DEVELOPMENT OF AN INFECTIVITY ASSAY FOR HUMAN HERPESVIRUS-8

by

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ABSTRACT

An accurate method for determining HHV-8 infectivity is lacking. The most common method currently used is the measure of encapsidated (i.e. DNAse-resistant) DNA genomes using quantitative real-time PCR. This method, while highly sensitive, does not distinguish between infectious and non-infectious virus particles. Immunofluorescence imaging of infected cells can provide some idea of infectivity but this method is subjective and accurate measurements are difficult to obtain. We have developed a cell culture assay using the HHV-8 cellular receptor DC-SIGN and a β-galactosidase gene under the control of the replication trans-activator (RTA) responsive promoter, T1.1. Infection of these cells with HHV-8 results in RTA production (from the infecting genome), which in turn drives the T1.1-β-galactosidase reporter gene. The T1H6 cell line containing the β-galactosidase gene under the control of the HHV-8 T1.1 promoter, was transfected in our lab with a plasmid expressing DC-SIGN under the control of the CMV IE promoter producing the cell line T1H6-DC-SIGN. Expression of DC-SIGN in T1H6-DC-SIGN cells was confirmed by IFA. β-galactosidase levels were determined using a chemiluminescent β-galactosidase detection kit (Clontech). Levels of β-galactosidase were compared between HHV-8-infected T1H6 and T1H6-DC-SIGN cells. $TCID_{50}$ values were determined using the Reed-Muench calculation. DNA copy numbers were determined using quantitative PCR specific

for HHV-8 DNA. Levels of β-galactosidase were significantly increased in infected T1H6-DC-SIGN cells compared to T1H6 cells supporting the role of DC-SIGN as a viral receptor. A ratio of $TCID_{50}$ values to DNA genome copy numbers demonstrated specific infectivity ranging from 10^{-4} to 10^{-6}. Validation of $TCID_{50}$ values was obtained by infection of immature dendritic cells using 1 and 2 $TCID_{50}$s. Using this assay, we compared replication kinetics in *de novo* HHV-8 infected activated B cells in two donors. In terms of public health, this is a more sensitive and specific assay for data that is needed to study HHV-8 infection. A potential clinical application using this assay involves determination of neutralizing antibody titers.

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-Sagar

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1.0   AN INTRODUCTION TO HHV-8

HHV-8, or human herpesvirus 8, is a γ-herpesvirus that belongs to the family herpesviridae, subfamily rhadinoviridae [1]. It is also referred to as Kaposi's sarcoma-associated herpesvirus (KSHV), since it is the etiologic agent in of all forms of Kaposi's sarcoma, as well as pleural effusion lymphomas and some forms of multicentric Castleman's disease [1]. HHV-8 DNA was identified in 1994 using representational difference analysis of a KS lesion from a patient with AIDS [2]. HHV-8 is seroprevalent in many countries worldwide [3], though highly active anti-retrovial therapy (HAART) has reduced overall incidence of HHV-8 related illnesses [4]. HHV-8 virus preparations are typically made using a HHV-8-positive/EBV-negative body cavity-based lymphoma cell line in which virus replication can be induced by phorbol esters such 12-O-tetradecanoylphorbol-13-acetate (TPA) [5, 6]. While there are several methods available to measure the presence of infectious virus or the amount of viral DNA in a sample, no method currently exists to accurately measure infectious HHV-8 titers.

1.1   ASSOCIATED ILLNESSES

1.1.1   Kaposi's Sarcoma

Kaposi's sarcoma (KS) was first reported in 1872 as an angioproliferative disorder of older men by Moritz Kaposi [7]. This form of KS is now referred to as classical KS. While in 1984 it had been reported that herpes-like virus particles were present in KS samples [8], it was not until 1994 that the virus DNA was identified in a lesion belonging to an AIDS patient [2]. In terms of structural pathology, infected cells take on a spindle shape with uncontrolled angiogenesis and inflammation to support the growth of the sarcoma [9, 10]. Variants of KS include classical KS, AIDS-associated KS, endemic KS, and iatrogenic KS [11-16].

1.1.1.1 Classical KS

This form of KS appears as an angioproliferative disease found in men of Mediterranean or Middle Eastern descent [12, 13]. Clinical manifestations are generally skin lesions on the legs, ankles, and feet [13, 14]. This form of KS presents more often in elderly men and is thought to be due to age-related, suppressed immune system, as well as the fact that they live in areas where HHV-8 infection is not uncommon [12, 13, 17, 18].

1.1.1.2 AIDS-associated KS

This is the most common form of Kaposi's sarcoma seen in the world appearing primarily in HIV-infected males. It is one of the AIDS-defining illnesses [1, 2, 19, 20]. Immune suppression is implicated in enabling for viral reactivation and subsequent replication, which may result in

development of KS lesions, primary effusion lymphoma, or multicentric Castleman's disease [2, 19, 21-24].

1.1.1.3 Endemic KS

This form of KS is also called African Kaposi's Sarcoma, as it is found in distinct parts of Africa where it is very common. It has been reported that transmission of HHV-8 in these regions may be through several different routes [13, 14, 25-33], and that viral infection tends to be in younger populations [25, 28, 29, 32, 33]. Immune suppression such as HIV/AIDS also contributes to the spread of endemic KS in Africa [11, 13, 14, 17, 27, 29, 31, 33].

1.1.1.4 Iatrogenic KS

This form of KS is found among recipients of solid-organ transplants [15, 16, 34]. The extreme immunosuppression associated with solid organ transplantation is felt to be responsible for allowing HHV-8 to break from latency resulting in productive infections [15, 16, 34, 35].

1.1.2 Other Clinical Manifestations of HHV-8 Infection

1.1.2.1 Primary Effusion Lymphoma

HHV-8 has also been implicated as the etiologic agent of primary effusion lymphoma, with the virus consistently shown to be present in individuals with this illness [1, 19, 22]. Primary effusion lymphoma is a B cell lymphoma [1, 22, 23], generally found in immune-suppressed individuals. This immune suppression is believed to be a prerequisite for disease development [19, 20, 22-24, 36].

1.1.2.2 Multicentric Castleman's Disease

Though HHV-8 is not required for the development of multicentric Castleman's disease (MCD), it is found in a subset of patients with HIV co-infection [1, 19, 21, 23]. The presence of HHV-8 results in a lymphadenopathy with high levels of serum IL-6, causing increased inflammation, tumorigenesis, and overall enlargement of lymph nodes in a systemic rather than localized manner. This systemic nature of the disease is related to failures in humoral immune responses [1, 19, 21-23].

1.2   EPIDEMIOLOGICAL FEATURES OF HHV-8 INFECTION

This section will discuss geographical distribution and transmission of HHV-8 and KS as well as the importance of HIV co-infection as another etiologic factor behind the development of KS and related illnesses.

1.2.1 Geographical Distribution of HHV-8
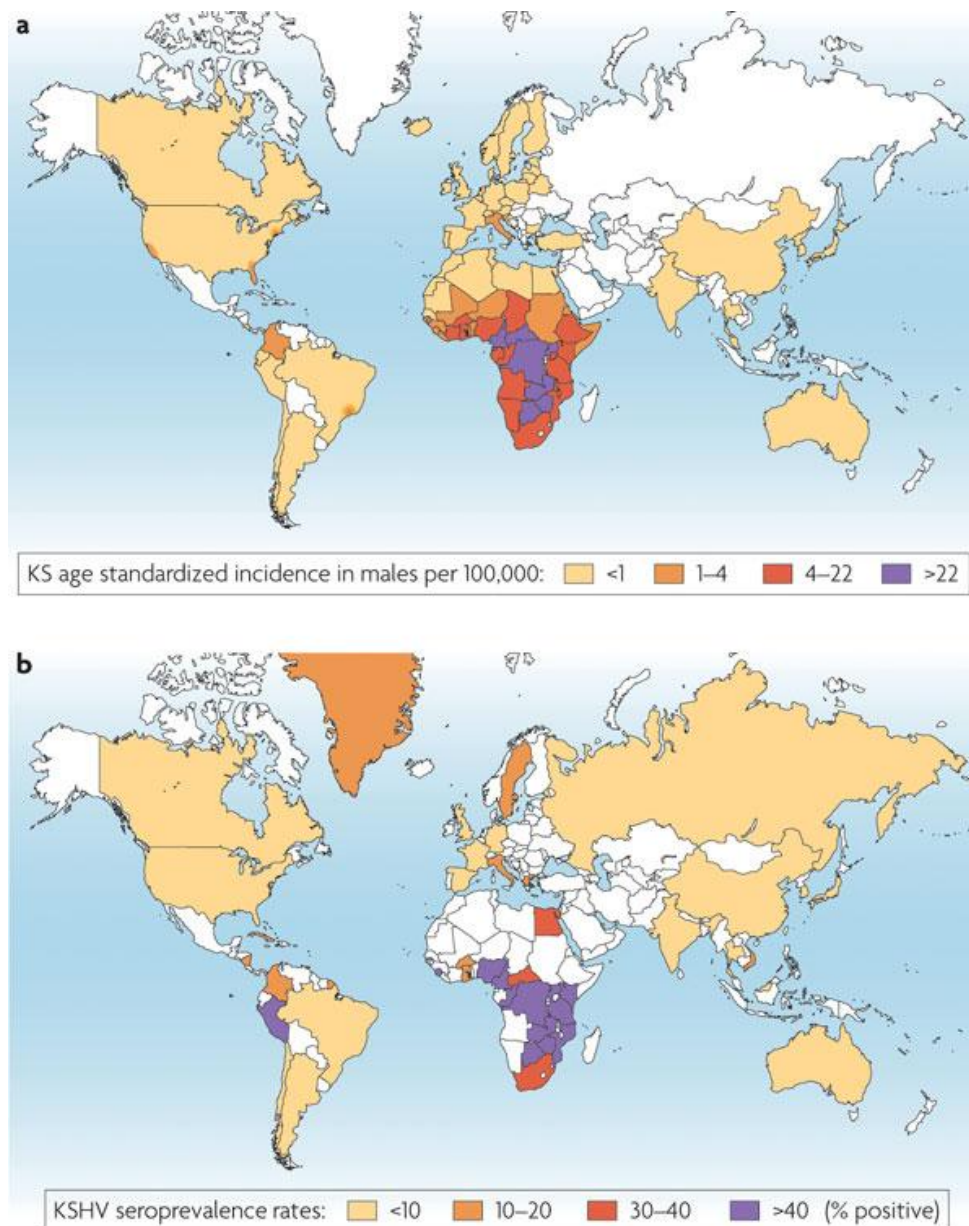
Figure 1: Incidence and Seroprevalence of KS: Age-standardized incidence of Kaposi's sarcoma in males shows cases primarily in sub-Saharan Africa, attributable to high HHV-8 seroprevalence [3].

1.2.2 Transmission of HHV-8

The single most effective method of HHV-8 transmission is direct contact with an infected individual, most often through sexual encounters. Immune suppression, like HIV co-infection, can support a more productive infection [1, 13, 27, 37-39]. Heterosexual transmission does occur, but apparently at smaller rates than homosexual exposure although the reasons for these differences are not clearly understood [11-13, 30, 37]. HIV seronegative males who are HHV-8 seropositive have a very low prevalence of KS [11, 17, 19, 37, 38, 40, 41], suggesting that HIV and its subsequent immunosuppression is an enabling factor in KS development [2, 37-41]. Sexual practices associated with HHV-8 transmission include deep kissing, giving and receiving oral sex, any given vaginal or anal sexual practices, oro-anal contact, or any genital contact with mucosal secretions [12, 13, 17, 26, 27, 30, 42, 43]. Some reports have also shown exchange of saliva as a mode of HHV-8 transmission [25, 27-29, 32, 33]. In sub-Saharan Africa, HHV-8 infection begins near childhood generally through salivary contact which occurs by mothers presumably by the pre-mastication of food before feeding a young child [25, 27-29, 32, 33].

1.2.3 Immune Suppression and KS Development

Endemic KS was prevalent in sub-Saharan Africa before the AIDS epidemic [14, 17, 29, 31, 33]. However, after the worldwide spread of AIDS, more cases of KS began to appear, though it can be argued that homosexual males participating in risky sexual practices were already seropositive for the virus; HIV and the disease AIDS simply allowed a break from viral latency [13, 35, 37-41]. Immune suppression can result in a latent HHV-8 infection reactivating to a productive infection; this is likely why patients undergoing chemotherapy or solid organ transplants may

also be at risk for the form of KS known as iatrogenic [15, 16, 34]. While HAART therapy (to fight HIV and thus immune suppression) has reduced the prevalence of KS, non-compliance with the therapy as well as factors not related to HIV will let HHV-8 persist and break from latency as an opportunist infection [4].

1.3   HHV-8 VIROLOGY

This section will discuss features of HHV-8 in terms of genomic structure, physical structure, reproductive cycle, stages of latency, and gene expression relevant to this research. HHV-8 is a member of the family herpesviridae, subfamily rhadinovirus [1].   As a member of the herpesvirus family, HHV-8 has an icosahedral capsid containing a double-stranded, linear DNA genome.   The capsid is encased by a lipid envelope and there is an amorphous structure containing both viral and cellular proteins located between the capsid and envelope termed the tegument [1].

1.3.1   Viral Tropism

HHV-8 can infect a variety of cell types such as endothelial cells, [1, 8, 9], activated B cells, dendritic cells and activated macrophages [5, 6]. In vitro studies have shown that among the known in vivo targets (B cells, endothelial cells, macrophages and dendritic cells), only B cell infection results in virus production [6].   Infection of the other cell types results in a non-productive, abortive type infection [35]. HHV-8 infection of immature dendritic cells has been shown in vitro to result in partial maturation of the DCs and a loss of antigen presentation

capability which may contribute to the ability of HHV-8 to evade a robust primary immune response [5].

1.3.2 Genomic Structure

The DNA genome of HHV-8 is approximately 160-170 kilobasepairs (kbp) in length [1, 35], with 145 of those coding for different open reading frames, many of which are similar to that of Epstein-Barr virus [1, 2]. The remaining base pairs make up terminal repeat sequences rich in G-C content [1]. There are about 75-80 different open reading frames (ORFs) in between the terminal repeats, which transcribe a variety of proteins for replication, latency, and immune modulation [1].

1.3.3 Characterization of Infection

1.3.3.1 Viral Entry

Herpes viruses use two types of receptors for viral entry; a binding receptor, which concentrates the virus on the surface of the cell and an entry receptor which is required for the virus to actually enter the cell through either fusion of its envelope with the cellular membrane of by endocytosis [1]. HHV-8, like several other herpesviruses, has been shown to use heparan sulfate as its binding receptor [1, 35]. At least two separate entry receptors have been identified. The first is dendritic cell-specific ICAM-3 grabbing nonintegrin (DC-SIGN) which is found on dendritic cells and activated B cells [5, 6], and the $\alpha3\beta1$ integrin which has been reported on other cell types [44].

1.3.3.2 Relevant Pathogenesis

Upon entry into the cell, the virus is delivered to the cytosol, releasing the tegument and capsid [1, 35, 45]. Like herpes simplex virus, the virus capsid uses microtubules to get to the outside of the nucleus where it is partially uncoated releasing the viral DNA genome [46]. Viral DNA enters the nucleus through nuclear pores and initiates viral replication and mRNA transcription [1, 46, 47].

Depending on the cell type, infection may result in lytic phase protein production or production of latent transcripts [1, 35, 47-49]. In context of this research, a notable lytic gene transcribed early in HHV-8 infection is ORF50, which encodes the replication trans-activator or RTA protein. RTA is necessary and sufficient for inducing the switch between a latent and lytic state of infection [48-52].

1.3.4   Difficulty in Studying HHV-8

A substantial challenge in studying HHV-8 is the task of determining infectious virus titers. During a productive infection, cell types permissive for replication produce both infectious and non-infectious particles. In studies where knowing the input of infectious virus is required, it is vital that we be able to distinguish infectious from non-infectious particles. The ability to determine an infectious titer permits in vitro experiments with defined multiplicity of infections (MOI) such as experiments investigating replication kinetics, development of latency, and neutralization assays, which has an important role in clinical settings.

1.3.4.1 Current Methodology for Determining HHV-8 Titer

Techniques to assay viral infectivity include determination of viral DNA copy numbers and fluorescence imaging of infected cells. Quantitative real-time PCR (to determine viral DNA copy number) is a great method for determining encapsidated (DNAse-treated) viral DNA copies, but the data do not indicate whether the virus is actually infectious. Antibody-based visualization assays are generally non-empirical, and determination of accurate infectious titer is difficult.

1.3.4.2 Current Studies

To address the question of how to determine infectious HHV-8 viral titer, some laboratories reported using recombinant mutant HHV-8 viruses that express different fluorescent proteins driven by either cellular or viral promoters. When using these mutant viruses, the number of fluorescent cells detected following infection is used to determine infectivity of the virus sample [51]. Although this is a novel cell culture-based technique for determination of viral titer, these studies inherently suffer from the fact that they require the use of mutant viruses and this procedure cannot be used with wild-type, non-mutant viruses. The assay developed in this thesis can be used to find infectious titers for any HHV-8 virus sample.

To study HHV-8 viral entry, a cell line called T1H6 was developed by Naoki Inoue at the CDC [52]. This is a 293T cell line with a plasmid containing the β-galactosidase gene under the control of the HHV-8 polyadenylated (PAN) RNA promoter (termed T1.1), which is a induced by the HHV-8 RTA protein [49]. Infection of these cells with HHV-8 results in production of the RTA protein from the infecting virus interacting with an RTA-response element (RRE) which drives the production of β-galactosidase through the action of the T1.1 promoter. β-galactosidase activity is measurable through a chemiluminescence-based β-galactosidase

detection kit. This is a novel way to quantitate infectious virus through the production of HHV-8 RTA as an indicator of lytic protein production. In the original study, Inoue and coworkers demonstrated the need to use polybrene to obtain efficient infection [52]. Since polybrene is used for receptor independent viral entry, this requirement can be viewed as proof that the T1H6 cells did not express a proper HHV-8 cellular receptor and diminishes the usefulness of these cells to measure infectious virus titers since infectivity should be based on natural (receptor-ligand binding) entry of virus into susceptible cells.

To address the problem requiring the use of polybrene with the T1H6 cells, we stably transfected the cells with a plasmid expressing DC-SIGN expressed under the control of a strong CMV immediate-early promoter. We have shown previously that DC-SIGN serves as an efficient entry promoter for HHV-8 [5, 6]. Expression of DC-SIGN on T1H6 cells should negate the need to use polybrene for viral entry. The construction of the T1H6-DC-SIGN cells has allowed for the development of the first in vitro assay to determine $TCID_{50}$ values with HHV-8.

2.0   SPECIFIC AIMS

2.1   AIM 1: DEVELOP A CELL CULTURE SYSTEM USING T1H6-DC-SIGN FOR DETERMINING TCID$_{50}$ VALUES FOR HHV-8

2.1.1   Determine Optimal Time for β-galactosidase Measurement

Determination of background levels of β-galactosidase and the optimal time post-infection for measuring β-galactosidase levels will be determined in T1H6-DC-SIGN infected and uninfected cells. This will demonstrate an appropriate time to measure β-galactosidase while also controlling for cell count.

2.1.2   Demonstrate Reporter Gene Specificity to HHV-8 ORF50 (RTA)

To demonstrate the specific ability of T1H6-DC-SIGN cells to produce β-galactosidase following expression of the HHV-8 RTA protein, T1H6-DC-SIGN cells will be transfected with increasing amounts of a plasmid expressing the HHV-8 ORF50 gene (which encodes for the RTA protein) and levels of β-galactosidase will be determined.

2.1.3   Demonstrate the Role of DC-SIGN as a Receptor for HHV-8 Entry

To demonstrate the role of DC-SIGN as a viral entry receptor, β-galactosidase levels will be compared between T1H6 and T1H6-DC-SIGN cells infected with increasing amounts of HHV-8.

2.1.4   Determination of HHV-8 TCID$_{50}$ values using T1H6-DC-SIGN cells

The utility of the T1H6-DC-SIGN β-gal assay for measuring TCID$_{50}$ will be demonstrated using different HHV-8 viral preparations.

2.1.5   Validation of TCID$_{50}$ Values

To validate TCID$_{50}$ values (determined in Aim 2.1.4), immature dendritic cells (which express DC-SIGN) will be infected at two different TCID$_{50}$ values. Demonstration of viral infection will be performed by immunocytochemistry using an antibody directed against the HHV-8 ORF59 protein.

2.2   AIM 2: SHOW UTILITY OF THE T1H6-DC-SIGN $TCID_{50}$ ASSAY

2.2.1   Determination of Specific Infectivity

Specific infectivity is defined as the ratio of infectious to non-infectious particles. To determine specific infectivity, the $TCID_{50}$ values will be converted to plaque forming units and divided by the number of encapsidated DNA genomes.

2.2.2   Examination of Replication Kinetics of HHV-8 in Activated B cells

The $TCID_{50}$ assay will be used to demonstrate the amount of infectious HHV-8 produced over time in activated B cells.

3.0   MATERIALS AND METHODS

3.1   CELL LINES

T1H6: T1H6 is a human embryonic kidney (HEK) 293T cell line containing a β-galactosidase gene under the control of the HHV-8 T1.1 promoter. T1H6 cells were grown in DMEM (CellGro #10-013-CV) with 10% heat inactivated FCS (Gemini #100-506), 1X Gentamicin (Lonza #17-518Z) and 100 µg/mL Hygromycin B (Clontech #631309). Cells were split using trypsin/EDTA (Cellgro #25-052-CI) when cell density was approximately 80% confluence.

T1H6-DC-SIGN: T1H6-DC-SIGN cells are T1H6 cells containing DC-SIGN under the control of a CMV immediate-early promoter. The plasmid expressing DC-SIGN was obtained from the NIH AIDS repository. Construction of these cells was performed by Dr. Heather Hensler in our laboratory by stably transfecting them with the DC-SIGN plasmid using a Lipofectamine 2000 kit according to manufacturer's protocol. Removal of T1H6 and T1H6-DC-SIGN cells from flasks for plating (prior to individual experiments) was performed using warm PBS instead of trypsin/EDTA to prevent cleavage of DC-SIGN from cell membranes.

BCBL-1: BCBL-1s are a B cell line that is HHV8+ and EBV-. Cells were grown in RPMI 1640 with L-glutamine (CellGro #10-040-CV), 10% FCS (Gemini #100-506), and 1X Gentamicin (Lonza #17-518Z). HHV-8 lytic replication can be induced in these cells using 12-O-tetradecanoylphorbol-13-acetate (TPA).

Monocyte-derived dendritic cells: To produce monocyte-derived dendritic cells (MoDCs), monocytes from 4×10^7 frozen PBMCs (graciously obtained from Dr. Pawel Kalinski's laboratory at the Hillman Cancer Center) were removed from other leukocytes by a plastic adherence assay. The PBMCs were thawed, and then spun briefly to remove most freezing media. They were then placed in T75 flasks with 10 mL serum-free IMDM (CellGro #10-016-CV) for 1 hour at 37˚ C. All cells that did not adhere were removed and the adhering cells were treated with 10 mL of IMDM + 10% FCS, as well as 1000 U/mL GM-CSF and 1000 U/mL IL-4 (gifts to Dr. Kalinski's laboratory from Schering Plough, Kenilworth, NJ). Media and cytokines were refreshed on day 4, and the immature dendritic cells were used on day 5.

3.2   METHODS

3.2.1   Preparation of HHV-8 Virus Stocks

Preparation of HHV-8 virus stock is the same as described in previous studies from our lab [5, 6]. In short, 1 L of BCBL-1s were grown to 2×10^5 cells/mL, then induced with 20 ng/mL of TPA for 5 days. Supernatant was harvested by spinning for 15 minutes at 5000 rpm at 4˚ C in a Sorvall SLA-1500 rotor and the cell pellets were lysed using multiple freeze-thaw cycles after the pellets were resuspended in 1X PBS+0.1% BSA. Supernatant from cell lysate was added to the previous supernatant, and 2.3% (w/v) NaCl was added. 7% PEG 8000 (w/v) was added to precipitate virus; this was left overnight at 4˚ C. The next day the Sorvall SLA-1500 rotor was used to centrifuge the previous supernatant for 30 minutes at 8000 rpm at 4˚ C. The pellet was resuspended and spun in Eppendorf tubes for 4 minutes at 13000 rpm. This solution was then put

through a 25% sucrose (w/v) in 1X PBS gradient and centrifuged for 2 hours at 28000 rpm at 4˚ C using a Beckman SW28 rotor and Beckman ultracentrifuge. The viral pellet then soaked overnight in PBS+0.1% BSA. The next day, virus was resuspended, put in 20 μL aliquots, and stored at −80˚ C.

3.2.2 Quantitative Real-Time PCR

To determine the amount of HHV-8 DNA genomes (defined as DNAse-resistant DNA) in virus stocks, a Qiagen QIAamp DNA Blood Mini kit was used to extract viral DNA (Qiagen #51106). Using a Quanta BioSciences master mix with dNTPs, Mg^{2+}, polymerase (Quanta #95051-100), reverse primer (IDT #50526394, 5'-AGTGAGCATGGCAGATGTTGCT-3'), forward primer (IDT #50526393, 5'-GTCTCTTGGACAAGCTCGCTGTT-3') a probe for HHV8 K8.1 glycoprotein (IDT #80176396, 5'/56-FAM/CGGTCTGTGAAACGGTCATTG), and a StepOne Plus thermocycler (Applied Biosystems), viral DNA copies were quantitated using a standard curve compared to a manufacturer's stock of pre-quantitated HHV-8 viral DNA (ABi #08-938-250).

3.2.3 Immunofluorescence Assays

1×10^5 cells/well of T1H6 and T1H6-DC-SIGN were plated in 8-well chamber slides with appropriate media. 4 days later, upon visual confluence, cells were washed in 1X PBS, fixed in 4% PFA, blocked and permeabilized in 0.3% saponin/0.5% BSA in 1X PBS, and blocked in 10% normal goat serum (Gibco #16210) in 1X PBS. Mouse-anti-DC-SIGN (R+D #MAB161) was used 1:100 in 10% normal goat serum and incubated for 1 hour at 37º C. After several 1X PBS

rinses, the cells were treated with goat-anti-mouse IgG-FITC 1:100 (Santa Cruz #sc-2010) for 1 hour at 37º C. Slides sat overnight (in the dark) in 1X PBS at room temperature. Expression of DC-SIGN was determined by visualization with a fluorescent microscope. To visually demonstrate HHV-8 infectivity, 8-well chamber slides were seeded with 3.5×10^6 immature DCs per well. HHV-8 was allowed to adsorb to the cells for 2 hours after which the media was changed, and the cells incubated at 37º C. 48 hrs post infection (hpi) expression of the HHV-8 ORF59 protein was determined using mouse-anti-ORF59 (ABi #13-212-100) as well as goat-anti-mouse IgG-FITC (Santa Cruz #sc-2010), both at 1:100 dilutions. 30 nM DAPI (Invitrogen #D1306) was used to counterstain the nuclei. Pictures were taken using a QImaging Retiga, using QCapture to acquire images. Images were merged using Adobe Photoshop's overlay and exclusion features.

3.2.4   β-galactosidase Measurements

For all experiments pertaining to background chemiluminescence, timing of β-galactosidase measurement, specificity of the cells response to HHV-8 ORF50 expression, and demonstration of DC-SIGN's importance in viral entry, 8×10^4 cells/well were plated in clear 48-well plates in replicates of 3. For purposes of determining $TCID_{50}$, 4×10^4 T1H6-DC-SIGN cells were plated in black, clear-bottom Corning CellBind 96 well plates in replicates of 6. T1H6 and T1H6-DC-SIGN were plated in DMEM (CellGro #10-013-CV) + 10%FCS (Gemini #100-506) + 1XGM (Lonza #17-518Z) + 100 µg/mL Hygromycin B (Clontech #631309). Plate layout for infection as well as measurement of chemiluminescence is shown below. 10-fold dilutions were set up using 10 µL of HHV-8 stock or supernatant and 90 µL of media, and 15 µL of each dilution was inoculated into each well.

Example: Experimental setup for plating and β-galactosidase measurement. Uninf. = uninfected, and numbers refer to dilutions of virus.

uninf.	uninf.	uninf.	uninf.	uninf.	uninf.	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-5}
10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}
10^{-2}	10^{-2}	10^{-2}	10^{-2}	10^{-2}	10^{-2}	10^{-1}	10^{-1}	10^{-1}	10^{-1}	10^{-1}	10^{-1}

48 hpi cells were harvested using 100 µL/well of CellStripper (CellGro #25-056-CI), by incubation at 37º C for about 15 minutes. The contents of each well was collected and a cell pellet harvested by centrifugation for 5 minutes at 13000 rpm, 4º C. Each pellet was washed 3 times with 100 µL/vial of cold 1X PBS. After each wash, the cells were pelleted by centrifugation for 2 minutes at 13000 rpm, 4º C. Utmost care was taken to aspirate as much PBS as possible without aspirating cells. Pellets were then resuspended in 120 µL of cold potassium phosphate/DTT lysis buffer to prevent protein oxidation damage. The potassium phosphate/DTT lysis buffer consisted of 915 µL 100 mM K_2HPO_4 in ddH_2O, 85 µL 100 mM KH_2PO_4 in ddH_2O, and 1 µL 1 M DTT in ddH_2O. The cell pellet solution was then subjected to 3 freeze-thaw cycles in dry ice with EtOH and a 37º C water bath to lyse the cells. Cell debris was removed by centrifugation at 13000 rpm for 10 minutes at 4º C, and lysates were carefully collected, avoiding the cell debris pellet by any means possible. 15 µL of lysate was plated per well, in replicates of 6. The Clontech β-galactosidase Chemiluminescence Detection Kit II (Clontech #613712) protocol recommends a maximum of 300 reactions; this uses 196 µL of manufacturer-provided β-galactosidase buffer and 4 µL of substrate per reaction, and this was used in all chemiluminescent measurements excluding $TCID_{50}$ determination. However, a productive, measurable reaction is possible using less of the manufacturer provided substrate and buffer, and

this was used in TCID$_{50}$ determination. 117.6 μL of buffer and 2.4 μL of substrate were plated per well in the dark. The substrate and buffer were mixed with the cell lysates, and the reaction proceeded for 1 hour at room temperature in the dark before measurement using a BioTek Synergy II plate reader; data was recorded in 10-second integrals at a sensitivity setting of 250. To determine positive vs. negative status of infection per well, the average of the uninfected cell lysate chemiluminescence readings (background average chemiluminescence), plus twice the background standard deviation value, was subtracted from each test value.

Example:

uninfected cell lysate chemiluminescence (hypothetical) = 400

standard deviation = 5

background value = 410

example test value = 415

True value = 5, 5 > 0, well is infected

If true value ≤ 0, well is uninfected

3.2.5 Mathematical Determination of TCID$_{50}$

The Reed-Muench calculation [53] was used to determine 50% endpoint titer of virus stocks as well as virions from infected activated B cells on T1H6-DC-SIGN.

Step 1: calculate proportionate distance (PD).

$$\frac{(\% \text{ of wells infected over } 50\%) - (50\%)}{(\% \text{ of wells infected over } 50\%) - (\% \text{ of wells infected less than } 50\%)}$$

Step 2: Solve and divide by inoculum size:

$$\text{TCID}_{50}/\text{mL} = 10^{(\text{PD}+(-\text{Log dilution greater than 50\% infected}))} / \text{inoculum volume (mL)}$$

To convert TCID_{50} values to plaque forming units/ml (PFU/ml) Poisson distribution was applied using the formula $P(0) = e^{(-m)}$ where $P(0)$ is the proportion of negative (uninfected) samples and m is the mean number of infectious units / mL (pfu/mL). Thus, for any TCID_{50} value, $P(0) = 0.5$ and solving for m results in $m = -\ln 0.5$ which is 0.7. Based on these results, the conversion of TCID_{50} to pfu/ml was achieved by multiplying the TCID_{50} value by 0.7.

3.2.6 Transfection Assay

A plasmid encoding HHV-8 ORF50 (RTA) was given to our lab as a gift by Dr. Yuan Chang and Dr. Patrick S. Moore at the Hillman Cancer Institute. A colony of E. coli containing the plasmid was grown on LB agar, selected, and let grown overnight in continuously agitated LB broth. Plasmid DNA was isolated using a cesium chloride density gradient. The DNA was dialyzed against 1X TE buffer overnight and precipitated with ethanol the next day. Using a Lipofectamine 2000 kit according to protocol, T1H6-DC-SIGN was plated at 8×10^4 cells/well in a 48 well plate, and 0.4, 0.8, and 1.6 mcg of ORF50 DNA were used to transfect the cells in replicates of 3. 48 hours later, the cells were harvested and β-galactosidase was measured.

3.2.7 ArrayScan IFA Evaluation of TCID_{50}s

On day 5 of immature MoDC preparation, 2×10^4 iDCs were removed from their flasks using CellStripper (CellGro #25-056-CI), counted, and plated in a black, clear bottom Corning CellBind 96 well plate in replicates of 4 using IMDM + 10% FCS. The next day, cells were set

up as uninfected, infected with 1 $TCID_{50}$, or infected with 2 $TCID_{50}$. Two separate virus stocks at 1 and 2 $TCID_{50}$s were used in these infections. The virus was allowed to adsorb for two hours and then media was replaced. 48 hpi, nuclei were stained using Hoechst 33342 (Invitrogen #H1399), and cells were stained for expression of ORF59 using a mouse monoclonal primary antibody for the viral protein ORF59 (ABi #13-212-100) and goat-anti-mouse IgG-FITC (Santa Cruz #sc-2010) as the secondary antibody, both used at 1:100. Stained cells were washed in 1X PBS overnight, and the next day they were read using a Cellomics ArrayScan VTi (read by William Buchser, Lotze DAMP Lab, Hillman Cancer Center). Thresholding of the scan was set to the primary antibody concentration that showed the clearest differences between infected and uninfected cells (1:100). Pictures were taken of various focal regions and used for visual analyses of the percentage of infected cells.

3.2.8   Statistical Analysis: T1H6 vs. T1H6-DC-SIGN

A 2-way ANOVA was performed to measure differences in HHV-8 infectivity (evaluated by measuring differences in β-galactosidase activity (RLUs) between T1H6 and T1H6-DC-SIGN cells. This assay was also performed on three different occasions, using both increasing volumes of virus as well as increasing dilutions of virus.

4.0   RESULTS

4.1   BACKGROUND EXPERIMENTATION

4.1.1   Demonstration that HHV-8 Virus Stocks are Infectious

4.1.1.1- qRT-PCR

Figure 2A

Figure 2B

Standard Curve

Target: HHV8 Slope: -3.047 Y-Inter: 40.03 R^2: 0.997 Eff%: 112.881
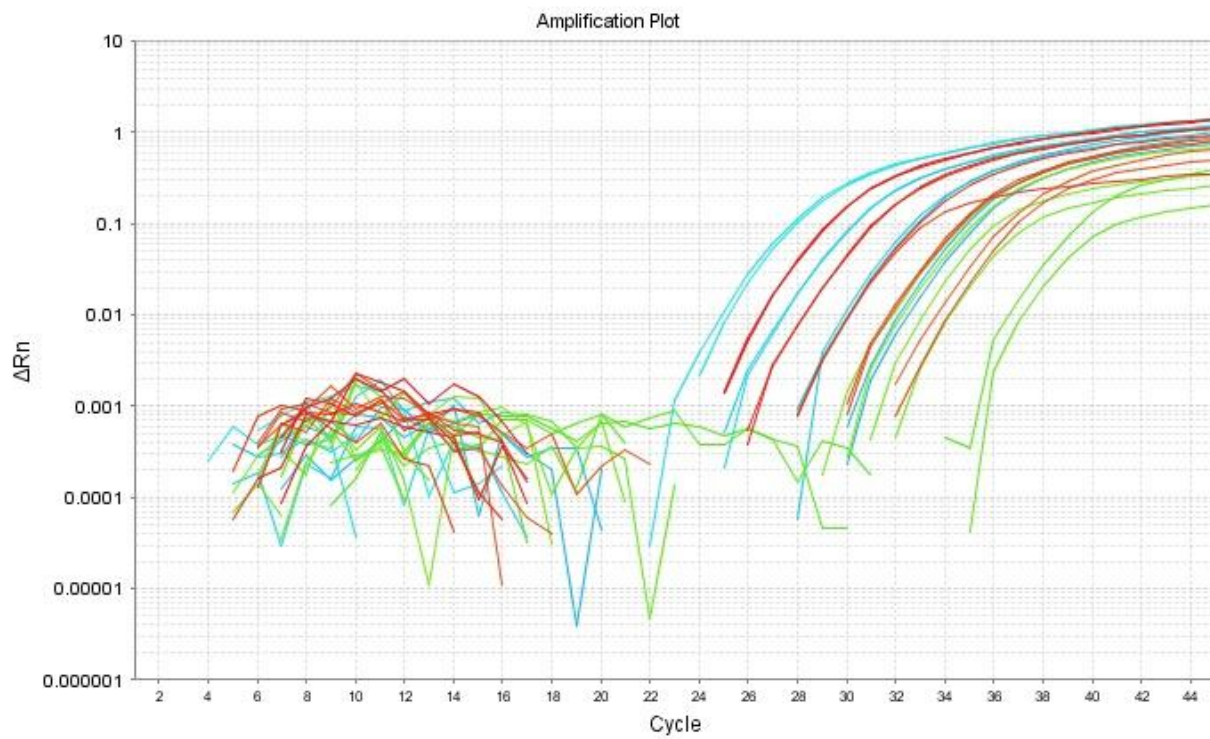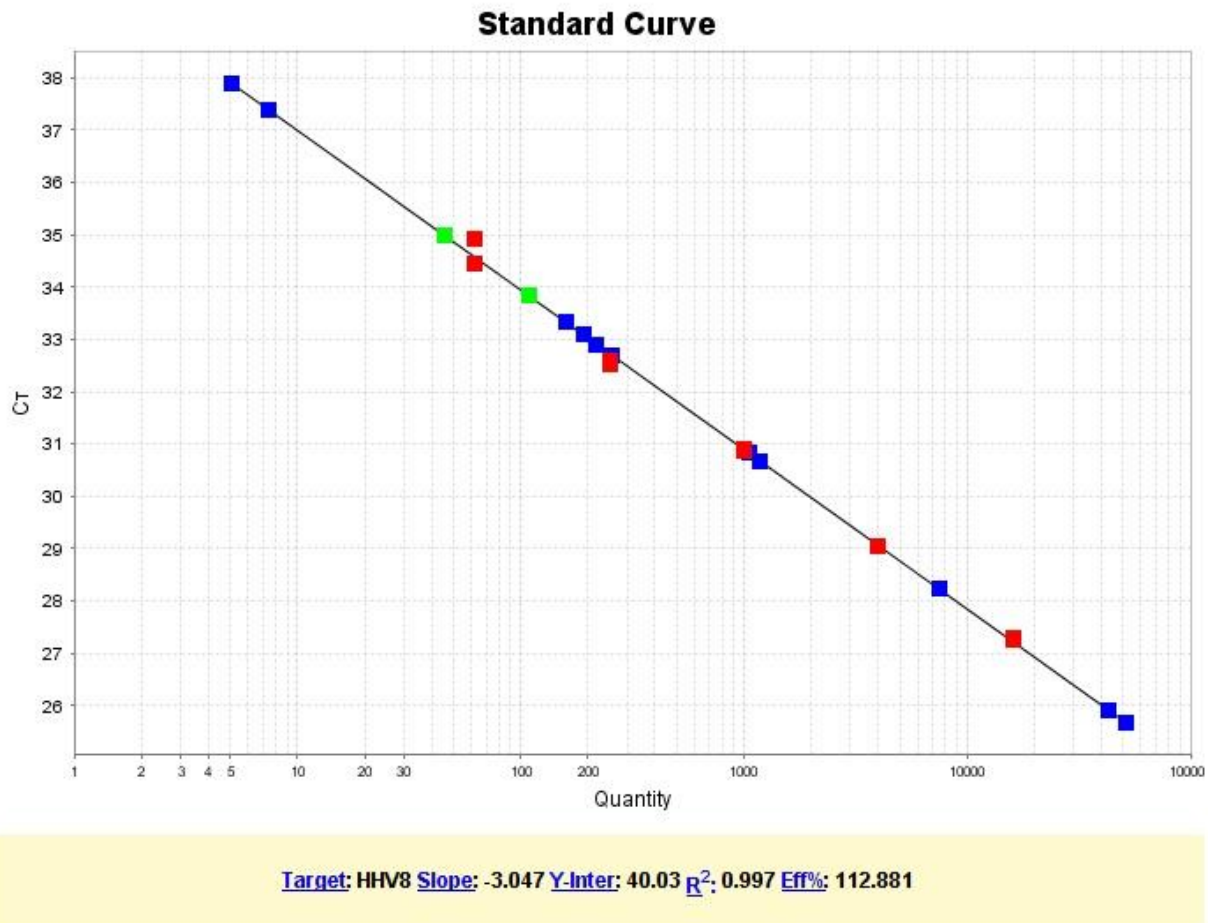
Figure 2: Determination of HHV-8 encapsidated genome copy numbers. qRT-PCR was performed on an HHV-8 preparation by Taqman chemistry using a primer set and probe for the K8.1 glycoprotein DNA. (A) Amplifcation plot. (B) Standard curve demonstrating the HHV-8 DNA standards (blue) and HHV-8 virus preparations (red and green).

The current method used in our laboratory to determine HHV-8 titers is by quantitative real time PCR (qRT-PCR) using DNAse-resistant viral DNA (which represents encapsidated DNA). As shown in Figure 2, this method is sensitive, allowing for the determination of viral genome numbers, but it cannot distinguish between infections and non-infectious virus.

4.1.1.2- IFA analysis

A second method for detecting viral infectivity is the use of immunofluorescence to detect the expression of viral proteins. An example of this is shown in Figure 3 demonstrating infection of immature DCs with HHV-8. While this is a non-quantitative method to assess HHV-8 viral stock infectivity, it is useful in characterizing infections.

Figure 3: Visual Confirmation of HHV-8 Stock Infectivity: Immunofluorescence assay on infected iDCs plated in chamber slides at $3.5 \times 10^6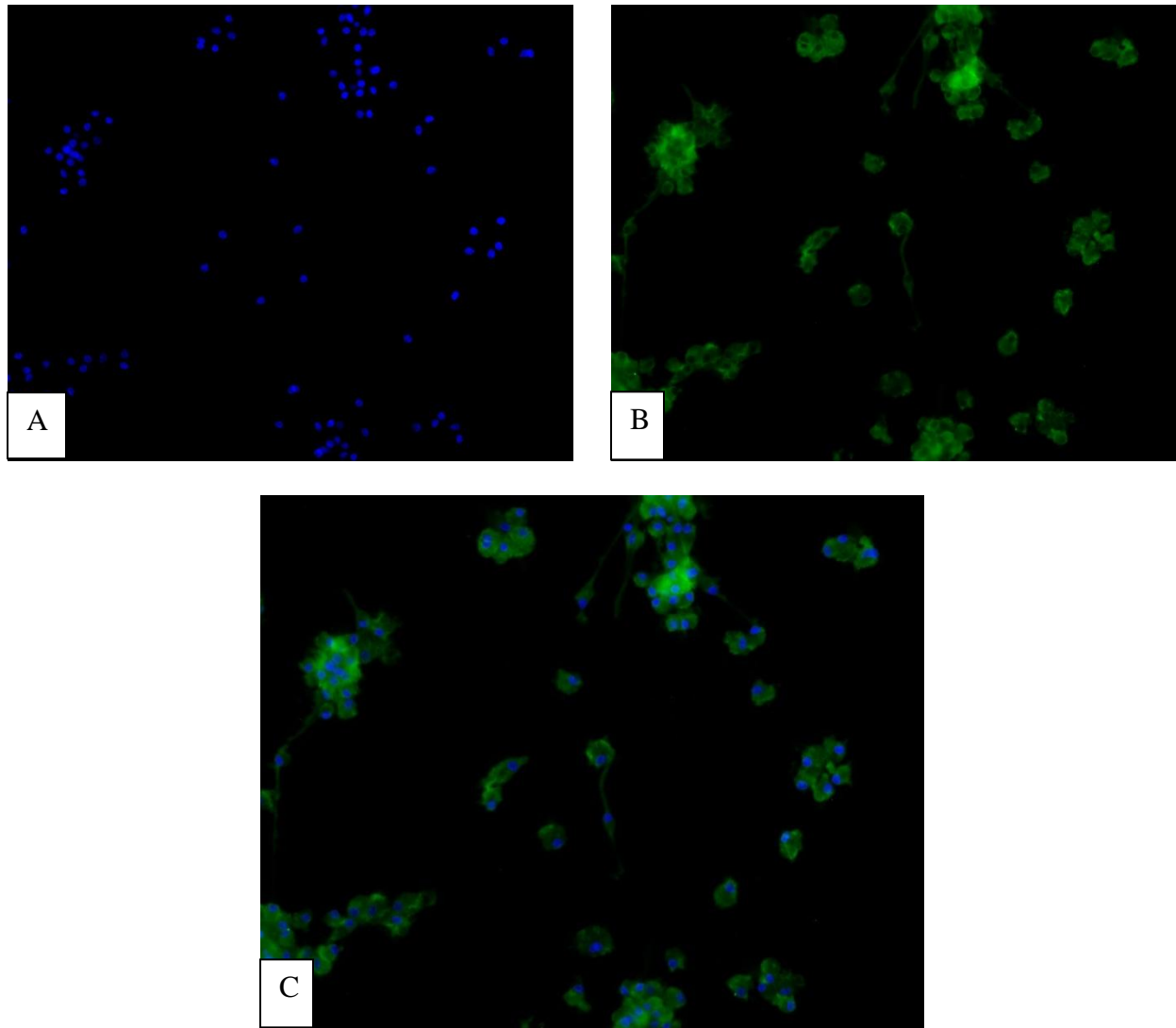$ cells/well. (A), DAPI staining of nuclei, (B) ORF59 staining detected by fluorescein-conjugated secondary antibody, (C) Merge of A and B.

4.2   AIM 1: DEVELOP A CELL CULTURE SYSTEM USING T1H6-DC-SIGN FOR

DETERMINING TCID$_{50}$ VALUES FOR HHV-8

4.2.1   Characterize T1H6-DC-SIGN Cells

T1H6-DC-SIGN cells were characterized by determining background β-galactosidase activity, differences in β-galactosidase levels following HHV-8 infection of T1H6 cells and T1H6-DC-SIGN cells, and demonstration that β-galactosidase activity is ORF50 expression dependent. The role of DC-SIGN as a receptor for HHV-8 entry was shown by infecting both T1H6 and T1H6-DC-SIGN cell lines with HHV-8 and comparing levels of β-galactosidase.

4.2.1.1 Background β-gal Production

To start charactering T1H6-DC-SIGN, cells were plated and over the course of 5 days lysates were harvested and tested for background β-galactosidase production. As seen in figure 4A, peak levels of β-galactosidase were seen at 72 hrs post plating. Beyond 72 hrs, the β-galactosidase levels dropped due to cell death caused by overpopulation of the wells. Figure 4B shows that 48 hpi is the best time for β-galactosidase measurement, as it allows for measureable increases in infection while simultaneously controlling for cell count. At 72 hours, there is no more control over cell count.

Uninfected T1H6-DC-SIGN Lysates

Figure 4A

β-Gal Chemiluminescence
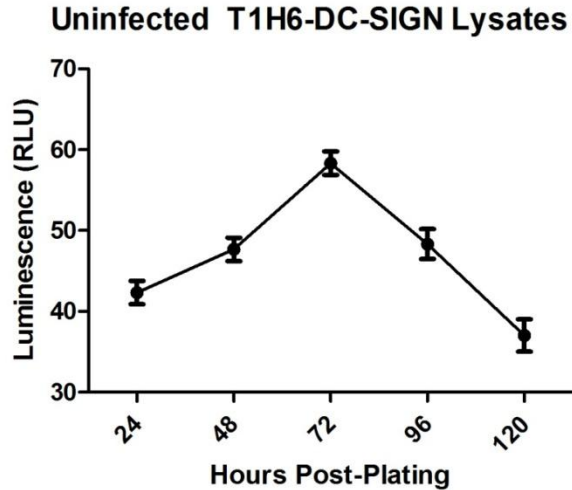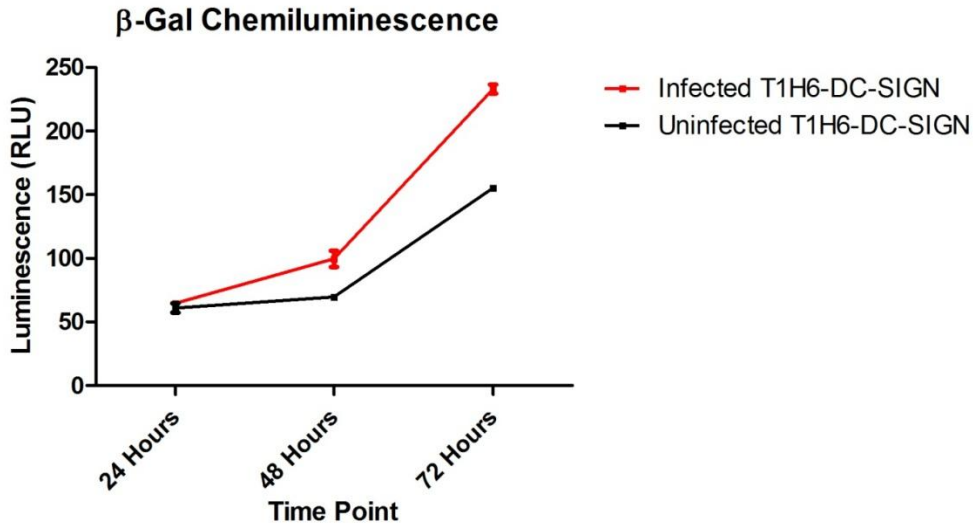
4B

Figure 4: Determining Optimal β-gal Measurement Timing: (A) Background levels of β-galactosidase activity were determined from uninfected cell lysates at days 1-5. (B) Levels of β-galactosidase were determined from uninfected or HHV-8-infected T1H6-DC-SIGN cells. Cells were infected with HHV-8 at a DNA MOI of 100, defined as a ratio of HHV-8 genome copy numbers to the number of cells plated. β-galactosidase levels were determined daily for three days following infection. Data are representative of mean chemiluminescence values from three wells and error bars indicate SEM.

4.2.1.2 Testing Dose-Dependent Reporter Gene Specificity to HHV-8 Production

To demonstrate that β-galactosidase activity in T1H6-DC-SIGN cells was due to expression of RTA (encoded by the ORF50 gene), a dose-dependent experiment was performed by transfecting T1H6-DC-SIGN cells with increasing dosages of a plasmid containing the ORF50 gene under the control of the strong CMV IE promoter. As shown in Figure 5, β-galactosidase levels were dose-dependent following ORF50 transfection.
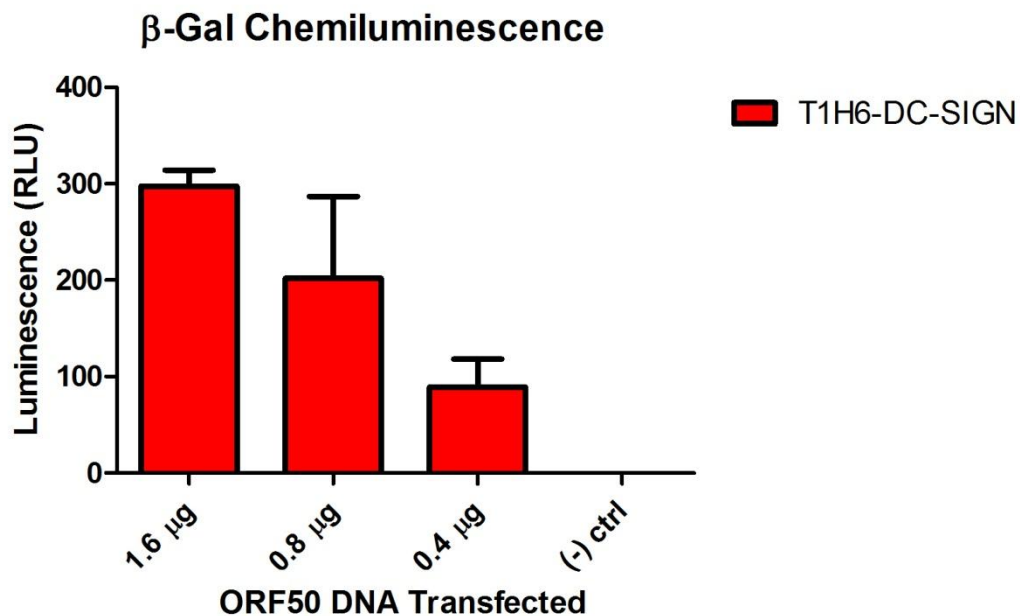
Figure 5: Specificity of Reporter Gene to HHV-8 ORF50: T1H6-DC-SIGN cells were transfected with Lipofectamine 2000 with increasing amounts of ORF50 DNA. β-galactosidase levels were determined 48 hrs post-transfection. Results represent average luminescence values from triplicates and error bars indicate SEM.

4.2.1.3 Visualization and Confirmation of DC-SIGN Expression

To confirm that T1H6 –DC-SIGN cells but not T1H6 cells expressed DC-SIGN, both lines were stained by immunofluorescence assay with a monoclonal antibody directed against DC-SIGN followed by a secondary goat anti-mouse antibody conjugated with fluorescein. Staining of T1H6 revealed no positive surface staining for DC-SIGN expression (Figure 6) while staining of T1H6-DC-SIGN cells revealed detectable surface expression of DC-SIGN (Figure 7). Morphological differences between the two cell lines are likely due to difficulties in cell adherence during staining and wash phases. As a positive control for DC-SIGN expression, immature monocyte-derived dendritic cells were also stained for DC-SIGN (Figure 8).
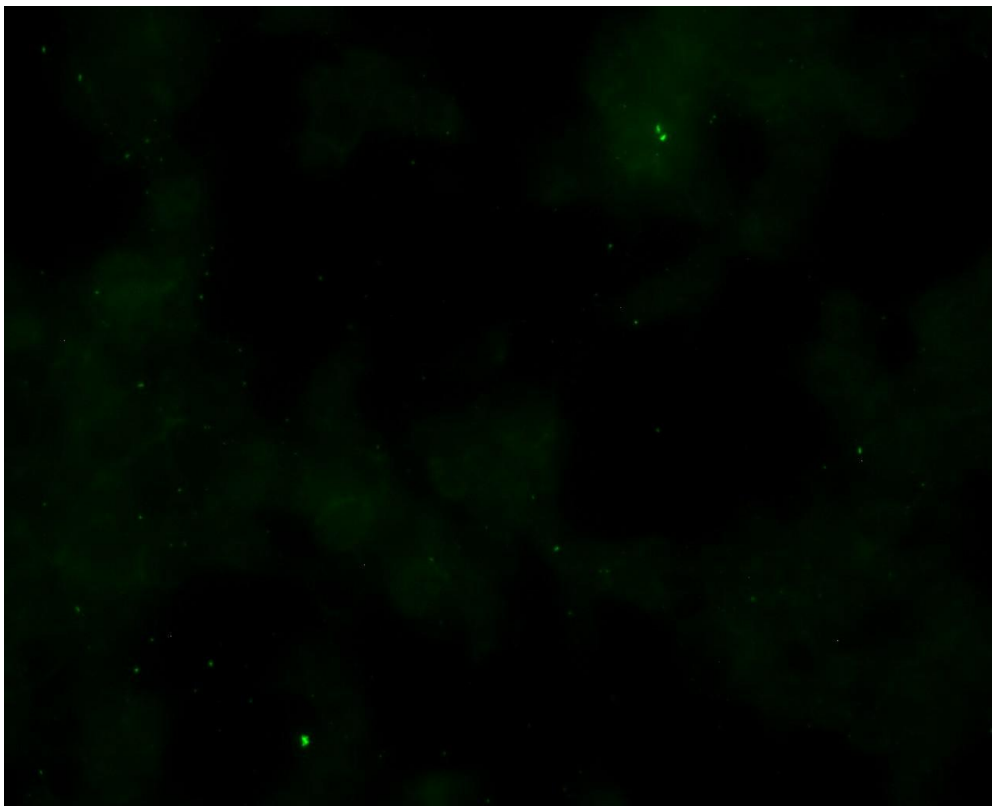
Figure 6: Confirming Lack of DC-SIGN Expression in T1H6: T1H6 cells were stained with a monoclonal antibody against DC-SIGN followed by a secondary goat-anti mouse antibody conjugated to fluorescein.
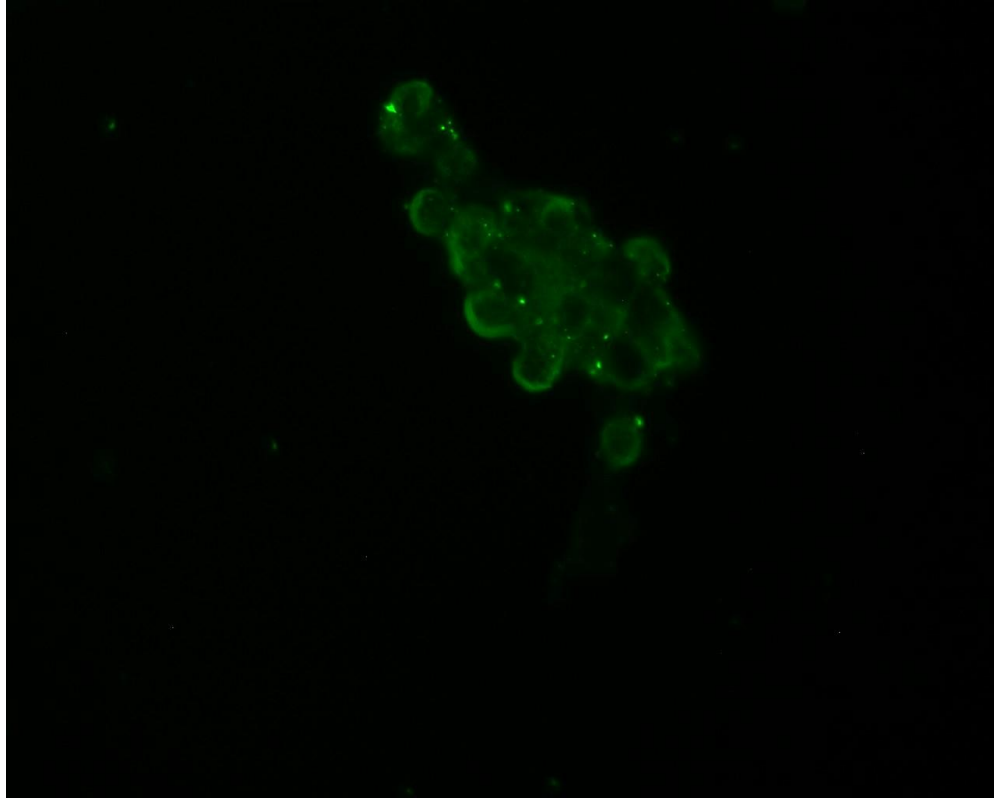
Figure 7: DC-SIGN Surface Expression in T1H6-DC-SIGN: T1H6-DC-SIGN cells were stained with a monoclonal antibody against DC-SIGN followed by a secondary goat-anti mouse antibody conjugated to fluorescein.
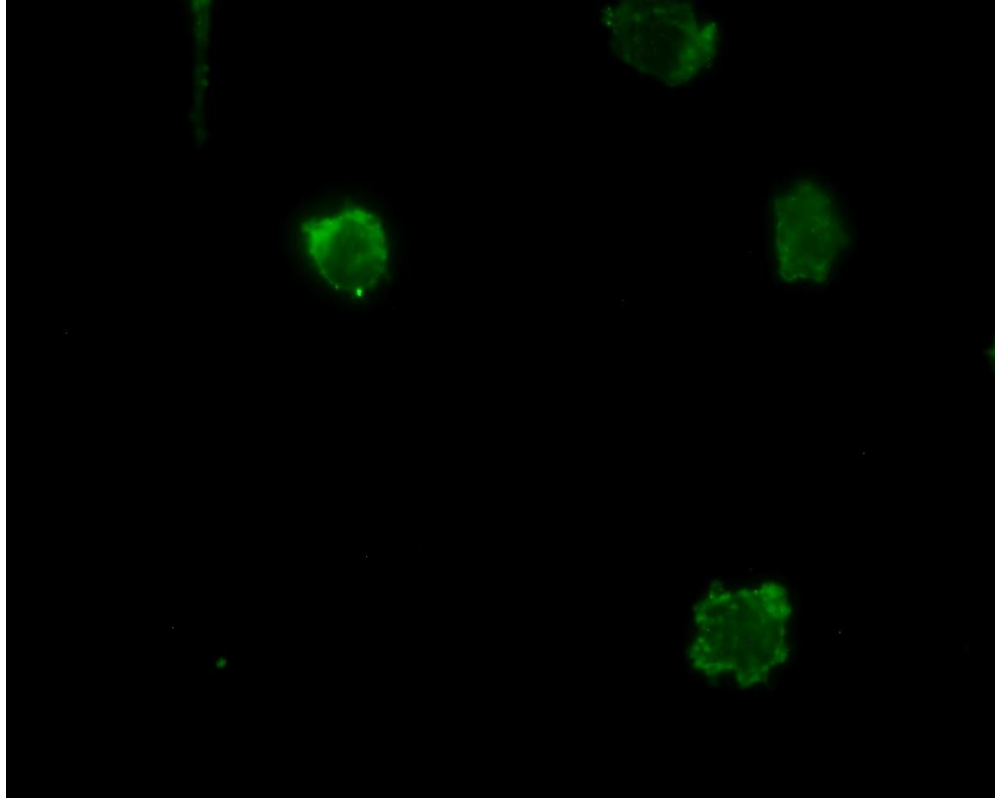
Figure 8: Positive Control for DC-SIGN Staining: Immature MoDCs grown from CD14+ monocytes and plated in chamber slides were stained for expression of DC-SIGN.

4.2.1.4 Compare T1H6 to T1H6-DC-SIGN Cells for Measuring HHV-8 Infectivity

To demonstrate the differences in β-galactosidase levels between HHV-8 infected T1H6 and T1H6-DC-SIGN cells, both cell lines were infected with increasing amounts of HHV-8 and levels of β-galactosidase activity were determined at 48 hrs post-infection. As shown in Figure 9, there was a significant increase in β-galactosidase levels in T1H6-DC-SIGN cells compared to T1H6 following infection with HHV-8 samples. The data shown in Figure 8 also suggest that there is an alternate mechanism of entry for HHV-8 in T1H6 cells since a small level of β-galactosidase above background was seen with the highest doses of HHV-8. In these experiments, PBS was used in place of β-gal substrate as a negative control, with β-galactosidase from the detection kit added to substrate for reaction and detection positive control.
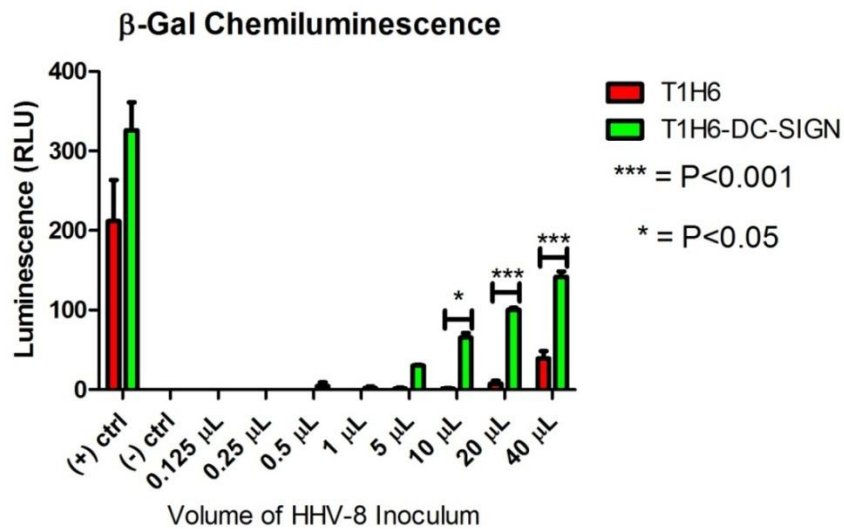
Figure 9: Comparison of T1H6 to T1H6-DC-SIGN: 8×10^4 T1H6 and T1H6-DC-SIGN were plated in triplicate and infected with varying dilutions of an HHV-8 viral preparation. β-galactosidase activity was measured at 48 hpi. Data are representative of 3 experiments showing mean chemiluminescence with error bars representing SEM. Asterisks denote differences with p values <0.05 as determined by 2-way ANOVA and Tukey post-test analyses.

4.2.1.5 Demonstrate utility of T1H6-DC-SIGN cells for determining HHV-8 TCID$_{50}$

The T1H6-DC-SIGN cells were used to determine HHV-8 TCID$_{50}$ values on several HHV-8

virus stocks using the assay developed in this thesis. The Reed-Muench calculation was used to

find TCID$_{50}$ [53]. Tables 1-4 show the results of TCID$_{50}$ determinations using four separate

HHV-8 virus stocks. Each dilution of virus was used to infect T1H6-DC-SIGN cells in replicates

of 6 wells and the plus/minus signs indicate positive or negative infection. These results were

later used to determine a TCID$_{50}$ value using the Reed-Muench calculation. Negative controls for

these experiments use PBS in place of β-gal reaction substrate. The average of uninfected cell

lysates plus 2 standard deviations were subtracted from each test value and if the resulting value

was greater than 0, the well was deemed as infected.

Table 1: Determination of TCID$_{50}$ in HHV-8 Stock #20

Dilution	Replicates					
10^{-1}	+	+	+	+	+	+
10^{-2}	-	+	+	-	+	+
10^{-3}	+	+	+	-	+	+
10^{-4}	-	+	-	+	+	+
10^{-5}	-	+	+	-	-	-
(-) ctrl	-	-	-	-	-	-

Using the Reed-Muench calculation (as described in Materials and Methods), the TCID$_{50}$ value

for HHV-8 virus stock #20 was 2.11×10^{6}/mL.

Table 2: Determination of TCID$_{50}$ in HHV-8 Stock #22:

Dilution	Replicates					
10^{-1}	+	+	+	+	+	+
10^{-2}	+	+	+	+	+	+
10^{-3}	-	+	+	+	+	-
10^{-4}	+	-	-	-	-	-
10^{-5}	-	+	-	-	-	-
(-) ctrl	-	-	-	-	-	-

Table 2 shows the results of a TCID$_{50}$ determination with a separate HHV-8 virus stock (#22)

The TCID$_{50}$ value for this virus stock was determined to be 1.46×10^5/mL.

Table 3: Determination of TCID$_{50}$ in HHV-8 Stock #24:

Dilution	Replicates					
10^{-1}	+	+	+	+	+	+
10^{-2}	-	+	-	-	+	+
10^{-3}	-	+	-	-	+	-
10^{-4}	-	+	-	-	-	-
10^{-5}	+	-	-	-	-	-
(-) ctrl	-	-	-	-	-	-

Table 3 shows the results of TCID$_{50}$ determination on HHV-8 virus stock 24, which was

6.67×10^3/mL.

Table 4: Determination of TCID$_{50}$ in HHV-8 Stock #25:

Dilution	Replicates					
10^{-1}	+	+	+	+	+	+
10^{-2}	+	+	+	+	-	-
10^{-3}	-	+	+	+	-	-
10^{-4}	+	-	-	-	-	-
10^{-5}	-	-	-	+	-	-
(-) ctrl	-	-	-	-	-	-

Table 4 shows the results of TCID$_{50}$ determination of HHV-8 virus stock 25 which was 6.67×10^4/mL.

4.2.1.6 Validate T1H6-DC-SIGN HHV-8 TCID$_{50}$ Values in Cell Culture

To validate the HHV-8 TCID$_{50}$ values obtained using the T1H6-DC-SIGN cell line, immature monocyte-derived DCs were infected with 1 or 2 TCID$_{50}$ of HHV-8 and 48 hrs post-infection, fixed and stained with an antibody directed against the HHV-8 ORF59 protein. Nuclei were stained with Hoechst. Immature dendritic cells were chosen as the target of HHV-8 infection as they are known and expected to express DC-SIGN on their surface (Figure 14). Pictures were taken of the infected cells. The pictures were blinded and given to three laboratory members who were tasked with counting the number of infected and uninfected cells (based on ORF59 staining). These experiments were repeated twice using two separate HHV-8 viral stocks (#20 and #23). Figures 10-13 represent the pictures of cells infected at 1 TCID$_{50}$ (figures 10 and 12) and 2 TCID$_{50}$ (figures 11 and 13). Tables 5-8 show the results of the cell counts of infected and uninfected cells by the three laboratory members. The results demonstrate that the TCID$_{50}$ values are valid in that infection of cells with 1 TCID$_{50}$ of either virus stock resulted in an average of 56% and 50% infected cells, while infection with 2 TCID$_{50}$ of either virus stock resulted in an average of 92% and 95% infected cells.
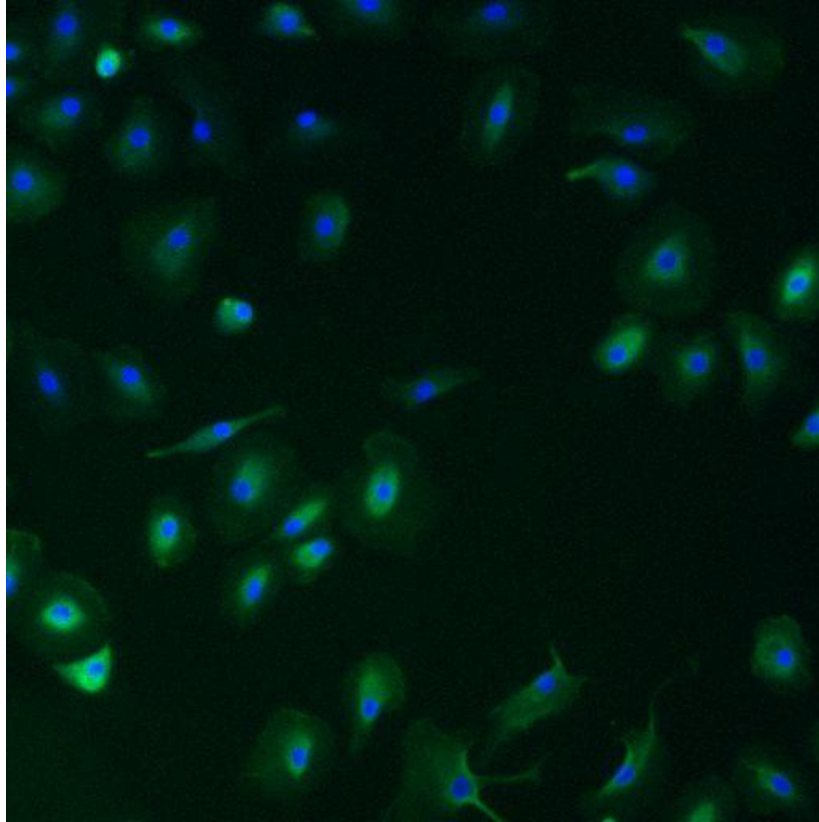
Figure 10: HHV-8 Stock #20 at 1 TCID$_{50}$: Immature monocyte-derived DCs were infected with HHV-8 viral stock #20 at 1 TCID$_{50}$ for 48 hrs, fixed and stained with anti ORF59 antibody (green) and Hoechst (blue).

Table 5: Validation of 1 TCID$_{50}$, HHV-8 Stock #20:

Stock 20	Counts:				
1 TCID$_{50}$	Counter 1	Counter 2	Counter 3	Mean	SD
Uninfected	14	14	26	18	7
Infected	21	24	22	22	2
Total	35	38	48		
% infected	60%	63%	46%	**56%**	9

Figure 11: HHV-8 Stock #20 at 2 TCID$_{50}$s: Immature monocyte-derived DCs were infected with HHV-8 viral stock #20 at 2 TCID$_{50}$ for 48 hrs, fixed and stained with anti ORF59 antibody (green) and Hoechst (blue).

Table 6: Validation of 2 TCID$_{50}$s, HHV-8 Stock #20:

| Stock 20	Counts:				
2 TCID$_{50}$s	Counter 1	Counter 2	Counter 3	Mean	SD
Uninfected	1	5	11	6	5
Infected	71	64	61	65	5
Total	72	69	72		
% infected	99%	93%	85%	**92%**	7
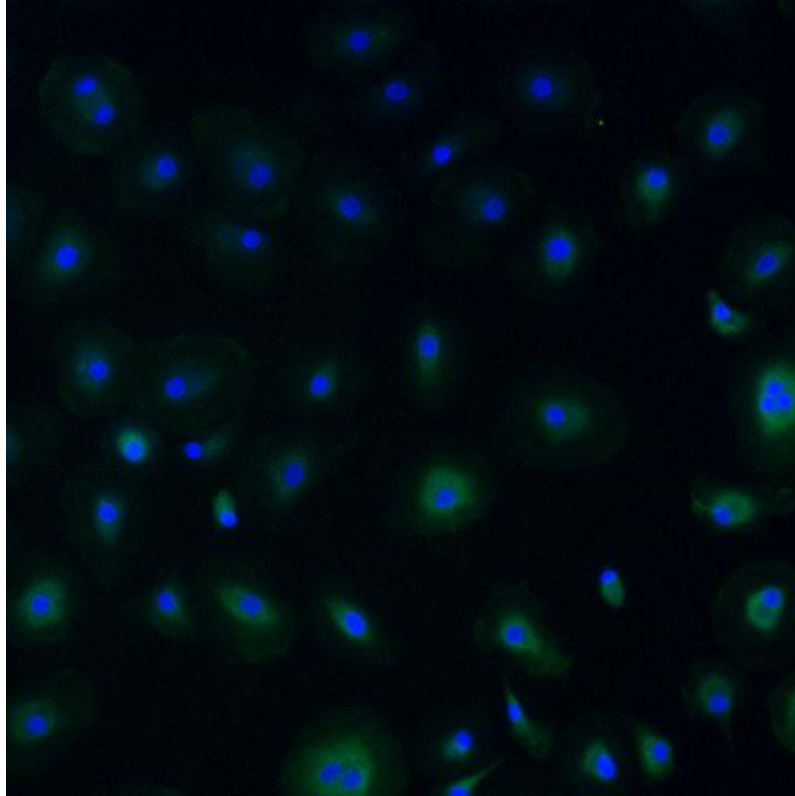
Figure 12: HHV-8 Stock #23 at 1 TCID$_{50}$: Immature monocyte-derived DCs were infected with HHV-8 viral stock #23 at 1 TCID$_{50}$ for 48 hrs, fixed and stained with anti ORF59 antibody (green) and Hoechst (blue).

Table 7: Validation of 1 TCID$_{50}$, HHV-8 Stock #23:

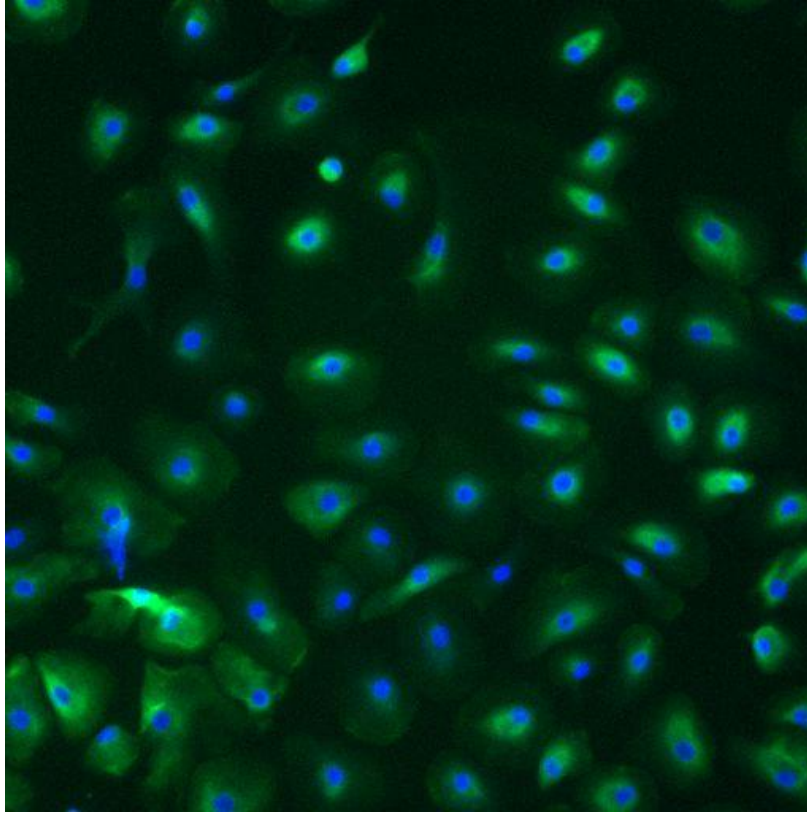| Stock 23					
1 TCID$_{50}$	Count 1	Count 2	Count 3	Mean	SD
Uninfected	21	16	24	20	4
Infected	23	24	20	22	2
Total	44	48	44		
% infected	52%	50%	45%	**50%**	3
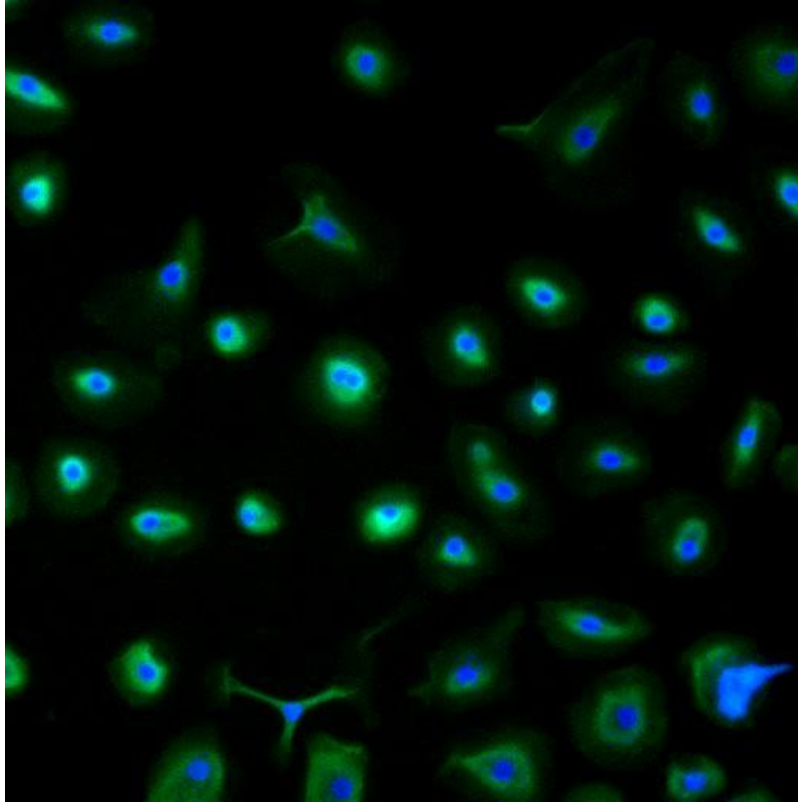
Figure 13: HHV-8 Stock #23 at 2 $TCID_{50}$s: Immature monocyte-derived DCs were infected with HHV-8 viral stock #23 at 2 $TCID_{50}$ for 48 hrs, fixed and stained with anti ORF59 antibody (green) and Hoechst (blue).

Table 8: Validation of 2 $TCID_{50}$s, HHV-8 Stock #23:

Stock 23					
2 $TCID_{50}$s	Count 1	Count 2	Count 3	Mean	SD
Uninfected	0	2	4	2	2
Infected	38	33	35	35	3
Total	38	35	39		
% infected	100%	94%	90%	**95%**	5

To further validate the $TCID_{50}$ values, immature monocyte derived DCs (from two separate PBMC donors) were also infected with HHV-8 using 1 and 2 $TCID_{50}$ values from two separate HHV-8 viral stocks. As shown in Table 9, the $TCID_{50}$ values were again validated with $TCID_{50}$ of 1 showing average infection of 49% and 52% of the cells while a $TCID_{50}$ value of 2 demonstrated an average infection of 98% and 92% of the cells.

Table 9: Repeat of $TCID_{50}$ Validation, Separate PBMC Donor:

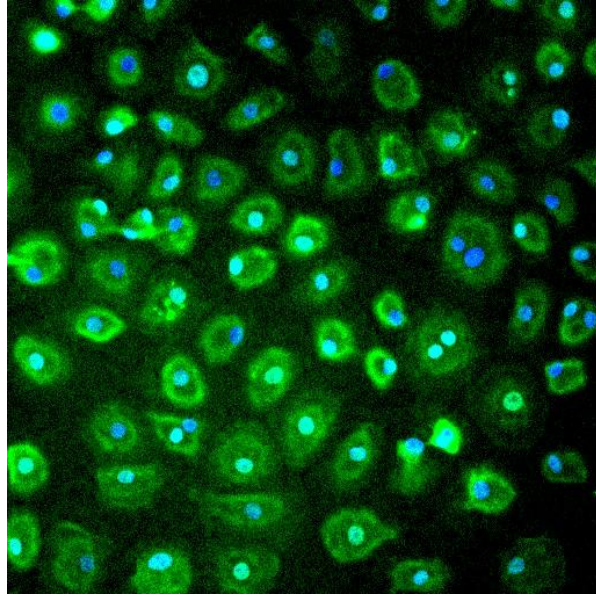| HHV-8 #20					
1 $TCID_{50}$	Count 1	Count 2	Count 3	Mean	SD
%inf	43%	61%	42%	**49%**	11
2 $TCID_{50}$s					
%inf	100%	100%	95%	**98%**	3
HHV-8 #23					
1 $TCID_{50}$	Count 1	Count 2	Count 3	Mean	SD
%inf	48%	59%	48%	**52%**	6
2 $TCID_{50}$s					
%inf	89%	93%	94%	**92%**	3

Figure 14: Confirming DC-SIGN Expression: DC-SIGN surface staining, validated by IFA in immature MoDCs used to test HHV-8 $TCID_{50}s$.
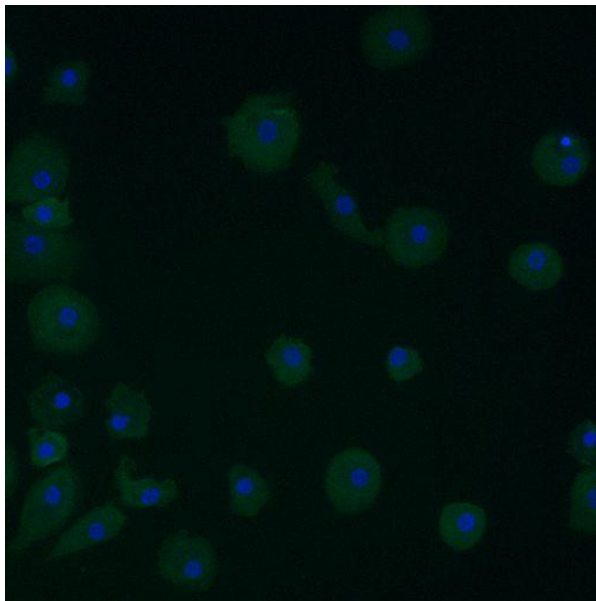

Figure 15: Negative Control for Staining of Viral Protein: Uninfected iDCs with both primary and secondary Ab for staining of ORF59 viral protein.

4.3 AIM 2: STUDIES DEMONSTRATING THE UTILITY OF THE HHV-8 T1H6-DC-SIGN TCID$_{50}$ ASSAY

4.3.1 Determine the Specific Infectivity of Different HHV-8 Stock Preparations

Specific infectivity is defined as the ratio of infectious virus particles to total particles. This is a useful measurement for determining the amount of infectious to non-infectious particles in a preparation. To determine specific infectivity the TCID$_{50}$ values must first be converted to infectious units such as plaque forming units / mL. This is accomplished (as described in the Materials and Methods section) by multiplying the TCID$_{50}$ value by 0.7. As shown in Table 10, conversion of HHV-8 viral stocks 13 and 15 from TCID$_{50}$ values to PFU/mL results in values of 4.28×10^2 and 4.28×10^5, respectively. For each stock preparation the number of encapsidated viral genomes (representing total virus particles) was 4.8×10^8/ml and 1.74×10^9/ml, respectively. The specific activities of these virus stocks therefore were 8.92×10^{-6} and 2.77×10^{-4}, respectively.

Table 10: Determination of Specific Infectivity:

	PFU/mL	DNA Copies/mL	Specific Infectivity
HHV-8 stock #13	4.28×10^2	4.80×10^8	8.92×10^{-6}
HHV-8 stock #15	4.28×10^5	1.74×10^9	2.77×10^{-4}

These results clearly demonstrate the majority of the virus particles in the stock solutions are non-infectious.

4.3.2 Use of the T1H6-DC-SIGN HHV-8 TCID$_{50}$ Assay to Examine HHV-8 Replication Kinetics in Infected, Activated B Cell Supernatants

To show HHV-8 replication kinetics in activated B cells, Emilee Knowlton of Dr. Charles R. Rinaldo's laboratory provided supernatant samples of *de novo* HHV-8 infected activated B cells. Each sample was tested for infectious HHV-8 using the T1H6-DC-SIGN TCID$_{50}$ assay. HHV-8 replication kinetics were determined from the B cells of several individual human donors. Figures 16A through 16H demonstrate the results of the TCID$_{50}$ assay on the supernatants of the infected B cells. In these experiments, UV-inactivated HHV-8 acted as negative control. As seen in these figures, HHV-8 infection could be detected in most of the B cell samples with viral titers increasing over time. These results demonstrate the ability of this assay to detect and titer infectious HHV-8 in vitro.

A

B

C

D

E

F

Donor 7

Donor 8

G

H
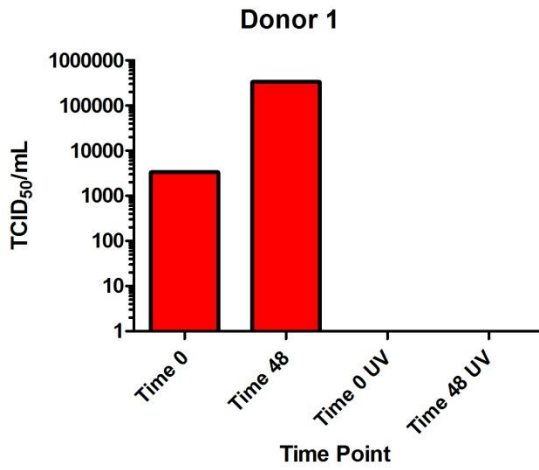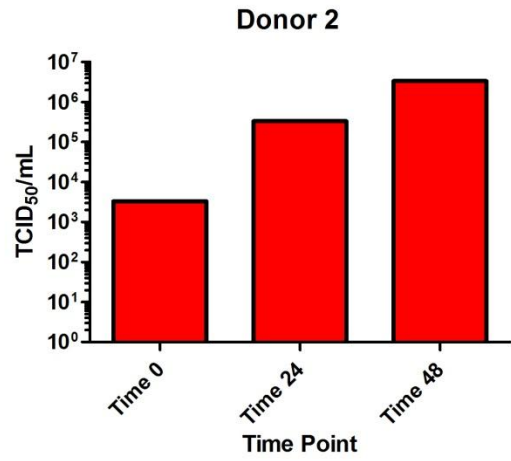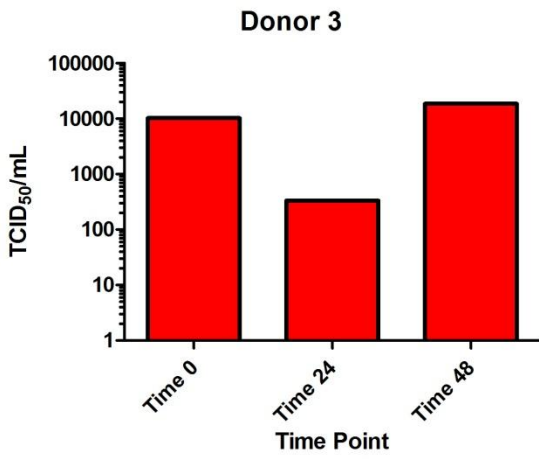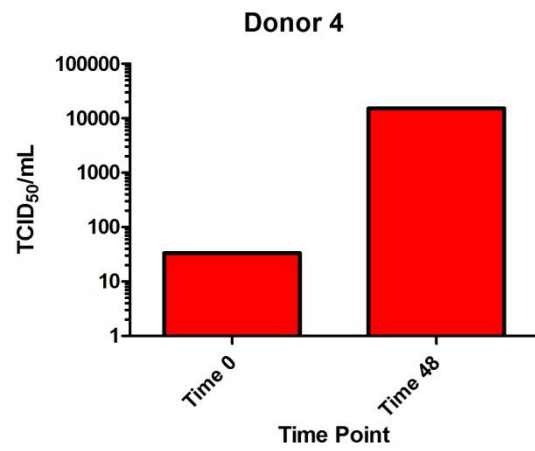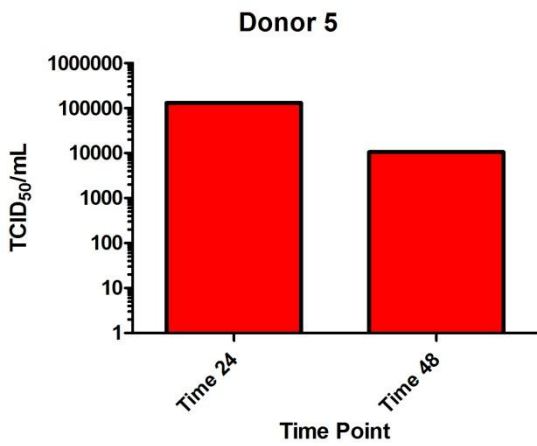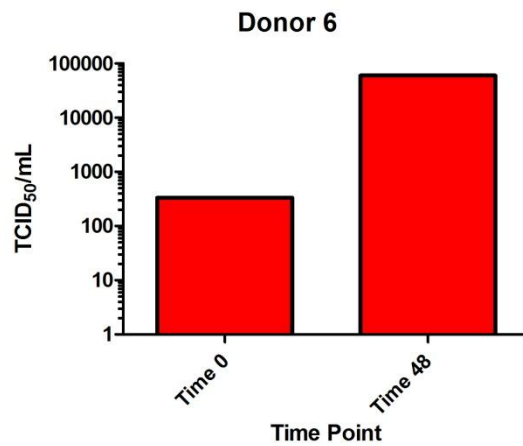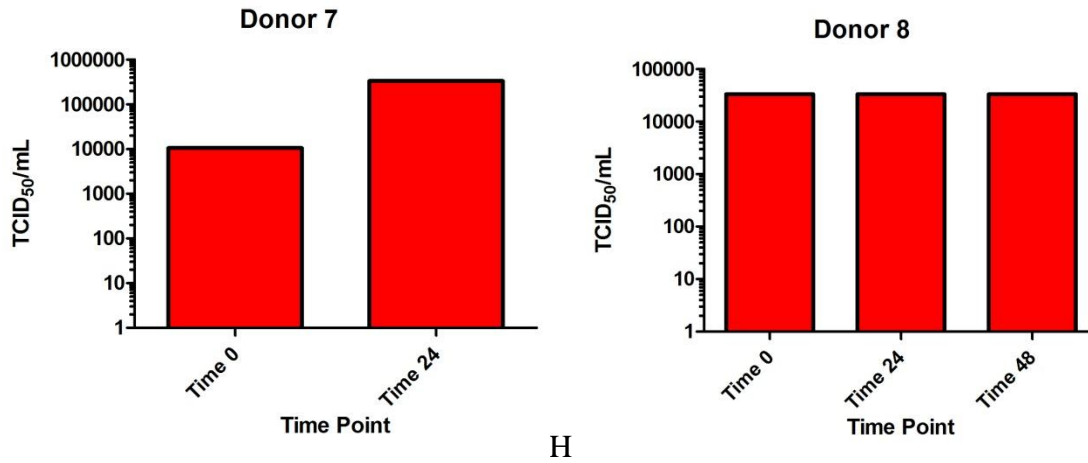
Figure 16: Time Course Showing Infectious HHV-8 Replication Kinetics in Activated B Cell Supernatants from Different Donors: Eight different donors' activated B cells were infected *de novo* with different HHV-8 virus samples; UV-inactived HHV-8 acted as a negative control (A). The supernatants were harvested and used as viral inoculum in the T1H6-DC-SIGN β-galactosidase assay. 48 hpi, T1H6-DC-SIGN cells in replicates of 6 were removed, washed, and lysates were plated to measure chemiluminescence. $TCID_{50}$ values were determined using the Reed-Muench 50% endpoint titer calculation.

5.0   DISCUSSION

5.1   EVALUATION OF THESIS AIMS

5.1.1   Background Work

The first factors to consider in developing an assay for HHV-8 infectivity include describing characteristics of virus in terms of infection using existing techniques. This includes the use of viral genome quantitation as well as imaging of infected cells. The qRT-PCR and IFA analysis together showed that the HHV-8 viral stocks used in developing this assay were infectious. Figure 2B showed quantitation of DNA copies comparing to a standard curve, and figure 2A showed specificity of the technique in terms of the HHV-8 genome; water as a negative control template showed no genomic amplification. The IFAs in figure 3 showed expression of early protein ORF59 which is expressed between 0 and 72 hpi [47, 50] as well as morphologically distinct dendritic cells; upon infection, immature dendritic cells become phenotypically mature and morphologically distinct; however, antigen presentation capability is curbed [5]. Immature, uninfected dendritic cells had slight autofluorescence but no indication of viral protein. Preparation of a viral stock from TPA-inducible BCBL-1s is a time-consuming process, but it does produce at least some infectious virus that could be tested with this assay.

5.1.2 Specific Aim I: Characterization of T1H6-DC-SIGN

5.1.2.1 Background β-galactosidase Measurements

The first objective of this aim was to take background measurements of β-galactosidase production in T1H6-DC-SIGN over a time course controlling for cell number. Figure 4A shows total β-galactosidase production over the span of 5 days in uninfected cell lysates; from this it can be inferred that 72 hours post-plating results in the highest background β-galactosidase, but by 72 hours the cells are at maximum confluence in a 48 well plate. By 96 hours T1H6-DC-SIGN is dying due to overconfluence. Figure 4B, at 48 hpi, shows differences between HHV-8-infected and uninfected T1H6-DC-SIGN lysate β-galactosidase chemiluminescence, and controls for cell count. 48 hpi was thus found to be the optimal time for measuring production of β-galactosidase and thus ORF50 (RTA) expression by HHV-8.

5.1.2.2 Specificity of Reporter Gene to HHV-8 ORF50 (RTA)

Next, the reporter gene (β-galactosidase driven by the HHV-8 T1.1 promoter) was tested for its specificity. Using a Lipofectamine 2000 kit according to protocol on 8×10^4 T1H6-DC-SIGN cells/well in triplicate, ORF50 DNA was transfected into the reporter cell line; as seen in figure 5, chemiluminescent β-galactosidase dose-response was visible 48 hours post transfection according to the amount of DNA transfected, and a negative reaction and detection control (PBS in place of β-galactosidase substrate and buffer) showed no chemiluminscent activity. The measurement of β-galactosidase activity was measured using manufacturer recommended volumes for β-gal substrate and buffer. Thus, the reporter in T1H6-DC-SIGN is specific to production of ORF50, or replication trans-activator (RTA), making expression of this protein a good way to determine infectious titers.

5.1.2.3 Visual Confirmation of DC-SIGN Expression on T1H6-DC-SIGN

To further characterize T1H6-DC-SIGN, fluorescence imaging was used to visualize surface expression of DC-SIGN. In figure 6, T1H6 was used as a negative control and had no visible staining beyond autofluorescence or background. Figure 7 shows clear surface expression of DC-SIGN on T1H6-DC-SIGN; a mouse monoclonal antibody to human DC-SIGN followed by goat-anti-mouse IgG FITC highlighted the surface receptor clearly. Figure 8 shows immature dendritic cells (provided by Dr. Pawel Kalinski's laboratory, Hillman Cancer Center) expressing DC-SIGN; downregulation of the receptor can also be seen in more matured dendritic cells. These IFAs together confirm expression of DC-SIGN in T1H6-DC-SIGN, and absence of expression in the parental cell line T1H6; immature dendritic cells worked as a positive control for staining.

5.1.2.4 Further Evidence for the Importance of DC-SIGN as a Receptor for HHV-8 Entry

A β-galactosidase assay was performed to compare the parental cell line T1H6 to T1H6-DC-SIGN using a virus stock that was found to be infectious via IFA on immature dendritic cells. Cells were plated as described and infected as seen in figure 9; 48 hours post-plating and infection, cells were harvested and β-galactosidase chemiluminescence was measured. A 2-way ANOVA showed strong differences in β-galactosidase activity between T1H6 and T1H6-DC-SIGN with larger volumes of virus, though T1H6 did also exhibit some dose-response. This could implicate other receptors for viral entry besides DC-SIGN for HHV-8. This experiment was repeated a total of 3 times, twice with increasing volumes of virus stock and once with sequentially diluted HHV-8 stock. Each experiment showed dose-response as well as large differences in infection attributable to DC-SIGN expression, further elucidating its importance as a receptor for HHV-8 entry.

5.1.2.5 Use of T1H6-DC-SIGN to Determine TCID$_{50}$ Values on Different Virus Stocks

Next, T1H6-DC-SIGN was used to determine TCID$_{50}$ values on various virus stocks, as shown in tables 1-4. Infections occurred in a dose-response according to viral dilutions, measured by β-galactosidase chemiluminescence. The mean of uninfected cell lysate β-galactosidase chemiluminescence plus 2 standard deviations was subtracted from each test value to show a well as infected or uninfected; this was applied to the Reed-Muench calculation as described in materials and methods [53], enabling determination of a 50% endpoint titer for several different HHV-8 stocks. Variation in TCID$_{50}$ shows inconsistency in terms of infectivity of different virus stock preparations, possibly attributable to the viability of stored viruses or slight differences in viral preparation procedures.

5.1.2.6 Validation of TCID$_{50}$ Values in Cell Culture

To validate TCID$_{50}$ values from the T1H6-DC-SIGN assay, $2x10^4$ monocyte-derived immature dendritic cells were plated in replicates of 4 in a 96-well plate and infected for 2 hours; media was then refreshed and staining was performed 48 hpi. The negative control was uninfected but all wells were stained for expression of HHV-8 early protein ORF59. Hoechst was used to stain the nuclei, and images were taken and processed in a Cellomics ArrayScan VTi by William Buchser of the Michael T. Lotze D.A.M.P. (Damage-Associated Molecular Patterns) laboratory at Hillman Cancer Center. DC-SIGN expression was confirmed in figure 15, showing surface receptor expression all around each cell. The images selected were representative of 1 and 2 TCID$_{50}$s (figures 10, 11, 12, 13) using 2 different virus stocks, as assessed by counting the number of infected vs. uninfected cells in a double-blinded study (tables 5, 6, 7, 8). The subjects counting the cells were also given the negative control in figure 16 as reference to assess the number of infected vs. uninfected cells; results are the average of the percent of cells infected

within the focal plane. This experiment was repeated using 2×10^5 dendritic cells in chamber slides stained for expression of the same viral protein, using DAPI as a nuclear counterstain; results are shown in table 9. This shows that the $TCID_{50}$ unit from the T1H6-DC-SIGN assay can be used to infect a pre-determined quantity of cells using a pre-calculated quantity of virus stock solution, as shown above with data and a repeat of the assay using a different PBMC donor.

No such unit existed for quantitating titers of infectious HHV-8, but this assay was appropriate to validate a controlled HHV-8 infection of dendritic cells in vitro. Expression of DC-SIGN on immature MoDCs provides further proof of DC-SIGN's importance for HHV-8 viral entry and establishing infection, meaning that using this receptor as a way to ascertain infectious titers for HHV-8 starting with viral entry is appropriate for this assay. Using HHV-8 ORF50 (RTA) as a measure of infection is also appropriate based on the reporter gene in T1H6-DC-SIGN, producing chemiluminescent β-galactosidase in response to productive HHV-8 infection. The titers ascertained from this assay show validation using two different virus stocks, two different PBMC donors, two different $TCID_{50}$ values, and a repeat of all of these in an external method.

5.1.3   Specific Aim II: Show Utility of the T1H6-DC-SIGN $TCID_{50}$ Assay

5.1.3.1 Determine the $TCID_{50}$ values in HHV-8 preparations and compare $TCID_{50}$ values with encapsidated DNA values

Using qRT-PCR to quantitate DNAse-treated encapsidated viral copies, it was possible to ascertain a ratio of these to $TCID_{50}$/mL (converted to PFU/mL) values from the T1H6-DC-SIGN assay; this ratio is called specific infectivity. Results shown in table 10 clearly show substantial differences in the number of DNA copies to infectious virions as produced by TPA-inducible

BCBL-1s using the process described in materials and methods. This shows that while qRT-PCR performed on DNAse-treated encapsidated viral copies is a good measure of DNA copy number, it does not distinguish between infectious and non-infectious particles as demonstrated by specific infectivity ratios. Therefore, any MOI ascertained from qRT-PCR may be highly inaccurate in contrast to the T1H6-DC-SIGN assay infectious unit. The use of DC-SIGN as a receptor for viral entry as well as assessment of ORF50 (RTA) production using a protein-specific promoter in a reporter cell line that drives production of β-galactosidase (T1.1, PAN RNA promoter) means that the T1H6-DC-SIGN β-galactosidase chemiluminescence assay can give far more sensitive and specific infectious titers for HHV-8 than qRT-PCR or any less quantitative technique such as fluorescence imaging or the use of mutant virus. The massive variations between infectious and non-infectious particles in different viral preparations may be attributed to a low level of TPA activation in BCBL-Is inducing HHV-8 replication, as well as the entire process required to make a virus stock; each step may cost logs of infectious virus.

5.1.3.2 Use the T1H6-DC-SIGN TCID$_{50}$ assay to look at HHV-8 replication kinetics in infected, activated B cell supernatants

Although a small subset of the total B cell population can be infected with HHV-8, tonsillar and peripheral activated B cells expressing DC-SIGN can permit HHV-8 entry and lytic replication [6]. However, no assay has been able to examine replication kinetics of HHV-8 over a time course in terms of infectious titer in a primary cell line permissive to HHV-8 replication. Activated, *de novo* HHV-8 infected B cells were provided by Emilee R. Knowlton of Charles R. Rinaldo Lab. Supernatants of infected cells were plated the same way as viral stock as described in materials and methods, and the TCID$_{50}$/mL was compared across various time points; this assay provided insight into the replication of infectious HHV-8 virions in *de novo* infected,

activated B cells in terms of an actual infectious unit. Figure 16A through 16H all demonstrate that TCID$_{50}$s can be determined for different HHV-8 stocks for different activated B cells across time points using this assay.

5.3   CONCLUSIONS

The T1H6-DC-SIGN TCID$_{50}$ assay can be used to determine infectious titers of HHV-8 in a far more sensitive and specific manner than pre-existing methods such as IFA on a permissive cell line or qRT-PCR on encapsidated, DNAse-resistant virions looking at DNA copy number. Any HHV-8 stock can be tested for infectious titers more accurately in vitro using this assay. DC-SIGN has already been shown to permit HHV-8 infection of dendritic cells and activated macrophages [5], as well as activated B cells [6]. As seen in figure 9, DC-SIGN is not the only receptor for HHV-8, but that receptor does bind the virus with high affinity in comparison to a non-DC-SIGN expressing cell line (T1H6); a 2-way ANOVA showed clear differences between the two cell lines with increasing quantities of virus. Upon viral entry and trafficking to the nucleus, immediate-early gene ORF50 is expressed [47]; replication trans-activator production results in a cascade of HHV-8 lytic protein production [47-50]. Because of the lytic transcripts produced by HHV-8 as a result of ORF50 production, the reporter cell line T1H6-DC-SIGN is a good indicator of infection in cells expressing DC-SIGN and the cell line was thus used as the basis for measurement of infectious vs. non-infectious viral stocks in this assay. β-galactosidase activity was proportional to the amount of virus added to cell culture; production of ORF50 (RTA) resulted in activation of an RTA response element, triggering T1.1, the polyadenylated (PAN) RNA promoter; this drove production of β-galactosidase, measurable by

chemiluminescence. Validation took place using DC-SIGN expressing immature dendritic cells, which then expressed phenotypic maturation in response to infection, seen in figures [10], [11], [12], and [13]. The applications in this thesis are a start to what can be done using this assay; looking at differences in specific infectivity between HHV-8 virus stocks shows tremendous variation, meaning a faster way to develop higher titer HHV-8 stocks should be considered. Observed replication kinetics can give ideas concerning the pathogenesis and progression of HHV-8 in many donors, and any parameter can be taken into consideration such as immune status, race/geographical location, HIV co-infection, and the like. Another potential application of this assay is to perform an antibody neutralization assay to see how much antibody is required to cut infection by 50%; this has tremendous application in the clinical setting as testing would be less expensive for evaluation and proper diagnosis of productive infection.

5.2   PUBLIC HEALTH SIGNIFICANCE

In context of public health, the clinical and research applications of this assay would be widely available in contrast to other technologies such as PCR, flow cytometry, etc. The only materials required are an HHV-8 stock, the T1H6-DC-SIGN cell line and all necessary elements of 293T care, potassium phosphate/DTT lysis buffer, a ClonTech β-galactosidase Chemiluminescence Detection Kit II, and a chemiluminescence plate reader. All of these alone would likely cost far less for far more sensitive data on HHV-8 concerning infectivity which has not been properly established as of today. Other techniques may be too expensive for certain labs or other countries where HHV-8 has high seroprevalence; this technique may be very helpful in determining neutralizing antibody titer in KS+ individuals rapidly and with high throughput. Hence, this

assay could save a substantial amount of money for labs studying HHV-8 as well as any given clinical setting in which the titering of infectious virus is necessary for diagnosis and application of treatment. The idea of a reporter cell line to measure infectious virions could be used in context of EBV as well since both HHV-8 and EBV are γ-herpesviruses with trans-activator proteins expressed immediate-early [1, 2, 54]**.**

6.0   FUTURE DIRECTIONS AND APPLICATIONS

The aim of this assay was to develop a way to find infectious titers for HHV-8 using a cell culture system that is the first of its kind. The major application shows differences in infectious virus in HHV-8 preparations and may lead to better techniques to isolate infectious virus particles. There is also the possibility of testing the same virus stock using this assay at several different times, to show how long any given virus stock will still be infectious when stored at – 80º C for any given time period. Having knowledge of the stability of virus stocks could lead to a better understanding of pathogenesis as it relates to multiplicities of infection, since infectious particles may reduce over time for HHV-8.

This assay already enabled insight into HHV-8 replication kinetics in a permissive primary cell line, with variation in infectious virus seen among different donors. The biggest application or direction with this assay is to test it in context of an antibody neutralization assay, to determine a titer of neutralizing antibody needed to reduce viral titers in a KS+ individual. To test this, an appropriate number of donors must be chosen for statistical power, and they could be matched on CD4+ T cell counts to control for HIV co-infection. This may lead to a substantially more rapid assessment of individual viral titers as they relate to KS development, for a far greater number of people considering this assay also reduces the time and cost needed for previously unattainable data needed in a clinical or diagnostic setting. Now that a system has been established to specifically quantitiate HHV-8 infectious titer, its effects on any given cell

type will become substantially more clear and interactions with the innate immune system can be studied closer; as shown by variations in specific infectivity, a MOI based on DNA copy may be off by up to 6 logs and this can make a difference in terms of innate immunity working with adaptive immunity in controlling HHV-8 infection. Studying differences between non-mutant and mutant virus may provide insight in terms of treatment based on specific ORFs made by HHV-8 to suppress immune function. Infection kinetics could also be properly studied in terms of single or multiple hit on multiple cell types.

Studies involving receptor expression will also be far more accurate, since this assay provides an infectious titer to use without polybrene; this may further elucidate receptors for HHV-8 entry into a variety of cell types expressing a variety of receptors. DC-SIGN and HHV-8 glycoprotein studies could also be pushed further in terms of assessing receptor affinity as it relates to infection and other factors like avidity of binding. In terms of virological study, a question to ask is how DC-SIGN binding affinity by HHV-8's envelope glycoproteins changes with different virus stocks or samples. In an alternate context related to HHV-8, perhaps the strength of HIV binding to DC-SIGN relating to dendritic cell antigen presentation implied by variation in envelope protein glycosylation [55] could imply there's much to be learned about HHV-8 endocytosis as it relates to processing in antigen presenting cells expressing DC-SIGN. Since DC-SIGN is not expressed on all cell types, perhaps the parental T1H6 could elucidate the role of α3β1 integrin, heparin sulfate, and xCT as methods of HHV-8 binding, entry, and pathogenesis.

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