria.

Recently, we have shown that HHV-8 replicates in activated B lymphocytes *in vitro*. This is in contrast to our findings shown here that HHV-8 does not replicate in MDDC or HMVEC (Figure 4[172]). In each of the latter two cases, viral proteins are expressed, but viral DNA does not replicate[172]. It is undetermined whether this is an *in vitro* phenomenon or whether both of these infections have a similar fate *in vivo*. In the context of KS, infected endothelial cells can be observed in states expressing either lytic or latent proteins[107,163]. However, it is not clear whether lytic cells in this context are representative of newly infected cells or cells that have reactivated from a latent state. *In vivo* infection of DC represents a potentially interesting aspect of virus biology as it would provide the virus with an attractive and efficient method for trafficking

through the body and granting access to sites outside of the original infection site. However, as far as DC are concerned, the evidence describing the *in vivo* infection of DCs is unclear[36,156,175]. Specifically, these studies have all focused at whether HHV-8-infected DC could be observed, and found mixed results. However, donors were not prescreened to determine their HHV-8 seropositivity and as a result, lack of positive data is potentially representative of the fact that donors were seronegative and not that DC were not infected. As a result, further studies are required to determine whether HHV-8 infection of DC is solely an *in vitro* phenomenon. For example, a more intriguing study would be one in which DC from both healthy seropositive donors and KS patients, who would have higher titers of HHV-8, were screened for HHV-8 infection such that lytic or latent state of infection could also be determined.

Also in this study, we have identified an HHV-8 glycoprotein that serves as a viral attachment protein responsible for binding to DC-SIGN. We have shown that gB is a binding partner for DC-SIGN, while K8.1 is not (Figure 6 & 7). While we cannot discount that other HHV-8 glycoproteins may bind DC-SIGN, this finding suggests that gB binds to DC-SIGN to allow the virus to be endocytosed into the cell, where it escapes from the endosome via a pH-dependent fusion with the membrane (Hensler and Jenkins, unpublished results,[171]). As a result, we can imagine an entry scheme such as the one depicted in Figure 12. Initially, HHV-8 attaches to the cell via interaction between heparan sulfate and either gB or K8.1. This allows concentration of the virus on the cell surface and puts the virus into proximity with subsequent binding partners. The virus then binds DC-SIGN through gB, inducing signaling which results in endocytosis of DC-SIGN and, likely, the production of cytokines. The virus is pulled into the vesicle, where it fuses with the membrane and escapes in a pH-dependent manner. It is unclear at what point during this process interaction with integrins or xCT might occur. As integrin binding induces FAK signaling and subsequent actin rearrangements[116,188], it is likely that integrin binding occurs near the time of DC-SIGN engagement in order to establish an environment which is conducive for entry, though this interaction could occur at any point in this

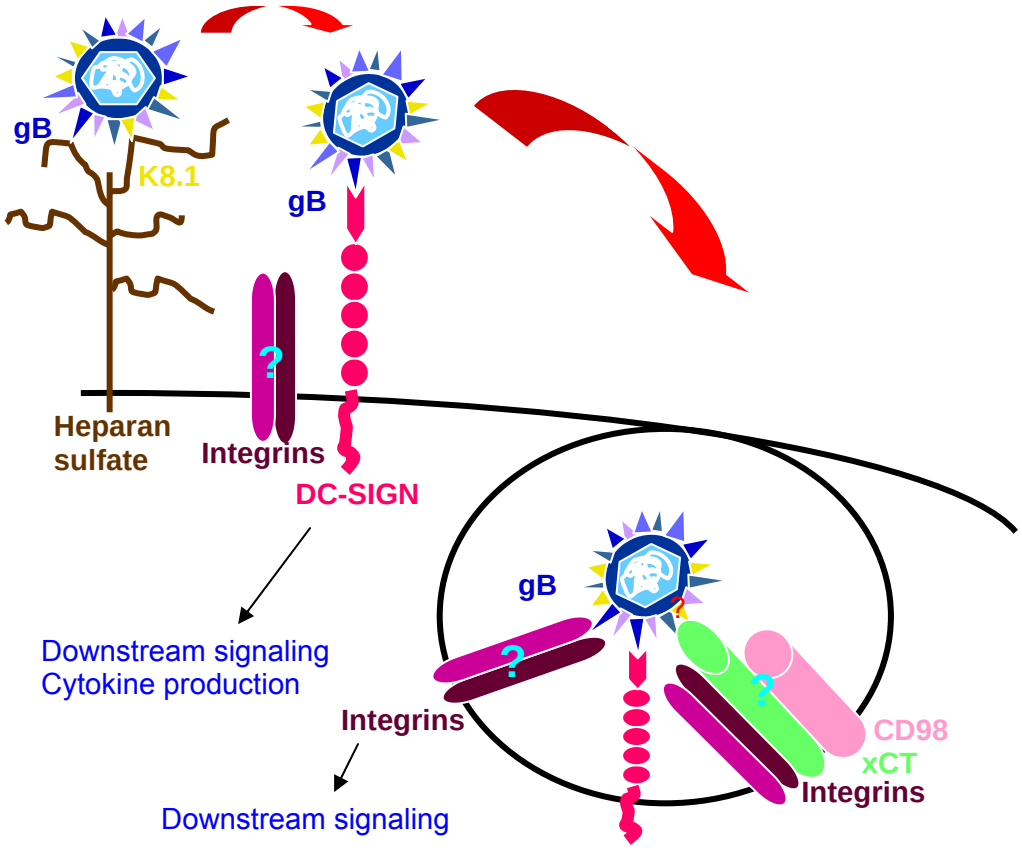

Figure 12. Proposed model for HHV-8 entry. HHV-8 binds to heparan sulfate through gB and K8.1A for attachment. HHV-8 binding to DC-SIGN through gB results in internalization and likely activates signaling pathways. The virus is endocytosed and escapes the vesicle in a pH-dependent manner. The virus interacts with xCT and integrins, but it is unknown when this interaction occurs in the process.

process. xCT has been shown to act as a fusion receptor for HHV-8[105], though its ubiquitous expression and lack of syncitia formation in viral glycoprotein-expressing cells makes it unlikely that this occurs independent of other receptors and at the cell surface as proposed by Kaleeba, et al.. It is possible that this interaction occurs within acidified vesicles, such that fusion does not occur without a pH-induced conformational change or without prior endocytosis via DC-SIGN. However, we and others have shown that blocking of xCT cannot inhibit infection[171,215], so it is unclear to what extent xCT may function in HHV-8 fusion.

We have also shown that HHV-8 infects cells by binding to the DC-SIGN CRD in regions that overlap both ICAM and gp120 binding sites, but appears to have its own slightly unique site (Figure 8). As our assay determined only those mutations that resulted in the loss of infectivity of DC-SIGN expressing cells, we cannot state that other mutations did not result in decreased binding efficiency of the virus. However, these results give an interesting insight into how DC-SIGN interacts with different ligands, and suggests that glycoproteins from different pathogens evolved separately to bind DC-SIGN, and as a result, have slightly different binding sites. Furthermore, this data suggests that targeted therapies which block pathogen interaction with DC-SIGN, yet do not interfere with ICAM binding, might be designed.

In this study, we have also shown that infection of DCs *in vitro* induces the release of pro-inflammatory cytokines (IL-6 and TNF-α), chemokines (MIP-1α/CCL3, MIP-1β/CCL4, IP-10/CXCL-10, RANTES/CCL5), and potentially T_H2-skewing cytokines (IL-10, Eotaxin/CCL11, IL-12p40 but not IL-12p70) (Figures 10 & 11). This is in agreement with published reports assessing *in vivo* cytokine and chemokine production in the context of HHV-8-related disease states[9,24,54,57,211]. Our findings imply that at least some of these responses are likely due to binding of the virus to DCs (such as IL-6 and TNF-α), which suggests that HHV-8 could have a detrimental effect on these cells without having to fully infect the cell. For example, as it remains unknown whether HHV-8 infects DCs *in vivo*, the virus would simply need to bind these cells in order to elicit a response, negating the requirement for the virus to fully enter the cell in order to have an effect.

We have results demonstrating that HHV-8 infection of immature DC, results in the production of IL-12p40, but not biologically active IL-12p70. As T_H1 induction is critical to the induction of efficient killing by macrophages and cytotoxic T lymphocytes and DC-mediated production of IL-12 is a prototypical T_H1-inducing cytokine, lack of bioactive IL-12 production by HHV-8 infected DC suggests a skewing towards a T_H2 phenotype. Furthermore, IL-12p40 has

been shown to form antagonistic homodimers in mice which bind the IL-12 receptor with a higher affinity than IL-12p70 and prevent binding and subsequent signaling by IL-12p70[125,224]. While this has not been yet demonstrated in humans, it is interesting to think that HHV-8 may have found a mechanism in which it prevents the production of bioactive IL-12p70 in addition to upregulating antagonistic IL-12p40 homodimers.

As one of its major functions, DC-SIGN acts to capture antigens, including viruses, in the periphery, process them and present them to T cells as part of the normal immune response. The process of antigen presentation requires the antigen (such as a virus) to be endocytosed and taken to lysosomes where the proteins are degraded and associated with MHC antigens. In addition to processing these proteins, the DC also produces cytokines which skew the helper T cell response to either a T_H1 or T_H2 response. In terms of viral infections, it has been suggested that a T_H1 response is desirable for clearing the infection while a T_H2 response is more advantageous for tolerance. Thus, if a virus can infect the DC, survive the endocytic pathway by preventing entry into a lysosome and cause the DC to elicit cytokines designed to modulate a T_H2 response, it would be less likely to be cleared and more likely to be able to persist in the host. As a result, some viruses have developed methods to subvert the immune response in this fashion and this subversion begins immediately with the interaction of the virus with entry receptors.[66,83,186] For instance, interaction of a viral glycoprotein with a cellular receptor may trigger signaling to allow the virus to be endocytosed, allowing the virus access to the cellular environment or may trigger other signals to allow the release of cytokines, allowing the infected cell to remain undetected by neighboring immune cells. Furthermore, DC-SIGN ligation has been shown to elicit the production of T_H2-skewing cytokines.[71,83,88,186] Therefore, given that HHV-8 binds DC-SIGN, our findings that HHV-8-infected DC produce T_H2-skewing cytokines have implications in the immune evasion strategies employed by the virus.

6.2 Future Directions

These studies have provided interesting findings regarding the initial interactions in HHV-8 infection of target cells, including MDDC, HMVEC-d, monocyte-derived macrophages, and activated B cells. Future studies will focus on expanding these results to include other known *in vivo* and *in vitro* target cells, such as HFF, keratinocytes, and CD34+ cells. It will be interesting to see whether HHV-8 infection is mediated by another receptor in these cell types, or whether the cells express a previously undescribed low level of DC-SIGN. Furthermore, our studies have focused on *in vitro* differentiated monocyte-derived dendritic cells and macrophages. Future studies are also needed to determine whether directly purified cells or *in vitro* differentiated cells from different precursor populations will behave in a similar manner upon HHV-8 infection.

Also, it will be important to further assess the interactions of gB and DC-SIGN. Our initial results suggest that DC-SIGN may bind to gB on a portion of the glycoprotein that is near the transmembrane region. This result is somewhat unexpected, as the large proportion of glycosylation sites on gB are located outside this region. Therefore, additional studies will be needed to determine whether this binding region is accurate, and more importantly, whether gB binds DC-SIGN through its sugars or if there is protein-protein interaction involved. Additionally, though it is not likely, studies are needed to determine whether any additional HHV-8 glycoproteins bind to DC-SIGN. In addition, we chose to map the HHV-8 binding site in the DC-SIGN CRD. This was chosen due to the fact that the HIV-1 gp120 and ICAM binding sites were demonstrated to be in this area, and crystal structures of DC-SIGN suggested this as a likely binding region[65,82,204]. However, we cannot discard the possibility that HHV-8 might also bind DC-SIGN in a region outside of the CRD. Thus, truncation mutants of DC-SIGN can be made to further delineate the minimal binding site for gB.

Our findings that HHV-8 interaction with DCs results in the expression of potentially T_H2-skewing cytokines shed some light on the mechanisms that the virus uses to evade the host

immune response. As our experiments suggest that binding of the virus may be sufficient for expression of the cytokines, future studies are planned to investigate whether the pattern can be replicated using UV-inactivated virus or soluble gB alone. Additionally, it is important to determine whether these and other immune evasion tactics are contributable to DC-SIGN binding or involve binding to any or all of the other surface proteins known to bind HHV-8. Furthermore, studies to determine whether other HHV-8 targets such as B cells, macrophages and endothelial cells produce similar or distinct cytokines upon interaction with HHV-8 are also planned.

Lastly, as previously mentioned, lack of IL-12 production by HHV-8 infected DC suggests a skewing towards a T_H2 phenotype. The importance of this lies in the fact that T_H1 environments promote cellular immunity, while T_H2 environments promote humoral immunity. More recently, this field has expanded to include a new set of T_H responses, the T_H17 subset. This subset is induced through TGF-β, TNF-α, and IL-23, a member of the IL-12 family which uses the IL-12p40 subunit and a unique p19 subunit, and is negatively regulated by IL-4 and IFN-γ. This subset was originally described based on its role in allergy and autoimmunity, but also appears to have a role in response to infection, although this role is less clear. For example, T_H17 responses appear to be protective against Klebesiella pneumoniae, but responsible for the immune-mediated tissue damage seen in Citrobacter rodentium infections[200]. Little has been studied as far as the effects of this subset in viral infection, although one recent study showed increased IL-17 production by these cells in the context of HIV infection, but the outcome of this induction was unclear.[138] Therefore, as we have shown that TNF-α and IL-12p40 are produced by HHV-8 infected DC, another interesting avenue of research would be to determine whether HHV-8 infection of DCs can push responses towards a T_H17 profile.

6.3 Public Health Significance

HHV-8 is the causative agent of several cancers, with Kaposi's sarcoma as the most frequently occurring of these. While the incidence of HIV-related KS is decreasing due to HAART therapy, KS incidences in developing nations where HAART therapy is not widely available are still relatively high[159]. In addition, endemic African KS, which arises independent of HIV co-infection has been estimated to have incidences between 9-25% of all cancers depending on the population analyzed[68,142,182,207]. Furthermore, KS represents a significant proportion of post-transplant cancers, specifically 0.5-5% of all cases in the US[139], but as high as 47.7% of all post-transplant cancers in other areas of the world[145]. Currently, therapy for HHV-8 related cancers centers solely on reducing the immunosuppression of the patient through HAART therapy or cessation, reduction or alteration in post-transplant immunosuppressive agents.

To design a targeted therapy to prevent primary infection and spread of HHV-8, it is important to understand the initial events during infection. Most notably, we need to know the identity of the cellular receptor(s) as well as the viral proteins that bind to these receptors. As the identification of viral and cellular binding partners has been used to design targeted therapies to prevent viral entry, (for example, Amantadine which blocks Influenza escape from intracellular vesicles, Pleconaril which blocks attachment of picornaviruses, and several HIV and HCV therapies which are currently under construction),[12,18,98,232] this information will permit future studies to develop anti-viral therapies designed to prevent viral infection and spread.

Furthermore, our results give insight into possible mechanisms that HHV-8 may use for immune evasion. Understanding of these mechanisms is not only important for the treatment of HHV-8, but also other pathogens that may employ similar techniques. For example, other pathogens that interact with DC-SIGN may induce similar signaling events that could lead to downstream skewed cytokine responses. For this reason, understanding of interplay between virus and host could result in prophylactic treatments for not only HHV-8, but other harmful pathogens.

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