CYTOKINE AND CHEMOKINE RESPONSES IN HUMAN HERPESVIRUS 8 INFECTION OF MONOCYTE DERIVED DENDRITIC CELLS AND B LYMPHOCYTES

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Emilee R. Knowlton

B.S., Marietta College, 2006

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by

Emilee R. Knowlton

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and approved by

Dissertation Advisor: Charles Rinaldo, PhD
Chairman and Professor, Department of Infectious Diseases and Microbiology
Graduate School of Public Health
Professor, Department of Pathology
School of Medicine
University of Pittsburgh

Committee Member: Frank Jenkins, PhD
Associate Professor
Department of Pathology
School of Medicine
University of Pittsburgh

Committee Member: Pawel Kalinski, MD, PhD
Professor
Department of Immunology
School of Medicine
University of Pittsburgh

Committee Member: Tianyi Wang, PhD
Associate Professor
Department of Infectious Diseases and Microbiology
Graduate School of Public Health
University of Pittsburgh

CYTOKINE AND CHEMOKINE RESPONSES IN HUMAN HERPESVIRUS 8 INFECTION OF MONOCYTE DERIVED DENDRITIC CELLS AND B LYMPHOCYTES

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Human herpesvirus-8 induces a wide range of inflammatory immune mediators known to contribute to its associated cancer, Kaposi's Sarcoma (KS). Soluble immune mediators, such as cytokines, chemokines and growth factors produced during HHV-8 infection have been associated with tumor-cell proliferation, angiogenesis and vascular permeability. We sought to determine immune mediator production by two antigen presenting cells (APC) that are susceptible to HHV-8 infection, i.e., monocyte derived Dendritic cells (MDDC) and B lymphocytes.

Dendritic cells abundantly express the HHV-8 receptor, type II C-type lectin, DC-specific ICAM-3 grabbing nonintegrin (DC-SIGN) resulting in viral entry, whereas only a small percentage of activated B cells express DC-SIGN *in vitro.* Despite this, HHV-8 infection of MDDC results in an abortive replicative cycle, whereas full-lytic cycle replication occurs in the B cells. I hypothesized that immune mediators produced by HHV-8 infected APC are unique between cell types and that HHV-8 infects a subset of B cells and initiates cytokine and chemokine production that contributes to HHV-8 replication, viral dissemination and initiation of KS and HHV-8 lymphomas.

I used a cytometric bead array to determine cytokine and chemokine production in B cells and MDDC, as well as qRT-PCR, $TCID_{50}$ assay and flow cytometry to determine HHV-8 replication in B cells. I identified significant differences in the quality and quantity of cytokine and chemokine profiles of HHV-8 infected APC. MDDC produced significant levels of MCP-1, MIP-1α, MIP-1β, RANTES, IP-10 and IL-10, while B cells produced significant levels of MIP-1α, MIP-1β, IL-6, TNF-α and IL-8. HHV-8 lytic replication in B cells resulted in polyfunctional immune mediator activity that may contribute to viral replication and proliferation of target cell populations in HHV-8 related cancers. The importance of this work was demonstrated by the detection of B cell-produced cytokines and chemokines in HHV-8/HIV co-infected individuals who developed KS. This is the first extensive, multiparameter, longitudinal study of HHV-8 infection of B cells and immune mediators in development of KS. This study provides novel targets for vaccine development and treatment options for KS, which could have great implications on Public Health.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

First, I would like to thank my advisor, Dr. Charles Rinaldo, for his extensive and ongoing support. He has greatly contributed to my development as a scientist and provided me with many great opportunities that have enhanced my academic career. It is with great sadness that I will finally delete the numerous voicemails that I've spent many mornings listening to regarding late breaking ideas and instructions for upcoming experiments.

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1.0 INTRODUCTION

Human Herpesvirus-8 (HHV-8), or Kaposi's sarcoma associated herpesvirus (KSHV), is the etiologic agent of Kaposi's sarcoma (KS) (50), a neoplasm of endothelial origin that occurs in four distinct epidemiologic forms (69, 135): classic or Mediterranean KS, epidemic or Acquired Immunodeficiency Syndrome (AIDS)-related KS, endemic or African KS, and iatrogenic or organ transplant-associated KS. KS is the most common cancer associated with Human Immunodeficiency Virus-1 (HIV-1) infection and AIDS (57). Although the incidence of KS in HIV-1 infected persons declined with the advent of antiretroviral therapy (ART) (98), KS can occur in persons on ART with suppressed HIV-1 infection (178). The success of ART in treating HIV-1 associated KS has been countered by the occasional occurrence of an immune reconstitution inflammatory syndrome (91). This is a severe, temporary enhancement of KS lesions due to an increase in inflammation and immunologic recovery after ART.

The discovery of HHV-8 and its causal role in KS development opened the potential for prophylaxis and treatment of the infection and cancer with antiviral drugs, and prevention of both with a vaccine. Strategies to achieve these ends require an intimate knowledge of the pathogenesis and immune control of HHV-8 infection. We postulate that host control of HHV-8 infection and development of KS is linked to T cell interactions with HHV-8 infected, professional antigen presenting cells (APC), i.e., dendritic cells (DC), monocytes/macrophages and B lymphocytes. Similarly, APC-T interactions are likely to be centrally involved in the HHV-8-associated B cell neoplasms multicentric Castleman's disease (MCD) (242) and primary effusion lymphoma (PEL) (42, 193).

HHV-8 has been reported to be transmitted to common marmosets and cause persistent infection with rare, KS-like skin lesions (49). However, there is as yet no consensus that this or other simian models (92, 137, 227) recapitulate human HHV-8 infection and development of KS or other cancers associated with this herpesvirus. Thus, although *in vitro* models are suspect to lacking certain *in vivo* characteristics, they offer the most relevant model of HHV-8 infection for this human species-specific herpesvirus.

As with the other human gamma herpesvirus, Epstein Barr virus (EBV) (202), HHV-8 targets APC both *in vivo* and *in vitro*. Indeed, the primary tropism of B cells by these gamma herpesviruses is uncommon among human virus infections. This sets the stage for development of their associated cancers both indirectly through alteration of host immunity dependent on APC function, and directly via neoplastic effects of the virus. HHV-8 is found in KS spindle cells, which are of mixed vascular and lymphatic endothelial cell and macrophage origin, monocytes that are found in proximity to KS lesions, and circulating B cells of KS patients (25, 33, 187, 219). In PEL, HHV-8 is found in immunoblastic cells expressing plasma cell markers, and in plasmablastic cells of a less terminally differentiated state in MCD (75). The intimate association of HHV-8 with such professional APC in the KS lesion and in other HHV-8 associated cancers suggests a major role for virus-APC interplay. Moreover, anti-HHV-8 T cell immunity that presumably is critically dependent on such virus-APC interactions is present in HIV-1 infected and uninfected persons who are seropositive for HHV-8 (226). Achieving a better understanding of the role of HHV-8 in inducing associated cancers could greatly benefit from a yet-to-be-developed *in vitro* model of primary HHV-8 infection of a natural target cell that consistently reflects virus lytic, latent and reactivation infections.

Infection of APC *in vitro* reveals different cycles of HHV-8 replication that are likely to relate to the pathogenesis of the virus. The first step in targeting and alteration of APC by HHV-8 is at the level of cell receptor. Herpesviruses use more than one receptor to infect the same cell (121). Use of these receptors by herpesviruses is hierarchical based largely on differential expression of the receptors in specific cell types and states of cell activation. Extensive *in vitro* evidence indicates that the ubiquitous cell surface proteoglycan heparin sulfate serves as an initial binding receptor for HHV-8 on endothelial cells and fibroblasts as well as APC (5, 6, 47, 141). Multiple integrins are subsequently involved in HHV-8 binding and entry (141). A third level of differential selection that has been identified from *in vitro* studies of the three major types of APC, i.e., monocyte-derived DC (MDDC), B cells and monocyte-derived macrophages (MDM), is the type II C-type lectin, DC-specific ICAM-3 grabbing nonintegrin (DC-SIGN; CD209) (215, 216, 263). A new entry receptor for HHV-8 on endothelial and epithelial cells (116), i.e., ephrin receptor tyrosine kinase A2, a tyrosine kinase that functions in neovascularization and oncogenesis, has not yet been assessed in HHV-8 infection of APC.

The relative contribution of each of level of HHV-8 binding to viral infection of APC is not clear. For example, the Raji B lymphoblastoid cell line (LCL) and the myeloblastoid K562 cell line constitutively express little or no DC-SIGN or $\alpha 3\beta 1$ integrin (216). Thus, these cell lines do not support detectable production of infectious virions (15, 24, 216). However, transfection of the cell lines with DC-SIGN renders them highly permissive for HHV-8 infection as measured by the production of viral proteins and DNA (216). Moreover, infection of these cell lines can be blocked by anti-DC-SIGN mAb, soluble DC-SIGN and mannan, a natural ligand of DC-SIGN. Interestingly, six B cell and T cell lines (BLAB, Ramos, BCBL1, JSC1, Jurkat and SupT1) were susceptible to infection through cell-mediated transmission with a doxycyline- inducible cell line

harboring recombinant HHV-8 (rKSHV.219), indicating that viral entry can be achieved despite lack of expression of a major HHV-8 receptor (192). There is also evidence that HHV-8 can infect $CD34^+$ stem cell precursors of DC *in vitro* by as yet undefined receptors (122, 154). It is likely that there are less prominent alternative receptors for HHV-8 that account for a small percentage of DC-SIGN negative APC and cell lines that can be infected by this virus.

Suggestive evidence that HHV-8 is B-cell tropic *in vivo* is that HHV-8 DNA is detected in B cells from patients with KS lesions (8) and some HIV-1/HHV-8 co-infected individuals (189). We speculate that this is related to DC-SIGN expression that is enhanced by an activated state in B cells. That is, once blood-derived B cells are activated to express DC-SIGN, HHV-8 can effectively establish infection and elicit full-cycle production of infectious virions in these cells (215). The fact that HHV-8 cannot infect Raji LCL and the K562 erythroleukemia cell line expressing DC-SIGN that lacks the transmembrane domain, supports DC-SIGN-mediated endocytosis of viral entry. Moreover, infection can be blocked by pretreatment of the B cells with anti-DC-SIGN mAb or mannan but not antibody specific for the amino acid transporter protein xCT (215). HHV-8 has been reported to use xCT for infection of surface adherent human cells (138), and in a post-entry stage of human endothelial cell infection as part of a complex of heterodimeric membrane glycoprotein CD98 and the α3β1 and αVβ3 integrins (265). Notably, HHV-8 infection is not restricted to blood-derived B cells, as tonsillar B cells constitutively express DC-SIGN and can be lytically infected with the virus *in vitro* (190, 215). It is probable that B cells in such tonsillar tissue are in an endogenously activated state resulting in enhanced expression of DC-SIGN.

HHV-8 infection of freshly derived blood and tissue B lymphocytes could provide an *in vitro* model for assessing HHV-8 lytic and latent infection. Our *in vitro* model for measuring

HHV-8 infectivity and replication supports the concept previously put forth that DC-SIGN is a major receptor for this virus (47, 215, 216). This adds to the wealth of evidence that shows that in addition to certain integrins (4-6, 22, 265), DC-SIGN is required for highly efficient infection of the natural APC targets with HHV-8, which is in contrast to previous reports (101). However, there is still need for improved reliable, quantitative measures of HHV-8 replication to better define B cell infection with HHV-8. This should be combinations of real time polymerase chain reaction (PCR) assays for cell-associated and non-cell associated copy numbers of HHV-8 encapsidated DNA, flow cytometry assays for enumerating the number of monoclonal antibody (mAb)-stained cells expressing viral lytic and latency cycle proteins, and most important, cell culture-based assays, e.g., a 50% tissue culture infectious dose assay, for quantitating the number of infectious virus particles.

It is postulated that HHV-8 infection drives B cells to an early plasmablast-like state in MCD and a preterminal plasma cell stage of differentiation in PEL (2, 42, 44, 45, 76, 79, 97, 119, 146, 176, 184, 194). Hassman *et al.*, recently showed that latency associated nuclear antigen $(LANA)^+$ B cells express Immunoglobulin (Ig) heavy chain M and the λ light chain at 2.5-3.5 days post-HHV-8 infection. These cells are plasmablast-like with increased IL-6R expression and increased proliferative response to Interleukin (IL)-6, with 7-36% expressing CD27 (119). This molecule is a member of the tumor necrosis factor (TNF)-receptor super family, and is involved in regulation of B cell activation. It is not known whether HHV-8 directly infects these IgM^+ memory B cells or a precursor of these cells. Also, there are no data on which subset of B cells supports a complete lytic cycle of replication with virion formation and death of the cell, or if this is abortive, leaving HHV-8 infected memory B cells that survive and maintain latent virus infection. Infection of naïve and IgM memory B cells may lead to establishment of latency in a

portion of cells, resulting in virus-driven plasmablast differentiation, while some cells support the viral lytic cycle. Activated B cells may support full lytic cycle replication, resulting in virion formation and cell lysis, or HHV-8 could abort the cycle prematurely and either enter latency or result in cell apoptosis.

Such definitive B cell targets for primary infection and lytic replication could be useful in studies of HHV-8 prophylactic and therapeutic vaccines. Currently, the main *in vitro* models to recapitulate HHV-8 infection in APC are cell lines persistently infected with the virus, particularly body cavity based lymphoma cells (BCBL-1), a B cell line derived from PEL, which is latently infected with HHV-8 and EBV negative (42, 50). In such cell line models, HHV-8 lytic and latent infections cannot be defined conventionally starting with the total absence of infectious viral particles, as there is always a low level of persistent virus production. However, latency can be disrupted, triggering the lytic cascade of viral replication and lytic genes expressed sequentially as immediate early genes, early genes and late genes, resulting in production of encapsidated virions. Such lytic viral replication is largely irreversible (47, 276). HHV-8 lytic gene profiling in these models has been extensively accomplished using tiling microarray (52), DNA microarray (2, 48, 167), and high-throughput real-time PCR (73, 89). However, most studies on latency-lytic reactivation of HHV-8 use various chemicals to induce viral replication (191). The question is whether such reactivation reflects natural HHV-8 viral lytic reactivation from latency, since chemical agents such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) have pleiotropic effects on host cell signaling and chromatin structure. How they affect cell signaling pathways is also unknown. Thus, the natural reactivated cascade of lytic transcripts of HHV-8 still waits to be revealed.

Instead of using chemical inducers, Nakamura *et al.*, (195) developed an engineered body cavity based lymphoma (BCBL-1) cell line that inducibly expresses the replication transactivator protein, RTA, encoded by open reading frame (ORF)-50, i.e., TREx-BCBL1-RTA. RTA has been shown to be necessary and sufficient for the switch between HHV-8 latency and lytic replication (71). In fact mutation of the RBP-Jk sites within the RTA promoter is enough to enhance latency in transformed-293 cells and peripheral blood mononuclear cells (PBMC) (169). In the TREx-BCBL1-RTA cell line, RTA expression is under the control of a doxycycline-inducible promoter and treatment of TREx-BCBL1-RTA cells with doxycycline (Dox) results in expression of RTA which in turn induces viral replication (195). While the role of RTA in causing a switch from latency to viral replication has been demonstrated by several laboratories, the mechanisms regulating coordinate induction of expression of most of the HHV-8 lytic genes during this reactivation have not been evaluated in a systematic fashion.

Virus reactivation events have been studied in primary and immortalized microvascular endothelial cells (MVEC) with the recombinant virus, rKSHV.219 (268). This virus expresses a green fluorescent protein (GFP) under an EF-1α promoter to indicate infection and a red fluorescent protein under a PAN promoter to indicate lytic transcription. This model should be considered for a more detailed evaluation of APC infection and reactivation.

Transcription of HHV-8 lytic genes occurs during either a primary infection of susceptible cells or during reactivation of latently infected cells. The question remains whether the kinetic gene activation in a chemically induced cell line (BCBL-1) or the naturally targeted RTA (TREx-BCBL1-RTA) will reflect the cascade events of natural infection of B cells or other APC. To identify the true gene transcription and reactivation events in HHV-8 infection, primary

cells susceptible to HHV-8 should be used. Only then can the observations from TPA induced BCBL-1 and DOX induced TREx-BCBL1-RTA cell lines be validated.

In 2005, a cluster of microRNA (miRNA) coded by HHV-8 was discovered (36, 232). This short, 22 nucleotide, non-coding miRNA silences mRNA expression through a silencing complex (miRISC). HHV-8 miRNAs are expressed during lytic and latency cycles of virus replication, and act on both cellular and viral transcriptomes (110). Studies of HHV-8 miRNA have utilized PEL cell lines, as well as foreskin fibroblasts and endothelial cells. These indicate a multifactorial role in maintaining viral latency, regulating lytic virus replication and enhancing cell survival. As miRNA activity is dependent on its level and targets within specific cell types, it is imperative that miRNA be assessed in primary B cells.

Finally, as HHV-8 is one of the few human viruses that primarily targets B cells, an in depth understanding of the effects HHV-8 infection has on these cells should be established. However, little data exist concerning B cell activation states or surface marker expression upon HHV-8 infection. Likewise, interactions between HHV-8 infected B cells and $CD4^+$ T helper cells are yet to be defined. Considering that the major function of B cells is production of Ab that prevent and ameliorate infection, there is need to assess the quality and quantity of Ab production over the course of HHV-8 infection and development of KS. Yet, there is no consensus assay for detecting or titering anti-HHV-8 Ab. Detection of anti-LANA Ab by immunofluorescence assays has low sensitivity (as low as 64%) among individuals with KS (61, 198, 212). ELISA and Western blot assays for anti-latent (LANA) and lytic (ORF65 or K8.1) Ab has higher sensitivities and is often used for serologic testing, yet can have low specificities (198). These conventional methods for serologic testing therefore lack standardization and can

be unreliable, underlining the necessity for more accurate methods of quantifying anti-HHV-8 Ab titers.

Given the vagaries of anti-HHV-8 Ab assessments, humoral immunity to HHV-8 infection has been described for several cohorts. A luciferase immunoprecipitation system that quantifies Ab response to multiple antigens was used to compare profiles of KS, MCD and PEL patients (34). The study showed significant differences in Ab responses among the groups, including higher anti-K8.1 Ab detected in PEL and MCD compared to KS and higher titers of ORF65 in PEL compared to KS. Likewise, higher Ab titers against v-cyclin were observed in KS and PEL compared to MCD, and higher anti-LANA Ab titers were detected in KS compared to MCD. An explanation for the difference in Ab responses in individuals with these HHV-8 associated cancers is currently unknown, but is likely a reflection of the differential expression of latent and lytic viral genes. The quality and quantity of anti-HHV-8 response may change over the course of disease progression or after anti-viral therapy. Following antiretroviral therapy, increases in Ab against both latent and lytic proteins have been observed for individuals with or without KS (29, 108, 253, 280).More in depth studies with larger cohorts and advanced testing methods should be performed, while *in vitro* models for HHV-8 infection in B cells and detection of antiviral Ab should be established.

Interestingly, there are only minimal data on neutralizing Ab in HHV-8 infection. The first such evidence was that rabbit polyclonal neutralizing Ab to gB prevent HHV-8 infection of primary human foreskin fibroblasts (4) and oral epithelial cells (82). Concurrently it was demonstrated that sera from persons who were seropositive for HHV-8 as shown by anti-LANA immunofluoresence assay also had neutralizing Ab that inhibited virus infection of transformed dermal microvascular endothelial cells (72). Using a recombinant HHV-8 (rKSHV.152) that

expresses GFP, Kimball, et al., (144) found significantly lower neutralizing Ab titers to HHV-8 in the serum of HIV-1 infected persons with KS compared to those without KS. This is in contrast to Inoue, et al., (129) who reported that there were no differences in neutralizing Ab titers between HIV-1 infected patients with or without KS. However, the latter study used an HHV-8 reporter cell line T1H6 treated with polybrene in their virus neutralization assay. Polybrene results in receptor-independent infection (67), thus potentially obscuring interpretation of virus neutralization assays. Finally, it is evident that is a need for in depth, longitudinal studies of neutralizing Ab and other antiviral Ab such as those that mediate Ab-dependent cell cytotoxicity, in relation to progression of HHV-8 infection and development HHV-8 related cancers.

In addition to B cells and MDDC, other APC have been shown to be susceptible to HHV-8 infection, including macrophages. Macrophages in several body compartments naturally express DC-SIGN (111, 139), as well as integrins including α3β1 (9), which presumably renders them susceptible to HHV-8 infection. An early report showed that MDM from normal donors that are stimulated *in vitro* with allogeneic PBMC can be infected by HHV-8, but this rarely results in complete, lytic replication (24). In addition, treatment of blood monocytes from KS patients with proinflammatory cytokines *in vitro* results in HHV-8 persistence (187).

MDM become susceptible to HHV-8 infection *in vitro* after activation with IL-13, which result in enhanced DC-SIGN expression (216). IL-13 is an anti-inflammatory, T helper (Th) 2 cytokine that promotes differentiation of B cells into antibody-secreting plasma cells. Importantly, non-IL-13 activated MDM express α3β1 integrin yet are not infected by HHV-8 *in vitro*, supporting the requirement of multiple receptors for efficient infection of APC by HHV-8. Indeed, when DC-SIGN is blocked in IL-13-activated MDM or the monocytic cell line THP-1,

HHV-8 can still bind using heparin sulfate but virus entry is reduced (141). HHV-8 establishes productive infection in THP-1 cells with an ordered expression of latency gene ORF73 and lytic gene ORF50. In fact, the HHV-8 genome was reported to persist for 30 days in these cells (141). Such limited expression of lytic genes together with the persistence of latency genes is believed to be unique for HHV-8 (148).

Of interest is that ORF K14 of HHV-8 encodes a surface glycoprotein vOX2 that is homologous to cellular OX2 (55), which inhibits macrophage function (96). The vOX2 glycoprotein could be central to HHV-8 immunopathogenesis in that it stimulates production of inflammatory cytokines IL-1β, IL-6, monocyte chemoattractant protein 1 (MCP-1), and TNF-α in primary monocytes, MDM and MDDC (55). Furthermore, expression of vOX2 on B cells stimulates monocytes to produce inflammatory cytokines. MDM transfected with vOX2 produce inflammatory cytokines and have enhanced phagocytic activity, while inhibiting the immunomodulatory effects of IFN-γ and down-regulating major histocompatibility complex (MHC) class I and class II expression on macrophages (231). It was recently reported that vOX2-transfected APC co-cultured with T cells results in suppressed interferon gamma (IFN-γ) production and mobilization of the cytolytic granule marker CD107a through inhibition of extracellular signal-regulated kinase (ERK1/2) phosphorylation (185).

Evidence of infection of human DC *in vivo* with HHV-8 has been limited (203, 221). When MDDC are infected *in vitro* with HHV-8, viral lytic proteins are produced with little viral DNA production (216), similar to abortive HHV-8 infection of vascular endothelial cells (5, 197, 214, 220, 267). Although HHV-8 infection does not significantly alter MDDC viability, it decreases MDDC function, i.e., lowers their capacity to activate antigen-specific $CD8^+$ T cell responses. Moreover, HHV-8 infected MDDC have impaired antigen uptake, with a significant

decrease in endocytic capacity and DC-SIGN expression within 24 hours after infection. DC-SIGN internalization in MDDC is associated with lytic HHV-8 gene expression (216). In addition to MDDC, HHV-8 *in vitro* infection of IL-13-treated MDM results in a loss of DC-SIGN surface expression, suggesting that HHV-8 binding to DC-SIGN triggers internalization. Hence, alteration of DC-SIGN expression could be a strategy used by HHV-8 to escape immune defenses and lead it to a non-robust immune response (273).

The skin and mucosa contain two major types of DC – Langerhans cells (LC) which reside in the epidermis in close contact with keratinocytes, and interstitial DC (iDDC) resident in the dermis and mucosal layers. LC and iDDC process cutaneous antigens and migrate to draining lymph nodes to present antigens to T and B cells. Because of the strategic position of LC and iDDC and their ability to capture pathogens, these cells could represent potential targets for HHV-8 infection. Furthermore, due to the expression of the C-type lectins, i.e., langerin (CD207) and DC-SIGN, on LC and iDDC, respectively, it is tempting to speculate that HHV-8 could utilize the same entry mechanisms as seen in MDDC (216). LC and iDDC can be generated from pluripotent cord blood $CD34^+$ cells (40) that could prove to be valuable tools to study HHV-8 infection and subsequent antigen process and presentation to T cells (59).

Plasmacytoid (pDC) are a lymphoid-lineage subset of APC that produce extraordinary amounts of the antiviral protein IFN-α in response to virus infection (166). DC-SIGN is not expressed by pDC, yet HHV-8 can infect human pDCs, up-regulate expression of the activation molecule CD83 and T cell co-receptor CD86, and induce production of IFN-α (278). Induction of IFN-α by HHV-8 occurs through activation of Toll-like receptor 9 (TLR9) signaling in pDC.

Several types of TLR expressed on different APC are emerging as important factors in the innate and adaptive immune response to HHV-8. Notably, triggering of C-type lectins,

including DC-SIGN, in combination with TLR triggering on DC induces signaling and cytokine responses, which in turn regulate T cell polarization that is central to host immune control of infections (264). In addition, TLR have also been implicated in reactivation of HHV-8. TRL7/8 could control reactivation of HHV-8 from latency in B cells, as demonstrated by agonists specific for TLR7/8 reactivating latent KSHV and inducing viral lytic gene transcription and replication in latently infected PEL cell lines of B cell origin (112). This has important implications for host control of HHV-8 infection, as signaling through the TLR1/2/6 complex, TLR7, TLR9 and TLR10 affects multiple stages of B cell activation, proliferation, cytokine secretion, terminal differentiation and antibody secretion in response to T cell-dependent antigens (16).

Cytokines and chemokines produced by inflammatory APC, as well as T cells, play a crucial role in HHV-8 replication and development of KS. Inflammatory changes occur early in KS, prior to the detection of the cancer (182). Proinflammatory processes drive early-stage KS to develop into mature, spindle cell lesions (222). Thus, KS tumors are comprised of spindle shaped cells of endothelial origin (219) in an environment rich in inflammatory cell infiltrates, including B cells, macrophages, monocytes and $CD8^+$ T cells (187). The infiltrating cells produce large amounts of Th1 polarizing, proinflammatory cytokines (e.g., IFN-γ, IL-1β, TNF-α and IL-6), chemokines (e.g., IL-8), and growth factors (e.g., vascular endothelial growth factor [VEGF]), which can induce the KS-like phenotype observed in activated endothelial cells (93, 187) (87, 187). IFNγ is the earliest and most abundant inflammatory cytokine observed in KS (93) and can be detected in KS lesions before evidence of HHV-8 DNA (187). IL-6 is also found at very high levels in both KS lesions and in circulation of patients with MCD (8). In MCD, IL-6 induces B cell proliferation and causes inflammatory clinical symptoms (237).

Observations from a transgenic mouse model demonstrate that mice expressing viral IL-6 but lacking mammalian IL-6 do not experience phenotypic changes (e.g., lymphoadenopathy, hypergammaglobulinemia, splenomegaly) associated with MCD (255). IL-6, as well as oncostatin M and IL-10, are also detected at high levels in PEL cells. Proliferation of PEL can be inhibited when receptors for the IL-6 pathway are blocked (74). Thus, an as yet minimally detailed imbalance in the Th1-Th2 milieu during HHV-8 infection appears to be closely linked to APC in driving the outgrowth of KS endothelial cells as well as PEL and MCD B cells.

Other cytokines and chemokines produced by APC, particularly IL-8 and MCP-1, are elevated in serum of KS patients and have been implicated in many cancers (179, 254). Enhanced expression of MCP-1, but not other NF-kβ activated cytokines (RANTES, IL-8 and TNF-α), is also detected in *in vitro* infected human umbilical vein endothelial cells (HUVEC) (39). When bound to its CCR2 receptor on endothelial cells, MCP-1 results in chemotaxis and mediates angiogenesis *in vitro* (100, 179). KS tumors are highly vascularized with abnormal angiogenesis, leading to enhanced blood flow to the tumor by expanding pre-existing blood vessels (182). IL-1β, TNF-α, IL-8 and IL-6 can also enhance tumor cell growth and vascularization (84, 86, 93) by inducing the expression of two angiogenic mediators, i.e., VEGF and fibroblastic growth factor (FGF) (58, 62, 84, 187). In addition to angiogenesis, inflammatory cells and cytokines can contribute to viral reactivation and replication. IFN-γ was shown to induce expression of the DNA polymerase processivity factor, ORF59 PF-8, in BCBL-1 (23) and reactivate latent HHV-8 in BC-3 PEL cells by activation of Pim-family kinases (53). Mercader *et al.*, showed oncostatin M, IFN-γ, and HGF/SF induced lytic cycle activation of BCBL-1 resulting in virion production(181). This principle has been demonstrated in HHV-8 infected PBMC, where inflammatory cytokines could maintain or increase viral load up to 10-

fold higher when the infected cells were cultured in the presence of inflammatory cytokines (187). A summary of host derived cytokines and their relationship to HHV-8 and/or associated cancers are shown in Table 1.

In addition to cellular cytokines and chemokines, HHV-8 encodes several proteins involved in inflammation and angiogenesis that contribute to the inflammatory environment observed in KS. Cytokines and chemokines encoded by HHV-8 have been the focus of numerous studies and reviews (105, 156, 182, 199, 230, 240). Thus, vIL-6 has 24% homology to human IL-6 and can induce expression of VEGF and MCP-1 (200). These in turn trigger angiogenic pathways. Elevated levels of vIL-6, as well as levels of human IL-6 and HHV-8 viral load, have been associated with a recently described syndrome of severe systemic inflammatory symptoms (262). The G-protein coupled receptor (vGPCR) is an early lytic phase gene homologous to the IL-8 receptor, CXCR-2 (12, 199). vGPCR constitutively signals and results in enhanced production of IL-1β, IL-8, MCP-1, IL-6 and VEGF that can have both autocrine and paracrine effects (107, 239). K1 and K15 are signal transducing proteins that induce VEGF, IL-6 and IL-8 (54). LANA, encoded by ORF73, and the viral flice inhibitory proteins (vFLIP) have been linked to enhanced cytokine production via activation of the mitogen activated protein kinase (MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathways, respectively (272). Viral interferon regulatory factor (vIRF3) expression inhibits MHC class II expression as well as IFNγ production (235). Finally, the viral macrophage inflammatory proteins (MIPs) (chemokine (c-c motif) ligand [vCCL1, vCCL2, vCCL3]) share homology to MIP1-α and regulated upon activation, normal T-cell expressed, and secreted (RANTES) and can induce monocyte chemotaxis and signal transduction (12, 196, 199). Given the plethora of such data derived from highly manipulated molecular and cell line models, the challenge is to

Table 1: Host cytokines and chemokines in relation to HHV-8

Immune Mediator	Produced by	Signaling pathway	Function	Relationship to HHV-8 and associated cancers
IFN-γ	Natural killer , CD8 and CD4 T cells	JAK/STAT NF-κB	Promotes NK cell activity, increases antigen presentation and macrophage function, activates iNOS, promotes Th1 differentiation	Most abundant inflammatory cytokine detected in KS tumors, induces expression of ORF59 in BCBL-1 and reactivates latent HHV-8 in PEL
IL-1β	Activated macrophages	NF-κB	Inflammatory cytokine that promotes, cell proliferation, differentiation and apoptosis	Elevated in serum of individuals with KS. Promotes tumor growth and vascularization through induction of VEGF and FGF. Induced by vGPCR
IL-6	T cells, B cells, macrophages	JAK/STAT NF-κB	Stimulates acute phase protein synthesis. Supports B cell growth. Antagonist to T regulatory cell functions.	Elevated in serum of individuals with KS. B cell proliferation factor, induces VEGF and FGF. Causes inflammatory symptoms in MCD. Induced by HHV-8 vGPCR, K1 and K15. Shares homology to HHV-8 encoded viral IL-6
IL-10	Monocytes, Treg, CD4 T cells and activated B cell subsets	JAK/STAT NF-κB	Enhances B cell survival, proliferation and antibody production. Regualtes JAK/STAT signaling. Downregulates Th1 cytokines	Detected at high levels in PEL
IL-12	Macrophages and DC	JAK/STAT NF-κB	Differentiation of naïve T cells into Th1 cells. Stimulates growth and function of T cells and production of IFN-γ /TNF-α from T and NK cells	Downregulated by HHV-8 in MDDC
TNF-α	Activated macrophages	NF-κB	Induces apoptosis, sepsis, cachexia, inflammation and inhibits tumorigenesis and viral replication	Elevated in serum of individuals with KS. TNF-α treatment in KS patients results in disease progression.
CCL2 (MCP-1)	Macrophages and endothelial cells	NF-κB	Recruits monocytes, T cells and DC to site of inflammation and mediates angiogenesis	Elevated in serum of individuals with KS. Induced by HHV-8 vGPCR. Produced by HHV-8 infected THP-1 monocytes through activation of TLR.
CCL3 (MIP-1α)	Activated macrophages	NF-κB	Activate granulocytes leading to inflammation. Induce synthesis and release of other pro-inflammatory cytokines (IL-1, IL-6 and TNFα) from macrophages	Induced by HHV-8 infected pDC and MDDC. Shares homology to HHV-8 encoded vCCL-1
CCL4 (MIP-1β)	Activated macrophages	NF-κB	Activate granulocytes leading to inflammation. Induce synthesis and release of other pro-inflammatory cytokines (IL-1, IL-6 and TNFα) from macrophages	Induced by HHV-8 infected pDC and MDDC.
CCL5 (RANTES)	T cells	NF-κB	Chemotactic for T cells and lymphocytes into inflammatory sites. Induces proliferation and activation of NK cells	Shares homology to HHV-8 encoded vCCL-2
CXCL8 (IL-8)	Produced by any cell with a TLR, especially epithelial cells and macrophages	NF-κB	Neutrophil chemoattractant and potent promoter of angiogenesis	Induces VEGF production. Signaling by vGPCR results in IL-8 production and IL-8 can bind to HHV-8 vGPCR to enhance signaling. Induced by HHV-8 K1 and K15
CXCL10 (IP-10)	Monocytes, endothelial cells and fibroblasts	NF-κB	Chemotactic for DC, T, NK and monocytes/macrophages. Promotion of T cell adhesion to endothelial cells and induction of angiogenesis	Produced by HHV-8 infected THP-1 monocytes through activation of TLR. Also produced by MDDC

link these unique HHV-8 factors directly to HHV-8 infection and development of cancers in natural targets of the virus.

In epidemic or AIDS-related KS, the immune dysregulation and induction of inflammatory cytokines acts to further enhance KS tumor growth. When BCBL-1 cells that are latently infected with HHV-8 are cultured with HIV-1 infected $CD4^+$ T cells, soluble factors secreted by the T cells cause the virus to enter lytic reactivation (181). Inflammatory cytokines induced by both HIV-1-infected and HHV-8-infected cells promote expression of receptors for HIV-1 Tat, which acts as a progression factor in KS development (14, 83) and increasing viral load (118). Indeed, serum and cell samples taken from KS lesions of HIV-1 infected individuals co-infected with HHV-8 show markedly increased levels of inflammatory cytokines, growth factors and angiogenic mediators (86, 209). Furthermore, treatment of KS patients with IFNγ, IL-2 and TNF causes KS progression (187).

HHV-8 has a broad cellular tropism *in vivo* including B cells, endothelial cells, monocytes, keratinocytes and epithelial cells that could result in production of inflammatory mediators (46). In monocytes, production of interferon inducible protein (IP-10), IFN-β1, MCP-1 and IRF-1 occurs in conjunction with an upregulation of TLR3 expression (277). Our lab has previously demonstrated that *in vitro* HHV-8 infection of MDDC, which do not support lytic virus replication, secrete IL-6, TNF-α, IP-10, MIP-1α and MIP-1β (123). While IL-12p40 expression increases post-infection, bioactive IL-12p70 is not detected in HHV-8 infected MDDC. This suggests a virus-related inhibition of constitutive production of IL-12p35, or a defect in complexing of these subunits into IL-12p70. Furthermore, the results support an

intentional skewing of cytokine production in HHV-8-infected MDDC towards induction of a Th2 response that could enhance development of KS.

Elevated levels of IL-1β, TNF-α, IL-6, IL-8 and IL-10 are detected in the serum of patients with EBV-associated diseases, while a less favorable outcome correlate with increases of IL-6 and IL-10 in Hodgkins lymphoma (90). Common strategies between EBV and HHV-8, such as NF-κB signaling pathway alterations (68, 120) and the expression of virokines (240), imply that an imbalance of immune mediators is associated with the oncogenesis of these gammaherpesviruses.

Although there are several studies that focus on HHV-8 induced and encoded cytokines, we know little regarding HHV-8-specific T cell-APC interactions and their role in controlling viral infection and disease. A key challenge is to adapt *in vitro* models using cell lines and HHV-8 constructs to systems that allow deciphering of the basic steps of natural HHV-8 infection, and antigen processing and presentation, in various types of APC. The interactions of APC with T cells that underlie the generation of anti-HHV-8 T cell immunity begin with DC of myeloid origin that take up viral antigen at local sites of infection, then travel to the draining lymphatics and induce antiviral T cell responses (260). There are specialized subsets of DC that populate different tissue sites and have distinct virologic interactions and immunologic functions. Myeloid-derived LC and dermal DC populate the epidermis and dermis respectively, and are associated with KS lesions. Interstitial or interdigitating tissue DC are similar in phenotype and function to dermal DC, and are linked to systemic KS lesions. Other DC subsets such as $CD141^+$ DC which are the human surrogates of mouse CD8α DC subsets (13, 136), could be natural targets for HHV-8. It is imperative that we assess transcription of HHV-8 ORFs in natural targets of the virus, in comparison to well documented immunomodulatory properties of HHV-8

expressed in cell lines and artificial constructs, such as persistently infected BCBL-1 and its variants (63).

Interactions of HHV-8 with DC subsets could be critical at the site of virus replication, and be centrally involved in generating T cell responses to the virus. Efficient activation of HHV-8 epitope-specific $CD8^+$ T cells requires presentation by peptide-loaded, autologous, mature MDDC (273). This is similar to optimal activation of anti-EBV cytotoxic T lymphocytes (CTL) by peptide-loaded DC (165, 217, 249-252, 279). Other studies have revealed polyfunctional $CD8^+$ and $CD4^+$ T cell reactivity and new MHC class I epitopes for HIV-1 Gag and Nef using peptide-loaded DC (128). Importantly, we have used this DC model to map epitopes of HHV-8 lytic and latency proteins with libraries of synthetic, 15mer peptides overlapping by 11aa (158). Nevertheless, it may be more practical to generate large numbers of CD40 ligand (CD40L)-activated, autologous B cells that favourably compare to DC as APC (238).

To date, relatively few $CD8^+$ and $CD4^+$ T cell epitopes within only 15 of the over 80 ORFs of HHV-8 have been identified, and most of these are restricted by human leukocyte antigen (HLA) A*0201 (226). Information is therefore needed on the broad range of potential antigenic sites in the virus that are restricted by other MHC class I and II haplotypes. Moreover, no studies have yet established a hierarchy of naïve and memory $CD8^+$ or $CD4^+$ T cell responses to HHV-8 epitopes in control of HHV-8 infection. There also are minimal data on whether alterations in anti-HHV-8 T cell responses are related to development of KS (114) and whether the lower incidence of KS in HIV-1 infected persons receiving ART is related to increases in anti-HHV-8 T cell responses (20, 29). Such information is important for development of prophylactic and therapeutic vaccines for HHV-8.

HHV-8 infection alters the capacity of DC to be recognized by and activate CTL. Both direct presentation using viral proteins endogenously produced in DC, and cross-presentation pathways using viral proteins from exogenous sources of virus are likely to be operative in HHV-8 infection. In fact, EBV does not replicate in MDDC, which instead activate anti-EBV $CD8^+$ T cells by an antigen cross-presentation pathway (124, 207, 251).

It is possible that HHV-8 infected, apoptotic endothelial cells, macrophages and B cells are recognized as "distressed" cells at local sites of infection and engulfed by LC and iDDC (260). These DC then migrate to local lymph nodes while processing the ingested viral proteins through alternative MHC class I pathways for presentation to $CD8^+$ T cells. Furthermore, several HHV-8 proteins, particularly those coded by ORFs K3 and K5, have intriguing properties of altering expression of MHC class I, T cell co-receptors and DC-SIGN. Interestingly, cytokines released by primary effusion lymphomas can interfere with the *in vitro* differentiation of immature MDDC from $CD14^+$ monocytes (56).

An intriguing recent discovery is that activated $CD4^+$ T cells suppress HHV-8 lytic replication in tonsillar B cells (190). The suppressive activity requires cell-cell contact. However, it is not a classic CTL response, as it can be mediated by T cells from HHV-8 seronegative persons, is not MHC restricted and does not lyse the B cell targets. This is proposed to be a pathway by which HHV-8 is driven into latency in B cells. These $CD4^+$ T cells are reminiscent of $CD8^+$ T cells that exhibit non-cytotoxic responses that suppress HIV-1 infection (142).

Presentation of HHV-8 proteins to both CD8 (MHC class I restricted) and CD4 (MHC class II restricted) T cells is impaired by HHV-8 infection. Evidence suggests that anti-HHV-8 $CD8^+$ T cell responses can be inhibited by K3 and K5 proteins that down-regulate MHC class I expression (64, 132). Interestingly, K5 encoded MIR2 down-regulates T cell co-stimulatory

molecules intracellular adhesion molecule (ICAM-1) and CD86 (65) and IFNγR1 (164) which could act to decrease T cell responses to HHV-8. Ishido *et al.,* showed that K5 dampens NK cell-mediated cytotoxicity by down-regulation of ICAM-1 and CD86 (131). The NK activating receptor, NKG2D, responsible for detecting infected cells, is down-regulated by HHV-8 K5 (258) via the release of the tumor-associated prostaglandin E2 (PGE2) from KS cells (81). This also results in inhibition of IL-15-mediated NK cell activation and survival, adding to the immune escape tactics employed by this virus (81). Likewise, infection of primary fibroblasts results in limited NK cell activation and subsequent killing activity (177). Brander, et al. (31), reported a decrease in lysis by HIV-1 peptide-specific CTL clones of cells infected with HHV-8. Thus, it is apparent that K3 and K5 potentially have multifactorial effects on immune control of HHV-8 infection. Of note is that the intracellular load of HHV-8 in infected endothelial cells is directly related to their loss of expression of MHC class I and ICAM-1, in association with expression of MIR2 (1). Interestingly, EBV infection also decreases recognition of latently infected cells by down regulation of MHC class I molecules, particularly in cells derived from Burkitt's lymphoma (127).

MHC class II recognition is dampened by HHV-8 infection. Sabbah *et al.*, reported that LCL, with an intact MHC class II processing pathway, could present LANA peptides to LANA-specific $CD4^+$ T cell clones, whereas PEL cells were not recognized in an IFN-γ ELIspot (229). PEL express vIRF3, a known inhibitor of the MHC class II master regulator, CIITA (class II transactivator) (235). When CIITA function was restored in PEL, $CD4^+$ T cell clone recognition was also restored (229), supporting a role for HHV-8 in the reduction of MHC class II expression. Interestingly, IFN-γ inducible expression of CIITA results in MHC class II expression on endothelial cells, and is impaired after HHV-8 infection through induction of

suppressor of cytokine signaling 3 (SOCS3) (35). This results in inhibition of the early events in the IFN-γ signaling pathway. In sum, various HHV-8 proteins appear to play a significant role in the disruption of antigen processing and presentation. However, further data are needed to understand the extent of viral protein function in immunopathogenesis of HHV-8 infection in APC.

Although immunity to HHV-8 is far less well defined than that to EBV, T cell immunity to HHV-8 likely plays a similar, critical role in viral control. First, there is an increase in $CD4^+$ and $CD8^+$ expanded T cells in patients with classic KS that share a T cell receptor (TCR)-β variable subunit bias (99), a phenomenon observed in response to chronic viral infections (259, 282). Second, $CD8^+$ T cell immunity to HHV-8 proteins is present in HHV-8 seropositive, healthy individuals. $CD8^+$ T cells specific for 5 HHV-8 lytic cycle proteins are present in blood in the first few months of primary HHV-8 infection of normal adults (274). This primary CTL and IFN-γ response to HHV-8 peaks within 2 years of infection, and wanes thereafter to low but detectable levels. Furthermore, KS does not commonly occur in individuals with a high $CD4^+$ T cell counts (248).

To date, however, there is little direct evidence for a role of T cell immunity in HHV-8 infection and control of KS (126). Lower $CD8^+$ T cell responses have been found in persons with KS compared to asymptomatic persons (114, 151). However, very modest increases in CD8 T cell responses to HHV-8 immunodominant peptides are found in persons on ART (29, 280). While progressive increases in HHV-8 load precede development of disease in HIV-1-infected persons (37, 153), evidence is lacking for a direct association between control of HHV-8 load and HHV-8 specific, T cell immunity (114). Nevertheless, an increased incidence of KS in organ transplant recipients and HIV-1-infected persons (69) suggests a role for T cell immunity

in prevention of KS, similar to T cell immunity in EBV-related cancers (109). Reduction of immunosuppressive regimens can result in spontaneous resolution of KS in organ transplant recipients (94). Similarly, the incidence of KS has declined after suppression of HIV-1 by ART (211), where T cell numbers and function are partially restored (17, 159, 223). There are also shorter incubation periods for development of KS after HHV-8 infection in HIV-1-infected men compared to men infected with HHV-8 prior to HIV-1 infection (104, 133). Primary infection with HHV-8 in immunosuppressed persons has a more severe outcome than reactivated HHV-8 infection. Finally, HHV-8 expresses many proteins that have immunomodulatory functions that could down-regulate T cell immunity (11).

The emerging biology of KS and HHV-8 infection presents intriguing factors that interrelate HHV-8 specific T cell immunity to control of the cancer. HHV-8 is found as a latent infection in most of the spindle cells in the KS lesion (27, 80, 95, 188). Since replication of herpesvirus in susceptible cells results in cell death, latency must be established either very soon after infection or possibly following an abortive (non-productive) infection. A small percentage of endothelial and KS spindle cells express a complete replication library of HHV-8 proteins early in the disease, whereas the majority of the transformed cells ultimately express only HHV-8 latency proteins. Circulating B cells and monocytes can be positive for HHV-8 DNA (8, 25), and HHV-8-infected macrophages are present in KS tissues(25). Th1 cytokines have been implicated in reactivation and persistence of HHV-8 in B cells and monocytes from KS patients (241). T cell infiltrates are common in KS tissues (25, 93). $CD8^+$ T cells in KS tissues produce IFN-γ and express HLA DR (93, 241), suggesting that tumor-infiltrating lymphocytes are responding to HHV-8 antigens.

Comprehensive longitudinal studies are needed to accurately assess the role of anti-HHV-8 T cell immunity in development of KS. T cell responses to HHV-8 could be directed at different lytic and latency proteins at different stages of infection and disease (109, 126). By comparison, in EBV the immediate early regulatory protein BMLF1 and other early and late lytic cycle proteins are targets for CD8 CTL during primary and latent infection (26, 127, 246). During mononucleosis, a primary symptomatic infection of EBV, both lytic and latency EBV-specific T cells are present, but responses to lytic epitopes tend to be stronger (168). In healthy EBV seropositive individuals, $CD8^+$ T cell responses are also found to be greater for lytic epitopes, with up to 3% of cells specific for a single lytic epitope and up to 0.5% for a single latency epitope (126). Anti-EBV CTL responses shift during latent infection to EBV nuclear antigens EBNA3 and LMP2, while still retaining specificity for some lytic cycle proteins (125). The hierarchy of CTL responses to immunodominant epitopes of EBV is related to a lower expression of latency proteins in infected cells (208). Although HHV-8 does not have genes homologous to EBNA and LMP, HHV-8 LANA, kaposin (T0.7 or ORF K12) and K1 are putative latency and transforming proteins that are targets for CTL (30, 158, 204).

Host selection of $CD8^+$ T cell epitopes within HHV-8 proteins could be based in part on the relative expression of viral proteins by the MHC class I endogenous pathway, comparable to EBV (161). However, evidence from the anti-EBV CTL field indicates that CTL reactivity to this gamma herpesvirus varies as to the HLA haplotype, with different MHC class I haplotypes exhibiting different CTL reactivity to the same EBV proteins (127). Perhaps HHV-8 has mechanisms similar to the Gly-Ala (Gar) (207) repeat domain in EBNA1 that inhibits proteosome processing of viral proteins through the MHC class I pathway (127, 160), thereby inhibiting generation of EBNA1 specific T cells. In fact, LANA1 can inhibit protein processing

in cis (149, 285). Bioinformatic analysis of HHV-8 sequences supports that latency proteins are likely to be poorer targets for CTL than immediate early or lytic proteins (266). However, it is not yet clear if the *in cis* function of LANA1 is directly involved in down-regulation of CTL lysis of HHV-8 infected cells, including how it compares to other putative, *in trans* inhibitors of CTL function such as K3 and K5. Moreover, the EBNA1-CTL inhibition concept has undergone major revision. First, the GAr domains of EBNA1 can inhibit mRNA translation, which may be more critical to lack of CTL recognition than inhibition of proteosomal processing (284). Second, EBNA1 infected cells express EBNA1 peptides that can be recognized by CTL when assessed in more sensitive assays (157). This indicates that the effects of LANA1 on pathways related to CTL function that use chimeric constructs, indicator cell lines, etc., need to be characterized in a natural context using $CD8^+$ CTL and natural targets that are specific for LANA1.

Similar to EBV, the $CD8^+$ T cell responses to HHV-8 tend to be directed more toward lytic antigens (224). While there are much fewer $CD8^+$ T cell epitopes known for HHV-8 than EBV, the majority of these epitopes are within the early and late-lytic proteins (225, 226). With regard to polyfunctionality, one study found that for both EBV and HHV-8, T cells specific for latency antigens were more polyfunctional than those specific for lytic antigens (21). The phenotype of these cells was also found to be different, with a greater proportion of effector memory T cells specific for latency antigens than lytic antigens for both EBV and HHV-8. In both EBV and HHV-8-associated malignancies, latency proteins are predominantly expressed, so it is thought that responses to latency proteins could be important in controlling these diseases (126, 256). Evidence suggests that there are higher levels of $CD8^+$ CTL specific for EBV and cytomegalovirus (CMV) than HHV-8 in the blood of seropositive individuals (114, 273). Higher

T cell responses to EBV and CMV antigens could be related to their greater viral load in persistently infected persons, with more turnover of viral antigen from latent, persistent reservoirs that maintains a greater level of memory CTL precursors.

Antigen-specific $CD8^+$ T cells occupy a lineage of naïve and memory compartments that are involved in the expansion, effector and contraction phases of $CD8^+$ memory T cells (117). Central memory and effector memory T cells are contrasted based on expression of surface molecules related to migration and differentiation. Patients with MCD have more $CD45RA^-CCR7^-CD27^-CD8^+$ IFN-γ^+ cells (a late memory T cell phenotype) and fewer $CCR7^-CD27^+CD45RA^-$ cells (early and intermediate T cell phenotype) than normal, HHV-8 seropositive controls. This phenotypic shift is not found for EBV-specific $CD8^+$ T cells. Interestingly, HHV-8 viral loads are negatively correlated with early and intermediate effector memory cells. The more differentiated T cell phenotype is associated with disease, rather than a loss of HHV-8 specific CD8 T cells or polyfunctional activity, as the HHV-8 specific T cells are similar in function (secretion of IFN-γ, TNF-α, MIP1-β, and/or CD107a) in infected patients and healthy controls (115).

In healthy, HHV-8 seropositive individuals controlling infection, there are both monofunctional and polyfunctional $CD8^+$ T cells present that are specific for HHV-8 proteins (158). This could have important implications in the immunopathogenesis of HHV-8 and for HHV-8-related disease development. In fact, patients who control KS have more polyfunctional $CD8^+$ T cells producing IFN-γ and TNF-α, while patients with progressive KS have weaker and less polyfunctional HHV-8-specific $CD8^+$ T cells (19). IFN-γ-producing CTL specific for some HHV-8 lytic and latency proteins also express CD107 and TNF-α (21). This is similar to polyfunctional CTL that produce multiple cytokines such as IFN-γ, IL-2 and MIP-1β that are

associated with enhanced control of HIV-1 infection (18, 173, 247). Also, $CD8^+$ CTL specific for EBV lytic and latency proteins differ in phenotype, including expression of programmed death-1 (PD-1) (127). PD-1 expression could act as a negative regulator of HHV-8 specific $CD8^+$ T cells during disease progression.

While both monofunctional and polyfunctional antiviral $CD8^+$ T cells are present in healthy HHV-8 seropositive individuals, a week-long DC-enhanced system was required to reveal these responses to HHV-8 proteins (273). Overall, the immune response to HHV-8 is relatively non-robust compared to T cell reactivity to other herpesviruses such as EBV (21, 158).This suggests that the number and/or functional capacity of circulating anti-HHV-8 T cells are relatively low. However, using direct, multimer staining, we have found that there is an average of 0.05-0.10% circulating, $CD8^+$ T cells specific for single, immunodominant MHC class I epitopes of HHV-8 in healthy, HHV-8 seropositive individuals (158). It is possible that these HHV-8-specific T cells are functionally down-regulated by T regulatory cells (Treg).

Treg are operative in peripheral tolerance and beneficial in preventing autoimmunity and tissue damage, through such activities as inhibitory cytokine secretion and suppression of DC function (270). However, Treg can also inhibit immunity needed to resolve infections. While little is currently known about Treg during HHV-8 infection and disease development, these cells have been found to be important during other viral infections, including EBV (162) and HIV (170). During primary EBV infection, patients with mononucleosis have less Treg than healthy seropositive individuals (281). In patients with Hodgkins lymphoma, Treg accumulated at tumor sites and those patients with higher Treg ratios had shorter disease-free survival (174, 236). Additionally, in these patients, several EBV epitopes stimulate Treg, and the increases in Treg numbers are associated with decreased EBV-specific $CD8^+$ T cell IFN-γ production (175).

During HIV-1 infection, HIV-1 specific $CD8^+$ T cell responses and cytolytic activity are repressed by Treg (145) and an increased Treg frequency effect $CD8^+$ T cell polyfunctionality that is restored with Treg depletion (171). In patients with nasopharyngeal carcinoma, large numbers of Treg are found both at tumor sites and in circulation (155, 163). As these cells could also be important in HHV-8-related disease development, such as KS, studies are needed to determine their exact role.

To succeed, a pathogen must be able to evade immune surveillance. In this review, we have described the effect of HHV-8 infection on cells of the immune system, with particular emphasis on professional APC and the effect of HHV-8 infection on T cell responses, and their relationship to the development of KS. Recognition that DC-SIGN expressed on DC, macrophages and B cells acts as a major receptor for HHV-8 has enhanced our ability to assess the effect of HHV-8 infection of these primary cells. This has revealed two distinct replication patterns of HHV-8 in APC, i.e., non-productive and productive, which could have direct consequences on viral pathogenesis. Furthermore, this should enable studies of virus gene transcription cascade in cells capable of supporting productive infection that are natural targets of HHV-8. Studies have also begun to elucidate the effect of HHV-8 infection on DC and B cell functions, as measured by cytokine and chemokine production and impairment of antigen presenting functions. The direct effect of HHV-8 infection of professional APC and its indirect effect on T cell control of infection are being tied together in a more revealing fashion to define the magnitude and breadth of T cell responses to HHV-8 antigens. T cell responses to HHV-8 antigens are not very robust as compared to EBV and CMV. This dampened immune response could be related to down regulation by Treg. Although evident in HHV-8 infection, it is not clear whether polyfunctional T cells are required to control progression of associated diseases.

Given that the most common route of HHV-8 transmission is through saliva, and KS lesions predominate in the skin and mucosa, APC at the mucosal site are the most likely to be critical in controlling HHV-8 transmission and pathogenesis. Understanding how these events are influencing the ability of APC to induce an effective immune response is essential in the development of therapeutic and preventative vaccine strategies.

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1.1 HYPOTHESIS

Human herpes virus 8 is the causative agent of Kaposi's sarcoma and is associated with two B cell cancers: Primary effusion lymphoma and multicentric Castleman's disease. The virus encodes 84 open reading frames, many of which represent human gene homologs, e.g., viral IL-6, and viral proteins that promote the production of host cytokines, chemokines, and growth factors (88, 134). These soluble immune mediators, including the host cytokines IL-6 and TNF-α, and the chemokine IL-8, are thought to play an important role in KS pathogenesis, including tumor-cell proliferation, angiogenesis, and vascular permeability (86, 134, 179, 254, 283). Indeed, development of KS has been associated with increases in these soluble factors in blood.

There is little information on the cellular source of these soluble host factors during HHV-8 infection, and how they might affect HHV-8 replication. Our lab has previously shown that HHV-8 infects two types of professional APC in vitro, i.e., monocyte-derived dendritic cells (216) and B lymphocytes (215), which are considered major targets of the virus *in vivo*. However, the virus undergoes productive infection in B cells but not DC.

I hypothesized that immune mediators produced by HHV-8 infected APC are unique between cell types and that HHV-8 infects a subset of B cells and initiates cytokine and chemokine production that contributes to HHV-8 replication, viral dissemination, and initiation of KS and HHV-8 lymphomas. Comparing the cytokine-chemokine response to the virus in these two cells following infection could be significant to our understanding the pathogenesis caused by HHV-8 infection, and could provide novel targets for vaccine development. I therefore proposed to evaluate cytokine and chemokine production in APC targeted by HHV-8 infection, and their relationship to HHV-8 replication.

1.2 SPECIFIC AIMS

1.2.1 Specific Aim I

We determined the cytokine and chemokine profiles in B cells compared to DC and defined HHV-8 replication within the B cells. MDDC and CD40L/IL-4-activated B lymphocytes were isolated from HHV-8 seronegative donors and infected with purified, live HHV-8 ("live" refers to fully replication competent virus). Supernatants were harvested at various time points up to 48 hours post-exposure (hpe) and screened for cytokines and chemokines by Cytometric Bead Array (CBA) (BD). In addition, down-regulation of IL-12p70 production was further investigated by examining the effect of HHV-8 infection of DC in parallel with stimulation by the TLR agonists lipopolysaccharide (LPS) and polyinosinic:polycytidylic acid (poly-I:C). Lytic HHV-8 replication was assessed in B cell pellets and supernatants by PCR quantification of HHV-8 lytic

cycle ORF K8.1 DNA, lytic protein expression by flow cytometry and infectious virion production by 50% tissue culture infective dose ($TCID_{50}$) assay.

1.2.2 Specific Aim II

We delineated the role of virus replication in B cell subsets in relation to HHV-8 induced cytokine and chemokine production. HHV-8 exposed cells were stained intracellularly to determine cytokine and chemokine expression. Live and ultraviolet light (UV-light) inactivated HHV-8, and soluble HHV-8 glycoprotein B were added to B cells to assess the role of viral replication in cytokine and chemokine production. The effect of IL-8 on HHV-8 replication in B cells was further studied by addition of recombinant IL-8 or neutralizing IL-8 mAb. The role of these immune mediators in the development of KS was also assessed in participants from the Pittsburgh Multicenter AIDS Cohort Study (MACS).

1.2.3 Specific Aim III

We determined B cell markers and signaling pathways that were activated/suppressed after HHV-8 infection in comparison to HHV-8 ORF expression. RNA was isolated from HHV-8 exposed and unexposed cells and used in microarray analysis and a qRT-PCR primer pair assay for HHV-8 ORFs (collaborative efforts with Dr. Jun Li of the Jenkins laboratory). The relationship between cytokine and chemokine signaling pathways and the host cell markers that were most significantly increased or decreased after HHV-8 infection were determined. Furthermore cellular gene activation was compared to the kinetics of HHV-8 gene expression by quantitative multiplex PCR.

2.0 HHV-8 INFECTION OF DENDRITIC CELLS

2.1 ABSTRACT

Human herpesvirus 8 induces a wide range of inflammatory immune mediators known to contribute to its associated cancer, Kaposi's Sarcoma, as well as the B cell lymphomas, multicentric Castleman's disease and primary effusion lymphoma. As dendritic cells abundantly express the DC-SIGN receptor and are susceptible to HHV-8 infection, we sought to determine the immune mediator profile of monocyte derived dendritic cells *in vitro.* We found significant increases of IL-10, MCP-1, MIP-1α, MIP-1β, RANTES and IP-10 in HHV-8 exposed DC that were initiated at the level of binding through HHV-8 glycoprotein B (gB). Furthermore, the Toll-like receptor-4 signaling pathway was involved in the down regulation of the antiviral cytokine IL-12p70.

2.2 INTRODUCTION

Cytokines and chemokines are small cell-signaling proteins used for cellular communication during both homeostasis and an active immune response. There are several families of these immune mediators, each with unique functions that make up a complex 'cytokinome'(66). Disruption of the immune mediator balance can result in inflammation, autoimmunity and

immunopathology (269). Several pathogens take advantage of the cytokine and chemokine network, including the *Herpesviridae* family of viruses. Herpes simplex virus (HSV) encode for proteins that interfere with chemokine function (269). Also, levels of chemokines are elevated in cerebral fluid of patients with Herpes simplex encephalitis (228). CMV induces a pro-inflammatory cytokine response at the blood brain barrier, including up-regulation of RANTES, IL-8, IL-6, CXCL-11 and IL-1β, that contribute to neuropathology (7). Both primate CMV and human EBV encodes for a viral IL-10 that serve several functions including; inhibition of DC maturation (213), down-regulation of MHC I on B cells (286), growth and transformation of B cells (186) and inhibition of Th1 cytokines (243).

Cytokines and chemokines also play a crucial role in the development of Kaposi's Sarcoma, a cutaneous tumor caused by Human herpesvirus 8 (188). KS tumors are highly vascularized with abnormal angiogenesis, leading to enhanced blood flow to the tumor by expanding pre-existing blood vessels (182). Inflammatory changes occur early in KS, prior to the detection of the cancer (182). Pro-inflammatory processes drive early-stage KS to develop into mature, spindle cell lesions (222). The infiltrating cells produce large amounts of Th1 polarizing, pro-inflammatory cytokines (e.g., IFN-γ, IL-1β, TNF-α and IL-6), chemokines (e.g., IL-8), and growth factors (e.g., VEGF), which can induce the KS-like phenotype observed in activated endothelial cells (87, 93, 187) Thus, imbalance of the cytokine and chemokine microenvironment created during HHV-8 infection appears to be closely linked to the outgrowth of KS endothelial cells.

When MDDC are infected *in vitro* with HHV-8, viral lytic proteins are produced with little viral DNA production (216), similar to abortive HHV-8 infection of vascular endothelial cells (5, 197, 214, 220, 267). Although HHV-8 infection does not significantly alter MDDC

viability, it decreases MDDC function, i.e., lowers their capacity to activate antigen-specific $CD8^+$ T cell responses. Moreover, HHV-8 infected MDDC have impaired antigen uptake, with a significant decrease in endocytic capacity.

Our lab has previously demonstrated that *in vitro* HHV-8 infection of MDDC secrete IL-6, TNF-α, IP-10, MIP-1α and MIP-1β (123). Furthermore, bioactive IL-12p70 is not detected in HHV-8 infected MDDC (123). We therefore sought to expand on previous findings and further evaluate the down-regulation of IL-12. Here, we show a more detailed examination of the DC cytokine and chemokine profiles and a possible involvement of the TLR-4 signaling pathway in the down-regulation of IL-12.

2.3 METHODS

2.3.1 Preparation of monocyte derived dendritic cells from blood

Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque density gradient separation. To obtain monocytes, PBMC were incubated with anti-CD14 mAb-coated immunomagnetic microbeads according to the instructions of the manufacturer (Miltenyi Biotec). $CD14^+$ cells were cultured in AIM-V medium (GIBCO) and treated with 1,000 U/ml of recombinant human IL-4/ml (R&D Systems) and GM-CSF for 5 days at 37ºC in 5% CO_2 to generate MDDC.

2.3.2 HHV-8 infection of MDDC

MDDC were resuspended at $1x10^6$ cells/ml in fresh AIM-V and left untreated or exposed to HHV-8 for 3 hours (h) at 37ºC in 5% CO_2. Unadsorbed virus was removed by washing the cells 2 times in AIM-V. Cells were resuspended at a final concentration of $1x10^6$ cells/ml and cultured at 37ºC in 5% CO_2, for up to 48 h. In some studies, MDDC were treated with 1µg/ml soluble glycoprotein B (gB) containing an arginine-glycine-aspartate (RGD) integrin binding motif (271) or gB mutant (gBm) containing an arginine-glycine-glutamic acid (RGE) motif (gift from Dr. Bala Chandran) for 3 h and then washed and recultured as described above. In some studies, purified HHV-8 was UV-light inactivated at 365nm wavelength at 10 cm for 20 minutes (m) and then treated with 1U/100µl DNase. Inactivation was verified in parallel B cell studies by viral DNA quantification, lytic protein expression and $TCID_{50}$ assay. In some studies, HHV-8 was passed through a 0.1µl filter and the resulting filtrate was used.

2.3.3 Supernatant collection and cytokine and chemokine detection

Supernatant samples were collected from unexposed and HHV-8 exposed MDDC at various times pre and post exposure. Supernatant samples were screened for IL-1β, -2, -4, -6, -7, -8, -10 and -IL-12p70, IFN-γ, TNF-α, lymphotoxin-α (LT-α), IP-10, MIP-1α, MIP-1β, MCP-1 and RANTES by CBA (BD) per manufacturer's instructions. Briefly, 50ul of cell culture supernatant was incubated with flex set kit capture beads for 1 h and then Phycoerythrin (PE) detection reagent for 2 h. Samples were read on an LSR-II flow cytometer (BD Immunocytometry Systems) and analyzed with Flow Cytometric Analysis Program (FCAP) Array Software (BD).

2.3.4 TLR stimulation

MDDC were exposed to HHV-8 for 3 h and recultured in AIM-V supplemented with a TLR-2 ligand *Porphyromonas gingivalis* LPS (Invivogen, 1µl/ml) and TLR-4 ligand LPS (Sigma, 1µl/ml) or a TLR-3 ligand poly-I:C (Sigma, 1µl/ml). Supernatant samples were collected and assayed by CBA to determine levels of IL-12p70 production.

2.3.5 Statistical analysis

We used the Student *t* test; assuming equal variance and paired for comparisons between groups, $P \leq 0.05$.

2.4 RESULTS

2.4.1 Effect of HHV-8 concentration on cytokine production

As DC are a target for HHV-8 infection and produce immune mediators known to be involved in KS, we sought to determine immune mediator production by HHV-8 infected DC. The induction of cytokines and chemokines was examined using primary MDDC and HHV-8 purified from BCBL-1. DC were infected with HHV-8 for 3 h and then washed thoroughly to remove any unadsorbed virions. DC were recultured for up to 48 h. Supernatants were collected at various times post exposure and tested for multiple analytes with CBA, including the cytokines; IL-1β, -2, -6, -7, -10, -12p70, IFN-γ, TNF-α, the chemokines; CCL2 (MCP-1), CCL3 (MIP-1α), CCL4

(MIP-1β), CCL5 (RANTES), CXCL8 (IL-8), CXCL10 (IP-10), LT-α and the growth factor VEGF, for a total of 17 immune mediators. These cytokines, chemokines and growth factors were selected based on their known association with KS and their availability in a newly developed BD assay. Of the 17 markers, 3 showed ≥ 2.5-fold increase at 24 (left) and 48 hpe (IL-6, MCP-1, MIP-1α) (**Fig. 1a**.). Additionally, at 24 hpe (left) IL-7, IL-10 and IP-10 were enhanced, while IL-1β, MIP-1β, RANTES and IL-8 were enhanced by 48 hpe. Of the markers that showed at least a 2.5-fold increase, we selected IL-6, IL-10, MCP-1, MIP-1α, MIP-1β, RANTES, IL-8 and IP-10 due to their corresponding elevated levels above unexposed DC (**Fig. 1b**).

To assure that the purified HHV-8 lacked non-viral contaminants that could trigger a nonspecific cytokine or chemokine response, DC were treated with HHV-8 or the filtrate of HHV-8 that had been passed through a 0.1μm filter. As shown in **Fig. 2**, unexposed and filtrate treated DC produced similar levels of cytokine and chemokines, which was substantially less than DC exposed to HHV-8, indicating that cytokine and chemokine production seen above background was due to virus infection rather than non-viral contaminating particles.

The effect of virus concentration on production of cytokines and chemokines was next investigated by infecting DC with 10-fold serial dilutions of HHV-8 to determine the optimal amount of virus required for maximum cytokine induction. We determined that a concentration of 10^7 copies of viral DNA used to infected $1x10^6$ MDDC generated the greatest analyte response by 24 hpe (**Fig. 3**) without causing cytopathic effects (viability >86%). This concentration was used for the remainder of experiments.

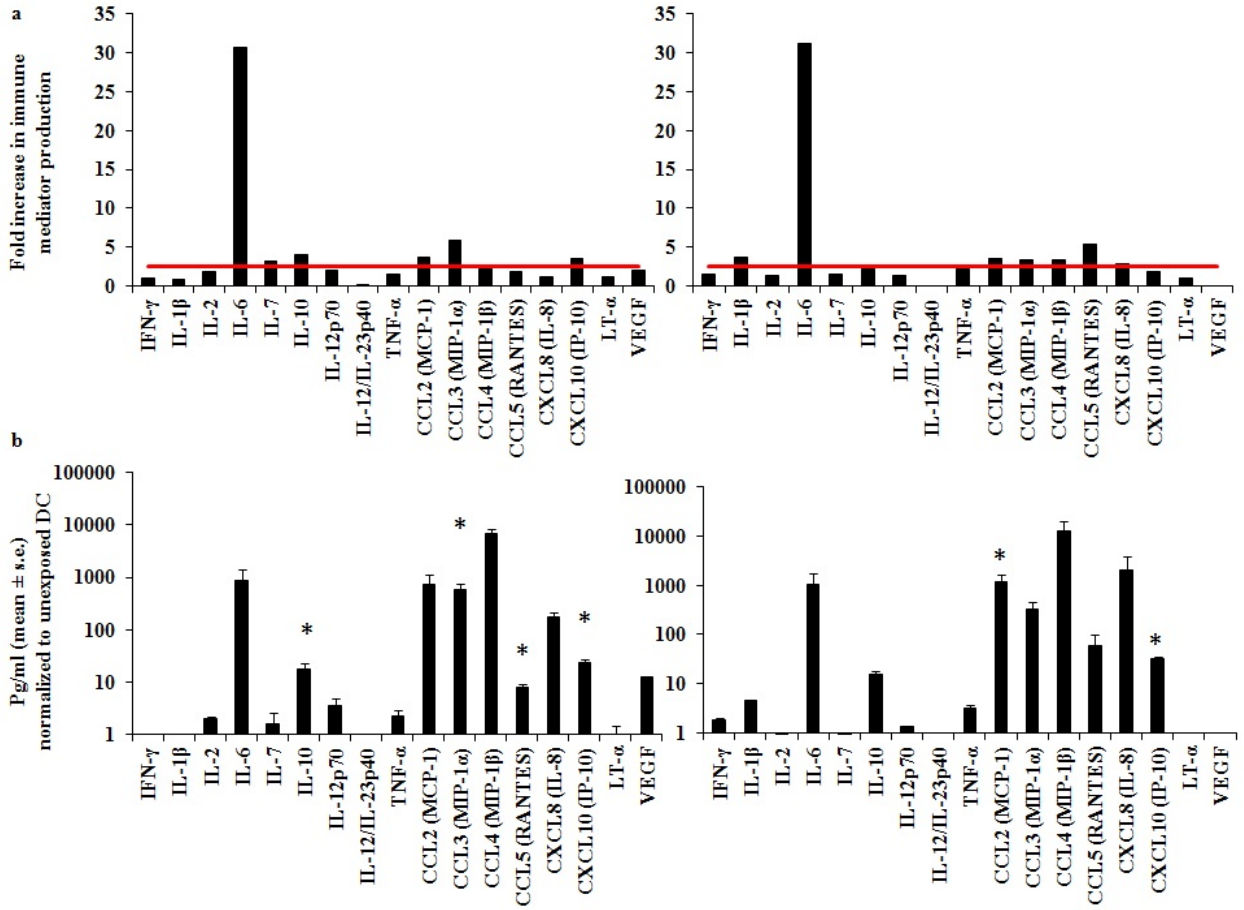

Figure 1: DC cytokine and chemokine screen

DC were left untreated or exposed to HHV-8. Supernatant samples were collected at 24 (left panels) and 48 (right panels) hpe and screened by CBA for 17 immune mediators. *(A)* Fold increase over unexposed DC and *(B)* normalized concentrations above unexposed DC (mean ± s.e.m., N = 1 [IL-1β, IL-12p40, VEGF], 2 [LT-α], 5 [IL-2, IL-7], 8 [TNF-α], 11 [IFN-γ], 16 [IL-12p70, RANTES], 17 [IP-10, IL-10], 18 [IL-6, IL-8], 21 [MCP-1, MIP1α] and 22 [MIP-1β].

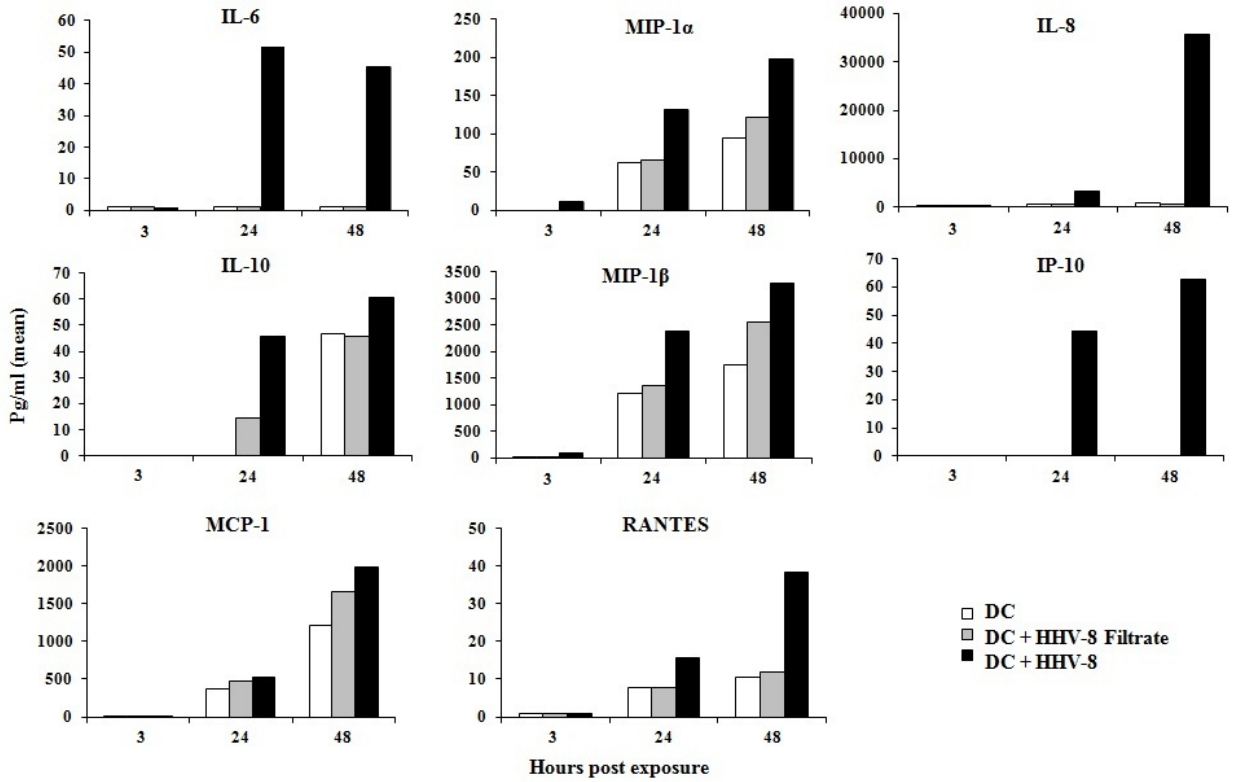

Figure 2: HHV-8 specific immune mediator response

DC were exposed to HHV-8 or the filtrate of virus that was passed through a 0.1µm filter. Supernatants were assayed by CBA at 3, 24 and 48 hpe. Data are representative of 2 individuals tested.

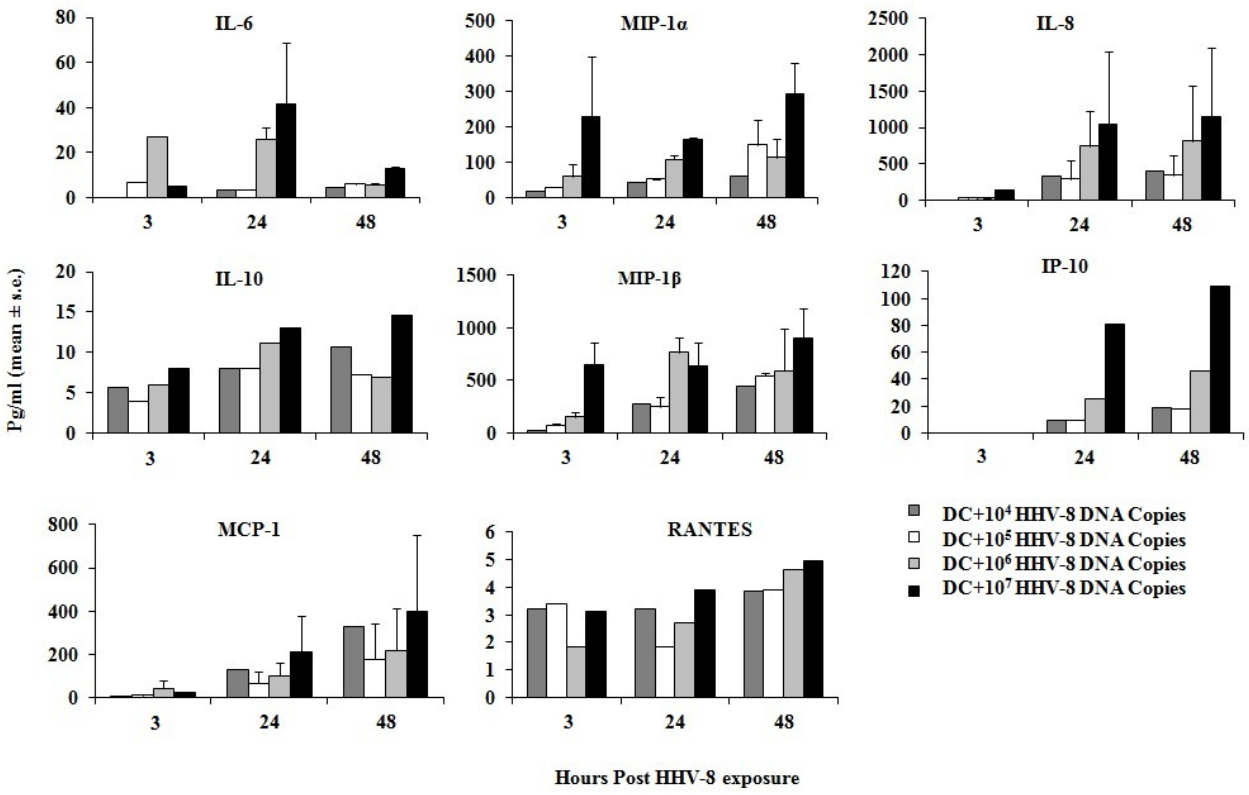

Figure 3: Immune mediator response is HHV-8 dose dependent

DC were exposed to 10^7 HHV-8 DNA copies or three 10-fold dilutions (10^6, 10^5, 10^4). Supernatants were assayed by CBA at 3, 24 and 48 hours post exposure (mean ± s.e.m., N =2).

2.4.2 HHV-8 cytokine and chemokine production in DC

As binding and entry of HHV-8 virions occurs rapidly *in vitro* and Hensler et *al.*, (123) showed induction of cytokine and chemokine production in DC as early as 2 h post infection, we next compared the length of adsorption period that allowed for the greatest cytokine and chemokine response. DC were exposed to HHV-8 for 1 or 3 h and supernatant samples were collected at various times post exposure (**Fig. 4**). Although results between a 1 h and 3 h adsorption were very similar, we determined that a 3 hr adsorption period resulted in the strongest induction of

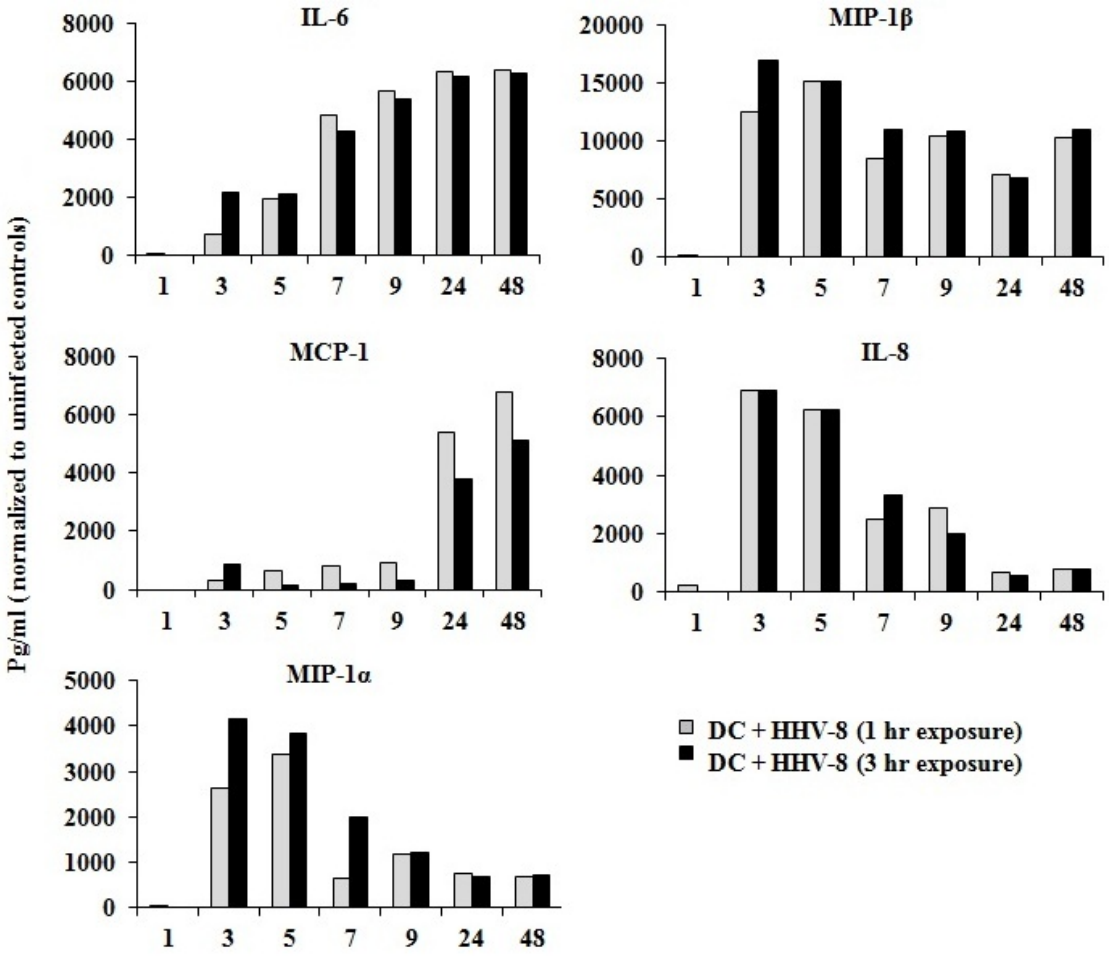

Figure 4: Three hour adsorption induces the best immune mediator response

DC were exposed to HHV-8 for 1 or 3 hours. Supernatants were collected at the end of each adsorption stage and at 1, 3, 5, 7, 9, 24 and 48 hpe for CBA analysis. Data are representative of 2 individuals tested.

cytokines and chemokines that was sustained for up to 48 h. Immediately following the 3 h adsorption period and prior to washing away unadsorbed virus, immune mediators production was enhanced, with significant levels of MIP-1α above unexposed DC cultures, consistent with previous reports (123), indicating a rapid immune mediator response upon HHV-8 exposure (**Fig. 5**).

After establishing the infection protocol and assay procedure that allowed for optimal detection of immune mediator production, we determined the cytokine and chemokine profiles of HHV-8 exposed DC. Nearly all cytokine and chemokine levels continued to rise until the last time point, 48 h, with the exception of the MIP chemokines that peaked in the first 24 h (**Fig. 6**). There were significant increases in IL-10, MCP-1, MIP-1α, MIP-1β, RANTES and IP-10 at 24 hpe with peak production of 24, 1,339, 282, 12,787, 80 and 62 pg/ml, respectively.

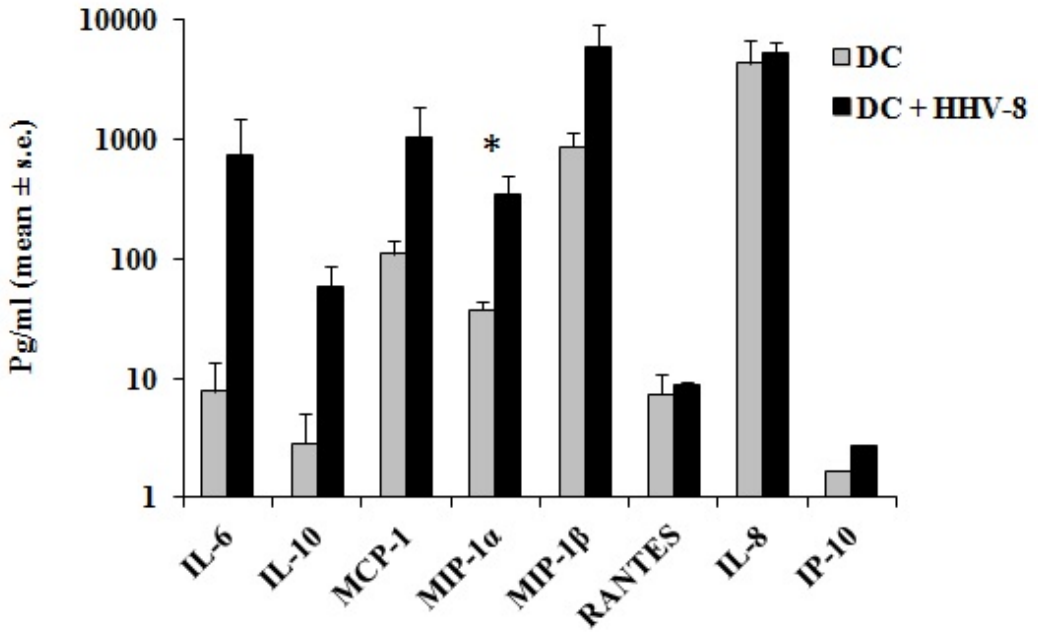

Figure 5: HHV-8 rapidly induces immune mediator production in DC

CBA was used to determine cytokine-chemokine production of HHV-8 infected DC after the 3 h virus adsorption period, prior to washing unadsorbed HHV-8 (mean ± s.e.m., N = 5, *P<0.05).

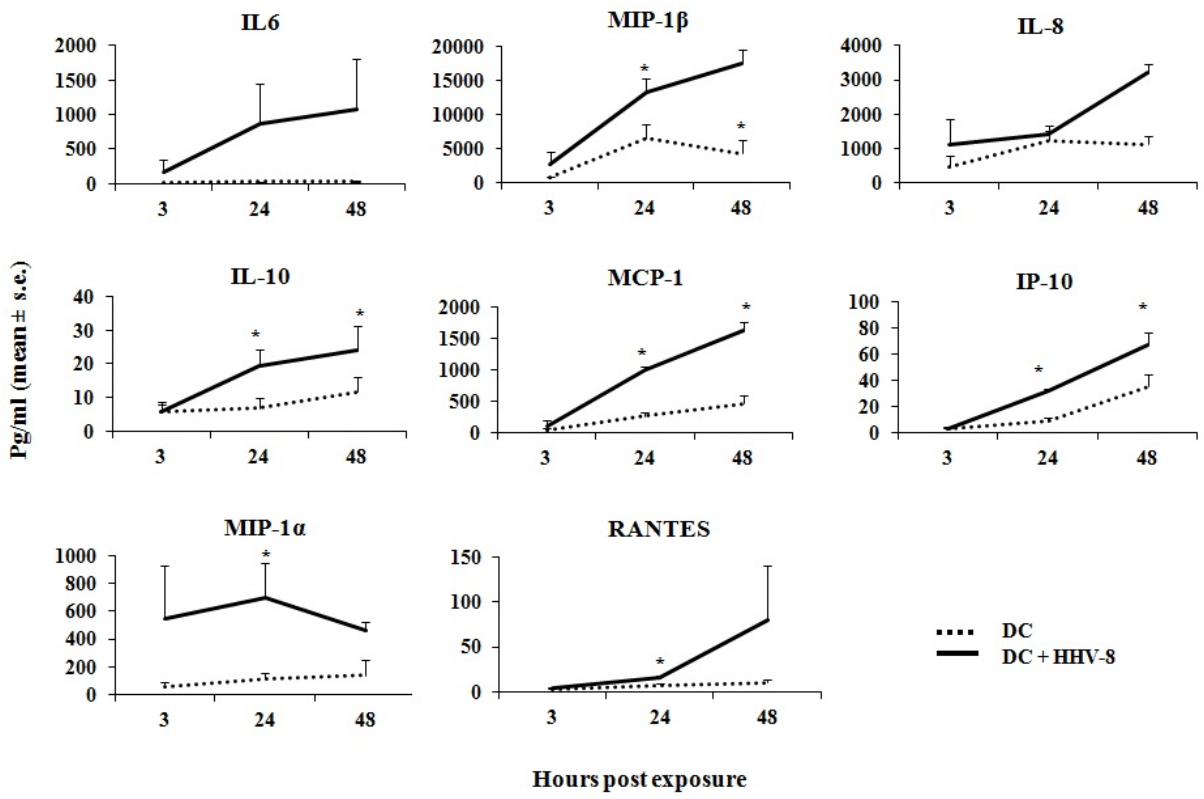

Figure 6: DC cytokine and chemokine kinetics

Supernatants were collected after the adsorption phase and subsequent washes (3h) and at 24 and 48 h and assayed by CBA to determine cytokine-chemokine production of unexposed and HHV-8 exposed DC (mean ± s.e.m., N = 13 [RANTES], 14 [IP-10, IL-10], 15 [IL-6, IL-8], 16 [MCP-1, MIP1α] and 17 [MIP-1β], *P<0.05.)

2.4.3 HHV-8 replication not required for immune mediator induction

As HHV-8 does not replicate in DC (216), we next sought to determine the effects of a replication incompetent HHV-8 on cytokine and chemokine induction. DC were treated with live HHV-8 or a UV-inactivated 'dead' HHV-8. To verify the success of the UV inactivation, UV-HHV-8 were used to infect B cells in parallel studies. B cells support lytic cycle replication and live HHV-8 showed increases in viral DNA, lytic protein expression and infectious virion

production, while dead HHV-8 did not (see **Fig. 15a-c in Chapter 3**). As expected, there were no significant differences in cytokine and chemokine production between live and UV-HHV-8 at 24 (left) or 48 h (**Fig 7**).

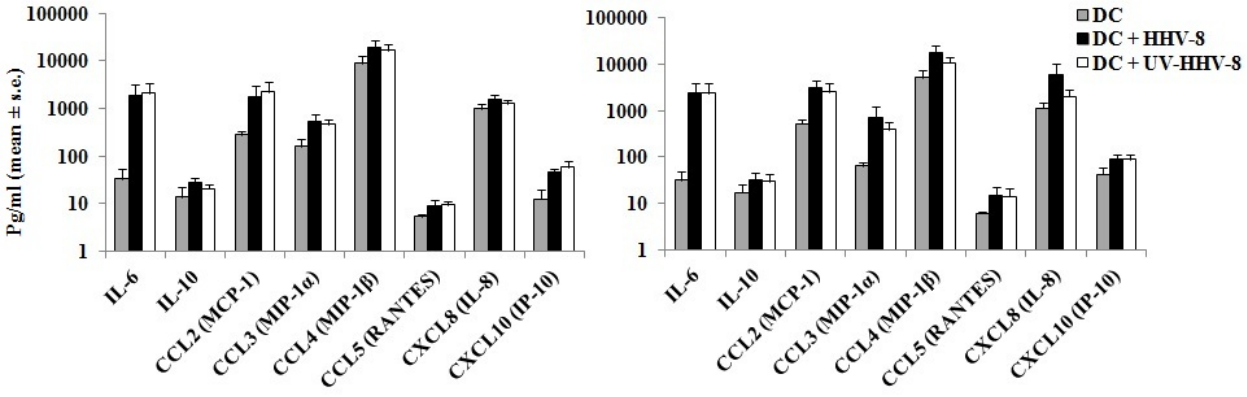

Figure 7: UV-inactivated HHV-8 induces immune mediator response

DC were exposed to live HHV-8 or UV-HHV-8. Supernatants were assayed by CBA at 24 (left) and 48 hpe (mean ± s.e.m., N =9).

As both live and dead HHV-8 elicited an immune mediator response in DC, we hypothesized that binding of HHV-8 to DC was sufficient to initiate cytokine and chemokine signaling cascades. To further elucidate the mechanism by which HHV-8 induces a cytokine and chemokine response in DC we used soluble HHV-8 gB. HHV-8 gB contains an RGD motif, a peptide region known to interact with cell surface integrins (271). At both 24 (left) and 48 hpe, there were several cytokines and chemokines that were enhanced by gB above unexposed cultures, indicating that early binding of HHV-8 to DC may initiate cytokine and chemokine responses (**Fig. 8**).

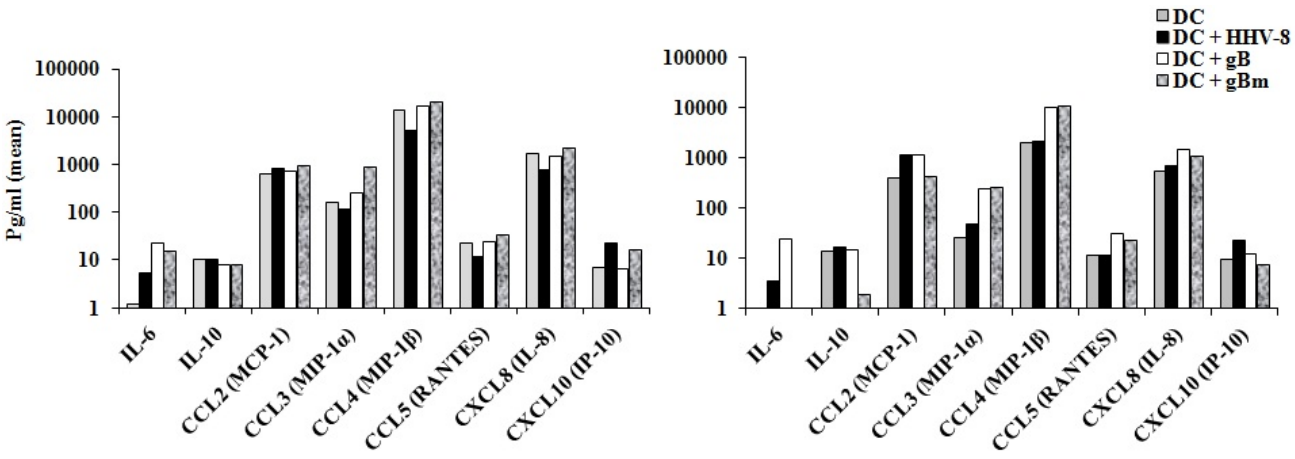

Figure 8: Soluble glycoprotein B induces immune mediator response

DC were exposed to HHV-8, soluble gB or soluble gBm and supernatants were assayed by CBA at 24 (left) and 48 h.p.e. (N =1)

Interestingly, when a soluble gB mutant (gBm) with an RGE motif that will not bind surface integrins was added, immune mediator induction was rarely diminished. Though gBm does not bind integrins, it maintains its ability to bind DC-SIGN (unpublished, Jenkins) and as DC-SIGN is prominently expressed on DC, this likely plays a key role in immune mediator induction.

2.4.4 HHV-8 down regulates IL-12p70 production via TLR-4 pathway

We have reported down regulation of IL-12 production by HHV-8 infected DC (123) assayed by ELISA, and in the present study confirmed this using CBA. We next tried to rescue IL-12 production using TLR agonists, as signaling through the TLR-4 pathway results in IL-12 production. As shown in **Figure 9**, DC treated with TLR-4 agonist LPS alone produced elevated

levels of IL-12p70 by 24 and 48 hpe. As expected, DC exposed to HHV-8 produced less than 20 pg/ml. Interestingly, production of IL-12 could not be restored when the DC were simultaneously exposed to HHV-8 and treated with the TLR-4 LPS, as levels remained less than 20 pg/ml. This inhibitory effect was not seen in HHV-8 exposed DC treated with the TLR-2 agonist LPS or the TLR3 agonist poly-IC, indicating that HHV-8 induced down-regulation of IL-12 production was specific to the TLR-4 pathway. Also, the production of IL-12 in the poly-IC treated HHV-8 exposed DC was higher than in poly-IC treated DC. DC exposed to UV-inactivated HHV-8 and then treated with TLR-4 agonist LPS also produced low levels of IL-12p70 production (data not shown), suggesting that full cycle HHV-8 replication is not required to modulate this immune function.

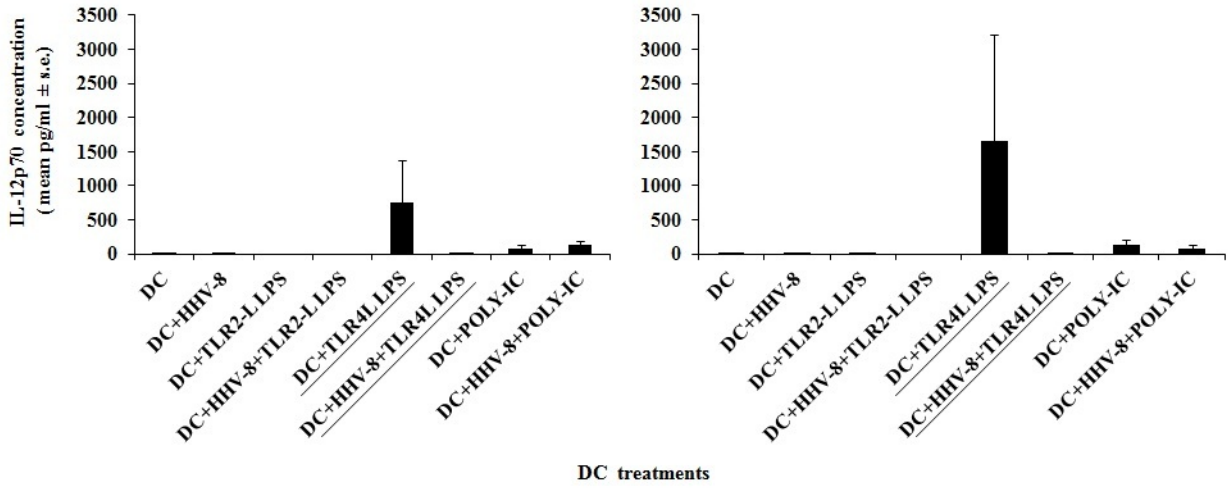

Figure 9: HHV-8 down-regulates IL-12p70 via the TLR-4 pathway

DC were infected with HHV-8 for 3 hours. Cells were washed and then treated with a TLR-4 ligand (TLR4L) LPS, TLR2 ligand (TLR2L) LPS or TLR3 ligand (TLR3L) poly-I:C. Supernatants were analyzed at 24 and 48 hpe (N=3, mean ± s.e.m.).

2.5 DISCUSSION

A delicate balance exists between protective immunity involving immune mediator production by host cells, and virus-driven induction of cytokines and chemokines that aid in the dissemination and spread of infection and mediate pathogenesis. Several of the mediators that are essential to the immune response and activation of lymphocytes can exacerbate infection and cause clinical symptoms when over produced in response to HHV-8 infection.

Here, we show a pro-inflammatory immune mediator profile in HHV-8 exposed DC, with significant increases of IL-10, MCP-1, MIP-1α, MIP-1β, RANTES and IP-10. The induction of these mediators occurred rapidly and was substantially elevated during the 3 hour exposure to HHV-8. The rapid induction of immune mediators implies early steps in HHV-8 infection may trigger an immune mediator response. This hypothesis was supported by two additional observations generated from gB and UV studies. Binding of gB to DC resulted in elevated levels of all 7 cytokines and chemokines above unexposed DC. It is difficult to directly compare the quantity of immune mediator production between the HHV-8 exposed DC and the gB exposed as we are unable to normalize the amount of soluble gB to the gB associated with the purified HHV-8 virions. Notably, the gB mutant that is unable to bind integrins could still induce a potent immune mediator response, indicating that there are other cell surface moieties involved in cytokine and chemokine signaling. Interestingly, triggering of C-type lectins, including DC-SIGN, in combination with TLR triggering on DC induces signaling and cytokine responses, which in turn regulate T cell polarization that is central to host immune control of infections (264). Furthermore, when UV-HHV-8 was used, production of the 7 immune mediators was nearly identical. This was expected as DC do not support lytic infection. These

data imply that binding of HHV-8 to DC surface receptors triggers an immune mediator response that does not require lytic replication.

The role of each cytokine/chemokine in HHV-8 infection and KS likely varies depending on their quantity and origin of production. In our study, with the exception of the MIP chemokines, immune mediator production increased over 2 days, with a peak on day 2. MCP-1 (3.8 fold increase) and IL-8 (3.6 fold increase) was greatly enhanced. These chemokines are also elevated in serum of KS patients and have been implicated in many cancers (179, 254). Additionally, when bound to its CCR2 receptor on endothelial cells, MCP-1 results in chemotaxis and has been shown to mediate angiogenesis in vitro (100, 179). IL-1β, TNF-α, IL-8 and IL-6 had 3.6, 2.2, 3.6, 32 fold increases, respectively and can enhance tumor cell growth and vascularization (84, 86, 93) by inducing the expression of two angiogenic mediators, i.e., VEGF and fibroblastic growth factor (FGF) (58, 62, 84, 187). IL-6 is also found at very high levels in both KS lesions and in circulation of patients with MCD (8).

In epidemic or AIDS-related KS, the immune dysregulation and induction of inflammatory cytokines acts to further enhance KS tumor growth. Indeed, serum and cell samples taken from KS lesions of HIV-1 infected individuals co-infected with HHV-8 show markedly increased levels of inflammatory cytokines, growth factors and angiogenic mediators (86, 209). Furthermore, treatment of KS patients with IFN-γ, IL-2 and TNF causes KS progression (187). The results of this study support an intentional skewing of cytokine production in HHV-8-infected MDDC towards induction of a pro-inflammatory response that could enhance development of KS.

We demonstrated a down-regulation of IL-12p70 production in DC, consistent with previous reports (123). IL-12 is a critical antiviral cytokine produced by DC to initiate a natural

killer and CD8+ cytotoxic T cell responses. A decrease in IL-12 production is detected in chronically infected HIV-1 positive individuals upon *in vitro* stimulation (51), resulting in a loss CD8 T cell cytotoxicity. Specifically, down-regulation of IL-12p70 is linked with HIV-1 Vpr (172). In addition, Herpesviruses, such as cytomegalovirus and HHV-6, down-regulate this cytokine as an immune evasion strategy.

HHV-8 down-regulates TLR-4 mediated signal transduction (150), a known pathway for induction of IL-12, in lymphatic endothelial cells. TLR-4 mRNA expression is decreased upon live and UV-killed HHV-8 infection with over 50% decrease in surface expression (150). The viral encoded G-protein coupled receptor (vGPCR) and interferon regulatory factor (vIRF1) decreases TLR-4 mRNA in a dose dependent manner (150). To determine if the down-regulation of IL-12 in our *in vitro* DC model was related to TLR-4 down-regulation, DC were exposed to HHV-8 and then treated with either a TLR-4 agonist LPS, a TLR-2 agonist LPS or the TLR-3 agonist poly-IC. Production of IL-12 could not be restored when DC were simultaneously exposed to HHV-8 and TLR-4 LPS. There was not a down-regulation observed for the other TLR agonists, indicating specificity for the TLR-4 pathway. Interestingly, addition of HHV-8 and poly-I:C increased IL-12 production nearly 2-fold, which supports findings by West et al., that TLR-3 expression is up-regulated during primary infection of monocytes (277). Additional studies to determine the proteins HHV-8 targets in these TLR pathways could provide valuable insight to the immune evasion strategies employed by this virus.

In conclusion, we determined that HHV-8 elicits a significant induction of pro-inflammatory cytokines and chemokines that could drive a Th2-skewed immune response resulting in a dampened CTL activation and HHV-8 persistence.

2.6 ACKNOWLEDGEMENTS

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3.0 HHV-8 INFECTION OF B LYMPHOCYTES

3.1 ABSTRACT

The role of human herpesvirus 8 infection of B lymphocytes in development of Kaposi's sarcoma is poorly defined. Here we found that cytokines and chemokines produced during HHV-8 infection of B cells were associated with development of KS. HHV-8 targeted activated, DC-SIGN expressing, IgM memory and naive B lymphocytes for complete, lytic replication. B cells infected with HHV-8 were predominately polyfunctional, producing combinations of 2-5 cytokines and chemokines. We observed corresponding elevated levels of these immune mediators in plasma and B cells of HIV-1-infected persons with KS compared to those without KS. Production of multiple cytokines and chemokines by HHV-8 lytic infection of IgM memory and naïve B cells could have a key role in viral dissemination and cell proliferation in KS.

3.2 INTRODUCTION

Infection of B lymphocytes with human herpesvirus 8 is significant to the development of Kaposi's sarcoma (8, 85, 86, 219) and the lymphoproliferative B cell cancers, primary effusion lymphoma (205) and multicentric Castleman's disease (8, 261). Cell proliferation, angiogenesis, and vascular permeability that are essential for development of these cancers could be driven at

least in part by cytokines and chemokines produced by B cells (85, 86, 209). Moreover, a recently described HHV-8-associated inflammatory cytokine syndrome without lymphoproliferation is defined by elevated levels of IL-6 and other cytokines (206).

The predominant association of HHV-8 with cells of its related cancers has been in the form of latent virus infection. Latent HHV-8 episomal DNA is present in a large percentage of cells within KS lesions (B cells, monocytes, and endothelial cells) (261) and B cells of HHV-8 lymphomas (38). Indeed, HHV-8 establishes latent infection *in vitro* in a subset of tonsillar IgMλ-expressing B cells with a plasmablast phenotype characteristic of MCD (119). Accumulating evidence, however, has incriminated lytic HHV-8 infection in driving HHV-8-associated cancers (103, 206). Thus, persistence of latent HHV-8 infection in KS cells is associated with ongoing lytic virus replication (113, 205, 245). Furthermore, many HHV-8 proteins encoded during lytic replication could have profound effects on inflammation and angiogenesis (102). HHV-8 viremia predicts subsequent development of KS (133), with persistence of HHV-8 requiring ongoing lytic replication and infection of new cells. Finally, therapy with monoclonal antibody rituximab that depletes $CD20^+$ B cells has profound antitumor effects in MCD (106), while antiviral drugs that block cytomegalovirus DNA synthesis decrease HHV-8 titers and prevent development of KS (103).

B cells from blood and tonsils of healthy adults that are pre-stimulated with surrogates of activated helper T cells, i.e., CD40L and IL-4, are permissive for HHV-8 lytic infection *in vitro* (215). However, evidence of lytic HHV-8 infection and induction of immune mediators in B cells and their relationship to HHV-8 cancers is lacking. In the present study we therefore examined the hypothesis that cytokine and chemokine production is enhanced upon lytic HHV-8 infection of B lymphocytes using a combined battery of new assays for viral DNA, protein, and

infectious virions. To link this to the immunopathogenesis of HHV-8 infection, we determined the state of B cell differentiation targeted during primary lytic HHV-8 infection *in vitro* by a newly developed, polyfunctional cytokine and chemokine profile. Here, we offer the first comprehensive evidence of HHV-8 lytic replication and cytokine and chemokine production in primary B cells. We show that HHV-8 predominately infects IgM^+ memory-like and naïve B cells that express DC-SIGN, and induces polyfunctional immune responses with combinations of IL-6, IL-8, TNF-α, MIP-1α and MIP-β. We found corresponding, elevated levels of HHV-8 DNA and all of these immune mediators except IL-6 in plasma of HIV-1-infected adults with KS compared to those without KS. $CD19^+$ B cells isolated from KS cases had elevated RNA levels of IL-6, MIP-1α, MIP-1β, IL-8 and VEGF compared to KS controls. Lastly, HHV-8-infected polyfunctional B cells were detected in the KS^+ cases. These data support that immune mediators induced by lytic HIV-1 infection of memory and naïve B cells are important in driving KS and HHV-8-associated B cell lymphomas.

3.3 METHODS

3.3.1 Preparation of B cells from blood

PBMC were isolated by Ficoll-Hypaque density gradient separation. $CD19^+$ B cells were collected by negative selection (B-Cell Isolation Kit II - Miltenyi Biotec) and cultured in Roswell Park Memorial Institute (RPMI)-1640 (GIBCO) medium supplemented with 10% heat-inactivated fetal calf serum (FCS) (GemCell). B cells were activated for 48 h at 37ºC, with 1 µg of soluble trimeric Mega CD40L/ml (Alexis) and 1,000 U of recombinant human IL-4/ml.

3.3.2 HHV-8 purification

HHV-8 was purified from body cavity based lymphoma (BCBL-1) cells latently infected with HHV-8 as previously described (41) with modification. Prior to sucrose cushion ultracentrifugation, supernatants were pooled and treated with 1U/100µl DNase (Sigma). Virus pellets were resuspended in 1 ml phosphate-buffered saline (PBS) (GIBCO) with 0.1% bovine serum albumin (BSA). DNA copies/ml (HHV-8 titer) was determined by PCR (described below).

3.3.3 HHV-8 infection of primary activated B cells

Activated B cells were suspended at $1x10^6$ cells/ml in fresh RPMI, with 10% FCS. B cells were left unexposed or exposed to 10^7 DNA copies of HHV-8 or UV-light inactivated HHV-8 DNA for 3 h at 37ºC in 5% CO_2. Cells were washed in fresh medium and centrifuged twice to remove any unadsorbed virus. Cells were resuspended in fresh RPMI with 10% FCS at $1x10^6$ cells/ml and cultured at 37ºC in 5% CO_2, for up to 48 h. For UV-inactivation, B cells were treated with equivalent doses of UV-HHV-8 as live HHV-8. In some experiments, B cells were treated with 1µg/ml soluble gB (271) for 3 h and then washed and recultured as described above.

3.3.4 HHV-8 K8.1 qRT-PCR in activated B cells

B cells were collected at various times post exposure and pelleted. 500,000 cells and 500ul of culture supernatant were assayed by PCR as previously described (210, 244). Samples were treated with 1µl DNase in 10 µl DNase buffer (Sigma) for 15 minutes then lysed in easyMAG

buffer (NucliSENS) and DNA was extracted on an easyMAG automated extractor (bioMérieux). Phocine herpesvirus (PhHV) was used as an internal control for DNA extraction (201). DNA was mixed with a primer set specific for HHV-8 K8.1(210) and the real-time PCR reaction was carried out on 7000 or 7500 ABI (Applied Biosystems). For KS^{-} and KS^{+} MACS samples, DNA was extracted from plasma and the reaction was carried out on ViiA7 (Applied Biosystems).

3.3.5 Microarray for B cell gene activation

B Cells were left unexposed or exposed to HHV-8 for 3 h, washed and recultured. 1 million cells per treatment were collected after the wash (3 h) and at 4, 6, 9, 15 and 27 hpe. Genomic DNA was digested by RNase free DNase and RNA was extracted using RNeasy Mini Kit (QIAGEN) and total concentration (ng/µl) were determined by NanoDrop 1000 Spectrophotometer (Thermo Scientific). 1µg RNA of each sample was given to the University of Pittsburgh Genomics and Proteomics Core Laboratory for RNA labeling and direct hybridization to Illumina HT12v4 microchips. Samples were run in duplicates to determine yield RNA expressions. Fold increases in HHV-8 exposed over unexposed B cells were calculated.

3.3.6 Supernatant collection and cytokine and chemokine detection

Supernatant samples were collected from unexposed and HHV-8-exposed B cells at various times post exposure. Supernatant samples were screened for IL-1β, -2, 4, -6, -7, -8, -10, IL-12p70, IFN-γ, TNF-α, IP-10, MIP-1α, MIP-1β, MCP-1, RANTES and VEGF by CBA (BD) as per manufacturer's instructions. Samples were read on an LSR-II flow cytometer (BD Immunocytometry Systems) and analyzed with FCAP Array Software (BD).

3.3.7 Flow cytometry and intracellular staining

$1x10^6$ cells were resuspended in 100 µl PBS per well in a 96 well V-bottom plate. Staining for various immune mediators was performed as previously described (18, 152) with modifications. For intracellular cytokine expression, cells were treated with Brefeldin A (5 µg/ml; Sigma) for 4 h at 37°C and then 20µl ethylenediaminetetraacetic acid (EDTA) (2mM) (GIBCO) for 10 m at room temperature. Cells were washed and resuspended in PBS containing 50 µl/ well of aqua viability dye (1ul dye in 500ul PBS) (Invitrogen) for 30 m in the dark, washed and resuspended in 5 µl of CD20-PE-Cy7 (BD) and 20µl of CD209-PerCP-CY5.5 (BD) for 30 m in the dark. After washing, cells were resuspended in 4% paraformaldehyde (PFA) for 10 m, washed and resuspended in 1x fluorescence-activated cell sorting (FACS) lysis solution (BD) for 10 m. Cells were washed and permeabilized for 10 m using 1X Perm solution (BD). Cells were washed 3 times and blocked with Super Blocking Buffer (Pierce) for 30 m. Cells were stained with 5µl/well of MIP-1β-allophycocyanin-H7, IL-6-V450, TNF-α-allophycocyanin and MIP-1α-PE and 20µl of IL-8-fluorescein isothiocyanate (FITC) (BD) for 30 m in the dark, then fixed in 1% PFA and analyzed with an LSR II flow cytometer.

To determine HHV-8 lytic protein expression, cells were additionally stained with anti-K8.1 or anti-ORF59 PF-8 mAb conjugated to Alexa fluor (AF)-680 using the Zenon conjugation kit as per manufacturer's specifications (Invitrogen). Purified mouse IgG_1 or IgG_{2B} (Sigma) were also conjugated with AF680 and used as controls. Isotype controls were used to gate for ORF59 PF-8 positive cells (consistently gated at approximately 1% background positivity).

For B cell phenotypic staining, cells were stained with CD20-AF405, DC-SIGN-AF488, IgD-PE, IgM- allophycocyanin, CD27- allophycocyanin-H7 CD23-PE, CD138- PerCPCy5.5 and/or CD38-PerCPCy5.5 (BD) for 30 m prior to fixing in 4% PFA.

3.3.8 Tissue culture infectious dose 50% ($TCID_{50}$) assay

A $TCID_{50}$ assay was developed using T1H6-DC-SIGN cells (Nadgir, submitted for publication, **see Appendix D**). 293T cells transfected with a pβgalBasic plasmid containing an RTA responsive element under a polyadenylated RNA promoter (130) were subcloned to express DC-SIGN and selected by hygromycin resistance. T1H6-DC-SIGN cells were harvested and resuspended in DMEM (Lonza) and 100μg/ml hygromycin (Clontech) at $4x10^4$ cells/100 μl and plated in a 96 flat bottom well plate. Supernatants at 3, 24, and 48 h post-B cell exposure were collected and diluted 1:10 in PBS for up to 5 10-fold dilutions. 30 μl of supernatant was added per well in sixlets. At 48 h cells were collected and centrifuged at 13,000 rpm for 1 m. Cells were washed with PBS 3 times and resuspended in lysis buffer (K_2HPO_4 +KH_2PO_4 + DTT) followed by 3 rapid freeze-thaw cycles. Cells were spun at 13,000 rpm for 10 m at 4°C. Supernatants were collected and 20 μl were plated with 196 μl β-galactosidase (β-gal) detection kit II buffer (Clontech) and 4 μl substrate per well. After 1 h, β-gal luminescence was determined using a Fluostar OPTIMA luminometer with a sensitivity of 200 at 5 second light intervals. Mean luminescence was determined by subtracting the mean of the control well (no virus added) + 2 standard deviations.

3.3.9 SPICE analysis of polyfunctional B cells

Flow cytometry data were analyzed using the FloJo (Tree Star) Software. Polyfunctional B cell responses to HHV-8 infection was assessed using the simplified presentation of incredibly complex evaluations (SPICE) program (Version 4.3, M. Roederer, Vaccine Research Center, National Institute of Allergy and Infectious Diseases [NIAID], NIH).

3.3.10 Study participants and samples

The *in vitro* studies were done using anonymous, adult blood donors who were determined to be HHV-8 antibody negative by an immunofluorescence microscopy assay (133). For *in vivo* studies, participants were chosen from the Pittsburgh site of the MACS, a longitudinal study of the natural history of HIV-1 infection in men who have sex with men that began in 1984 (140). Thirty MACS participants were chosen based on HIV-1 and HHV-8 status, as well as the presence or absence of Kaposi's sarcoma. An indirect immunofluorescence assay was used to detect HHV-8 serum antibodies (273). HHV-8 viral load was determined by PCR as described above. HIV-1 viral load in the plasma was determined using Roche Ultrasensitive RNA PCR assay (Hoffman-LaRoche). T cell levels were determined using flow cytometry (234). The 30 participants were classified into two HIV-1-positive/HHV-8-positive and KS-negative or KS-positive groups, with fifteen participants in each group. Serum samples were chosen within a year of KS development and the corresponding time point for the controls that did not develop KS. Two additional time points were chosen 2-5 and 6-10 years prior to KS development. All participants were Caucasian men, with an average age of 32.1 (range of 23 to 46) at the first visit.

3.3.11 Serum biomarker assay

Serum samples were tested for the presence of several biomarkers using an electrochemiluminescence Meso Scale Discovery (MSD) multi-array assay. Ultra-sensitive kits for human IL-6, IL-8, TNF-α, MIP-1β and MIP-1α and a human serum kit for VEGF (MSD) were used according to the manufacturer's protocol for serum samples. Both samples and

standards were performed in duplicate. Plates were read on a SECTOR Imager 2400 electrochemiluminescence machine (MSD) and data were analyzed using the Discovery Workbench (version 3, MSD).

3.3.12 cDNA synthesis and real-time RT-PCR

A two-step RT-PCR assay was used to measure the levels of expression of immunomodulatory host mRNAs as previously described (233). Gene expression was normalized to the endogenous control mRNA, β-glucuronidase, and the values presented were calculated as $2^{-\Delta Ct}$

3.3.13 Statistical Analysis

We used the Student *t* test; two-sample assuming equal variance and paired for comparisons between groups.

3.4 RESULTS

3.4.1 B cells support HHV-8 lytic replication

B cells infected with HHV-8 could be a major source of cytokines and chemokines. To examine this in depth, we developed new, multiparameter assays to measure HHV-8 infection of B cells. We first established a flow cytometry assay for measuring production of HHV-8 lytic proteins K8.1 and ORF59 PF-8 using DC-SIGN transfected RAJI and K562 cell lines. We have previously shown that DC-SIGN is predominately found on a subset of B cells (ranging from 8-

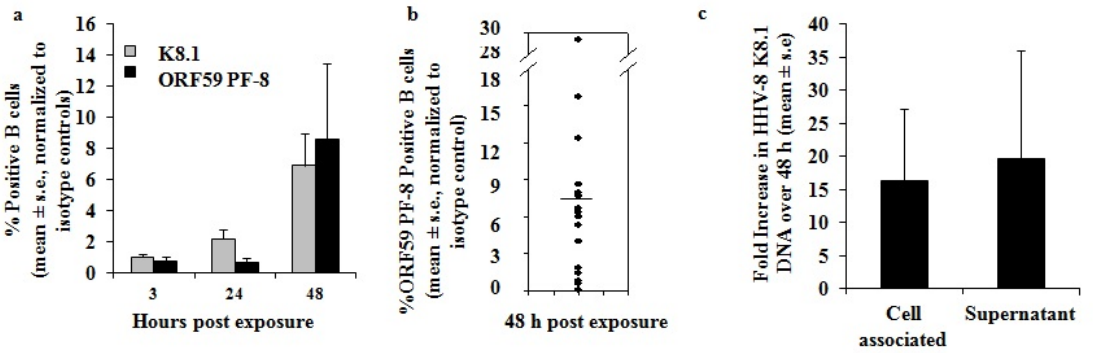
18%) after activation. Moreover, we showed that DC-SIGN serves as an entry receptor for HHV-8 on B cells using fluorescent microscopy to detect DC-SIGN^{+} cells expressing HHV-8 proteins (215). To our knowledge, there is no published method for detection and quantization of HHV-8 lytic protein expression by flow cytometry in primary cells. We used K562 cells stably transfected with DC-SIGN (K562$^{DC\text{-}SIGN}$ cells) and exposed these to varying concentrations of purified HHV-8. At 48 hpe K562$^{DC\text{-}SIGN}$ cells were collected and stained for viability, as well as permeabilized and stained intracellularly for HHV-8 using mAb specific for ORF59 PF-8. With the undiluted pool of virus (10^7 DNA copies), 11% of K562$^{DC\text{-}SIGN}$ cells were positive for ORF59 PF-8 (**Fig. 23a in Appendix B)**. As the number of HHV-8 DNA copies used to infect K562$^{DC\text{-}SIGN}$ cells decreased, so did the detection of HHV-8 ORF59 PF-8, with undetectable levels at 10^2 DNA copies.

Next, we used a different cell line, RAJI$^{DC\text{-}SIGN}$ cells, to further explore the sensitivity of the flow cytometry assay. RAJI$^{DC\text{-}SIGN}$ cells were mixed with non-DC-SIGN transfected RAJI cells at 5:1, 1:1 and 1:5 ratios. DC-SIGN surface expression of each ratio was measured by flow cytometry (**Fig. 23b in Appendix B**). Cells were exposed to HHV-8 and stained for K8.1 at 48 hpe (**Table 2 in Appendix B**). As the surface expression of DC-SIGN was diluted using non-transfected RAJI cells, the percent of HHV-8 K8.1^{+} cells diminished. Taken together, these data indicate that our flow cytometry method is a suitable assay to quantify HHV-8 infected cells, using both ORF 59 PF-8 and K8.1 as markers for lytic infection.

We next applied the intracellular staining (ICS) and flow cytometry assay to blood-derived B cells. Initial studies showed that peak levels of lytic virus proteins of 6.9(±2.06)% (mean±se) for K8.1 and 8.6(±4.8)% for ORF59 PF-8 were detected at 48 h (**Fig. 10a**). Based on this, we continued our experiments focusing on ORF59 PF-8 expression, detecting 7(±1.6)%

ORF59 PF-8^{+} B cells at 48 h (n = 18) (**Fig. 10b**). Notably, these flow cytometry studies were done by gating on viable B cells, with a viability of 79%±5.3 by 48 h in the HHV-8 exposed B cells. However, as herpesvirus lytic replication leads to cell lysis, we also examined the dead singlet lymphocyte population. Similar to viable B cells, 7.2%±1.5 of HHV-8-exposed, dead B cells were positive for ORF59 PF-8 (data not shown), indicating that full lytic cycle replication had occurred in a subset of HHV-8 infected cells.

Production of lytic cycle proteins proves that HHV-8 can infect B cells, but is not definitive evidence that the cells produce infectious virus. We therefore next assessed HHV-8 DNA production by quantitative PCR as a parameter of HHV-8 lytic virus replication. We found a broad range of cell-associated DNA levels in the infected B cell cultures, with a mean fold increase of 16.2 in cell associated DNA copies and 19.7 of non cell-associated DNA (in the supernatant), of 9 donors (**Fig. 10c**). To verify that HHV-8 DNA detected in the B cell cultures corresponded to infectious virions, we developed a $TCID_{50}$ assay using T1H6-DC-SIGN cells. Briefly, T1-H6 cells contain a T1.1 polyadenylated nuclear RNA promoter (PAN) controlling β-gal (130). Therefore, if a productive infection occurs, the number of β-gal positive cells can be enumerated and used to calculate a $TCID_{50}$ (Nadgir, submitted for publication). B cell supernatants were tested at 5 dilutions in 6 different wells to calculate the $TCID_{50}$ (**Fig. 10d**). Production of infectious virus was evident in B cell cultures from 100% (8/8) donors, with peak virus titers ranging from $1.52x10^{4}$ to $3.33x10^{6}$ $TCID_{50}$ by 48 h (**Fig. 10e**). Collectively, these data indicate that B cells from healthy, HHV-8 seronegative adults can be lytically infected with HHV-8 and produce infectious virus.



d

Dilution Factor	3 Hours Post Exposure						24 Hours Post Exposure						48 Hours Post Exposure					
-1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
-2	+	+	+				+	+	+	+	+	+	+	+	+	+	+	+
-3	+	+	+				+	+	+	+	+	+	+	+	+			
-4							+	+	+				+	+	+	+		
-5							+						+	+	+			
	$TCID_{50}$ 3.33E4/ml						$TCID_{50}$ 3.33E5/ml						$TCID_{50}$ 3.33E6/ml					

e

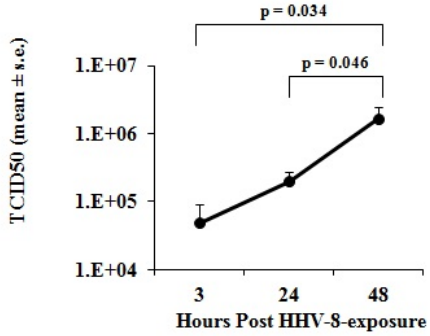


Figure 10: HHV-8 lytic proteins K8.1 and ORF59 PF-8 detected by flow cytometry

B cells were infected with HHV-8 for 3 h then washed repeatedly. Cells and supernatants were collected at 3, 24 and 48 hpe *(A-E). (A)* Cells were stained intracellularly for HHV-8 proteins K8.1 and ORF59 PF-8 and percentages of HHV-8 K8.1 or ORF59 PF-8 (mean±s.e.m., N=6) positive B cells were determined. *(B)* Percentages of HHV-8 ORF59 PF-8 (mean±s.e.m., N=18) positive B cells at 2 days post infection. *(C)* Cell pellets and supernatants were treated with DNase and lysed to determine HHV-8 K8.1 DNA copies by qRT-PCR. The mean fold increase in HHV-8 DNA are shown. (N=9). *(D)* T1H6-DC-SIGN$^+$ cells were infected for 48 h with supernatants collected at 3, 24 and 48 h from HHV-8 exposed B cells. Each supernatant was assayed in 10- fold dilutions in 6 wells. T1-H6-DC-SIGN$^+$ cells were collected, lysed and treated with β-galactosidase substrate for 1 h and read on a luminometer. The number of infected wells was determined by subtracting luminescence of control wells. Representative data are shown for the number of infected wells for each dilution, with $TCID_{50}$ calculated for each time point. *(E)* $TCID_{50}$ (mean±s.e.m.) was determined (N=8) (P values as shown; one and two tailed, paired two sample for means t-test).

3.4.2 HHV-8 lytic infection rapidly induces a cytokine-chemokine response

To evaluate immune mediators induced by HHV-8 infection of B cells, we quantified protein levels using a fluorescent bead-based immunoassay and determined mRNA expression using a genome-wide microarray. We initially screened levels of 16 cytokines, chemokines and growth factors, i.e., IFN-γ, IL-1β, 2, -4, -6, -7, -10, -12, TNF-α, MCP-1, MIP-1α, MIP-1β, RANTES, IL-8, IP-10 and VEGF. These immune mediators were selected based on their known association with KS and their availability in a newly developed BD assay. Of the 16 markers, 6 showed ≥ 4-fold increase at 24 (left) and 48 hpe (**Fig. 24a in Appendix B**), with significant production of IL-6, IL-10 (24 hpe only), TNF-α, MIP-1α, MIP-1β and IL-8 above unexposed B cells (**Fig. 24b in Appendix B**). In addition, mRNA analysis by microarray revealed ≥1.5 fold increase in 8 of these immune mediators (**Fig. 11 and continued in Appendix C).** We continued our study examining the cytokines and chemokines that had a ≥ 2-fold increase in RNA expression or ≥ 4-fold increase in secreted protein levels, i.e., IL-6, TNF-α, MIP-1α, MIP-1β and IL-8.

Microarray data showed that TNF-α, IL-6 and MIP-1α had a ≥2 fold increase in mRNA by 4-6 h post-HHV-8 exposure compared to unexposed B cells, with peak fold increases of 2.06, 2.13 and 10.8, respectively (**Fig. 12a**) The CCL-3-like (CCL3L) and CCL-4-like (CCL4L) genes also had enhanced expression after infection with HHV-8 (7.6, 16, 10.4 and 7-fold for CCL3L1, CCL3L3, CCL4L1, CCL4L2, respectively). The increases in mRNA levels corresponded with increases in protein levels detected in the supernatant of HHV-8 exposed cultures. During virus adsorption, HHV-8-exposed B cells produced significant levels of TNF-α, IL-6 and the 2 MIP chemokines ($P<0.05$) (**Fig. 12b**), implying a rapid induction upon virus

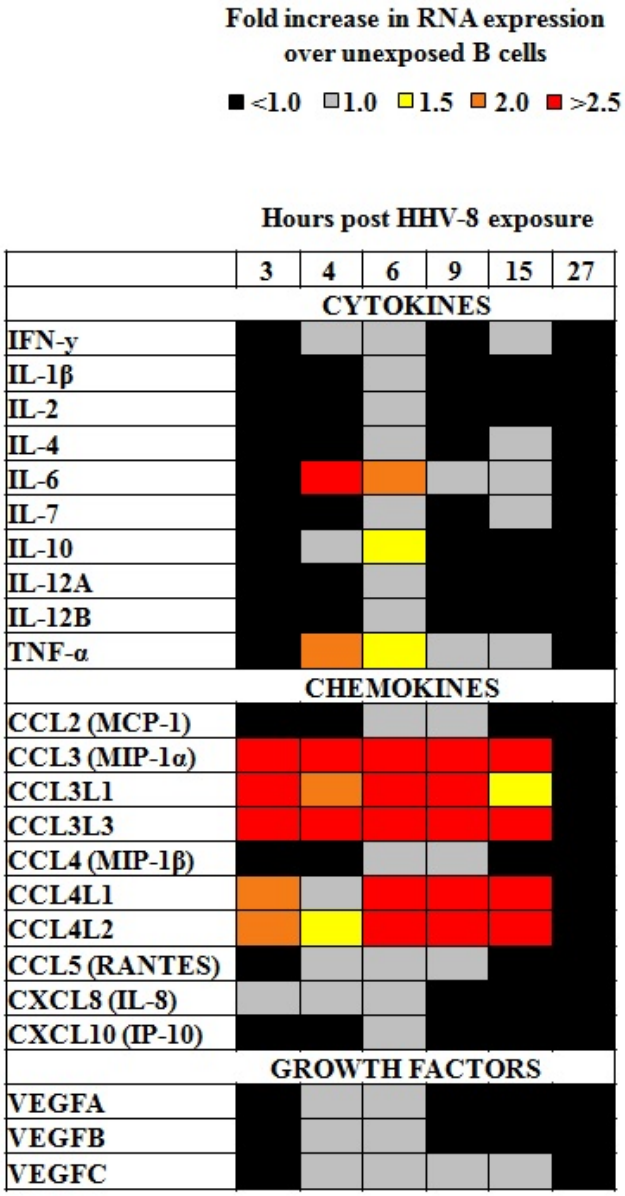


Figure 11: B cell cytokine and chemokine screen

B cells left untreated or exposed to HHV-8 were collected after 3 h of HHV-8 exposure and 4, 6, 9, 15 and 27 h post exposure. RNA was extracted and hybridized to Illumina HT12v4 microchips. Samples were examined in duplicate. Fold increases in RNA expression in HHV-8 exposed over unexposed were calculated (<1.0, 1.0, 1.5, 2.0, >2.5). Genes examined in parallel CBA studies are depicted in the heat map.

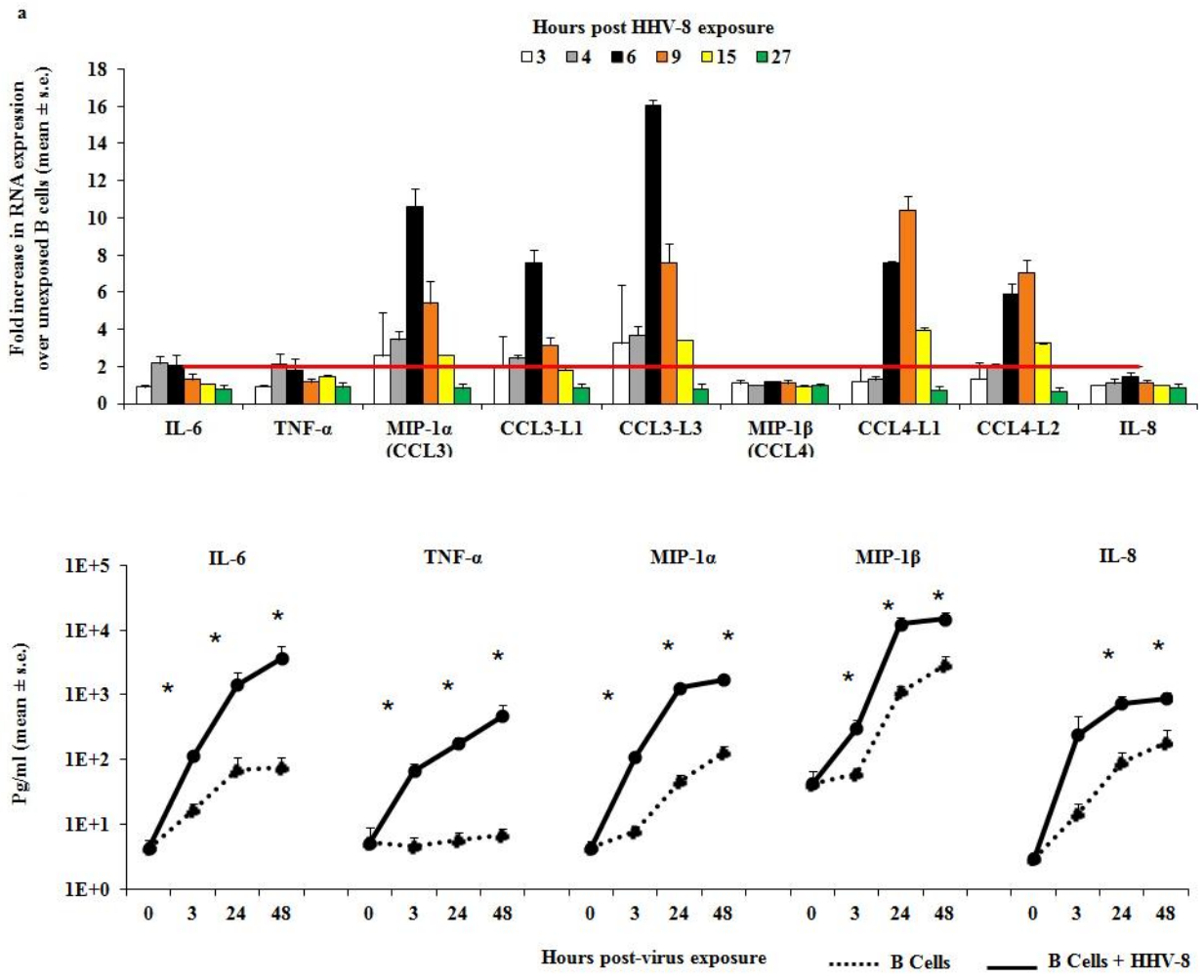


Figure 12: Kinetics of immune mediator production in HHV-8 infected B Cells

(A) B cells left untreated or exposed to HHV-8 were collected after 3 h of HHV-8 exposure at 4, 6, 9, 15 and 27 hpe. RNA was extracted and hybridized to Illumina H12Tv4 microchips. Samples were examined in duplicate. Fold increases in RNA expression in HHV-8 exposed over unexposed were calculated (mean±s.e.m.) and shown for cytokines/chemokine selected from screening. *(B)* Supernatants were collected prior to and after the 3 h adsorption phase and at 24 and 48 hpe and used in an immunobead fluorescence assay to determine cytokine-chemokine production of unexposed and HHV-8 exposed B cells (mean ±s.e.m., N=23, **P*<0.05).

exposure. Results at 24 and 48 hpe revealed significant increases of all 5 mediators above unexposed B cell cultures (*P*<0.05). To confirm the induction of immune mediators was specific

to HHV-8 rather than non-viral contaminating particles, we conducted an HHV-8 dose response (**see Fig. 25 in Appendix B**) and used a 0.1μm filter (**see Fig 26 in Appendix B**) to determine cytokine and chemokine levels. As detected with DC, 10^7 HHV-8 DNA copies induced the greatest immune mediator response that was not detected when the virus filtrate was used.

Taken together, these results indicate that HHV-8 generates a rapid, selective cytokine and chemokine response in HHV-8-exposed B cells that is sustained for 48 h post-infection, parallel to the lytic cycle replication of HHV-8 in B cells.

3.4.3 HHV-8 lytically infected B cells are polyfunctional

We next examined the induction of cytokine and chemokines in relation to the average 6-to-9% of B cells that support HHV-8 lytic infection at the single B cell level. CD40L/IL-4 activated B cells were left unexposed or exposed to HHV-8 for 48 h, then stained extracellularly for CD20 and intracellularly for IL-6, IL-8, TNF-α, MIP-1α and MIP-1β and the two lytic markers, HHV-8 K8.1 or ORF59 PF-8 (representative gating strategy, **Fig. 13**). Data were analyzed to determine the percentage of single cells that were monofunctional (cells that only produce one of the 5 mediators) and polyfunctional (cells that produce combinations of 2-to-5 mediators).

Comparing the entire B cell population by this method showed similar percentages of cells producing one or more immune mediators in the unexposed versus the HHV-8-exposed cultures (**Fig. 14a**). We next focused on the immune mediator production within the HHV-8 exposed virus positive (ORF59 PF-8^+ or K8.1^+) and exposed virus negative (ORF59 PF-8^- or K8.1^-) populations. HHV-8-exposed B cells that were negative for ORF59 PF-8 or K8.1 shared an immune mediator functional pattern similar to the unexposed B cells, whereas B cells positive for ORF59 PF-8 or K8.1 were far more polyfunctional. Indeed, >99% of the exposed, HHV-8

positive B cells were producing at least one cytokine or chemokine, compared to 80% in the exposed HHV-8 negative B cells. A larger portion of the exposed virus negative B cells were monofunctional (28% ± 3) compared to the exposed virus positive B cells (5.7% ± 0.5).

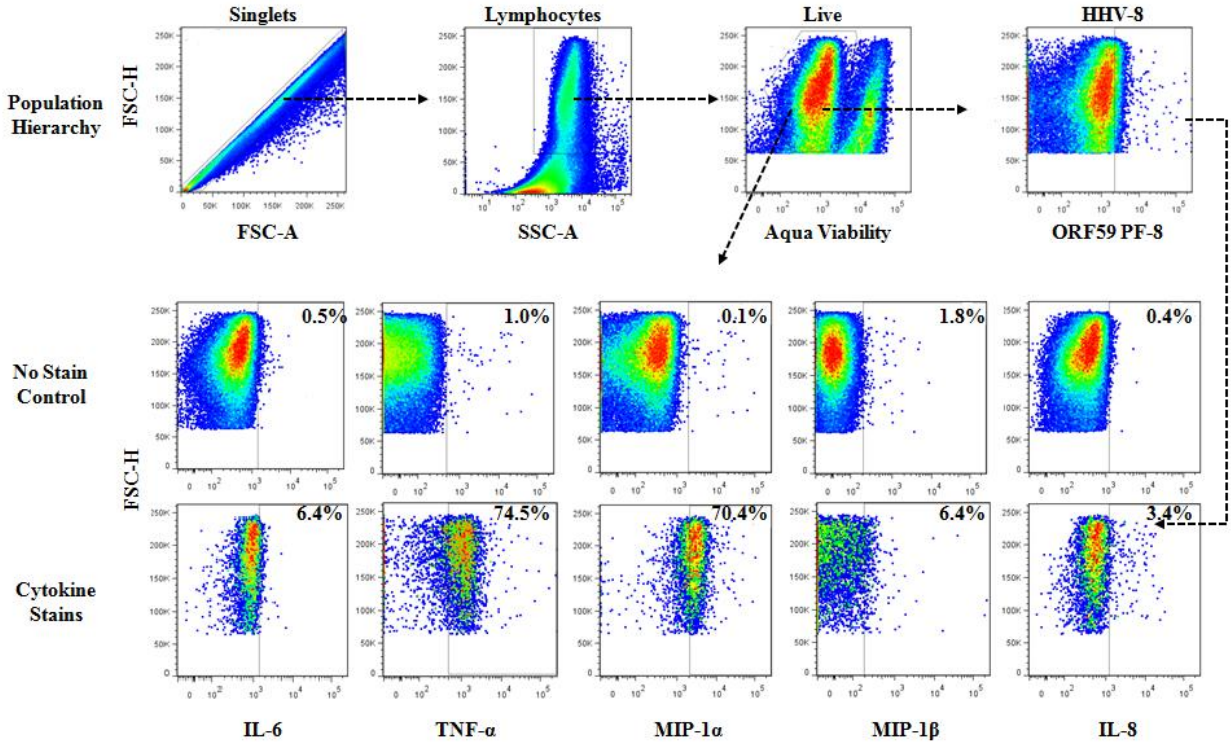


Figure 13: Representative cytokine gating strategy

Singlet populations were determined by forward scatter height (FSC-H) and area (FSC-A), then gated against FSC-H and side scatter-area (SSC-A) to select the lymphocyte population. Singlet lymphocytes were gated against an aqua viability dye (AF-430) to determine live cells. Live singlet lymphocytes with no stains were used to set the gate for cytokine and chemokines (middle panel). Live singlet lymphocytes were also gated against AF680 for ORF59 PF-8 or K8.1 expression. An IgG control conjugated with AF-680 (not shown) was used to set the gate for ORF59 PF-8 or K8.1 positive cells. The HHV-8 (K8.1 or ORF59 PF-8) positive cells were gated against each cytokine (bottom panel) and compared to exposed-HHV-8 negative cells (data not shown) for SPICE analysis. Each cytokine was gated against FSC-H (representative data).

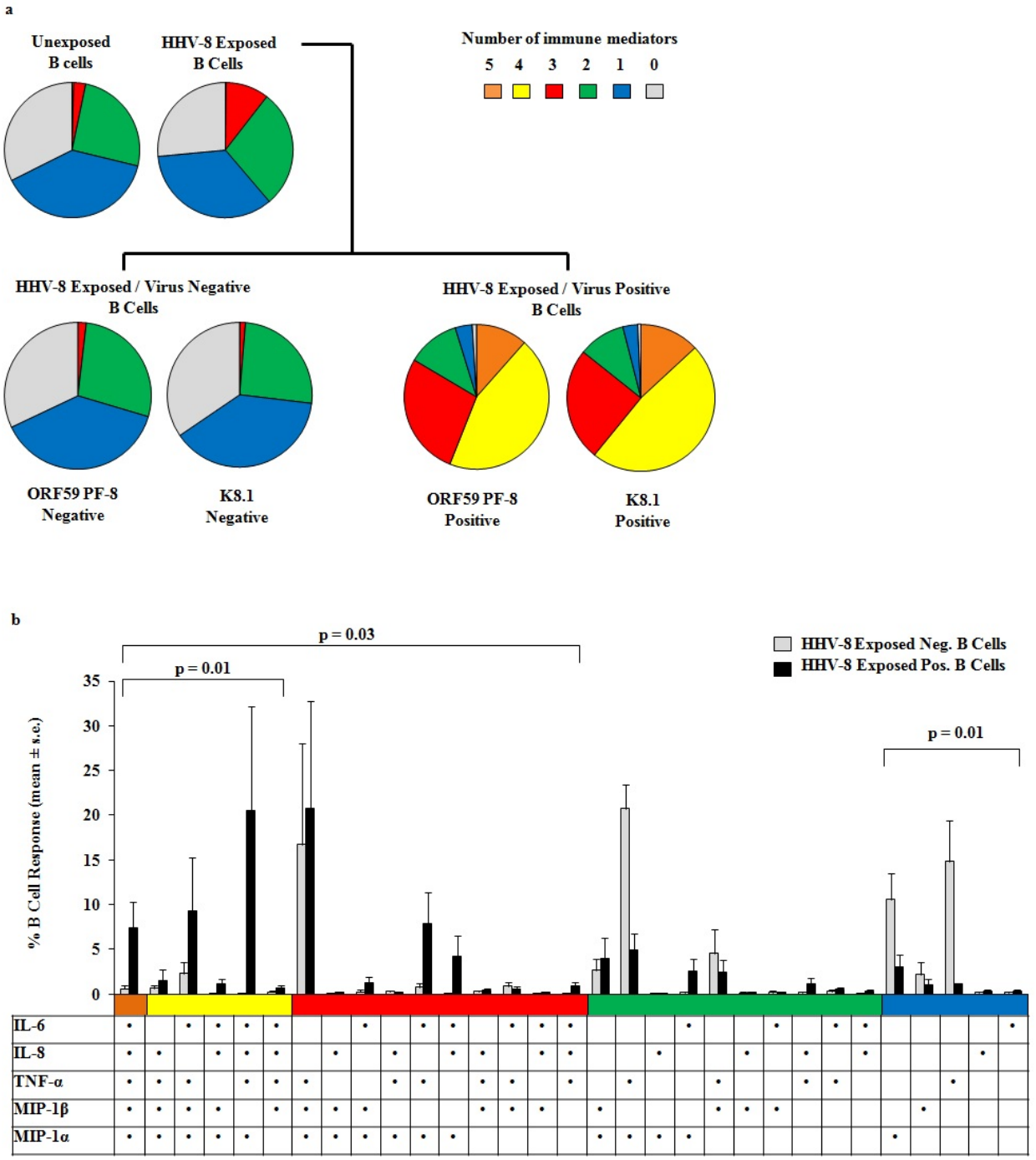


Figure 14: HHV-8 infected B cells are polyfunctional

B cells were left unexposed or exposed to HHV-8 for 3 hours and then cultured for 48 h. B cells were intracellularly stained for HHV-8 K8.1 or ORF59 PF-8 and the 5 immune mediators. Responses were measured by polychromatic flow cytometry for unexposed and HHV-8 exposed B cells. HHV-8 exposed cells were further separated into HHV-8 ORF59 PF-8 or K8.1 positive and negative cells and the percentage of each cytokine producing cell was determined using Flow Jo software. Data are representative of N=4. *(B)* Percent responses diagrams were generated using SPICE (mean±s.e.m., N=4, **P*<0.05 for comparison between exposed pos. and exposed neg. within brackets).

Importantly, 93%±1.1 of the HHV-8-exposed, virus positive B cells were polyfunctional, compared to only 52%±1.0 of exposed virus negative B cells. Moreover, 7.4 %±2.9 of HHV-8-exposed, virus positive B cells produced all 5 mediators compared to the <1% of HHV-8-exposed, virus negative cells. These polyfunctional patterns were similar between $K8.1^+$ and ORF59 PF-8^+ B cells, further validating the specificity of this assay for lytic protein detection.

To further assess B cell production of immune mediators in relation to HHV-8 infection, multiparameter analysis was performed. HHV-8-exposed, virus positive responses were heavily weighted to combinations of 3-to-5 immune mediators, whereas most responses for the HHV-8-exposed, virus negative B cells had 1-to-2 immune mediators (**Fig. 14b**). A significant percentage of exposed-positive cells produced 5 (P=0.03) or 4 (P=0.02) mediators (or a combination, P=0.01) compared to exposed-negative cells. Conversely, exposed-negative cells were significantly more monofunctional (P=0.01) or lacked cytokine production (P=0.02). Several combinations of immune mediator production were detected within the HHV-8-exposed, virus positive group, including the combined 5 immune mediators that accounted for ≥5% of the B cell responses. Nearly 21% of cells produced a foursome combination of IL-6, TNF-α, MIP-1α and MIP-1β, while nearly 10% of cells produced a triad of IL-6 and the 2 MIP chemokines. In the HHV-8-exposed, virus negative group, the highest responses were detected as production of single immune mediators, including 21% for IL-6 and 15% for MIP-1α alone. This is the first evidence of polyfunctional B cells within or without the context of virus pathogen-exposure. These intracellular data extend evidence derived from our quantitation of extracellular cytokines and chemokines by revealing that a significant subpopulation of HHV-8-exposed B cells is highly polyfunctional in direct relation to lytic virus infection.

3.4.4 Immune mediator induction in B cells exposed to inactivated HHV-8

We next determined the mechanism of induction of immune mediators by HHV-8-exposed, uninfected B cells. We reasoned that binding of virus alone, or paracrine effects of the virus-infected B cells, could initiate signaling cascades in uninfected B cells resulting in cytokine and chemokine production. B cells were therefore exposed to replication competent HHV-8 or UV-HHV-8, (replication incompetent). We observed a mean increase of $6.4x10^6$ copies/ml of B cell-associated DNA for HHV-8 and no increase for UV-HHV-8 over 48 h (**Fig. 15a**). $TCID_{50}$ results revealed a 4 log_{10} increase over 48 h for HHV-8 (**Fig. 15b**), and very low, background levels ($<5x10^2$) for the UV-HHV-8 that did not increase over time. Intracellular staining data showed 8.6% ORF59 PF8-positive cells for HHV-8 and 0.6% positive cells for the UV-HHV-8 at 48 hpe (**Fig. 15c**). These data support that UV-HHV-8 did not replicate in B cells.

We next determined bulk production of cytokines and chemokines in response to HHV-8 and UV-HHV-8. We found decreases of 35%, 34%, 35%, 46% and 21% in immune mediator production in supernatants of UV-HHV-8-exposed B cell cultures compared to HHV-8-infected cultures for IL-6, TNF-α, MIP-α, MIP-1β and IL-8, respectively (**Fig. 15d**). These data support that HHV-8 replication accounts for a portion of the production of cytokines and chemokines in B cells, while B cell activation with CD40L/IL-4 and non-replicating virus particles are associated with the residual production of these immune mediators.

We postulated that the mechanism by which inactivated, replication incompetent HHV-8 induced immune mediator responses in B cells was related to virus binding to the cells. To examine this, we studied recombinant HHV-8 gB, containing an RGD motif. By 48 h post-treatment, soluble gB induced levels of cytokines and chemokines above that in untreated B cells and similar to cells exposed to HHV-8 (**Fig. 15e**).

These data imply that the initial binding step between HHV-8 and cell surface receptors is sufficient to induce an immune mediator response, and supports our results with UV-inactivated virus. They also indicate that binding of HHV-8 (active or inactivated) via gB to B cells initiates an immune mediator response that is enhanced upon HHV-8 entry and replication.

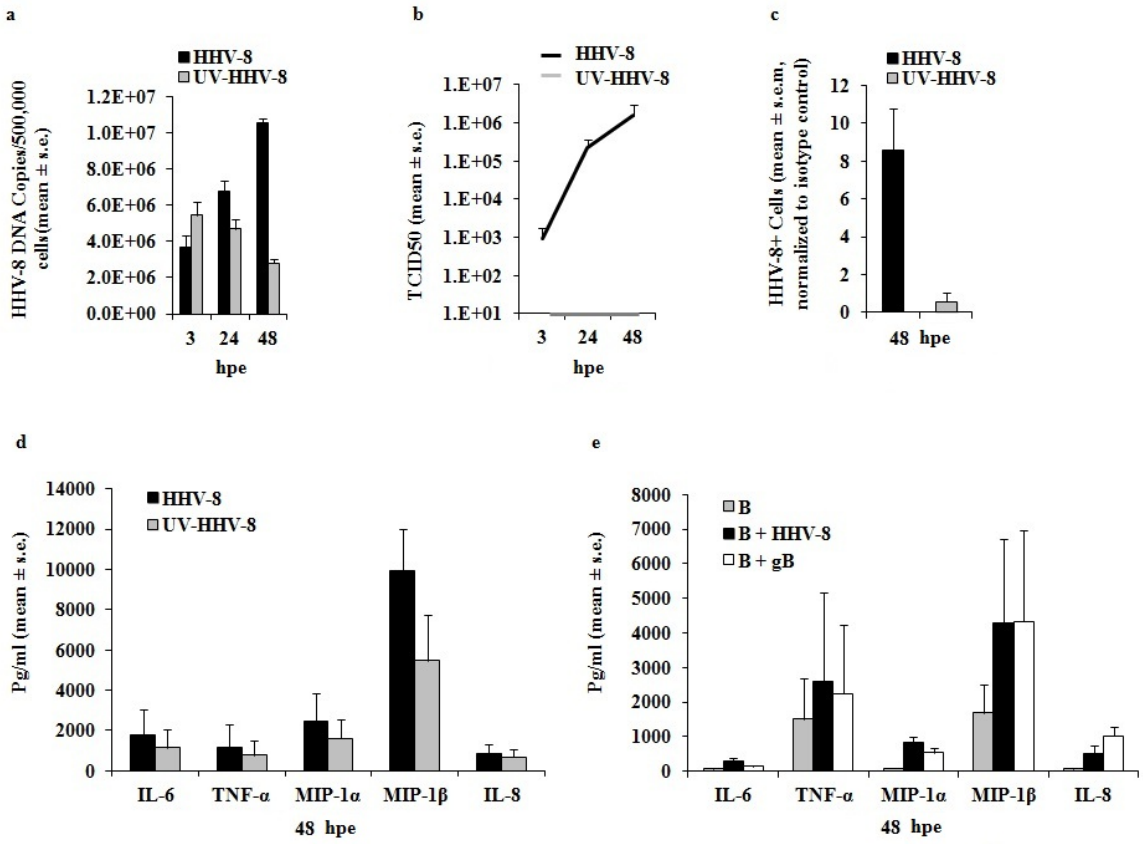


Figure 15: UV-light inactivation results in reduced immune mediator production

B cells were exposed to HHV-8 or UV-HHV-8 and cells and supernatants were collected at various time points post-exposure. *(A)* HHV-8 K8.1 DNA Copies were determined in cell pellets for HHV-8 and UV-HHV-8 exposed B cells over 48 h (mean±s.e.m., N=4). *(B)* $TCID_{50}$ was determined for T1H6-DC-SIGN^{+} cells infected with both HHV-8 and UV-HHV-8 exposed B cells. $TCID_{50}$ could not be determined above background for UV-HHV-8 exposed B cells due to sporadic positivity (mean±s.e.m., N=4). *(C)* B cells exposed to Live or UV-HHV-8 were collected at 48 hours post infection and stained for intracellular HHV-8 K8.1 or ORF59 PF-8. (mean±s.e.m., N=5). *(D)* Supernatants collected from HHV-8 or UV-HHV-8 exposed B cells at 48 hpe were assayed by CBA (mean±s.e., N=4). *(E)* Supernatants collected from HHV-8 or soluble gB exposed B cells at 48 hpe were assayed by CBA (mean±s.e.m., N=4, *$P \leq 0.05$).

3.4.5 IL-8 enhanced HHV-8 replication

As there was a 4-fold increase in IL-8 in HHV-8-exposed B cells and IL-8 can serve as ligand for HHV-8 vGPCR, resulting in VEGF, IL-6, GRO-α and additional IL-8 production (54, 283), we sought to determine the role of IL-8 on HHV-8 replication. We therefore added IL-8 or neutralizing IL-8 antibodies to B cell cultures at the time of HHV-8 exposure. As a mean of 3 individual experiments, detection of HHV-8 K8.1 or ORF59 PF-8 expression by flow cytometry was enhanced by 4% when IL-8 was added to cultures compared to HHV-8 alone (**Fig. 27a in Appendix B**). A 4% decrease was observed when neutralizing antibodies were added (mean±s.e., N=3). Also, cell-associated DNA increased from 0 to 48 hours in the HHV-8 treated B cells, but the addition of IL-8 resulted in an increase in viral DNA, indicating IL-8 was contributing to viral replication (**Fig. 27b in Appendix B**). This was not observed for supernatant levels as addition of IL-8 resulted in the lowest amount of supernatant levels of HHV-8 DNA. B cells treated with neutralizing IL-8 antibodies showed a large spike in cell-associated DNA at 24 hours that was 3.5 million copies more than in cells treated with IL-8. There were also elevated levels of supernatant HHV-8 DNA. The increase in viral DNA and percent of HHV-8 lytic protein positive B cells in cultures treated with IL-8 suggest that the chemokine may contribute to HHV-8 replication through the vGPCR enhanced expression of the ORF50 promoter.

3.4.6 HHV-8 lytic cycle replication in DC-SIGN$^+$ IgM$^+$ B cells.

We next determined the B cell subset targeted for HHV-8 lytic infection and immune mediator production. B cells were exposed to HHV-8 and stained at 48 hpe for IgD and IgM heavy chain

isotypes, as well as CD20, CD27, CD38, DC-SIGN and intracellular ORF59 PF-8 expression. We first gated on ORF59 PF-8 positive cells, followed by delineation of immunoglobulin expression (**Fig. 16a**). Cells expressing IgM and IgD were then gated on CD27 to delineate naïve and memory B cells (147). 47±16% were detected in the IgM memory population ($IgM^+IgD^+CD20^+CD27^+$) compared to 38±13% (mean±s.e.) of ORF59 PF-8 positive cells in the naïve B cell population ($IgM^+IgD^+CD20^+CD27^-$) (**Fig. 16c**). We found that some $CD27^+$ cells lacked CD20, a B cell pan marker that is lost upon plasma cell differentiation. We reasoned that these were early stage plasmablasts. Indeed, $CD27^+CD20^-$ cells also expressed the plasma-stage marker CD38. Finally, we quantified the DC-$SIGN^+$ cells within each of these populations and found that 71%, 95% and 100% of naïve, IgM memory and plasmablasts $ORF59^+$ cells expressed DC-SIGN (**Fig. 16b and c**).

As HHV-8-exposed pos. B cells were the major polyfunctional B cells detected; we next determined polyfunctional cytokine and chemokine production in B cell subsets. We stained the B cells for surface expression of CD20, CD27, IgM and DC-SIGN and intracellular expression of ORF59 PF-8, IL-6, IL-8 and MIP-1β. Both the $IgM^+CD20^+CD27^+$ (IgM memory) and $CD20^+IgM^+CD27^-$ (naïve) populations expressing DC-SIGN showed enhanced expression of IL-6, IL-8 and MIP-1β compared to the HHV-8-exposed neg. population (**Fig. 16d**). Likewise, polyfunctional analysis revealed that both the HHV-8 pos. naïve and IgM memory cells displayed polyfunctional properties, whereas the majority of HHV-8 exposed neg. cells lacked cytokine or chemokine production (**Fig. 16e**).

These data indicate that subsets of DC-$SIGN^+$ B cells expressing IgM and variable levels of the CD27 memory marker are responsible for both monofunctional and polyfunctional cytokine and chemokine production in HHV-8-exposed, virus infected B cells.

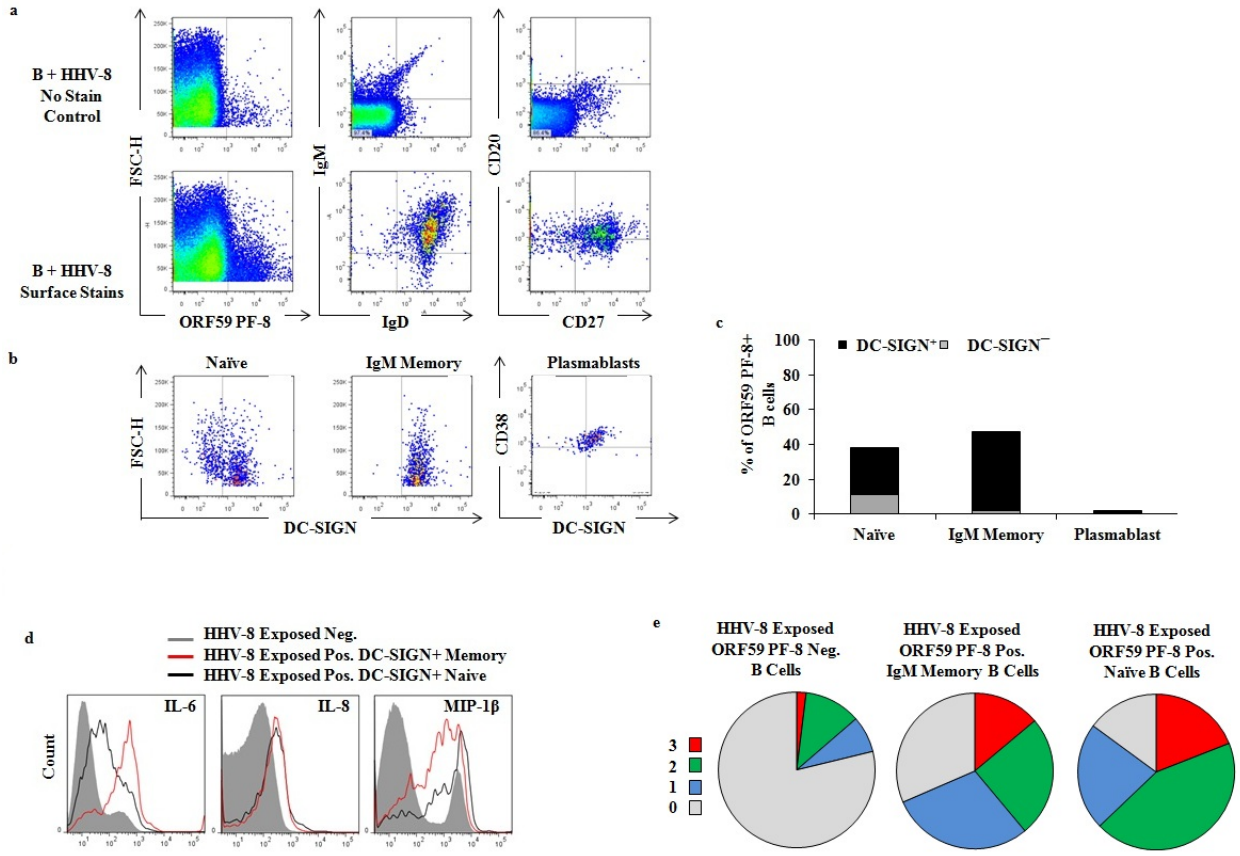


Figure 16: HHV-8 targets DC-SIGN^{+}IgM^{+} naïve and memory B cells

Cell samples were collected at 48 h post-infection and stained for B cell surface marker expression and intracellular HHV-8 ORF59 PF-8. *(A)* Flow cytometry gates were set using HHV-8 infected B cells stained for control IgG. HHV-8 positive cells were first gated on IgM/IgD expression. Double positive cells were then gated against CD20/CD27. *(B)* IgM^{+}IgD^{+}CD20^{+}CD27^{-} (naive) and IgM^{+}IgD^{+}CD20^{+}CD27^{+} (IgM memory) were next gated against DC-SIGN expression. IgM^{+}IgD^{+}CD20^{-}CD27^{+} were further gated against CD38/DC-SIGN (plasmablasts); data are one representative of 4 individuals tested. *(C)* Total percentages of naïve, IgM memory and plasmablasts (sum of black and gray bars) and DC-SIGN expression from each population (gray bars) were determined (mean, N=4). *(D-E)* B cells were stained for DC-SIGN, IgM, CD20 and CD27 surface expression and then ORF59 PF-8, IL-6, IL-8 and MIP-1β intracellular expression. ORF59 PF-8^{+} B cells expressing DC-SIGN, IgM, CD20 and either CD27^{+} (IgM memory) or 27^{-} (naïve) were selected and the expression of IL-6, IL-8 and MIP-1β were determined compared to ORF59 PF-8^{-} populations *(D)*. Percentages of cells producing 3, 2, 1, or no immune mediators were determined *(E)*. Data are one representative of 4 individuals tested.

3.4.7 B cell immune mediators are enhanced in patients with KS

To investigate the role of B cell immune mediators in HHV-8 disease development, we examined serum levels of TNF-α, IL-6, MIP-1α, MIP-1β and IL-8, as well as the KS-related growth factor VEGF(10), in participants from the Multicenter AIDS Cohort Study (MACS) who were co-infected with HIV-1 and HHV-8 for similar periods of time, and who did (cases) or did not (controls) develop KS prior to the advent of effective antiretroviral therapy (ART). The MACS controls were classified as slow progressors, based on their CD4 counts, to represent an opposite extreme in the natural history of HIV-1 infection and AIDS. There were lower numbers of $CD4^+$ T cells, and higher numbers of $CD8^+$ and $CD3^+$ T cells in KS cases compared to controls 2-5 years before KS development (**Fig. 17a**). Plasma viral loads for HIV-1 (*P*=0.007) and HHV-8 (*P*= non-significant [NS]) were higher within the year prior to KS development in cases compared to controls (**Fig. 17b**).

We noted a similar pattern of lower levels of IL-8, MIP-1α, MIP-1β and VEGF at 6-10 years pre-KS diagnosis in cases compared to controls, with a progressive increase in levels of these immune mediators in cases 2-5 years pre-KS and within 1 year of KS diagnosis (**Fig. 17c**). IL-8 and MIP-1α levels were nearly 2-fold higher and significantly increased (*P*=0.01 and 0.008, respectively) in KS cases compared to controls within the year prior to KS diagnosis. Levels of MIP-1β and VEGF increased over time in the KS cases and decreased in the controls, with higher levels evident in the cases the year prior to KS diagnosis (*P*=NS). TNF-α levels were higher at all time points in the KS cases compared to the controls. Both TNF-α and VEGF approached significance (*P*=0.07) in cases. In contrast to these 5 immune mediators, IL-6 levels were slightly higher in the controls than cases over the total 6-10 years of follow-up.

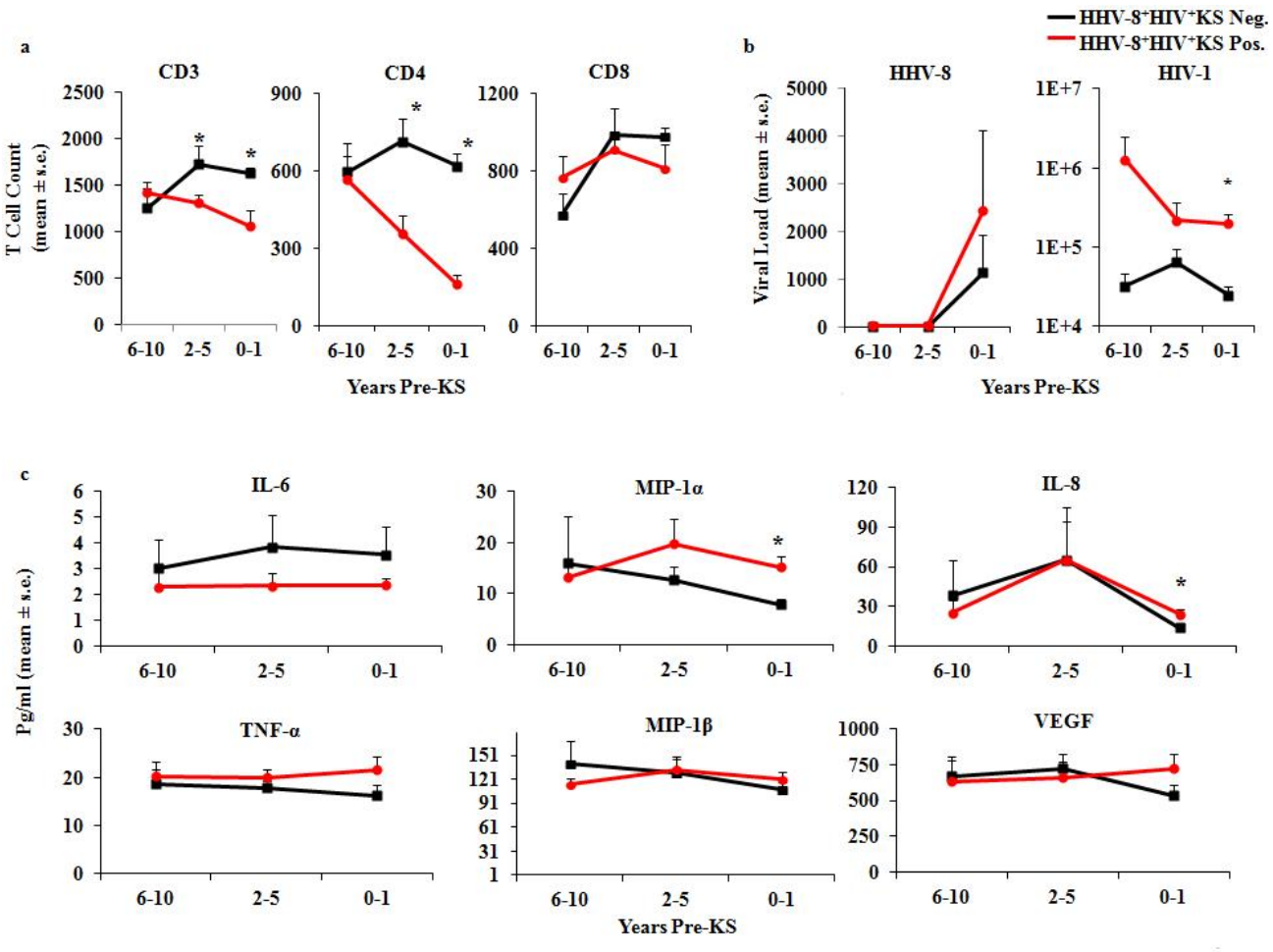


Figure 17: T cell counts, viral loads and serum biomarkers in MACS participants

15 HIV-positive, HHV-8-positive, KS-negative (controls) and 15 HIV-positive, HHV-8-positive, KS-positive (cases) subjects were selected from the Pittsburgh MACS. 3 visits were chosen, with one visit within a year of KS development in the cases and the corresponding visit in the controls, a second visit 2-5 years prior to KS and a third visit 6-10 years prior to KS. *(A)* CD3, CD4 and CD8 T cell counts at each visit were determined by flow cytometry. (B) HHV-8 and HIV viral loads at each visit were determined by PCR. *(C)* For each visit, frozen serum samples were thawed and concentrations of TNF-α, IL-8, MIP-1α, IL-6, MIP-1β and VEGF were determined at 3 time points using an electrochemiluminescence MSD assay (mean±s.e.m., N=10 at years 2-10 prior, N=15 at years 0-1 prior, per group, **P*<0.05.)

Collectively, these data show that levels of IL-8, TNF-α, MIP-1α, MIP-1β and VEGF, but not IL-6, are increased within a year of KS diagnosis. We next determined whether B lymphocytes were producing these biomarkers. We conducted cross sectional studies on 3 KS

cases and controls at the time of KS diagnosis (cases) or corresponding times in the controls. $CD19^+$ B cells were purified from PBMC and assayed for viral DNA and immune mediator mRNA. KS controls and two KS case had undetectable levels of HHV-8 in their $CD19^+$ B cells, while one KS case had a viral load of 183,306 copies/100,000 cells. Expression of IL-6, MIP-1α, MIP-1β, VEGF and IL-8 mRNA was elevated in KS cases compared to controls, with 1.6, 4.1, 2.7 and 3.5 and 2.1-fold increases, respectively (**Fig. 18**). TNF-α was enhanced in KS controls. These data indicate that HHV-8 infected B cells produce more pro-inflammatory cytokines, chemokines, and growth factors in HIV-1 infected individuals who are developing KS compared to those without KS.

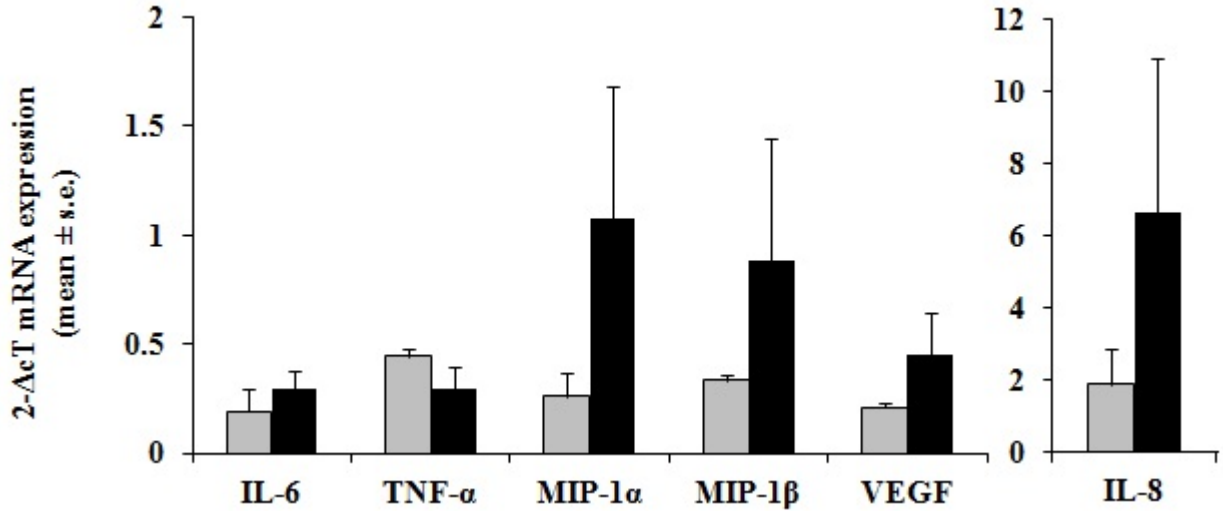


Figure 18: Cytokine and chemokine B cell RNA

CD19+ cells were isolated from PBMC of 3 HIV-positive, HHV-8-positive, KS-negative (controls) and 3 HIV-positive, HHV-8-positive, KS-positive (cases) subjects from the Pittsburgh MACS at 0-1 years prior to KS development. RNA was extracted and used in a real-time RT-PCR assay to determine mRNA expression of immune mediators (mean±s.e.m., N=3 per group, *$P \leq 0.05$).

We next determined the polyfunctional profile of the $CD19^+$ B cells from the 3 KS^+ cases. HHV-8 ORF59 PF-8 negative and positive cells were selected and the intracellular expression for each cytokine and chemokine was determined. We detected 6.61, 1.14 and 2.91% ORF59+ cells among KS cases (**Fig. 19a**) with a mean $ORF59^+$ population of 3.73% (±1.47) (data not shown). There were a higher percentage of polyfunctional B cells detected in the HHV-8 infected cells compared to the uninfected cells, with a mean of 72% of HHV-8 infected cells producing 2 or more immune mediators compared to only 36% of uninfected cells (**Fig. 19b**), supporting our *in vitro* model.

Taken together, these data indicate that HHV-8 infected B cells of individuals that develop KS produce more pro-inflammatory cytokines, chemokines, and growth factors than individuals that do not develop KS.

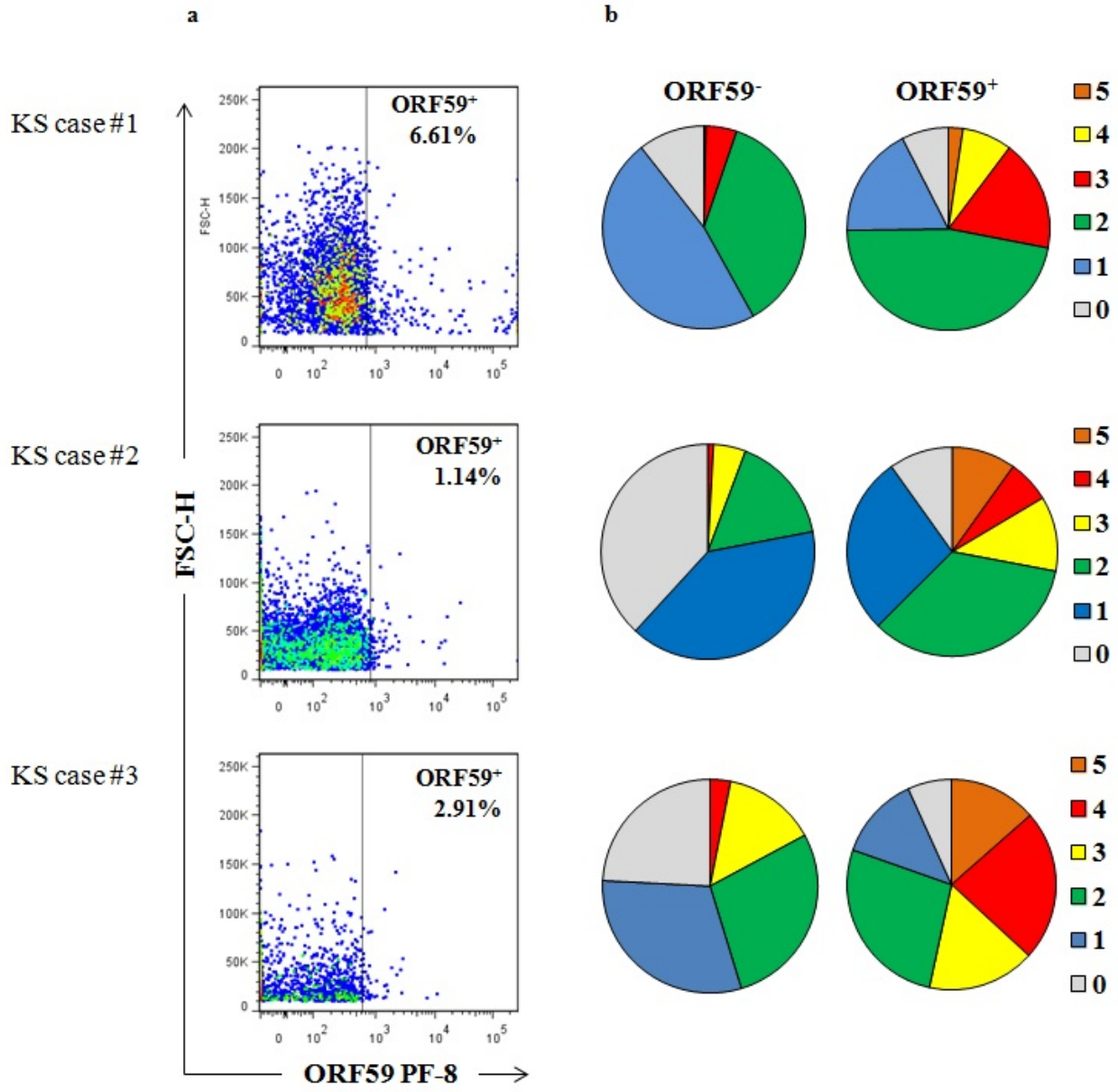


Figure 19: Polyfunctional B cells in KS cases

CD19+ cells were isolated from PBMC of 3 HIV-positive, HHV-8-positive individuals with KS (cases) from the Pittsburgh MACS at 0-1 years prior to KS development. Cells were stained for ORF59 PF-8 and the 5 immune mediators previously used in the *in vitro* studies. Percentages of cells producing 1-5 immune mediators within the ORF59 PF-8 negative and positive populations were calculated.

3.5 DISCUSSION

HHV-8-infected B cells are likely a major source of infectious virus and immune mediators that drive the oncogenic process of KS and the HHV-8-associated B cell lymphomas MCD and PEL. However, there is little direct evidence linking HHV-8 infection of B cells and induction of soluble immune mediators to HHV-8 pathogenesis and oncogenesis. To address these relationships in depth, we developed new measures for HHV-8 proteins, DNA and infectious virions, and B cell mRNA and protein analysis for quantitation of immune mediator production. Here we show that activated B cells derived from the blood of healthy, HHV-8 seronegative adults, supported lytic replication of HHV-8 as demonstrated by increases in HHV-8 DNA, ORF59 PF-8 or K8.1 positivity, and infectious virus. An in depth examination of the cytokines and chemokines produced by the infected B cells revealed multiple characteristics that could be important in HHV-8 pathogenesis and oncogenesis. Overall, we found that HHV-8 infection of B cells induced the greatest amounts of mRNA and protein for 2 cytokines (IL-6 and TNF-α) and 3 chemokines (MIP-1α [and CCL3L], MIP-1β [and CCL4L] and IL-8) among 16 different immune mediators screened. Production of these immune mediators occurred at three different levels, i.e., first level: B cell activation by CD40L and IL-4, surrogates of activated $CD4^+$ T cells; second level: exposure of the activated B cells to HHV-8 and binding of virus to cell surface moieties; third level: HHV-8 infection and lytic replication **(Fig.20)**.

The first level of immune mediator induction by HHV-8 was polyfunctional activity and relatively low level of production of the 5 immune mediators in activated B cells. This level represents production of cytokines and chemokines during T-B cell activation and host inflammation. The second level was a result of HHV-8 binding to B cell surface moieties, with non-replication-dependent induction of cytokines and chemokines. In some cases, cytokine and

chemokine mRNA and protein levels were elevated at the earliest time measured, i.e., 3 h after exposure to HHV-8. This implies that binding of HHV-8 very early in B cell infection initiates signaling cascades involved in immune mediator production. This was supported by our data that both UV-HHV-8 and soluble HHV-8 gB elicited similar cytokine-chemokine profiles and levels above the unexposed, activated B cells as did replication competent HHV-8. Notably, the amount of gB associated with our purified HHV-8 and the soluble gB was not normalized in the B cell cultures, so comparisons of cytokine and chemokine levels among these different B cell cultures must be viewed with caution.

We defined the third level of immune mediator production among the lytically infected B cells as modeled on studies of HIV-1 antigen-stimulated T cells (152). This revealed for the first time B cells producing HHV-8 lytic proteins while distinguishing virus exposed-infected and exposed-uninfected B cells, and determining among these which cells were producing combinations of each cytokine and chemokine. We found that approximately 8% of the HHV-8-exposed B cell cultures were lytically infected with virus as shown by expression of the lytic proteins K8.1 or ORF59 PF-8. Remarkably, within this virus positive B cell population, 99% of cells produced at least one cytokine or chemokine, with 76% of cells being polyfunctional, i.e., producing 2-to-5 immune mediators. Among the remaining 92% of the B cells that were HHV-8 exposed and virus negative, 80% produced at least one immune mediator, but only 52% were polyfunctional. This concurs with our findings that binding of HHV-8 or gB to B cells elicits monofunctional and polyfunctional immune mediator responses, albeit at lower levels than lytically infected B cells. Thus, HHV-8 lytically infected B cells have the broadest range in polyfunctional cytokine and chemokine activity.

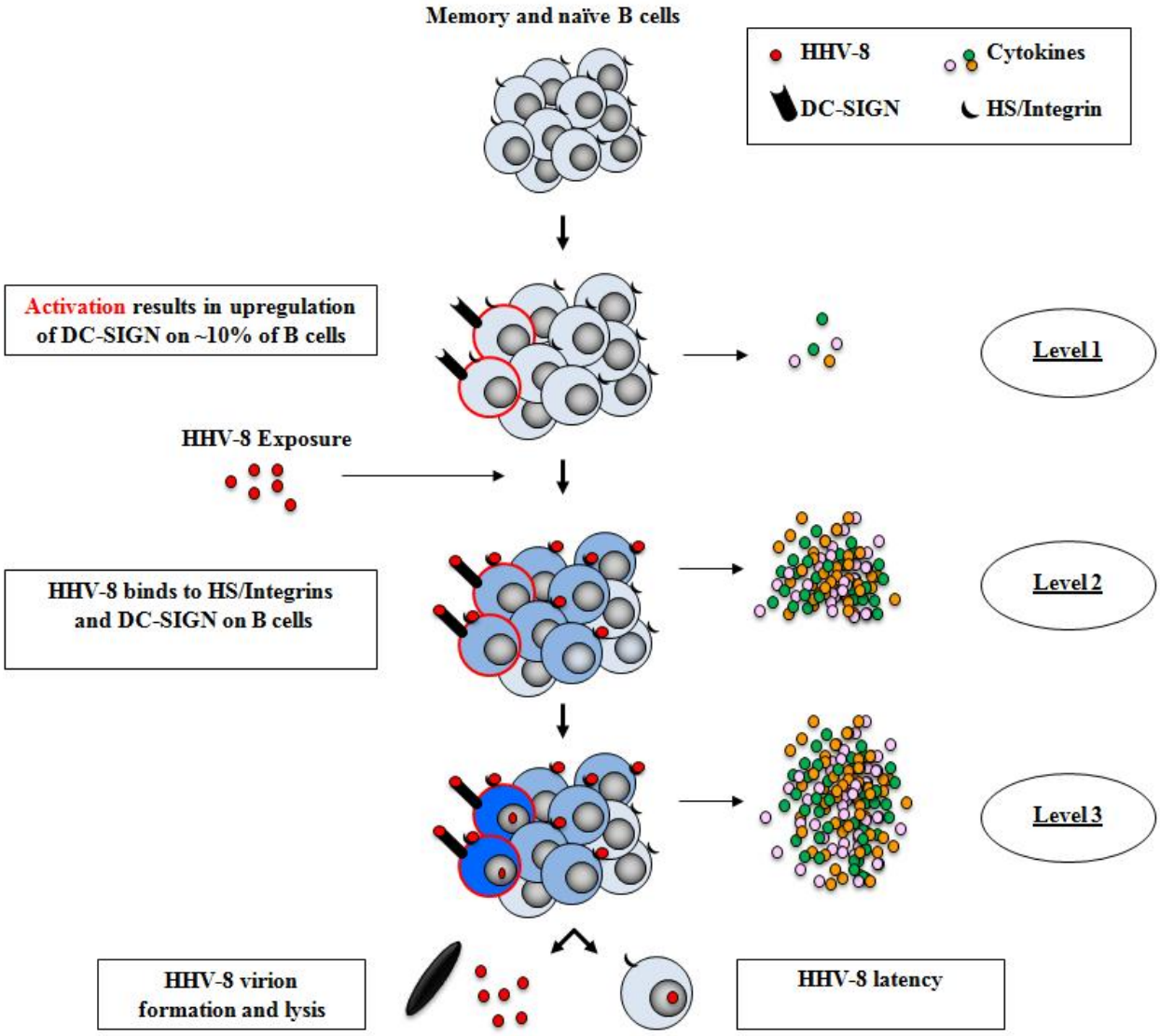


Figure 20: Three tiers of B cell immune mediator production

Activation (red outline) of B cells results in immune mediator production and upregulation of DC-SIGN on a subset of cells (~10%). HHV-8 binding to heparin sulfate, integrins and DC-SIGN upon exposure initiates a more substantial production of immune mediators. Subsequent HHV-8 infection and lytic cycle replication in DC-SIGN+ naïve and memory B cells induces a polyfunctional cytokine and chemokine response.

At present, the role of these polyfunctional B cells is unclear. The term "polyfunctional" has been used to describe T cells that are important in control of HIV-1(18) and in the progression of HHV-8 infection and related cancers (21, 115, 158). The presence of polyfunctional B cells in HHV-8 infection could have detrimental rather than beneficial outcomes. In response to HHV-8 infection, B cells produced elevated levels of MIP-1α and β, which are chemokines involved in B cell recruitment, activation and immunoglobulin production (143, 257). MIP-1α and β could increase the activated B cell population most capable of replicating HHV-8 both locally and systemically. Enhanced IL-6 production, a B cell proliferation factor, could also increase targets for HHV-8 replication (237) as well as block the suppressor effect of $CD4^+CD25^+$ T regulatory cells (78). Furthermore, IL-6 is a proinflammatory cytokine that enhances TNF-α, which together can create a rich inflammatory microenvironment, promoting KS tumor growth and vascularization (183). The generation of anti-HHV-8 effector T cells and subsequent production of IFN-γ could also be influenced by TNF-α (180).

Finally, there was a four-fold increase in IL-8 in HHV-8-exposed B cells. Bottero et al. (28), showed that vGPCR expression can up-regulate the promoter for the lytic switch protein, leading to ORF50 expression in PEL cells. We speculate that IL-8 can act as an autocrine or paracrine factor to enhance HHV-8 replication via vGPCR signaled enhancement of the ORF50 (**Fig. 28 in Appendix B**). Interestingly, IL-8 was shown to enhance the replication of another herpesvirus, cytomegalovirus (CMV) when added to cultures of human endothelial fibroblasts (HEF). Furthermore, neutralizing IL-8 mAb reduced CMV replication (189). Addition of IL-8 in this study resulted in an increase in $HHV\text{-}8^+$ cells as well cell-associated DNA. The increase in supernatant and cell-associated HHV-8 DNA in the cultures treated with neutralizing

antibodies does not necessarily dispute this finding as vGPCR signaling is constitutive, meaning that even in the absence of IL-8, vGPCR can still signal and enhance ORF50. The presence of IL-8 would therefore be sufficient to enhance replication rather than necessary. Further studies to determine the effects of IL-8 in combination with other synergistic growth factors, such as VEGF should be conducted.

Given that B cells are a primary target for very few human viruses, and that B cell subsets have a variety of specialized functions, we examined B cell phenotypic characteristics to determine if specific subsets were lytically infected with HHV-8 and if this was related to production of cytokines and chemokines. We found that the majority (47%) of ORF59 PF-8^+ cells were detected in the $IgM^+IgD^+CD20^+$ $CD27^+$ IgM-memory (or marginal zone [MZ]-like(275)), compared to 38% $IgD^+IgM^+CD20^+CD27^-$ naïve B cell population and 2% plasmablasts. Our findings fit with classic KS where the pre-immune/natural effector B cell compartment, including MZ-like ($IgD^+IgM^+CD27^+$) and naïve ($IgD^+IgM^+CD27^-CD5^-$) B cells, is expanded compared in healthy controls, with a resting state of activation. (70) Of note is that most of the HHV-8-infected IgM memory and naïve B cells expressed DC-SIGN. This corresponds to our previous evidence that binding of HHV-8 to DC-SIGN is an essential step in productive infection of B cells (215). Expansion of such B cell populations would provide targets for initial HHV-8 infection and full lytic cycle replication.

HHV-8 lytic cycle replication was coupled with monofunctional and polyfunctional immune mediator production in IgM memory and naïve B cells. Thus, there were a greater proportion of ORF59 PF-8^+ IgM memory B cells producing various combinations of IL-6, IL-8 and MIP-1β compared to ORF59 PF-8^- B cells. A similar predominance of polyfunctional activity was observed in the HHV-8-infected naïve B cells. Memory B cells are known to

produce more IL-6, TNF-α, LT-α (77, 78) and MIP-1α and MIP-1β (3) than naïve B cells, which produce greater amounts of IL-10. Overall, our data indicate that HHV-8 targets DC-SIGN$^+$ IgM$^+$ B cells expressing variable levels of CD27 to create a cytokine and chemokine milieu conducive to oncogenic cell proliferation.

It is believed that HHV-8 infection drives B cells to an early plasmablast-like state in MCD and a preterminal plasma cell stage of differentiation in PEL (45, 119, 146, 237). In our study, 2% of the ORF59 PF-8$^+$CD27$^+$ B cells lacked expression of CD20, which is lost as B cells differentiate into plasmablasts or plasma cells. These cells also expressed the plasmablast marker CD38. Hassman *et al.*,(119) determined that cells expressing the HHV-8 latency gene LANA also expressed IgM and the λ light chain at 60-84 h post-infection. These cells were considered plasmablast-like, as a high proportion were blasting or dividing and had increased IL-6R expression. We examined HHV-8 infection at an earlier time point *in vitro* when lytic replication predominates (215). Our detection of HHV-8 lytic infection in what appear to be early stage plasmablasts extends the Hassman *et al.* (119) findings and offers evidence of a memory or naïve B cell target for lytic virus replication (**Fig.21**). Further studies are needed to determine transitional states among these lytically and latently infected B cells.

A balance exists between protective immunity initiated by host cells via cytokine and chemokine production and virus-driven induction of cytokines and chemokines that serve to disseminate virus infection and mediate pathogenesis. Several of the immune mediators that are essential to the immune response and activation of lymphocytes can exacerbate infection and cause clinical symptoms when over produced in response to HHV-8 infection. In assessing clinical correlates of these *in vitro* data, we found elevated levels of TNF-α, MIP-1α, MIP-1β, and IL-8, as well as VEGF, in the plasma of HIV-1/HHV-8 co-infected MACS subjects

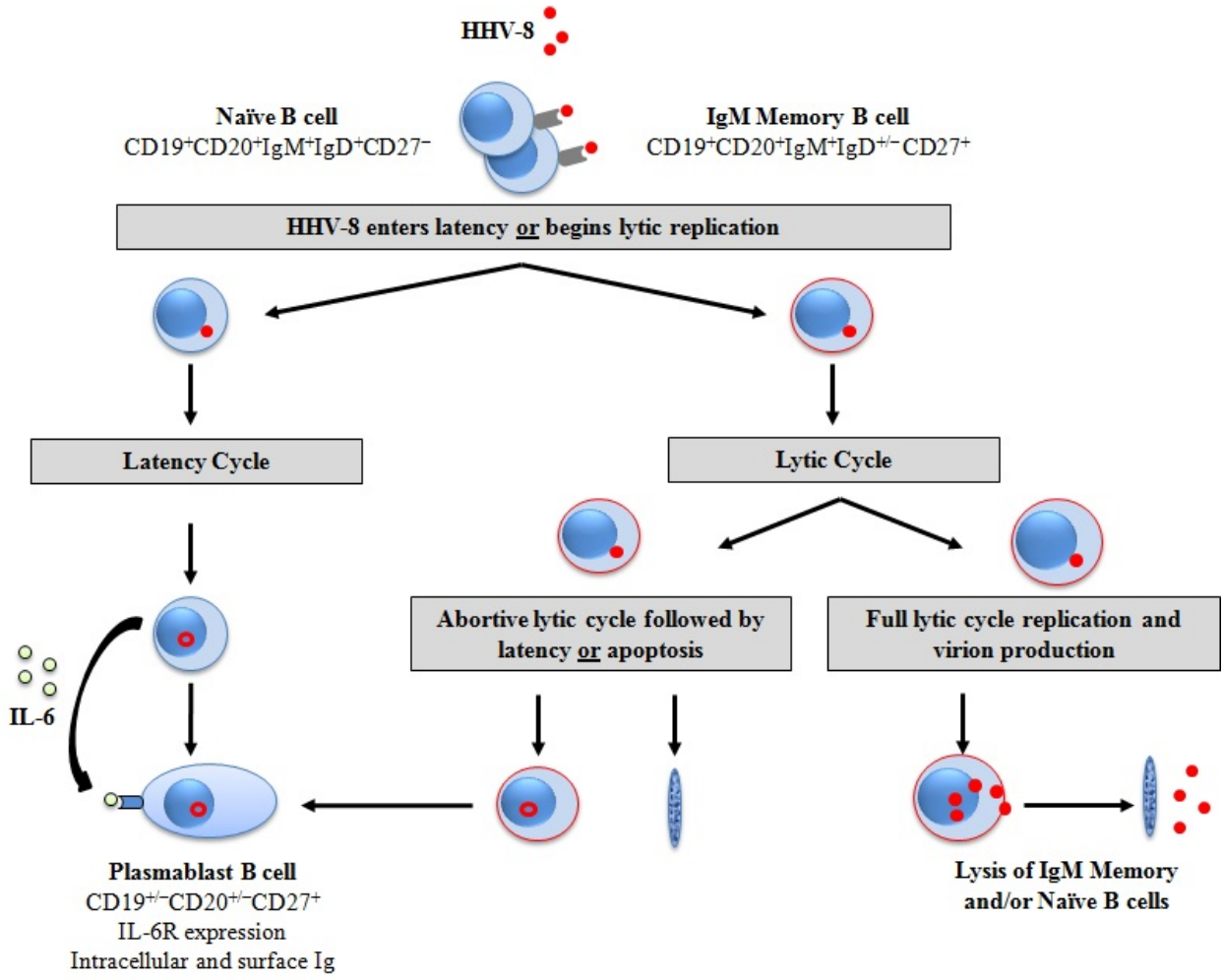


Figure 21: HHV-8 targets B cell subpopulations for infection

The B cell target for HHV-8 infection is unknown. However, evidence suggests that naïve and/or IgM memory B cell subsets are susceptible to HHV-8 infection. HHV-8 is endocytosed after binding to cell surface entry receptors. The virus then enters latency (left) or initiates lytic replication (215). Latently infected cells drive differentiation toward a plasmablast phenotype that is responsive to the proliferative cytokine, IL-6. The alternative pathway is the entry of HHV-8 into the lytic cycle to begin transcription of lytic-associated proteins in activated B cells (red outline). The lytic cycle may stop prior to virion production, resulting in an abortive replicative cycle as seen in DC, endothelial cells and fibroblasts. The virus in these cells likely enters latency or may result in B cell apoptosis. Some cells, however, will support full lytic cycle replication, resulting in lytic protein synthesis and increases in viral DNA that correspond to infectious HHV-8 progeny and subsequent release through cell lysis.

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coincident with their diagnosis of KS, but not during many years prior to KS. These data support a role for these immune mediators in development of KS. Interestingly, circulating levels of IL-6 were low throughout the course of HIV-1/HHV-8 co-infection in MSM who eventually developed KS and those that did not. This is in agreement with previous studies in the MACS showing very low levels of IL-6 in the blood of HIV-1 infected subjects (32). The function of these cytokines and chemokines in HHV-8 infection and KS likely varies depending on the quantity and origin.

Using HHV-8 seronegative B cells, we detected a major shift toward polyfunctionality upon HHV-8 infection *in vitro* that could be critical in viral replication and dissemination, proliferation of target cells, and induction of KS and HHV-8-related lymphomas. Indeed, we found enhanced mRNA expression of 5 immune mediators in circulating B cells of KS cases compared to non-KS controls within 1 year prior to KS diagnosis. Furthermore we detected ORF59-PF-8$^+$ B cells by flow cytometry that had enhanced polyfunctional activity in the KS$^+$ cases. These data imply HHV-8-driven B cell production of immune mediators contributes to development of KS and provide a foundation for more in depth studies of polyfunctional cytokine and chemokine production by B lymphocytes in disease.

In conclusion, our study shows for the first time that activated, DC-SIGN expressing, IgM memory and naïve B cells serve as prime targets for HHV-8 lytic replication with production of infectious virus. The HHV-8 infected, IgM memory and naïve B cells exhibited enhanced production of multiple pro-inflammatory cytokines and chemokines that have been linked to viral pathogenesis, KS and HHV-8-associated lymphomas. Several of these immune mediators were elevated in the plasma and CD19$^+$ B cells of HHV-8/HIV-1 co-infected individuals who developed KS. These polyfunctional B cells likely play a significant role in

viral replication, dissemination of HHV-8, and proliferation of target cell populations that drive HHV-8 cancers.

3.6 ACKNOWLEDGEMENTS

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4.0 DISCUSSION

HHV-8 infection of professional APC could demonstrate an evolutionary mechanism to establish viral latency in cell types responsible for initiating T cell adaptive immune responses. Although valuable conclusions have been drawn from immortalized cell lines as surrogates for these APC, a primary cell model such as blood and tonsil B lymphocytes provides a more natural accounting of the quality of HHV-8 infection, better reflecting the mechanisms of latency and abortive and non-abortive virus replicative cycles.

I expanded on the B cell replication model introduced by Rappocciolo *et al.*, to include additional methods of virus replication quantitation. I enumerated HHV-8 lytic protein positive cells using intracellular staining and flow cytometry, and incorporated a newly developed $TCID_{50}$ assay to verify infectious virion production by HHV-8 infected B cells. These data, in conjunction with a qRT-PCR assay showed that *in vitro* infection of primary B lymphocytes from HHV-8 seronegative donors resulted in an increase in HHV-8 DNA, lytic protein expression and infectious particle production.

The question remains as to why HHV-8 infection can result in an abortive replicative cycle for some cell types, such as DC, and full-lytic cycle replication, as observed in B cells. As an *in vitro* model of HHV-8 replication in B cells has been established, a genome-wide study to determine cellular gene activation and/or suppression was conducted. One aspect under examination is the induction of immune mediators that could contribute to HHV-8 replication.

I hypothesized that immune mediators produced by HHV-8 infected APC is unique between cell types. Utilizing several techniques, I showed exposure of B cells to HHV-8 results in a large-scale production of several immune mediators, including significant levels of IL-6, TNF-α, MIP-α, MIP-β and IL-8, which is distinct from immune mediators induced during parallel infections of MDDC, which resulted in significant levels of IL-10, MIP-α, MIP-β, MCP-1, RANTES and IP-10. Whether the production of these immune mediators may aid or hinder virus replication is the focus of future studies.

Three levels of immune mediator production were detected in the B cell model, using a CBA assay for secreted proteins. CD40L/IL-4 activated B cells produced low levels of immune mediators, which likely represent production of cytokines and chemokines during T-B cell activation and host inflammation. The second level occurred upon virus binding to cell surface receptors as demonstrated by UV-HHV-8 and the soluble HHV-8 glycoprotein B. The third and highest level occurred upon HHV-8 entry and subsequent lytic replication. These data were further supported using a highly sensitive flow cytometry assay that allowed for quantification of the percentage of cells that produce 0, 1, or a combination of 2-5 immune mediators. Activated, uninfected B cells demonstrated low levels of polyfunctional activity, whereas the majority of the HHV-8 infected population was polyfunctional. This represents the first data regarding polyfunctional B cells in response to pathogen exposure and further supports the notion that B cells are not simply antibody producing cells, but rather, play an integral role in shaping the immune response via production of effector cytokines and chemokines.

A delicate balance exists between protective immunity involving cytokine and chemokine production by host cells, and virus-driven induction of cytokines and chemokines that aid in the dissemination and spread of infection and mediate pathogenesis. Several of the mediators that

are essential to the immune response and activation of lymphocytes can exacerbate infection and cause clinical symptoms when over produced in response to HHV-8 infection. The role of each cytokine/chemokine in HHV-8 infection and KS likely varies depending on their quantity and origin of production, which in turn is controlled by signaling pathway activation states. We observed activation of several pathways upon HHV-8 exposure, including B cell receptor signaling, and the JAK/STAT, MAPK/ERK and NF-κB pathways. Activation of these pathways occurred immediately after exposure to HHV-8 (3 hours) and contained the most up-regulated genes at either 6 or 9 hours post HHV-8 exposure. Every immune mediator within the observed DC and B cell cytokine and chemokine profiles can be transcribed via the NF-κB pathway, while several are also produced by the JAK/STAT pathway (reviewed in Table 1). In addition, one of the most frequently observed canonical pathways that were activated upon virus exposure included the communication between innate and adaptive immune cells, further supporting the significant role of immune mediators upon HHV-8 infection.

I also hypothesized that HHV-8 infects a subset of B cells and initiates cytokine and chemokine production that contributes to HHV-8 replication, viral dissemination and initiation of KS and HHV-8 lymphomas. In this study, I showed an *in vivo* correlation with the *in vitro* work using specimens from the Multicenter AIDS Cohort Study. I showed that levels of 4 cytokines and chemokines (TNF-α, MIP-α, MIP-β and IL-8), as well as VEGF were enhanced in the plasma of individuals who are HHV-8^+, HIV^+ who developed KS compared to those individuals who are HHV-8^+, HIV^+ and did not develop KS. These data suggest that up-regulation of these immune mediators may aid in driving the development of the cancer. Furthermore, B cells isolated from the individuals who did develop KS had elevated levels of IL-6, MIP-α, MIP-β, IL-8 and VEGF RNA, compared to those who did not develop KS, indicating that B cells were

directly contributing to the production of the enhanced levels of immune mediators detected in the plasma. Lastly, I determined that the HHV-8 infected B cells from these individuals were polyfunctional by flow cytometry, further supporting the importance of HHV-8 driven immune mediator production in B cells.

From these studies we propose that latently infected DC and lytically infected B cells produce several proinflammatory cytokines and chemokines that can spread HHV-8 infection, enhance HHV-8 driven plasmablast formation and IL-6 receptor expression and enhance spindle shaped endothelial cell growth and vascularization (**Fig. 22**).

4.1 PUBLIC HEALTH SIGNIFICANCE

Although there is an abundance of data regarding HHV-8 pathogenesis, lytic replication and cytokine production from cell lines that utilize vector over expression systems, genetically mutated recombinant viruses and chemically induced reactivation events, I provide a comprehensive study focusing on two cell types naturally targeted by HHV-8 and the replicative/abortive replicative events that follow. I have used the information obtained from the *in vitro* studies to evaluate a relationship to the progression of KS in the patient population. This is the most extensive natural model to date regarding lytic infection to determine natural reactivation events and the cascade of HHV-8 gene activation in transition from latency to lytic replication. This is also the first evidence of polyfunctional B cells in the context of pathogen exposure. Therefore, the role of polyfunctional B cells in other diseases can now be explored. A more defined role of polyfunctional B cells in KS development could be achieved using additional MACS groups.

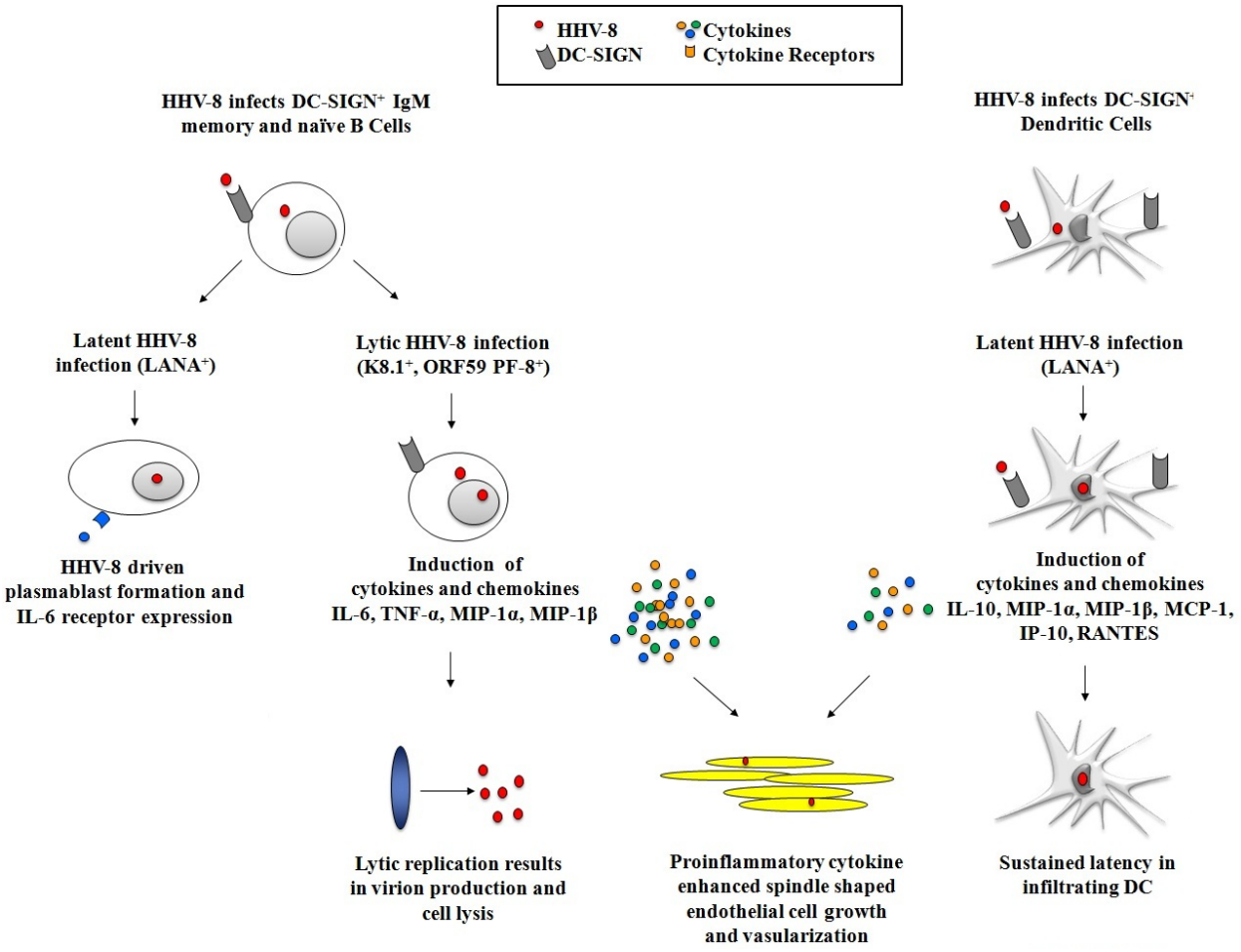

Figure 22: Model for immune mediator responses in latent and lytic HHV-8 infections

HHV-8 uses DC-SIGN as an entry receptor on both activated IgM memory and naïve B cells as well as MDDC. Upon infection, HHV-8 can enter a latent state in both B cells and DC, resulting in the expression of LANA. In B cells, latent HHV-8 infection drives the formation of plasmablasts, with enhanced expression of the IL-6 receptor (119). However, HHV-8 can also enter a lytic replication cycle in B cells that result in viral DNA synthesis, lytic protein expression and production of infectious virions. Within the lytically infected B cells there are a significant percentage of polyfunctional B cells that result in enhanced production of IL-6, TNF-α, MIP-1α, MIP-1β and IL-8. This is in contrast to the immune mediator profile detected in latently infected MDDC, in which significant quantities of IL-10 and the MIP1α, MIP-β, MCP-1, IP-10 and RANTES chemokines are secreted. Production of these immune mediators by HHV-8 infected B cells and DC within KS lesions may contribute to KS growth and vascularization. Furthermore, chemokines produced by HHV-8 infected cells could attract monocytes and macrophages to the KS lesion to help spread latent infection and maintain viral persistence, while IL-6 and TNF-α drive could plasmablast formation and IL-6 receptor expression on latently infected plasmablast-like cells (119).

This is also the first extensive, multiparameter, longitudinal study of HHV-8 infection of B cells and immune mediators in development of KS. Finally, this model for HHV-8 infection of B cells resulting in cytokine and chemokine production could be adapted to create an *in vitro* model for KS spindle cell formation. B cell derived, HHV-8 driven-immune mediators may initiate KS spindle cell formation from normal endothelial cells, which could have implications in studying the development of this cancer. These data may provide several new models and targets of HHV-8 infection and induction of immune mediators for assessing anti-HHV-8 therapies and vaccines.

APPENDIX A: ABBREVIATIONS

AIDS: Acquired Immunodeficiency Syndrome
AF: alexa-fluor
APC: antigen presenting cells
ART: antiretroviral therapy
BCBL-1: body cavity based lymphoma cells
BCR: B cell receptor
β-GAL: beta galactosidade
BSA: bovine serum albumin
CBA: cytometric bead array
CCL: chemokine (c-c motif) ligand
CCL-L: CCL-like
cT: cycle threshold
CXCL: chemokine (C-X-C motif) ligand
CXCR: C-X-C chemokine receptor
CD40L: cluster differentiation 40-ligand
CIITA: class II transactivator
CMV: cytomegalovirus
CTL: cytotoxic T lymphocyte
DC: dendritic cells
DC-SIGN: type II C-type lectin, DC-specific ICAM-3 grabbing nonintegrin
DOX: doxycycline
EBV: Epstein Barr virus
EDTA: ethylenediaminetetraacetic acid
ERK: extracellular signal-regulated kinase
FACS: fluorescence-activated cell sorting
FCAP: flow cytometric analysis program
FCS: fetal calf serum
FGF: fibroblast growth factor
FITC: fluorescein isothiocyanate
FLICE: FADD-like interferon converting enzyme
FSC-A: forward scatter-area
FSC-H: forward scatter-height
gB: glycoprotein B
gBm: glycoprotein B mutant
h: hours
hpe: hours post exposure

HGF/ SF: hepatocyte growth factor/ scatter factor
HHV-8: Human herpesvirus-8
HIV: Human Immunodeficiency virus
HSV: Herpes simplex virus
HLA: human leukocyte antigen
ICAM: intercellular adhesion molecule
ICS: intracellular staining
iDDC: interstitial-dermal DC
IFN: interferon
Ig: immunoglobulin
IL: interleukin
IP-10: interferon inducible protein
JAK/STAT: Janus kinase-signal transducer and activator of transcription
KS: Kaposi's Sarcoma
KSHV: Kaposi's sarcoma associated herpesvirus
LANA: latency associated nuclear antigen
LC: langerhans cells
LCL: lymphoblastoid cell line
LPS: lipopolysaccharide
m: minutes
mAb: monoclonal antibody
MACS: Multicenter AIDS cohort study
MCD: multicentric Castleman's disease
MDDC: monocyte derived Dendritic cells
MDM: monocyte-derived macrophages
MAPK: mitogen activated protein kinase
MCP-1: monocyte chemoattractant protein-1
MHC: major histocompatability complex
MIP: macrophage inflammatory protein
MSD: meso scale discovery
MZ: marginal zone
NIAID: National Institute of Allergy and Infectious Diseases
NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells
NS: non-significant
NIH: National Institutes of Health
ORF: open reading frame
PAN: polyadenylated nuclear RNA promoter
PBMC: peripheral blood mononuclear cells
PBS: phosphate buffered saline
pDC: plasmacytoid DC
PD-1: programmed death
PE: phycoerythrin
PEL: primary effusion lymphoma
PFA: paraformaldehyde
PGE2: prostaglandin E2
PhHV: Phocine herpesvirus

Poly-I:C: polyinosinic:polycytidylic acid
P: significance
RANTES: regulated upon activation, normal T-cell expressed, and secreted
RPMI: Roswell Park Memorial Institute
RT-PCR: real-time PCR
rKSHV.219: recombinant HHV-8
RTA: replication transactivator protein
S.E.M.: standard error of the mean
SOCS: suppressor of cytokine signaling
SPICE: simplified presentation of incredibly complex evaluations
SSC: side scatter
$TCID_{50}$: 50% tissue culture infective dose
TCR: T cell receptor
Th: T helper
TNF: Tumor necrosis factor
TPA: 12-*O*-tetradecanoylphorbol-13-acetate
TLR: Toll-like receptor
TREG: T regulatory cells
UV: ultraviolet
VEGF: vascular endothelial growth factor
vFLIP: viral FLICE-inhibitor protein
vGPCR: viral G-protein coupled receptor
vIRF3: viral Interferon regulatory factor

APPENDIX B. SUPPLEMENTAL FIGURES

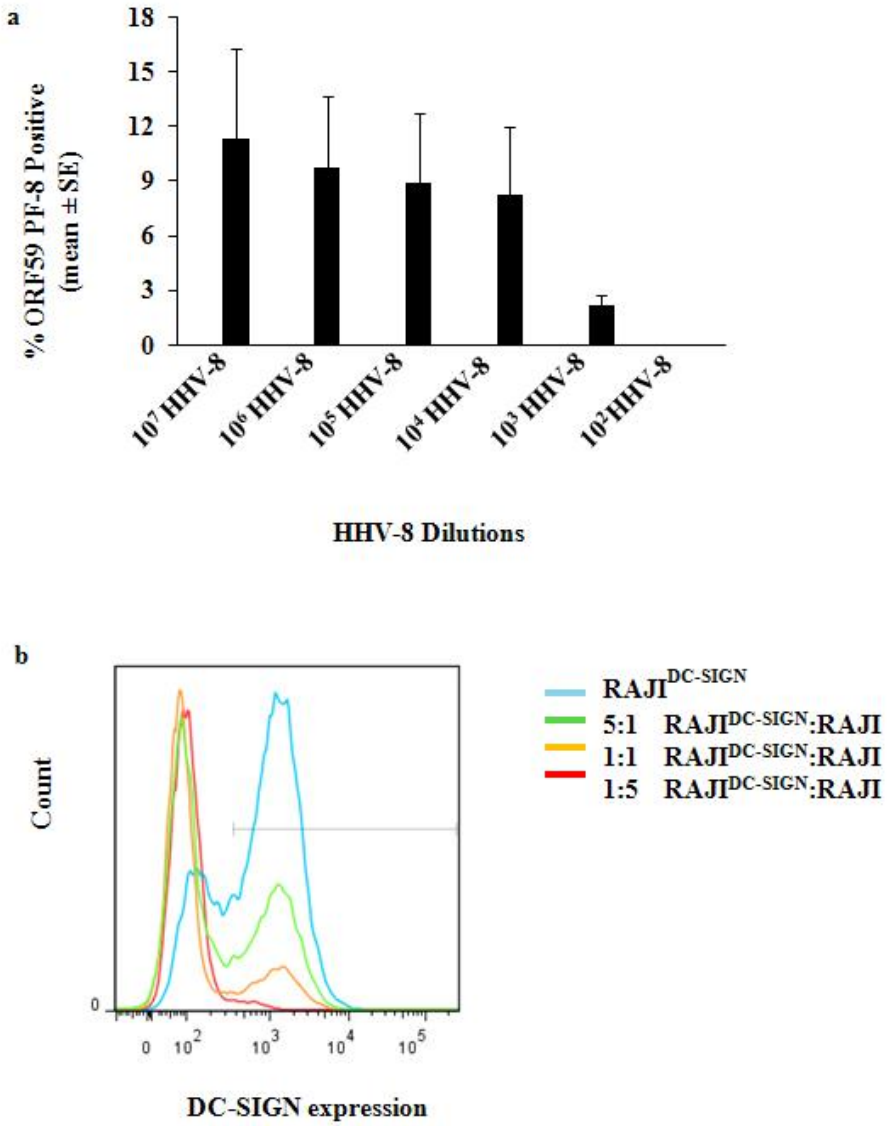

Figure 23: Detection of HHV-8 in DC-SIGN transfected RAJI and K562 cell lines

(A) K562$^{DC\text{-}SIGN+}$ were exposed to 10^7 copies of HHV-8 and 5 subsequent 10-fold dilutions. The percentage of ORF59 PF-8$^+$ cells were determined by flow cytometry at 48 hpe (mean±s.e.m., N=3). *(B)* RAJI and RAJI $^{DC\text{-}SIGN+}$ cells were mixed in different ratios and expression of DC-SIGN was determined by flow cytometry.

Table 2: DC-SIGN dependent detection of K8.1

Cells	HHV-8 K8.1 % Positive
RAJI$^{DC\text{-}SIGN}$	29.4%
5:1 RAJI$^{DC\text{-}SIGN}$: RAJI	26.3%
1:1 RAJI$^{DC\text{-}SIGN}$: RAJI	5.9%
1:5 RAJI$^{DC\text{-}SIGN}$: RAJI	1.3%

Different ratios of RAJI and RAJI $^{DC\text{-}SIGN+}$ cells were exposed to HHV-8 for 3 h. Cell samples were collected 48 hpe and stained intracellularly for HHV-8 K8.1 (mean, N=3).

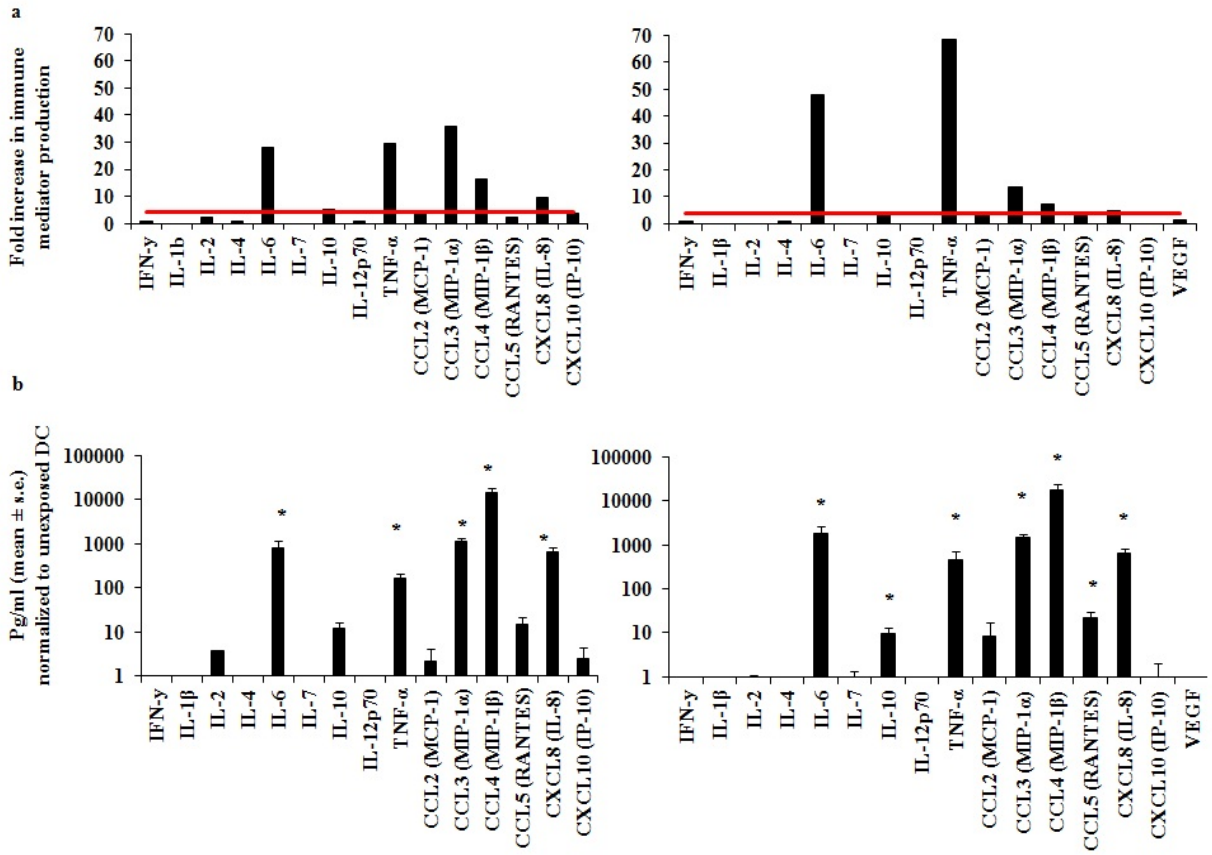

Figure 24: B cell cytokine and chemokine screen

B cells were left untreated or exposed to HHV-8. Supernatant samples were collected at 24 (left panels) and 48 (right panels) hpe and screened by CBA for 16 immune mediators. *(A)* Fold increase over unexposed B cells and *(B)* normalized concentrations above unexposed B cells (mean ± s.e.m., N = 1 [IL-1β, IL-4, IL-7], 3 [IL-2, MCP-1, IL-12p70, IP-10], 7 [RANTES, IFN-γ], 9 [IL-10], 11 [VEGF], 22 [IL-8], 23 [TNF-α, IL-6, MIP1α and MIP-1β].

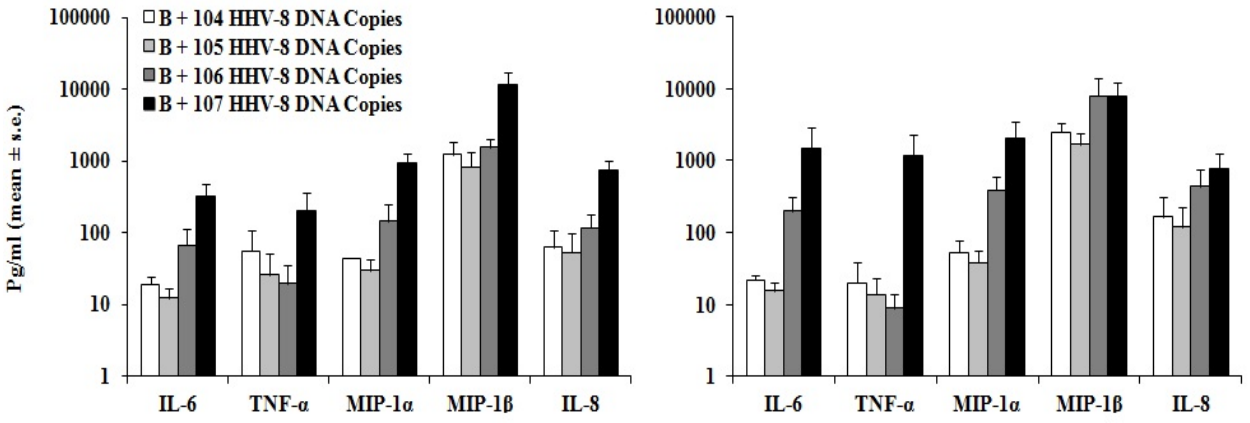

Figure 25: Immune mediator induction is HHV-8 dose dependant

B cells were exposed to 10^7 copies of HHV-8 and 3 subsequent 10-fold dilutions. The concentration of immune mediators were determined by CBA 24 (left), and 48 h.p.e. (mean±s.e.m., N=3).

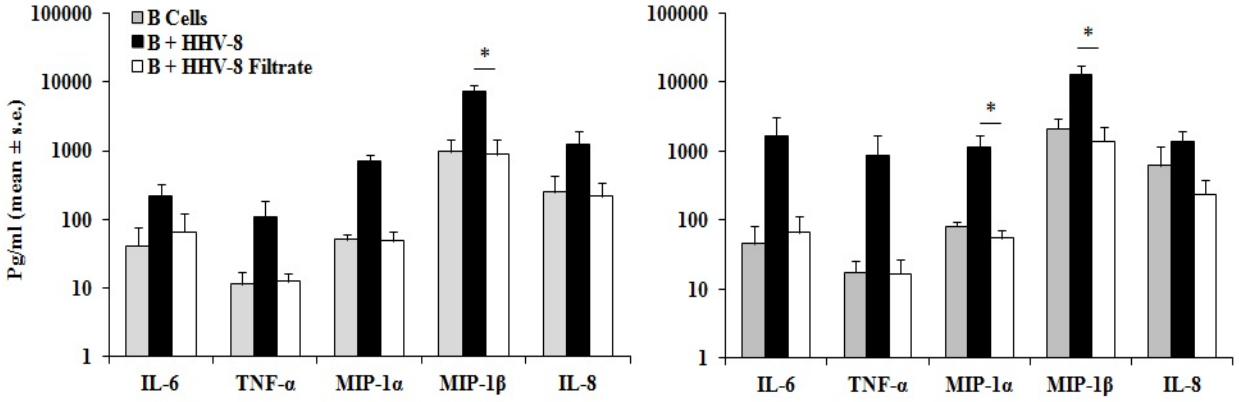

Figure 26: HHV-8 filtrate does not induce immune mediator response

B cells were exposed to HHV-8 or HHV-8 that had been passed through a 0.1µm filter. The concentration of immune mediators were determined by CBA 24 (left) and 48 hpe (mean±s.e.m., N=4, **P*<0.05).

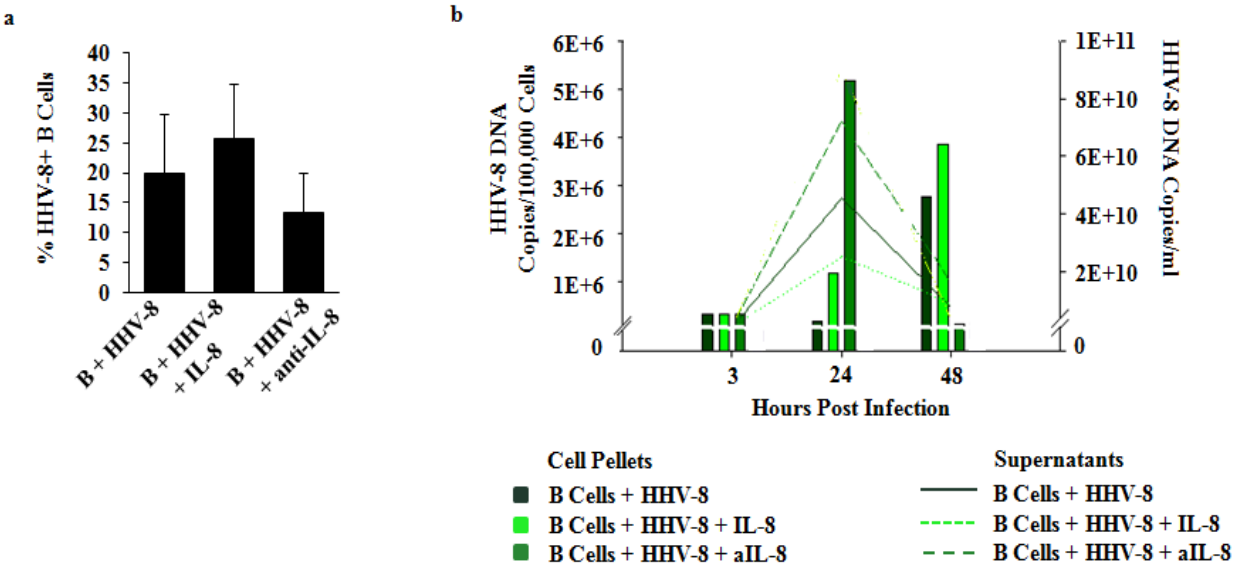

Figure 27: IL-8 enhanced HHV-8 replication

B cells were exposed to HHV-8 alone or in the presence of recombinant IL-8 or IL-8 neutralizing antibodies (anti-IL-8). *(A)* B cells were stained at 48 hpe for ORF59 PF-8 or K8.1 expression (mean±s.e.m., N=3). *(B)* Cells were collected at 3, 24 and 48 hpe and assayed by qRT-PCR for K8.1 DNA (data are representative of 2 individuals tested).

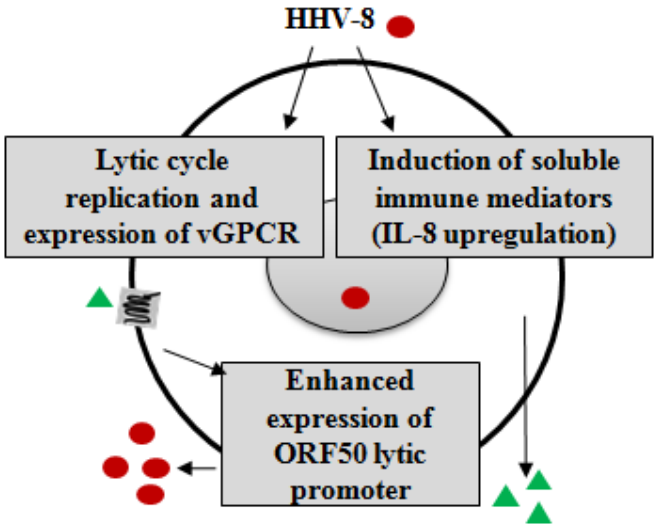

Figure 28: Model for IL-8 induced HHV-8 replication

Hypothetical role of IL-8 in HHV-8 replication binding to HHV-8 vGPCR to enhance ORF50 promoter expression resulting in virion formation

APPENDIX C. MICROARRAY DATA

We exposed B cells to HHV-8 for 3h and then washed unadsorbed virus from the culture using 2 centrifugations as described in section 3.2.5. Cells were collected at 3 (immediately after the wash), 4, 6, 9, 15 and 27 h post HHV-8 exposure. RNA was extracted and used in an Illumina HT12v4 whole-cellular genome microarray and a quantitative real-time PCR assay using primer-pair sets for HHV-8 open reading frames.

Microarray data was received from the Genomics and Proteonomics Core Lab as absolute gene values on duplicate slides. Fold increases in gene expression from unexposed to HHV-8 exposed B cells was calculated for both slides and averaged. Raw data for 34,592 genes is linked as an xcell file. To minimize the number of genes in focus, a filter was placed on the data to determine the number of genes that had at least a 1.5-fold increase or decrease in gene expression for each of 6 time points (**Fig. 29**). A total of 2,030 genes were up regulated upon HHV-8 exposure over the course of one day compared to unexposed B cells, whereas less than 32 were down regulated. Genes displaying ≥1.5-fold increase were used in additional analysis using through the use of IPA (Ingenuity Systems, www.ingenuity.com) to determine the most prominent biological functions, canonical pathways, molecules and upstream regulators for each time point post virus-exposure. Graphs and tables were generated in IPA software. A summary for each time point in described in **Tables 3-8.**

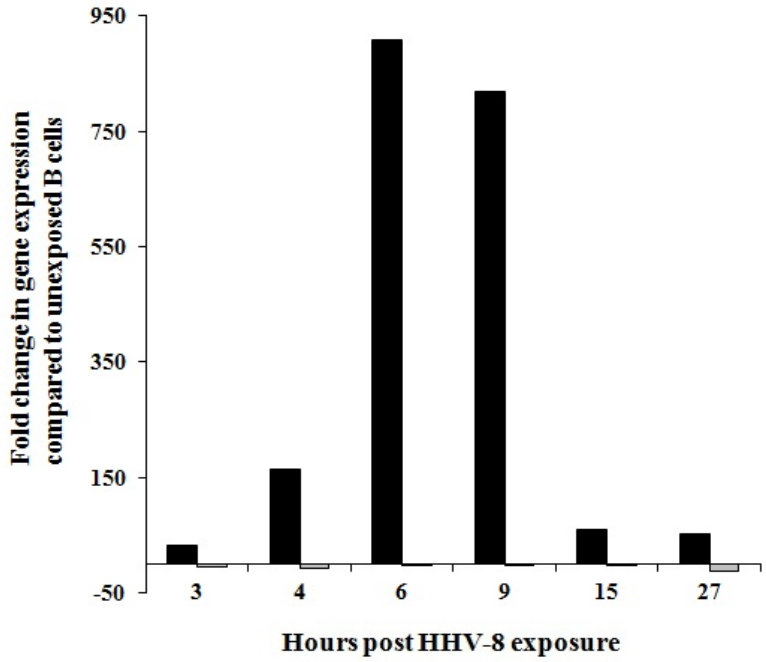

Figure 29: Fold change in B cell gene expression upon exposure to HHV-8

Unexposed and HHV-8 exposed B cells were used in an Illumina HT12v4 microarray for cellular gene expression at 6 time points. A 1.5-fold cutoff was placed on the data to determine the number of genes up or downregulated upon HHV-8 exposure.

Many of these top pathways included direct links to immune mediators, such as; communication between innate and adaptive immune cells, immune cell trafficking, cellular movement, inflammatory response and inflammatory disease. As immune mediator signaling was the focus of this aim, we determined the activation states of various pathways known to interact with or result in cytokine and chemokine production (reviewed in Table 1). A 1.5 fold filter was placed on all genes and applied to the Janus Kinase-Signal Transducer and Activator of Transcription (JAK/STAT) signaling pathway. The JAK/STAT pathway is a signaling mechanism for a wide array of cytokines and growth factors. JAK activation stimulates cell proliferation, differentiation, cell migration and apoptosis. At 6 hours post HHV-8 exposure, the

most molecules within the JAK/STAT pathway was enhanced, including, STAT, SHP1, PI3K, STAT3, c-FOS and IL-6 (**Fig30)**. At the same time post HHV-8 exposure, the ERK/MAPK signaling pathway also possessed the highest number of enhanced molecules (**Fig. 31**). The ERK/MAPK signal transduction pathways respond to various extracellular stimuli, ranging from growth factors and cytokines to cellular stress. Genes that were up-regulated within the ERK/MAPK pathway included PKA, PKS, Src, PI3K, PAC1, MKP 1/2/3/4, c-MYC/N-Myc, NFATc1, MP1, ATF-1, CREB, cFOS and STAT 1/3. There was also enhanced expression of molecules in the NF-κB pathway, several of which are activated by other herpesviruses (EBV, HSV), including PKC, PI3K, HVEM and IκB (**Fig 32).** NF-κB is involved in cellular responses to stimuli such as stress, cytokines and bacterial or viral antigens.

As expected, B-cell receptor signaling was activated, with 7, 9 and 11 molecules enhanced in HHV-8 exposed B cells at 4, 6, and 9 hours, respectively (**Fig. 33-35).** CD19, MEKK, Bam32, SHIP, CaM, Egr-1 and Bcl-6 were up-regulated at 4 hours, SHP-1, PI3K, SHIP, MALT1, RP2B, NFAT, IκB, Egr-1 and CREB were up-regulated at 6 hours, while CD22, SHP1, BCAP, SHP2, MEKK, MKK3/4/6, MKK4/7, MALT1, Cam, IκB and Egr-1 were up-regulated at 9 hours.

As we originally determined proteins levels of 16 different cytokines, chemokines and growth factors in our CBA assay, we determined the relative fold increase for each of these markers at the RNA level (**Fig. 12a**). The majority of genes, including; IFN-γ, IL-1β, IL-2, IL-4,

Table 3: 3h microarray analysis

3 Hours Post HHV-8 Exposure	P value	Number of Molecules or Ratio	Fold	Predicted activation state
TOP NETWORKS				
Connective Tissue Disorders, Immunological Disease, Inflammatory Disease				
Hematological Disease, Infectious Disease, Cell Death				
Cell-To-Cell Signaling and Interaction, Cell-mediated Immune Response, Cellular Movement				
TOP BIOLOGICAL FUNCTIONS				
Diseases and Disorders				
Connective Tissue Disorders	4.42E-08 - 2.58E-02	7		
Immunological Disease	4.42E-08 - 1.88E-02	7		
Inflammatory Disease	4.42E-08 - 2.58E-02	7		
Skeletal and Muscular Disorders	4.42E-08 - 2.58E-02	9		
Neurological Disease	5.66E-05 - 2.23E-02	9		
Molecular and Cellular Functions				
Cellular Movement	2.77E-05 - 2.81E-02	8		
Cell Death	6.99E-05 - 2.60E-02	12		
Cell-To-Cell Signaling and Interaction	8.68E-05 - 2.81E-02	9		
Cellular Growth and Proliferation	1.08E-04 - 2.81E-02	12		
Gene Expression	1.25E-04 - 2.81E-02	10		
Physiological System Development and Function				
Behavior	6.24E-06 - 2.92E-02	8		
Skeletal and Muscular System Development and Function	2.77E-05 - 2.69E-02	7		
Hematological System Development and Function	4.81E-05 - 3.04E-02	10		
Immune Cell Trafficking	8.68E-05 - 3.02E-02	5		
Connective Tissue Development and Function	1.08E-04 - 2.02E-02	9		
TOP CANONICAL PATHWAY				
Glucocorticoid Receptor Signaling	2.15E-04	4/294 (0.014)		
ERK5 Signaling	2.40E-03	2/64 (0.031)		
CCR5 Signaling in Macrophages	2.97E-03	2/94 (0.021)		
CDK5 Signaling	4.67E-03	2/94 (0.021)		
Communication between Innate and Adaptive Immune Cells	5.21E-03	2/110 (0.018)		
TOP MOLECULES				
Fold Change Up-regulated				
CCL3L1/CCL3L3			6.48	
CCL3			5.13	
BAG3			3.67	
NR4A2			3.51	
RGS2 (includes EG:19735)			3.05	
EGR1			2.76	
CCL4L1/CCL4L2			2.42	
EGR2			2.25	
HSPA6			2.19	
FOSB			2.15	
TOP UPSTREAM REGULATORS				
CREB1	3.15E-09			inhibited
FOSL1	3.53E-08			inhibited
NR3C2 (includes EG:100534796)	3.25E-07			inhibited
CREBBP	6.51E-07			inhibited
ELK4	9.33E-07			inhibited

Table 4: 4h microarray analysis

4 Hours Post HHV-8 Exposure	P value	Number of Molecules or Ratio	Fold	Predicted activation state
TOP NETWORKS				
Antimicrobial Response, Infectious Disease, Hematological System Development and Function				
Cell-mediated Immune Response, Cellular Development, Cellular Function and Maintenance				
Protein Synthesis, Cardiovascular Disease, Cellular Compromise				
Cancer, Hematological Disease, Connective Tissue Disorders				
Cellular Growth and Proliferation, Cell Cycle, Inflammatory Response				
TOP BIOLOGICAL FUNCTIONS				
Diseases and Disorders				
Connective Tissue Disorders	1.14E-14 - 4.62E-04	23		
Immunological Disease	1.14E-14 - 1.32E-04	27		
Inflammatory Disease	1.14E-14 - 4.62E-04	26		
Skeletal and Muscular Disorders	1.14E-14 - 4.66E-04	31		
Cancer	8.90E-10 - 4.45E-04	33		
Molecular and Cellular Functions				
Cellular Movement	4.24E-11 - 3.85E-04	35		
Cellular Growth and Proliferation	4.24E-11 - 4.25E-04	36		
Cell Death	9.32E-11 - 4.25E-04	34		
Cell Cycle	3.56E-09 - 3.68E-04	15		
Cellular Function and Maintenance	4.56E-08 - 3.91E-04	26		
Physiological System Development and Function				
Hematological System Development and Function	3.88E-12 - 4.57E-04	28		
Tissue Morphology	3.88E-12 - 4.62E-04	28		
Hematopoiesis	8.08E-10 - 2.85E-04	20		
Lymphoid Tissue Structure and Development	8.21E-09 - 4.68E-04	19		
Embryonic Development	2.31E-08 - 3.37E-04	20		
TOP CANONICAL PATHWAY				
TNFR2 Signaling	6.28E-08	4/34 (0.17)		
TNFR1 Signaling	2.85E-05	4/53 (0.075)		
Differential Regulation of Cytokine Production in Macrophages and T Helper Cells by IL-17A and IL-17F	3.72E-05	3/18 (0.167)		
Altered T Cell and B Cell Signaling in Rheumatoid Arthritis	2.81E-04	4/92 (0.043)		
Crosstalk between Dendritic Cells and Natural Killer Cells	3.20E-04	4/95(0.042)		
TOP MOLECULES				
Fold Change Up-regulated				
SNORD3A			6.96	
FOS			5.55	
EGR1			4.36	
CCL3L1/CCL3L3			3.66	
CCL3			3.41	
CD69			3.36	
RGS1			2.47	
FOSB			2.46	
CDKN1A			2.36	
EGR2			2.34	
TOP UPSTREAM REGULATORS				
NFkB (complex)	1.57E-11			inhibited
STAT5A	5.30E-11			inhibited
ZFP36	1.22E-10			inhibited
CREB1	2.76E-10			inhibited
FOSL1	4.60E-10			inhibited

Table 5: 6h microarray analysis

6 Hours Post HHV-8 Exposure	P value	Number of Molecules or Ratio	Fold	Predicted activation state
TOP NETWORKS				
RNA Post-Transcriptional Modification, Cancer, Cell Death				
Cell-mediated Immune Response, Cellular Development, Cellular Function and Maintenance				
Cellular Assembly and Organization, DNA Replication, Recombination, and Repair, Small Molecule Biochemistry				
Developmental Disorder, Hereditary Disorder, Skeletal and Muscular Disorders				
Cellular Development, Connective Tissue Development and Function, Inflammatory Disease				
TOP BIOLOGICAL FUNCTIONS				
Diseases and Disorders				
Connective Tissue Disorders	9.44E-09 - 4.22E-03	38		
Immunological Disease	9.44E-09 - 6.32E-03	62		
Inflammatory Disease	9.44E-09 - 6.32E-03	50		
Skeletal and Muscular Disorders	9.44E-09 - 7.29E-03	64		
Cancer	2.38E-06 - 7.70E-03	104		
Molecular and Cellular Functions				
Cellular Development	2.51E-14 - 7.15E-03	96		
Cellular Growth and Proliferation	2.51E-14 - 6.91E-03	109		
Cell Death	2.82E-12 - 7.29E-03	108		
Cellular Function and Maintenance	2.65E-07 - 7.39E-03	60		
Gene Expression	2.89E-07 - 3.48E-03	74		
Physiological System Development and Function				
Hematological System Development and Function	4.83E-14 - 7.75E-03	76		
Tissue Morphology	4.83E-14 - 7.75E-03	60		
Cell-mediated Immune Response	3.41E-07 - 7.75E-03	29		
Hematopoiesis	5.40E-07 - 6.91E-03	39		
Lymphoid Tissue Structure and Development	5.40E-07 - 6.91E-03	40		
TOP CANONICAL PATHWAY				
Communication between Innate and Adaptive Immune Cells	1.28E-05	9/110 (0.082)		
Crosstalk between Dendritic Cells and Natural Killer Cells	7.19E-05	8/95 (0.084)		
Dendritic Cell Maturation	4.93E-04	10/207 (0.048)		
Systemic Lupus Erythematosus Signaling	2.43E-03	10/248 (0.04)		
Antigen Presentation Pathway	2.50E-03	4/40 (0.10)		
TOP MOLECULES				
Fold Change Up-regulated				
CCL3L1/CCL3L3			17.011	
CCL3			10.82	
CL4L1/CCL4L2			8.48	
EGR2			4.07	
SGK1			3.33	
ID3 (includes EG:15903)			3.16	
NR4A2			3.08	
FOSB			3.08	
LRMP			3.01	
RGS2 (includes EG:19735)			2.96	
TOP UPSTREAM REGULATORS				
CREB1	5.68E-11			
FOSL1	1.32E-06			
REL	3.36E-06			
NFATC2	4.83E-06			
NR3C1	8.69E-06			

Table 6: 9h microarray analysis

9 Hours Post HHV-8 Exposure	P value	Number of Molecules or Ratio	Fold	Predicted Activation State
TOP NETWORKS				
RNA Post-Transcriptional Modification, Gene Expression, Cell Cycle				
Post-Translational Modification, Lipid Metabolism, Nervous System Development and Function				
RNA Post-Transcriptional Modification, Humoral Immune Response, Protein Synthesis				
Cellular Development, Nervous System Development and Function, Visual System Development and Function				
DNA Replication, Recombination, and Repair, Cell Cycle, Cellular Growth and Proliferation				
TOP BIOLOGICAL FUNCTIONS				
Diseases and Disorders				
Inflammatory Response	4.64E-07 - 1.13E-02	41		
Connective Tissue Disorders	5.31E-06 - 1.05E-02	35		
Immunological Disease	5.31E-06 - 1.16E-02	61		
Inflammatory Disease	5.31E-06 - 1.05E-02	29		
Skeletal and Muscular Disorders	5.31E-06 - 1.05E-02	58		
Molecular and Cellular Functions				
Cellular Development	1.44E-10 - 1.16E-02	119		
Cellular Growth and Proliferation	1.44E-10 - 1.16E-02	123		
Gene Expression	1.08E-07 - 8.53E-02	87		
Cell Death	4.01E-07 - 1.16E-02	107		
Cell-To-Cell Signaling and Interaction 4.64E-07 - 9.99E-	4.64E-07 - 9.99E-02	42		
Physiological System Development and Function				
Hematological System Development and Function	1.75E-10 - 1.16E-02	71		
Tissue Morphology	1.06E-08 - 1.16E-02	61		
Hematopoiesis	2.30E-08 - 1.16E-02	51		
Humoral Immune Response	1.11E-07 - 1.16E-02	40		
Immune Cell Trafficking	4.64E-07 - 1.08E-02	37		
TOP CANONICAL PATHWAY				
Cell Cycle: G1/S Checkpoint Regulation	9.00E-04	6/65 (0.092)		
RAN Signaling	3.69E-03	3/24 (0.125)		
GADD45 Signaling	5.11E-03	3/22 (0.136)		
CDK5 Signaling	5.96E-03	6/94 (0.064)		
Communication between Innate and Adaptive Immune Cells	7.79E-03	6/110 (0.055)		
TOP MOLECULES				
Fold Change Up-regulated				
CCL4L1/CCL4L2			11.692	
CCL3L1/CCL3L3			8.96	
CCL3			6.48	
LRMP			4.52	
EGR2			4.50	
RGS2 (includes EG:19735)			2.95	
PHACTR1			2.91	
MYC			2.69	
TRIB1			2.62	
STAT5A			2.50	
TOP UPSTREAM REGULATORS				
MYC	4.95E-06			activated
EGR1	8.15E-06			activated
ZBTB17	1.01E-05			activated
NFATC2	2.63E-05			activated
MYCN	6.28E-05			activated

Table 7: 15h microarray analysis

15 Hours Post HHV-8 Exposure	P value	Number of Molecules or Ratio	Fold	Predicted Activation State
TOP NETWORKS				
Antigen Presentation, Cell-To-Cell Signaling and Interaction, Hematological System Development and Function				
Cancer, Developmental Disorder, Hematological Disease				
Cellular Movement, Skeletal and Muscular System Development and Function, Connective Tissue Disorders				
TOP BIOLOGICAL FUNCTIONS				
Diseases and Disorders				
Cancer	4.01E-06 - 8.85E-03	15		
Developmental Disorder	4.01E-06 - 5.32E-03	4		
Hereditary Disorder	4.01E-06 - 8.85E-03	4		
Renal and Urological Disease	4.01E-06 - 8.85E-03	5		
Reproductive System Disease	4.01E-06 - 6.81E-03	4		
Molecular and Cellular Functions				
Cell-To-Cell Signaling and Interaction	1.09E-06 - 8.85E-03	11		
Cellular Growth and Proliferation	1.09E-06 - 8.85E-03	19		
Cellular Movement	3.29E-06 - 8.85E-03	11		
Cellular Development	7.53E-06 - 8.85E-03	17		
Cellular Function and Maintenance	1.26E-05 - 8.85E-03	10		
Physiological System Development and Function				
Connective Tissue Development and Function	1.09E-06 - 8.82E-03	7		
Hematological System Development and Function	2.12E-06 - 8.85E-03	14		
Immune Cell Trafficking	2.12E-06 - 8.85E-03	9		
Tissue Development	2.12E-06 - 8.85E-03	13		
Skeletal and Muscular System Development and Function	3.29E-06 - 8.82E-03	8		
TOP CANONICAL PATHWAY				
Cell Cycle: G1/S Checkpoint Regulation	1.60E-04	3/65 (0.046)		
GADD45 Signaling	5.10E-04	2/22 (0.091)		
Communication between Innate and Adaptive Immune Cells	5.65E-04	3/110 (0.027)		
p53 Signaling	5.83E-04	3/96 (0.031)		
Colorectal Cancer Metastasis Signaling	7.75E-04	4/257 (0.016)		
TOP MOLECULES				
Fold Change Up-regulated				
CCL4L1/CCL4L2			3.956	
EGR2			3.545	
CCL3L1/CCL3L3			3.429	
LRMP			3.119	
PHACTR1			2.831	
MYC			2.815	
CCND2			2.70	
CCL3			2.59	
SERPINE2			2.24	
LMO2			2.19	
TOP UPSTREAM REGULATORS				
EGR1	1.14E-06			
CTNNb-LEF1	6.90E-06			
FOSL1	1.07E-05			
STAT1	1.83E-05			
STAT3	1.98E-05			

Table 8: 27h microarray analysis

27 Hours Post HHV-8 Exposure	P value	Number of Molecules or Ratio	Fold	Predicted Activation State
TOP NETWORKS				
Cellular Assembly and Organization, Nervous System Development and Function, Cell Death				
Nucleic Acid Metabolism, Small Molecule Biochemistry, Molecular Transport				
Tissue Morphology, Cellular Movement, Immune Cell Trafficking				
TOP BIOLOGICAL FUNCTIONS				
Diseases and Disorders				
Hereditary Disorder	1.42E-04 - 2.92E-02	12		
Neurological Disease	1.42E-04 - 1.67E-02	10		
Skeletal and Muscular Disorders	1.42E-04 - 2.28E-02	8		
Dermatological Diseases and Conditions	4.63E-04 - 2.60E-02	7		
Inflammatory Disease	4.63E-04 - 2.60E-02	4		
Molecular and Cellular Functions				
Small Molecule Biochemistry	2.59E-05 - 4.98E-02	13		
Post-Translational Modification	1.92E-04 - 4.19E-02	8		
Cell Death	2.69E-04 - 4.98E-02	10		
Cellular Development	3.27E-04 - 4.12E-02	11		
Cellular Growth and Proliferation	3.27E-04 - 4.51E-02	11		
Physiological System Development and Function				
Digestive System Development and Function	1.07E-03 - 1.63E-02	4		
Embryonic Development	1.65E-03 - 4.67E-02	6		
Nervous System Development and Function	1.65E-03 - 4.98E-02	7		
Organismal Development	1.65E-03 - 3.56E-02	6		
Skeletal and Muscular System Development and Function	1.65E-03 - 3.72E-02	4		
TOP CANONICAL PATHWAY				
Arginine and Proline Metabolism	2.27E-04	3/177 (0.017)		
Methionine Metabolism	1.03E-03	2/78 (0.026)		
Urea Cycle and Metabolism of Amino Groups	1.25E-03	2/78 (0.026)		
Valine, Leucine and Isoleucine Degradation	5.07E-03	2/107 (0.019)		
Tyrosine Metabolism	6.36E-03	2/198 (0.01)		
TOP MOLECULES				
Fold Change Up-regulated				
LRMP			2.519	
NME1 (includes EG:18102)			2.264	
PHACTR1			2.203	
FABP5			2.194	
CCND2			2.139	
ZBED2			2.042	
DHRS9			2.03	
IL4I1			1.99	
NPW			1.99	
RPL29 (includes EG:367874)Δ			1.95	
TOP UPSTREAM REGULATORS				
MYC	9.75E-08			activated
MYCBP	1.10E-04			activated
MYCN	1.44E-04			activated
MXD1	2.34E-04			activated
MNT	8.77E-04			activated

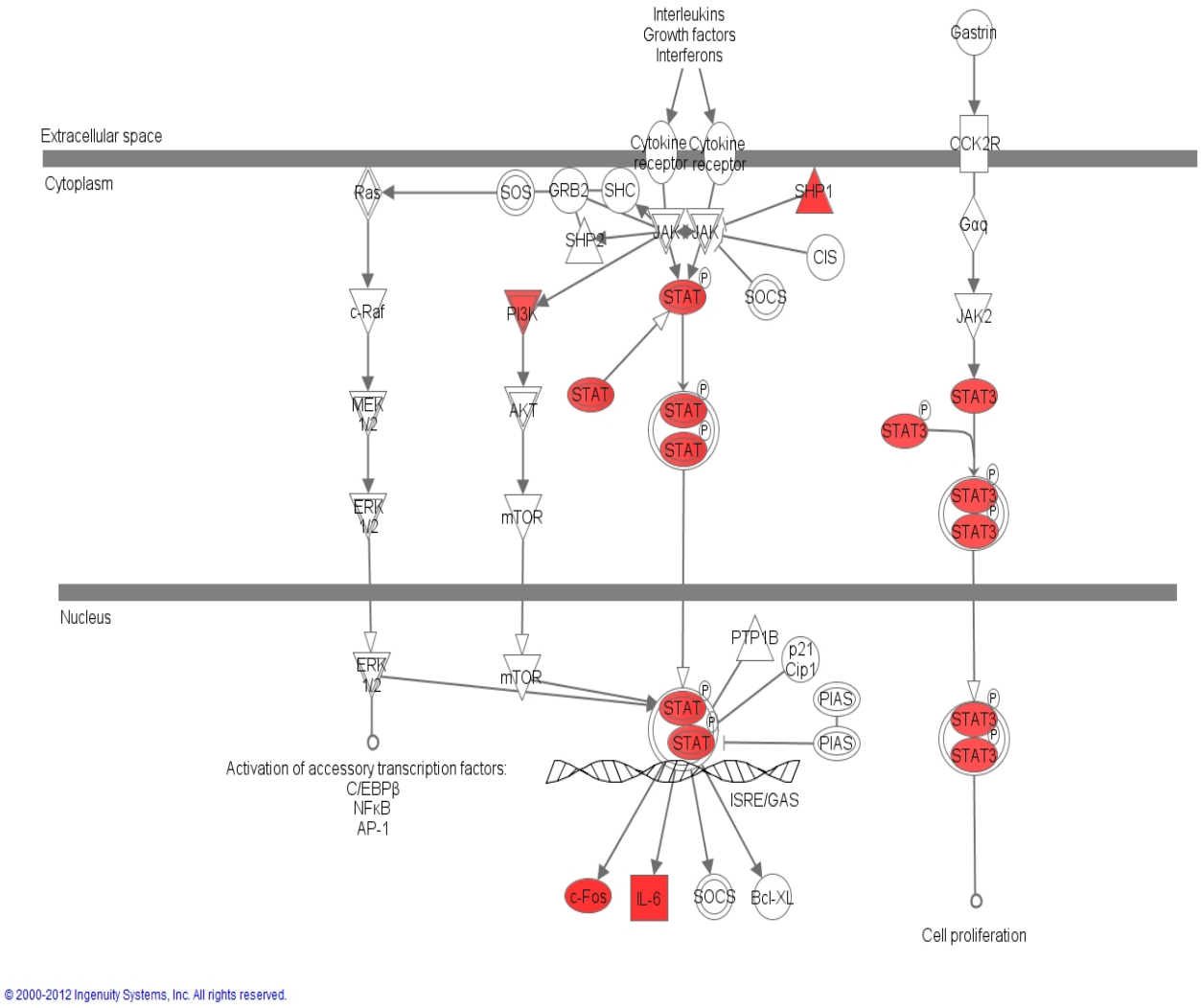

Figure 30: JAK/STAT signaling activation at 6 hours

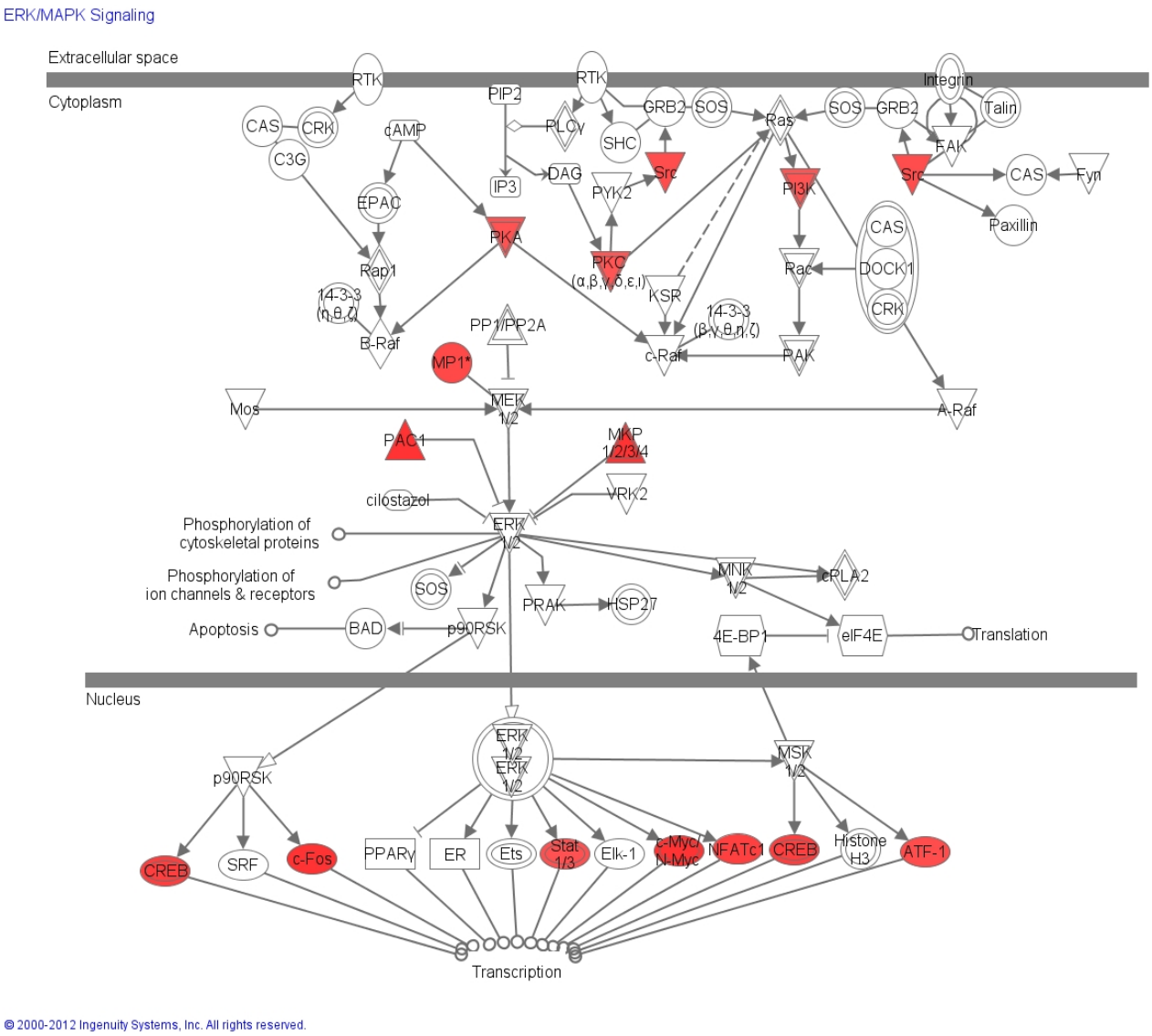

Figure 31: ERK/MAPK signaling activation at 6 hours

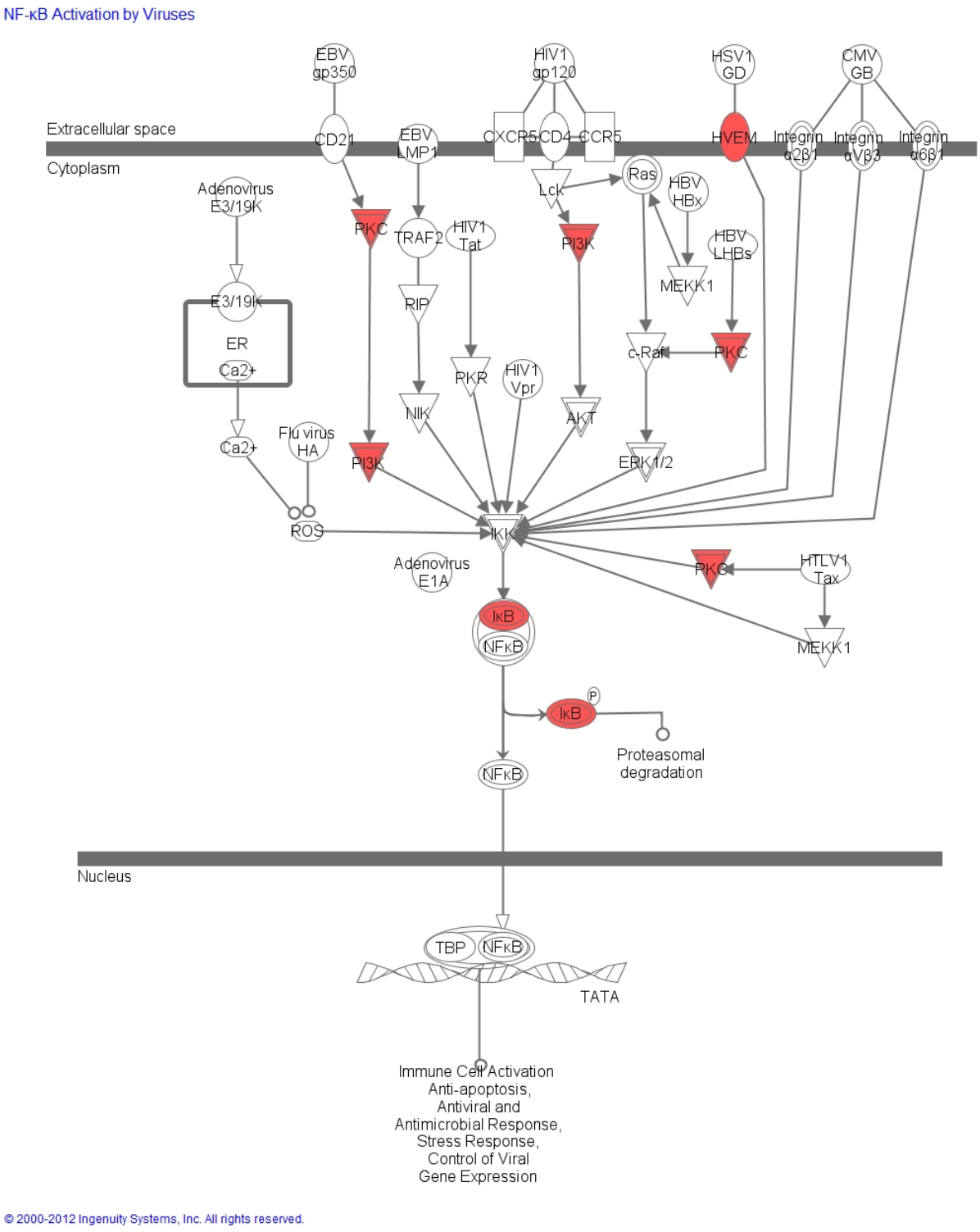

Figure 32: NF-κB signaling activation at 6 hours

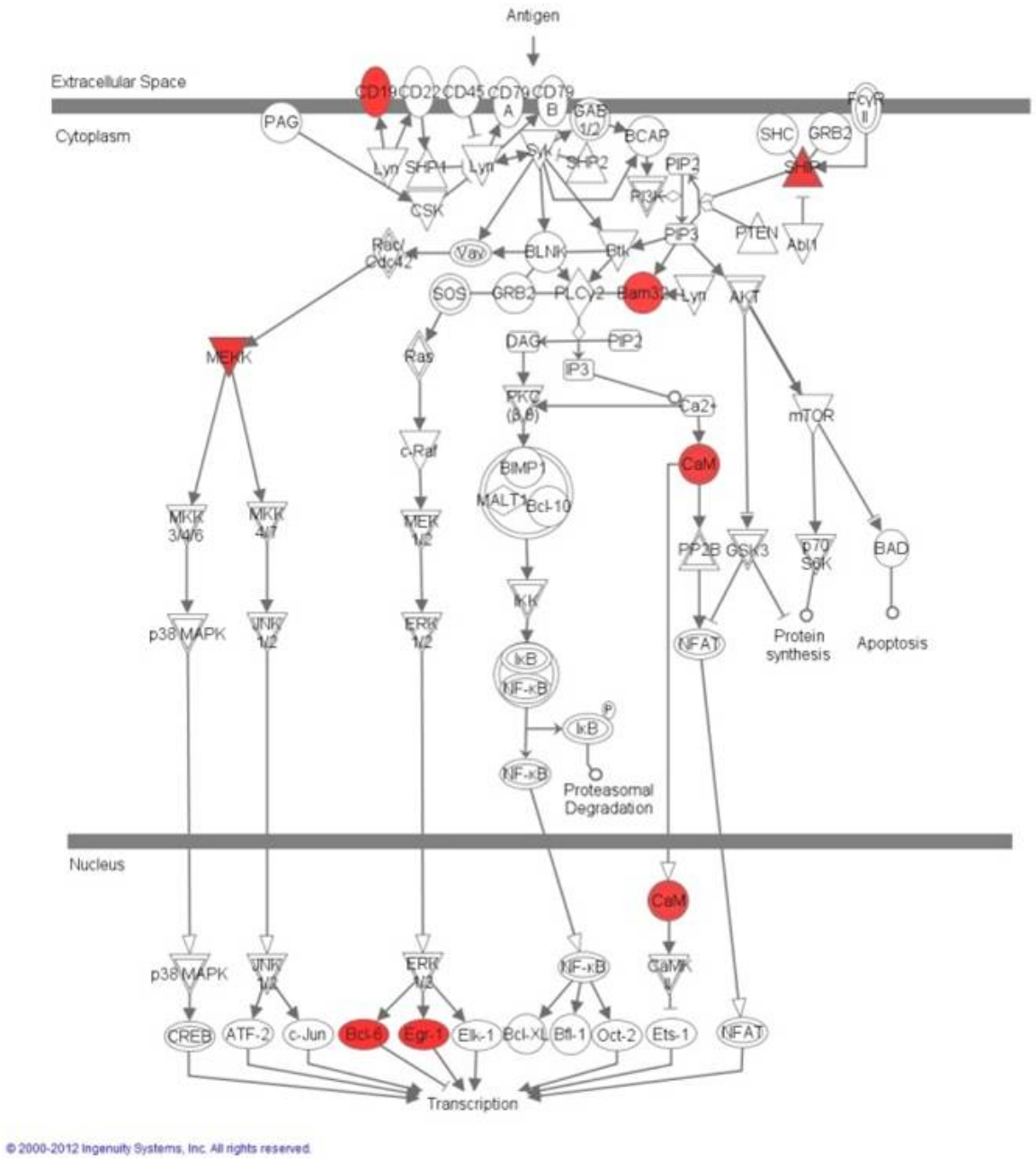

Figure 33: B cell receptor signaling at 4 hours

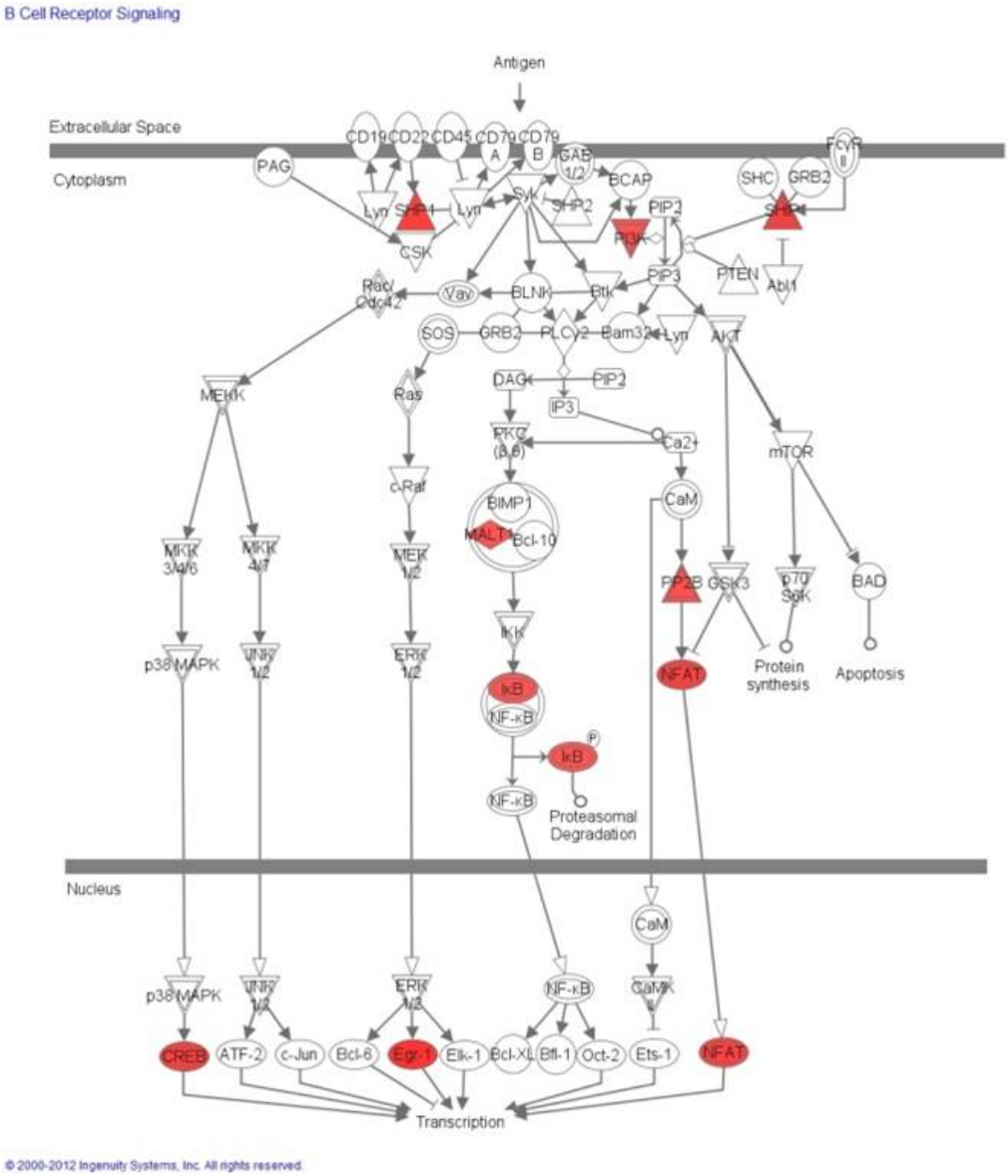

Figure 34: B cell receptor signaling at 6 hours

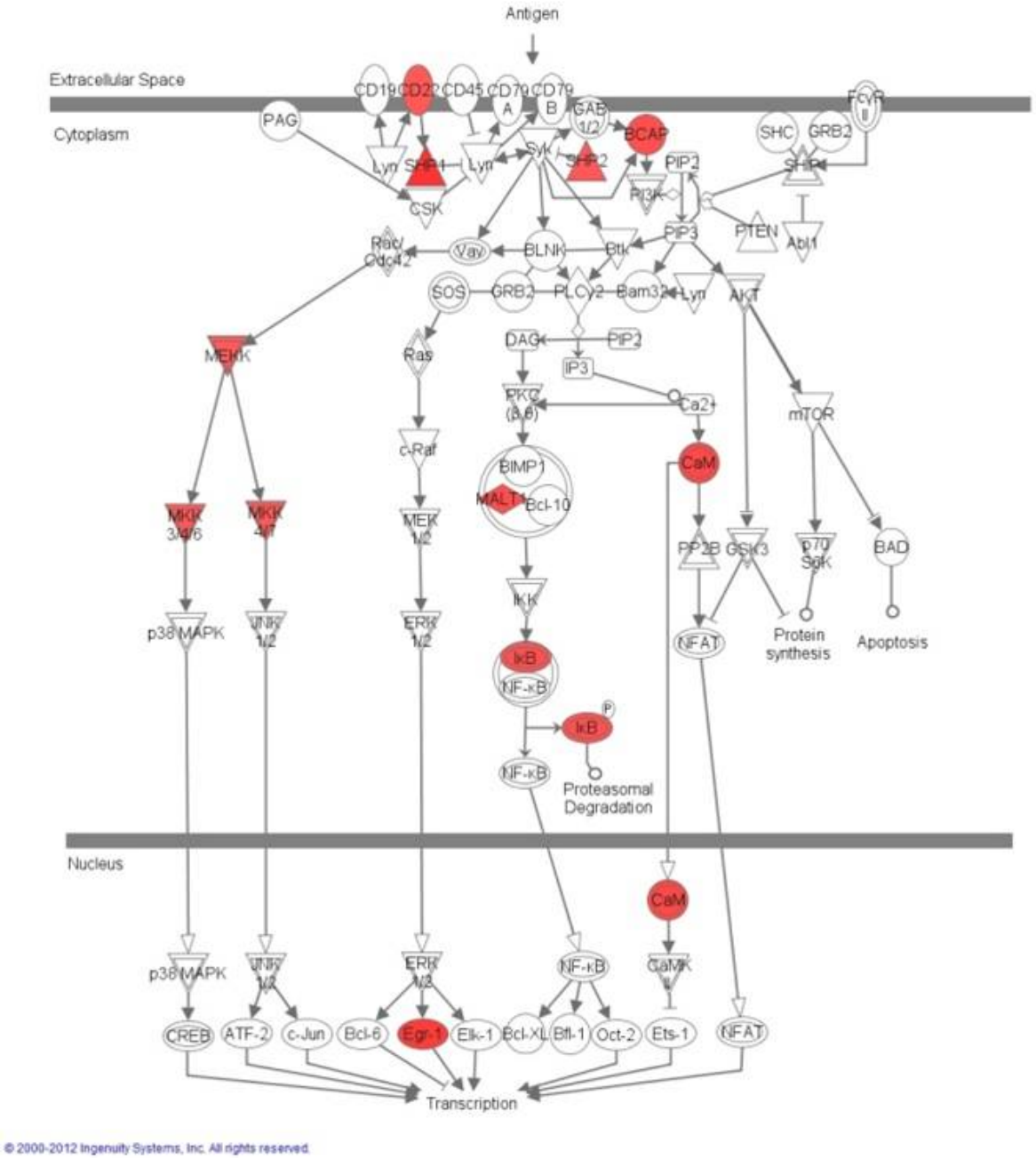

Figure 35: B cell receptor signaling at 9 hours

IL-7, IL-12A/B, CCL2 (MCP-1), CCL4 (MIP-1β), CCL5 (RANTES), CXCL8 (IL-8), CXCL10 (IP-10) and VEGF-A/B/C, did not have ≥1.5-fold increase in expression in the HHV-8 exposed compared to unexposed B cells. For most genes this was consistent with protein assays. However, RNA levels for IL-8 and MIP-1β were not enhanced, whereas protein levels were greatly enhanced above background levels. Interestingly, there was a 1.5, 2 and >2.5 fold increase detected in the two MIP-1β like genes, CCL4-L1 and CCL4-L2. There is approximately 95% homology shared between the CCL4 and CCL4-Like genes at both the genomic and amino acid level (60), implying the CBA protein assay may detect CCL4 as well as the CCL4-Like genes. The same sequence identity homology is found between the CCL3 and CCL3-Like genes (60). CCL3 and its like genes had enhanced RNA expression of ≥1.5-fold for all time points, with the highest fold increase detected for CCL3-L3 of 16 fold (**Fig. 12a**.) Enhances were also detected for IL-6, TNF-α and IL-10.

C.1 APPENDIX SECTION

Parallel studies on unexposed and HHV-8 exposed B cells were conducted by Dr. Jun Li of the Jenkins Laboratory, University of Pittsburgh, Department of Pathology. HHV-8 ORF primer-pair quantitative RT-PCR (89) was used to determine the kinetics of HHV-8 gene expression in B cells. The axis is represented as ΔCt. This refers to the normalized value between the target gene and the internal control gene, β-actin. The smaller the cT value, the more abundant the gene expression. ORF59 and K8.1 gene expression were elevated immediately after HHV-8 exposure, peaking at 4 hours (**Fig. 36**). The expression of these genes is consistent with

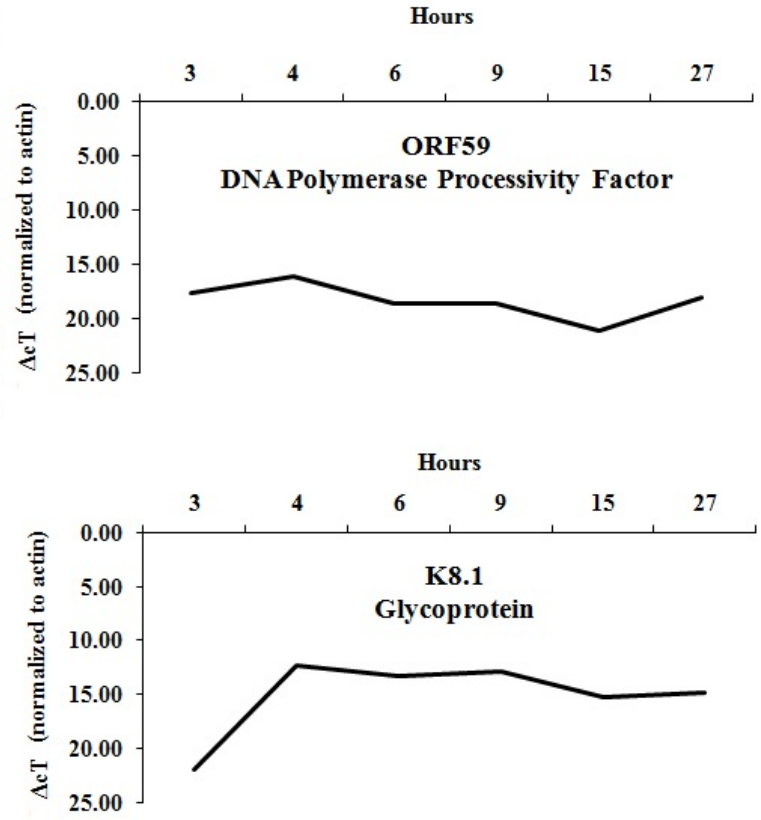

Figure 36: HHV-8 K8.1 and ORF59 gene expression kinetics

B cells were exposed to HHV-8 for 3 h and then collected after 2 washes and at 4, 6, 9, 15 and 27 hpe. RNA was extracted from B cells and used in a qRT-PCR assay using primer pairs for ORF K8.1 and ORF59.

the detection of HHV-8 K8.1 and ORF59 PF-8 proteins by intracellular staining and flow cytometry as early as 24 hpe.

As cytokine and chemokine induction was one of the main focuses of this study, it is important to note that HHV-8 encodes for a cytokine (vIL-6), 3 chemokines (vCCLI, II, III) (200) and a chemokine receptor (vGPCR) (12). Expression of vIL-6 increased over time with peak expression at 9 hours (**Fig. 37**). There is no primer pair for HHV-8 vCCL-III and vCCL-II only had detectable cT values at 6 h, but vCCL-I showed peak expression at 3 hours. vGPCR,

which greatly contributes to cytokine and chemokine induction and is a homologue of the IL-8 receptor(43), CXCR2, had peak expression at 4 hours post HHV-8 exposure.

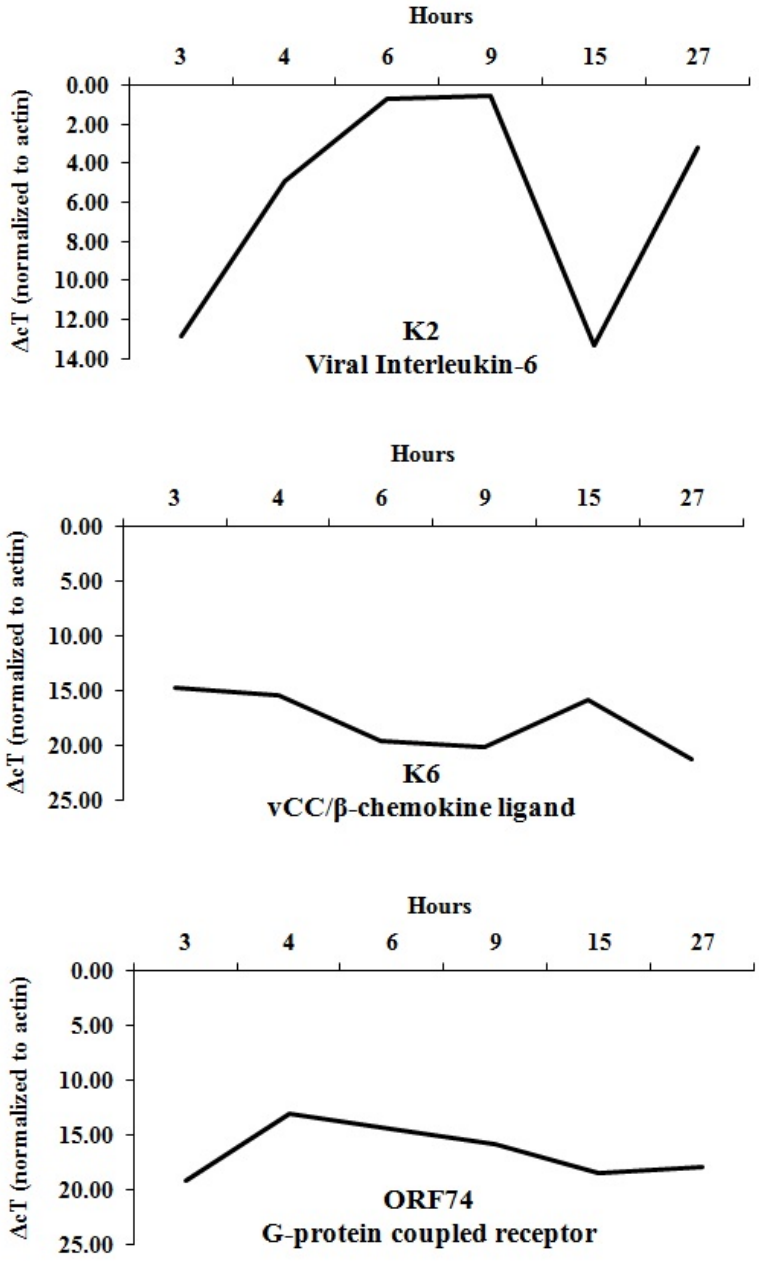

Figure 37: HHV-8 cellular homologue gene expression kinetics

B cells were exposed to HHV-8 for 3 h and then collected after 2 washes and at 4, 6, 9, 15 and 27 hpe. RNA was extracted from B cells and used in a qRT-PCR assay using primer pairs for ORF K2, ORF K6 and ORF74.

APPENDIX D. $TCID_{50}$ ASSAY DEVELOPMENT

A reliable assay to determine the infectivity of HHV-8 virion preps is lacking. A 293T cell line previously described (130) (see section 2.3.8) was transfected with DC-SIGN by Dr. Hensler of the Jenkins laboratory, resulting in T1-H6 DC-SIGN$^+$ cells. T1-H6 DC-SIGN$^+$ cells were next used to develop a $TCID_{50}$ assay to determine the number of infectious particles per HHV-8 DNA copies by Sagar Nadgir as his master's thesis. A $TCID_{50}$ is defined as the median tissue culture infective dose that produces pathological change in 50% of cell cultures inoculated. For purified HHV-8 preparations, a $TCID_{50}$ was first calculated using the Reed-Muench (218) formula for purified HHV-8 preparations. HHV-8 was then used to infect DC at 1 or 2 $TCID_{50}$ to shown a difference in infectivity (**Fig. 38).**

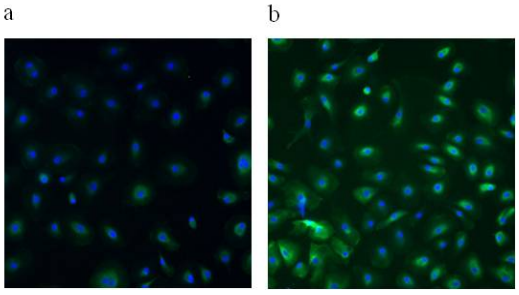

Figure 38: $TCID_{50}$ in DC

Expression of HHV-8 ORF59 protein in immature dendritic cells infected with *(A)*1 $TCID_{50}$ or *(B)* 2 $TCID_{50}$ of HHV-8.

Table 9: Validation of $TCID_{50}$ values

Table 1. Validation of $TCID_{50}$ Values						
Monocyte Donor	HHV-8 Viral Preparation	$TCID_{50}$	% Infected			Mean ± SD
			Count 1	Count 2	Count 3	
1	1	1	60	63	46	56 ± 9
		2	99	93	85	92 ± 7
	2	1	52	60	45	52 ± 8
		2	100	94	90	95 ± 5
2	1	1	43	61	42	49 ± 11
		2	100	100	95	98 ± 3
	2	1	48	59	48	52 ± 6
		2	89	93	94	92 ± 3

MDDC from 2 donors were used to determine $TCID_{50}$ validity. Each donor was exposed to two different HHV-8 viral preparations and β-gal positive cells (% infected) were determined.

A summary of the percentage of infected cells per TCID50 used is shown for 2 viral preps and 2 donors in **Table 9**. The $TCID_{50}$ resulted in means of 56, 52, 49 and 52 infected cells, while two times the $TCID_{50}$ (#2) resulted in 92, 95, 98, and 92 infected cells. These data validate the use of the T1-H6 DC-SIGN$^+$ cells in a $TCID_{50}$ assay.

To test the application of the $TCID_{50}$ assay, supernatants from B cells exposed to HHV-8 or UV-HHV-8 were transferred to the T1-H6 DC-SIGN$^+$ cells for detection of infectious virions released as a result of lytic infection. Supernatants collected at 3 time points from 2 individual experiments were used and B-galactosidase luminescence for 5 dilutions was determined (**Fig. 36a, c**). Does responses were evident at all 3 time points for both donors in supernatants collected from HHV-8 treated B cells, whereas supernatants collected from UV-HHV-8 treated

B cells resulted in sporadic β-gal. production at various dilutions. Due to this, a $TCID_{50}$ could not be calculated for the UV samples. Live virus, however, showed a 2-log increase between 0 and 48 hours (**Fig. 39b)** and a 4-log increase between 0-24 hours (**Fig. 39d**). Additional samples were tested using this method and the mean of 8 donors is shown in Figure 10e.

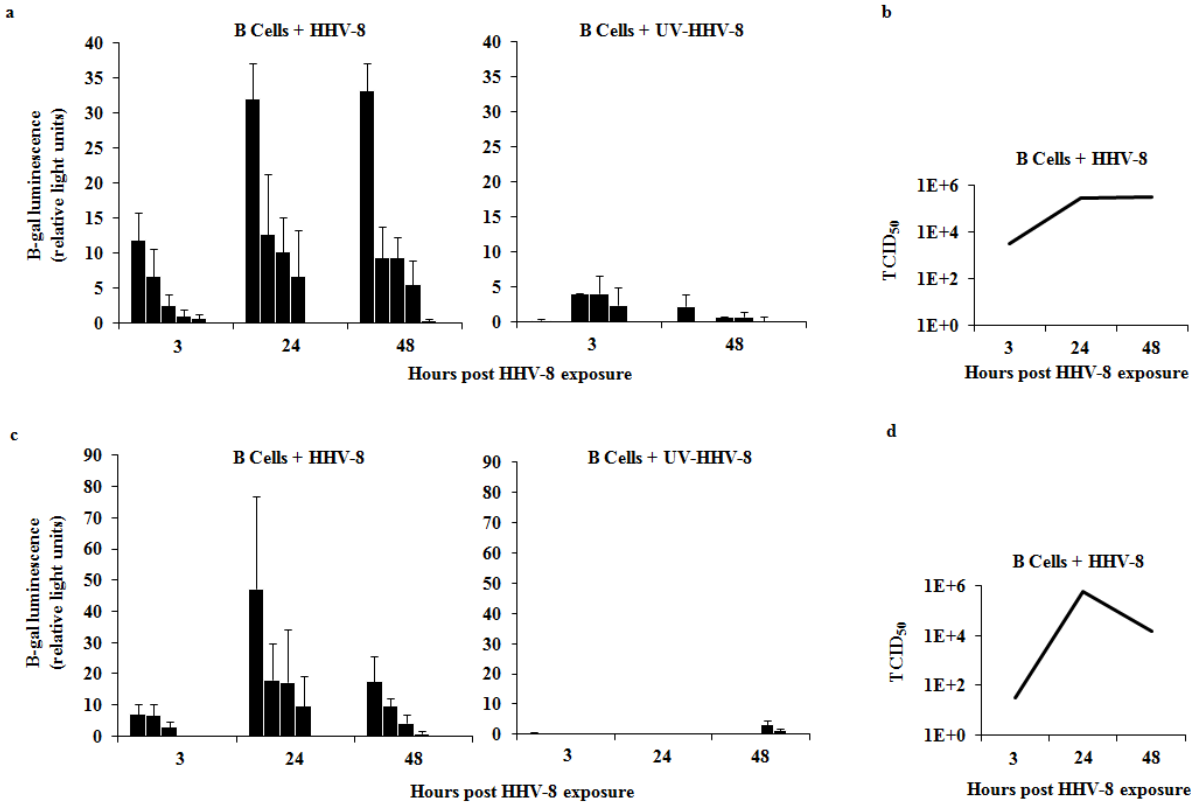

Figure 39: B cell supernatants used to determine $TCID_{50}$ application

(A, C) Supernatants collected at 3, 24 and 48 hpe from HHV-8 and UV-HHV-8 exposed B cells from 2 separate donors were used to infect T1-H6 DC-SIGN^{+} cells at 5 10-fold dilutions. β-gal luminescence was determined at 48 hpe for each donor (mean ± s.e.m. of 6 wells for each dilution). *(B, D)* The number of β-gal positive wells per dilution was used to calculate a TCID50 for each donor .

Submitted for publication: $TCID_{50}$ Assay for titering infectious human herpesvirus 8 (HHV-8). S. Nadgir, H. Hensler, E. Knowlton, C. Rinaldo and F. Jenkins.

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