# MOLECULAR AND CELLULAR INTERACTIONS BETWEEN THE HOST AND HERPESVIRUSES

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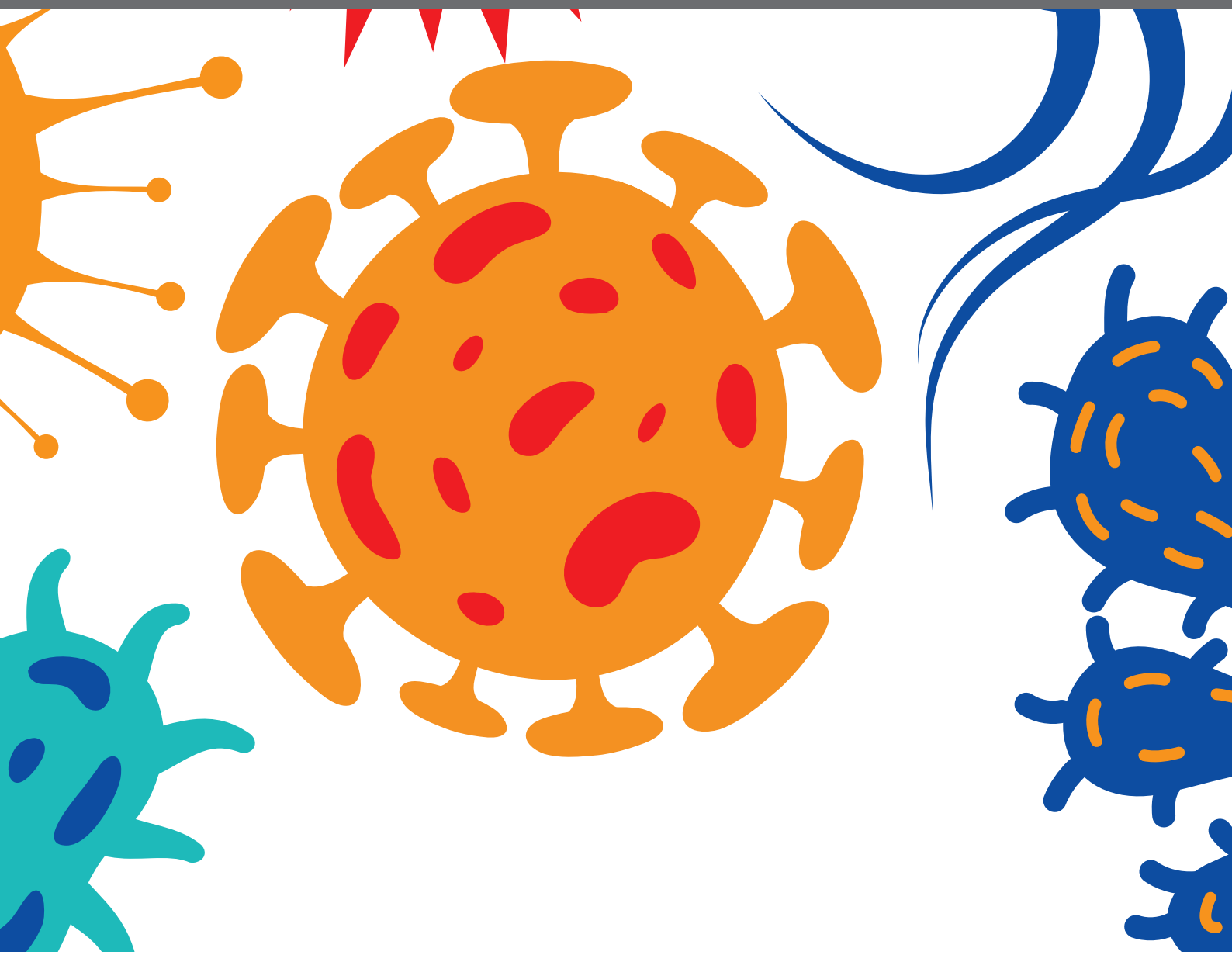

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# MOLECULAR AND CELLULAR INTERACTIONS BETWEEN THE HOST AND HERPESVIRUSES

Topic Editors:
**Hem Chandra Jha,** Indian Institute of Technology Indore, India
**Subhash C. Verma,** University of Nevada, Reno, United States

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EDITORIAL
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# Editorial: Molecular and Cellular Interactions Between the Host and Herpesviruses

*Hem Chandra Jha*[1*†] *and Subhash C. Verma*[2*†]

[1] Infection Bioengineering Group, Department of Biosciences and Biomedical Engineering, Indian Institute of Technology Indore, Indore, India, [2] Department of Microbiology and Immunology, University of Nevada, Reno, NV, United States

**Keywords: herpesvirus, epstein - barr virus, kaposi sarcoma associated herpesvirus, herpes simplex virus, viral pathogenesis**

**Editorial on the Research Topic:**

**Molecular and Cellular Interactions Between the Host and Herpesviruses**

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***Edited and reviewed by:***
*Curtis Brandt,*
*University of Wisconsin-Madison, United States*

***Correspondence:**
*Hem Chandra Jha*
*hemcjha@iiti.ac.in*
*Subhash C. Verma*
*scverma@med.unr.edu*

*[†]These authors have contributed equally to this work*

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Herpesviruses are among the most widely prevalent viruses in humans and associate with multiple diseases including cancer. These viruses can cause different diseases because of their propensity to infect a variety of cell types and establish latent (chronic) infection. Herpesviruses use multiple host's cell receptors to enter the target, which determines their cellular tropism. Interestingly, virus entry and the establishment of latent infection orchestrates through various host's and pathogen's intricate mechanism. One such well-known mechanism is the DNA damage responses, which is efficiently manipulated by the herpesviral proteins for the establishment of viral infection and the induction of viral pathogenesis. Importantly, cells infected with herpesvirus displays two types of viral genes expression profiles, latent and lytic. The latent phase is known to be associated with the expression of minimal viral genes which are associated with maintaining viral genome and the modulation of cellular pathways. During the lytic phase, majority of the viral genes are expressed, leading to the production of progeny virions and the lysis of the infected cells. The quiescent (latent) phase, which is the predominant phase in immune competent individuals, enters into the lytic phase due to multiple cellular/environmental triggers including favorable microenvironments, immunosuppression and other associated co-infection, etc. Evidently virus infection leads to disease pathogenesis by modulating various cellular pathways, including the host cell energy metabolism pathways, for their advantage. Importantly, energy metabolism pathways such as Carbon are regulated differentially in different cells based on the cellular microenvironments. Therefore, targeting these pathways for eradicating virus associated pathogenesis is an important explorative area. Hence therapeutic agents capable of blocking host proteins, gene expression, multiple transcription factors, cellular signal pathways, immune cell activation, transcription factors, cytokines, angiogenesis, invasion, and metastasis, can be effective in preventing pathogenesis.

In this Research Topic Madavaraju et al. summarized how Herpes simplex virus (HSV) can infect a broad range of hosts that leads to human diseases. They demonstrate the importance of surface glycoproteins of HSV, which are evolutionarily conserved and show an astonishing

capability to bind more than one receptor on the host cell surface. They also highlighted the importance of cellular pH in viral entry and subsequent modulation inside the host.

Next, Ohsaki and Ueda reviewed the importance of DDR activities during virus infection. They explained how virus utilizes this mechanism to make conducive microenvironments for its advantages. Further Munz has demonstrated the roles of lytic and latent genes in cancer progression. He showed the importance of lytic genes capable of making a favorable microenvironment for tumorigenesis.

Further Liu et al. reported the importance of energy metabolism in cancer progression, which included aerobic glycolysis, glutaminolysis, and fatty acid synthesis pathways. They connected the metabolism pathways with viral replication, viral survival, and proliferation in the host.

Hutcheson et al. emphasized on common cellular mutations in EBV mediated Burkitt's lymphoma tumors and discussed how those can be targeted for the expression of viral genes that are no longer expressed, which may help in improving the treatment of EBV mediated BLs.

Bhowmik and Zhu discussed strategies utilized by herpesviruses for evading the DNA-induced immune responses, which are important in the development of vaccines and antivirals for herpesvirus-associated diseases.

Journo et al. demonstrated the footprints of cellular CpG DNA methylation in Kaposi Sarcoma tissues, which revealed a dramatic change in global methylation patterns in KS development. Further, Pardamean and Wu emphasized the studies on both SOX and ORF10 in elucidating their roles in KSHV induced disease. Authors also discussed the relatedness of these proteins in rodent virus, murine gammaherpesvirus-68 (MHV-68), and their importance in understanding KSHV pathogenesis.

Li et al. presented the importance of gene UL26.5 in Herpes simplex virus type 2. They demonstrated the immunodominance of ICP35, which can be used for designing an integrated antigen with other viral glycoproteins. Additionally, Rodriguez showed how different Sp100 isoforms are manipulated during herpesviruses HSV1, VZV, HCMV, EBV, and KSHV infection.

Dollery et al. used chronically infected derivative of TIME (telomerase immortalized endothelial) cells, which maintains latent virus by constitutively expressing (eGFP). Identity of the iTIME.219 cells was validated both phenotypically and genetically, and thus proposed to be used as a cell culture model of the KSHV replication. Further, Aalam and Totonchy evaluated KSHV biology in the lymphocyte compartment, and presented mechanisms unique to B lymphocytes.

Gou et al. investigated the metabolic networks in PK-15 cells infected with a variant virulent or classical attenuated pseudorabies virus (PRV). Authors showed that an inhibition of the pentose phosphate pathway (PPP) leads to a decline in viral titers, however the prevention of oxidative phosphorylation in the tricarboxylic acid (TCA) cycle had a minimal effect.

Barrett et al. discussed how KSHV manipulates the activities of IL-1 signaling pathway to facilitate its associated disease progression. Authors also discussed therapeutic potential of IL-1 blockade against KSHV-related diseases and several unsolved questions of the field.

Overall, this Research Topic covered viral entry, propagation inside various cells, signaling mechanism and potential therapeutics for blocking the herpesviruses associated malignancies.

## AUTHOR CONTRIBUTIONS

The authors contributed equally to this work. HJ and SV co-wrote the manuscript. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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# Role of Interleukin-1 Family Members and Signaling Pathways in KSHV Pathogenesis

*Lindsey Barrett[1], Jungang Chen[1], Lu Dai[1], Karlie Plaisance-Bonstaff[2], Luis Del Valle[3]* and Zhiqiang Qin[1]**

*[1] Department of Pathology, Winthrop P. Rockefeller Cancer Institute, University of Arkansas for Medical Sciences, Little Rock, AR, United States, [2] Department of Medicine, Louisiana State University Health Sciences Center, Louisiana Cancer Research Center, New Orleans, LA, United States, [3] Department of Pathology, Louisiana State University Health Sciences Center, Louisiana Cancer Research Center, New Orleans, LA, United States*

Kaposi's sarcoma-associated herpesvirus (KSHV) represents the etiological agent for several human malignancies, including Kaposi's sarcoma (KS), primary effusion lymphoma (PEL), and multicentric Castleman's disease (MCD), which are mostly seen in immunocompromised patients. In fact, KSHV has developed many strategies to hijack host immune response, including the regulation of inflammatory cytokine production. Interleukin-1 (IL-1) family represents a major mediator for inflammation and plays an important role in both innate and adaptive immunity. Furthermore, a broadening list of diseases has revealed the pathologic role of IL-1 mediated inflammation. In the current mini-review, we have summarized recent findings about how this oncogenic virus is able to manipulate the activities of IL-1 signaling pathway to facilitate disease progression. We also discuss the therapeutic potential of IL-1 blockade against KSHV-related diseases and several unsolved questions in this interesting field.

**Keywords: Kaposi's sarcoma-associated herpesvirus, Kaposi's sarcoma, primary effusion lymphoma, multicentric Castleman's disease, interleukin-1**

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***Edited by:***
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***Reviewed by:***
*Neelam Sharma-Walia, Rosalind Franklin University of Medicine and Science, United States*
*Qiliang Cai, Fudan University, China*

****Correspondence:***
*Zhiqiang Qin*
*zqin@uams.edu*
*Luis Del Valle*
*ldelva@lsuhsc.edu*

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## INTRODUCTION

Kaposi's sarcoma-associated herpesvirus (KSHV) infection causes several human cancers including Kaposi's sarcoma (KS), primary effusion lymphoma (PEL), and multicentric Castleman's disease (MCD) (Broussard and Damania, 2019). These KSHV-associated malignancies develop mainly in immunocompromised patients, especially those infected with human immunodeficiency viruses (HIVs) (Vangipuram and Tyring, 2019). Further, the morbidity rate of patients with KSHV-associated diseases is much higher in patients with compromised immune systems compared to those with competent immune systems (Mesri et al., 2010).

KSHV has two alternating life-cycle programs following primary infection of host cells, the latent and lytic phases, which are characterized by different patterns of viral gene expression (Mesri et al., 2010). During latency, viral genomes persist as circular episomes with no progeny virion production and only a limited number of latency-associated genes expressed, including latency-associated nuclear antigen (LANA), viral Fas-associated protein with death domain (FADD)–like interleukin-1β-converting enzyme (FLICE)-like inhibitory protein (vFLIP), viral cyclin (vCyclin), as well as

some viral microRNAs (Uppal et al., 2014). Once entering the lytic phase, which is caused by various stimuli, almost all viral genes are highly expressed, followed by genomic DNA replication and mature virion release (Ye et al., 2011). KSHV is known to hijack many aspects of the host's immune response such as viral detection and cytokine production. Interleukin-1 (IL-1) is an inflammatory cytokine family of 11 distinct proteins that has a wide array of functions in innate immunity processes. The IL-1 superfamily contains many pro-inflammatory cytokines (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, and IL-36γ) and a few anti-inflammatory cytokines (IL-36Ra, IL-37, and IL-38) (Boraschi and Tagliabue, 2013). Among them, IL-1 is the defining member of this family and its physiology and relationship to pathology has been thoroughly studied and reported. IL-1 includes two activator cytokines, IL-1α and IL-1β, and one inhibitory factor, the IL-1 receptor antagonist (named as IL-1Ra). The main function of IL-1 is to respond to tissue damage caused by pathogen-associated molecular patterns (PAMPs) such as viral products, or damage-associated molecular patterns (DAMPs) such as adenosine 5'-triphosphate (Xu et al., 2019). Upon stimulation, IL-1α and IL-1β both bind to the type I IL-1 receptor (IL-1R1) which then recruits the IL-1 receptor accessory protein (IL-1RAP), as well as the adaptor protein MyD88, which are necessary for triggering signal transduction. Once the IL-1 receptor complex is formed, a downstream signaling cascade is activated which then stimulates a collection of related immune responses and/or inflammatory genes (Jensen, 2017). Dysregulation of the IL-1 pathway has been shown to be linked to a number of autoinflammatory and autoimmune diseases, such as atherosclerosis and systemic sclerosis, respectively, as well as cancers like gastric carcinoma and lung cancer (El-Omar et al., 2001; Bhat et al., 2014; Ridker et al., 2017; Xu et al., 2019).

KSHV infection has been found to induce the production of a variety of host pro-inflammatory cytokines. For example, primary KSHV infection in monocytes can increase the release of IL-1α, IL-1, and IL-6 (Host et al., 2017). These cytokines have been suggested to regulate early KS lesion progression and have been found at high levels in the sera of KS patients (Ensoli and Stürzl, 1998). Other IL-1 family members such as IL-33 have recently been shown to play a role in KSHV pathogenesis by regulating chromatin compaction through nucleosome-nucleosome interactions (Roussel et al., 2008). Therefore, in this mini-review, we will summarize recent findings about the relationship between KSHV and the IL-1 family members. We will try to highlight how KSHV may utilize the IL-1 signaling pathway to facilitate disease progression and how potential immunotherapies could target such mechanisms.

## THE IL-1 SIGNALING PATHWAY

IL-1 is a major mediator for inflammation and plays an important role in both innate and adaptive immunity. IL-1α and IL-1β both signal through the cell surface receptor, IL-1R1 (Jensen, 2017). Upon ligand binding, the transmembrane IL-1R accessory protein, IL-1RAP, is recruited to the site. This heterodimer formation leads to intracellular recruitment of the adaptor protein, MyD88, and mobilization of IL-1R-associated kinases (e.g., IRAK1, IRAK2, and IRAK4). These kinases, along with additional signaling factors, lead to the phosphorylation and degradation of nuclear factor κB (NF-κB) inhibitor IκB. The end of this signaling pathway results in the translocation of activated transcription factors, such as activator protein 1 (AP-1) and NF-κB, to the nucleus where specific gene expression is activated. IL-18 and IL-33 also stimulate gene expression through the same intracellular pathway using their receptor-accessory protein complexes (IL-18R1/IL-18RAP and IL-1R-like 1/IL-1RAP). The other cytokines, IL-36α, IL-36β, and IL-36γ, bind to the receptor IL-1R-like 2 (IL-1RL2 or IL-36R), which then uses IL-1RAP as its receptor-accessory protein and stimulates the same signaling cascade as IL-1, IL-18, and IL-33 (Dinarello, 2019). Interestingly, KSHV has developed strategies to manipulate the functions of b1these different IL-1 signaling molecules after invading host cells.

## IL-1α/β AND RECEPTORS

Several studies have reported that KSHV infection or viral protein infiltration can upregulate IL-1α and/or IL-1β expression. For instance, one study showed that ectopic expression of viral macrophage inflammatory protein-II (vMIP-II) within endothelial cells upregulated multiple proangiogenic factors, including IL-1α, resulting in enhanced angiogenesis (Cherqui et al., 2007). Another viral protein, vOX2, a glycosylated cell surface protein, was found to dramatically stimulate primary monocytes, macrophages, and dendritic cells to produce IL-1β (Chung et al., 2002). On the other hand, KSHV Orf63, encoding a viral homolog of human NLRP1 (NACHT, LRR, FIIND, CARD domain and PYD domains-containing protein 1), was found to reduce IL-1β expression and related signaling through inhibition of the inflammasome (Gregory et al., 2011). In fact, many studies suggest that inflammatory and angiogenic cytokines including IL-1β contribute to the pathogenesis of KS by causing abnormal proliferation, angiogenesis, and a KS-like phenotype independent of KSHV (Ensoli et al., 1992). For example, IL-1β was markedly elevated in most KS lesions (Samaniego et al., 1997). Furthermore, IL-1β was elevated during initial KSHV-MCD flares compared with remission (Polizzotto et al., 2013). Interestingly, our recent study demonstrated that IL-1β was required for the upregulation of PD-L1 expression by viral lytic reactivation from KSHV-infected tumor cells (Chen et al., 2019), which may represent a novel mechanism for virus-associated tumor cell immune escape. It remains unclear about the situation of receptor and accessory proteins of IL-1 in KSHV-infected cells. Our recent data indicate that KSHV infection significantly upregulates IL1R1 and IL1RAP from endothelial cells (**Figure 1A**). Moreover, both proteins are found highly expressed in KS tumor cells, especially IL1R1 (**Figure 1B**).

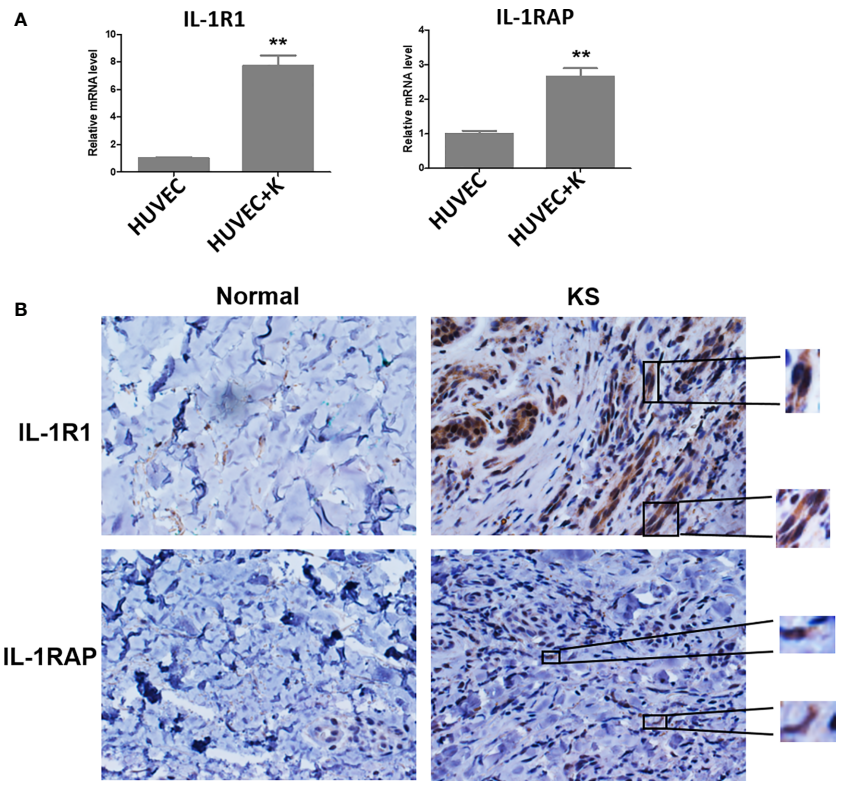


**FIGURE 1** | IL-1R1 and IL-1RAP are upregulated by KSHV and highly expressed within AIDS-KS tumor tissues. **(A)** Primary human umbilical vein endothelial cells (HUVEC) were infected with KSHV (MOI~10) or not for 48 h, followed by qRT-PCR analysis. The data were normalized to the β-actin housekeeping gene expression. Error bars represent the S.D. for three independent experiments. **$p < 0.01$. **(B)** Expression of IL-1R1 and IL-1RAP in representative formalin-fixed paraffin-embedded KS tissues from HIV+ patient without treatment were determined by immunohistochemical staining (40×). The higher magnification for IL-1R1 and IL-1RAP detection in KS tumor cells were also shown (60×). Normal skin tissues were used as a control.

## IL-18, IL-33, AND IL-36

KSHV latency, especially viral FADD-like interleukin-1-β–converting enzyme [FLICE/caspase 8]-inhibitory protein (vFLIP), was found to induce the expression of IL-18 (as well as IL-1β) in an NF-κB dependent manner (Singh et al., 2013). In contrast, one of the viral lytic products, KSHV polyadenylated nuclear RNA (PAN RNA) decreased the expression of IL-18 (Rossetto and Pari, 2011). Similar inhibitory effects on IL-18 were observed with KSHV Orf63 in the study mentioned above (Gregory et al., 2011).

The functional roles of IL-33 and IL-36, and their regulatory mechanisms in KSHV-infected cells remain mostly unclear. One very recent study reported that the plasma IL-33 concentrations were higher in individuals with KS in Uganda, Africa (Byakwaga et al., 2020), implying that this cytokine and its related signaling may also play role in KSHV pathogenesis. Interestingly, IL-33 has also been demonstrated as a chromatin-associated factor in the nucleus of endothelial cells, which has a short chromatin-binding peptide that shares similarities with a motif found in KSHV-encoded latency-associated nuclear antigen (LANA) (Carriere et al., 2007; Roussel et al., 2008). As we know, LANA is responsible for the attachment of the viral episome to mitotic chromosomes (Barbera et al., 2006); thus, this IL-33 peptide can also dock into the acidic pocket formed by the H2A-H2B dimer at the nucleosomal surface and regulate chromatin compaction through nucleosome-nucleosome interactions. However, the association between IL-33 and KSHV latency and lytic reactivation remains unknown.

## MYD88 AND IRAKs

One study using X chromosome-targeted sequencing identified 34 common missense mutations in 100% of PEL cases, including a Phe196Ser change in the IRAK1 protein. Moreover, IRAK1 was constitutively phosphorylated in PEL and required for tumor cell survival (Yang et al., 2014). By using CRISPR/Cas9 knockout technology, the same group recently reported that established PEL cell lines were able to circumvent the loss of IRAK1, IRAK4, and MyD88, while the deletion clones were deficient in IL-10 production (Seltzer et al., 2020). Due to the suppression of T cell function by IL-10, the authors suggest that the IRAK pathway may contribute to early-stage development of PEL. KSHV

encodes 12 pre-microRNAs (pre-miRNAs), which are processed into 25 mature microRNAs (miRNAs) (Qin et al., 2017). Interestingly, Abend et al. reported that IRAK1 and MyD88 were directly targeted by several KSHV-microRNAs, particularly miR-K12-9 and miR-K12-5, respectively (Abend et al., 2012). The presence of miR-K12-9 and miR-K12-5 inhibited the production of IL-6 and IL-8 upon IL-1α stimulation of endothelial cells. In another study, Lingel et al. reported that KSHV-encoded replication and transcription activator (RTA) was able to bind to MyD88 RNA and suppress its RNA synthesis (Lingel et al., 2016). Another group recently found that KSHV RTA downregulated MyD88 expression at the protein level by degrading MyD88 through the ubiquitin (Ub)-proteasome pathway (Zhao et al., 2015).

## IL-1 BLOCKADE

Since IL-1 is a master cytokine of local and systemic inflammation, pharmacological blockade of IL-1 activity has been applied in a variety of inflammatory diseases that results in a rapid and sustained reduction in disease severity. There are three major categories of IL-1 blockers which have been approved by the Food and Drug Administration (FDA) for clinical treatment: **1)** The IL-1 receptor antagonist (e.g., Anakinra), blocks the IL-1 receptor and therefore reduces the activity of IL-1α and IL-1β; **2)** The soluble decoy receptor (e.g., Rilonacept, also known as IL-1 Trap), a dimeric fusion protein consisting of the ligand-binding domains of the extracellular portions of IL-1R1 and IL-1RAP linked in-line to the fragment-crystallizable portion (Fc region) of human IgG1 that binds and neutralizes IL-1; **3)** The neutralizing monoclonal anti-IL-1β antibody (e.g., Canakinumab), which directly targets IL-1β (Dinarello and van der Meer, 2013; Dinarello, 2013). There are other neutralizing monoclonal antibodies targeting IL-1α or the IL-1 receptor in different clinical trials (Dinarello and van der Meer, 2013). Interestingly, Boehringer Ingelheim Company recently developed a new IL1RAP antibody, BI-5041, which targets a unique epitope on IL-1RAP and therefore blocks IL-1, IL-33, and IL-36 signalling. Currently, there is limited data about IL-1 blockade therapy in KSHV-related malignancies (El-Osta et al., 2010). Two case reports detailed the successful treatment of MCD by Anakinra for two patients, although their KSHV infection status remains unclear (Galeotti et al., 2008; El-Osta et al., 2010).

## CONCLUSION AND PROSPECTIVE

Current research reveals that KSHV has developed different strategies to manipulate IL-1 signaling activity (summarized in **Figure 2**) in order to balance the host's inflammatory response or help the virus escape the host's immune response. The virus-encoded latent and lytic proteins and even viral non-coding RNAs can target multiple components of the IL-1 signaling pathway. However, there are still many questions in this field waiting for further investigation. For example, the functions and regulatory mechanisms of certain IL-1 family members (e.g., IL-36, IL-37, IL-1Ra, and IL-1RAP) during KSHV infection or virus-induced tumorigenesis remain unknown. It is also unclear whether the intermediates of IL-1 signaling may affect KSHV replication, especially the "latency to lytic" switch. Furthermore, the efficacy

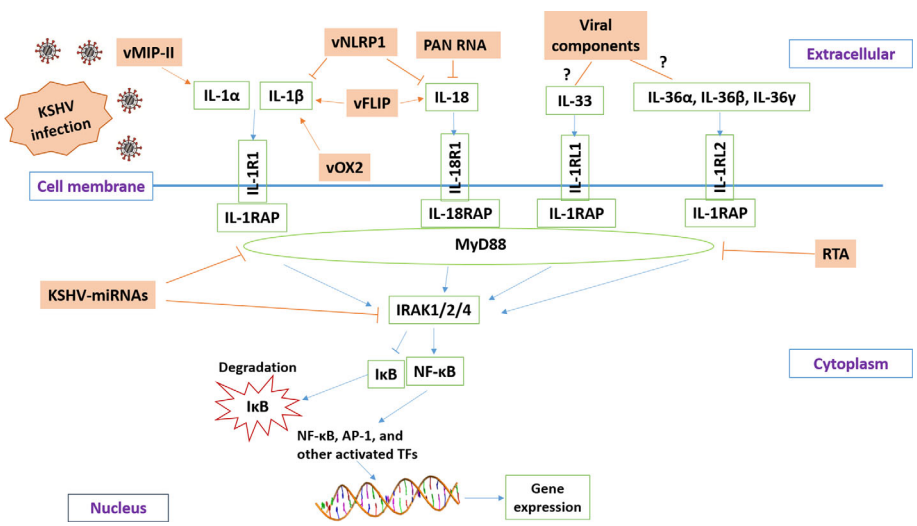


**FIGURE 2** | Schematic diagram of potential mechanisms for KSHV manipulating IL-1 signaling pathway. The brown and green rectangles represent viral and host genes, respectively. The arrows and bars represent the activation and inhibition, respectively. Notably, the mechanisms of KSHV regulation of IL-33 and IL-36 signaling remain mostly unknown.

of IL-1 blockade therapy either alone or combined with other therapies for KSHV-related malignancies needs to be tested. Fully understanding these questions will shed light on the molecular mechanisms of KSHV pathogenesis and tumorigenesis and facilitate the development of more efficacious antiviral and anticancer treatments.

## AUTHOR CONTRIBUTIONS

LB wrote the manuscript. JC, LD, and KP-B participated in figure preparation and discussion. LV and ZQ reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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# Molecular Virology of KSHV in the Lymphocyte Compartment—Insights From Patient Samples and *De Novo* Infection Models

*Farizeh Aalam and Jennifer Totonchy**

*Biomedical and Pharmaceutical Sciences, Chapman University School of Pharmacy, Irvine, CA, United States*

The incidence of Kaposi's sarcoma-associated herpesvirus (KSHV)-associated Kaposi Sarcoma has declined precipitously in the present era of effective HIV treatment. However, KSHV-associated lymphoproliferative disorders although rare, have not seen a similar decline. Lymphoma is now a leading cause of death in people living with HIV (PLWH), indicating that the immune reconstitution provided by antiretroviral therapy is not sufficient to fully correct the lymphomagenic immune dysregulation perpetrated by HIV infection. As such, novel insights into the mechanisms of KSHV-mediated pathogenesis in the immune compartment are urgently needed in order to develop novel therapeutics aimed at prevention and treatment of KSHV-associated lymphoproliferations. In this review, we will discuss our current understanding of KSHV molecular virology in the lymphocyte compartment, concentrating on studies which explore mechanisms unique to infection in B lymphocytes.

**Keywords: KSHV, HHV8 (KSHV), virus-host interaction, B lymphcytes, immune evasion, hematological malignancies**

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***Correspondence:**
*Jennifer Totonchy*
*totonchy@chapman.edu*

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## INTRODUCTION

KSHV (HHV8) belongs to the gamma-herpesvirus family and is associated with both lymphoid and non-lymphoid cell tumors in humans (Chang et al., 1994). KSHV-associated malignancies occur primarily in the context of immunodeficiency. KSHV is the etiologic agent of Kaposi's sarcoma, as well as the B cell lymphoproliferative disorders, primary effusion lymphoma (PEL), and multicentric castleman disease (MCD) (Chang et al., 1994; Cesarman et al., 1995) as well as the recently discovered KSHV inflammatory cytokine syndrome (KICS) (Uldrick et al., 2010).

Despite nearly three decades of research, not much is known regarding the early stages of development for KSHV lymphoproliferative disorders and the person-to-person transmission of KSHV. This can be partly explained by the host range limitation and broad *in vitro* cellular tropism of KSHV (Blackbourn et al., 2000; Bechtel et al., 2003). During the latent phase of infection, viral gene expression is limited and KSHV is maintained as an extrachromosomal episome; persisting for the lifetime of the individual (Ueda, 2018). Like other herpesviruses, KSHV can become lytic under some physiological conditions (Grundhoff and Ganem, 2004; Li et al., 2014; Johnston et al., 2019; Wei et al., 2019). The process by which the lytic switch occurs and the relative contributions of

lytic/latent phases to KSHV persistence are poorly understood. This is partly because expression and activity of the KSHV regulatory proteins appear to be cell type and tissue-specific and *in vivo* niches for persistence in humans remain poorly characterized (Rivas et al., 2001; Koch et al., 2019). There are significant gaps in our understanding of how KSHV targets B cells for infection and how the virus manipulates B cell physiology in the development of PEL and MCD (**Figure 1**). Further study of KSHV molecular virology in the lymphocyte compartment is needed to understand the pathogenesis of KSHV-associated lymphoproliferation so that effective treatment paradigms can be developed. In this review, we explore our current understanding of KSHV biology in B cells concentrating on studies which use *de novo* infection of human B cells, analysis of patient samples from KSHV lymphoproliferative disease, and relevant lymphoma cell lines. We have intentionally omitted discussion of KSHV manipulation of cytokine expression and signaling from this work as it is complex, and we have recently reviewed the topic comprehensively elsewhere (Alomari and Totonchy, 2020). Moreover, we have omitted discussion of humanized mouse models for KSHV infection as they have also been reviewed very recently (Münz, 2020).

# B LYMPHOCYTE-SPECIFIC MOLECULAR VIROLOGY OF KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS

## Entry Into B Cells

HHV-8 DNA is detectable in the B cells from both HIV+ and HIV-PEL and MCD cases (Dupin et al., 1999). Interestingly, KSHV isolated from EBV+PEL cells is able to infect B cells from seronegative patients (Mesri et al., 1996). Phylogenetic analysis and the association of KSHV infection with pathological lymphoproliferations are sufficient to characterize KSHV as a lymphotropic gamma-herpesvirus. However, primary B cells and B lymphoma cell lines show poor susceptibility to KSHV infection *in vitro* compared to adherent cell lines (Bechtel et al., 2003). The extensive *in vitro* susceptibility of adherent cell lines can partly be explained by the presence of various cellular receptors used by the viral glycoproteins for attachment and entry (Akula et al., 2001a; Akula et al., 2001b; Akula et al., 2002; Rappocciolo et al., 2008; Hahn et al., 2009; Chen et al., 2019; Großkopf et al., 2019; Muniraju et al., 2019)

KSHV virion attachment to adherent cells can be facilitated through heparan sulfate proteoglycans on the host cell surface

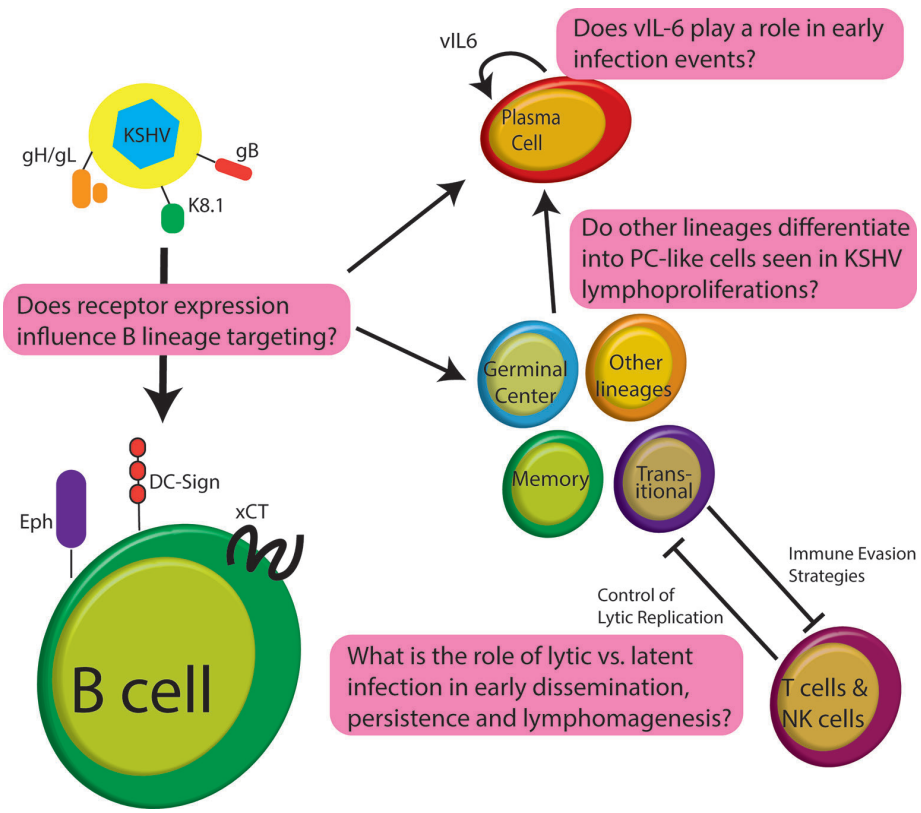


**FIGURE 1** | Schematic of early infection events for KSHV in B lymphocytes highlighting some of the significant questions that remain unanswered in the field.

(Akula et al., 2001a), and the low *in vitro* susceptibility of B cells has been attributed to a lack of HS expression. This theory is supported by the observation that restoration of cell surface HS in B cell lines results in increased susceptibility to infection (Jarousse et al., 2008; Jarousse et al., 2011; Dollery et al., 2019). The lectin DC-SIGN has also been implicated as an attachment factor for KSHV entry into B cells. Approximately, 8% of CD19 +CD20+ peripheral blood B cells and 26% of tonsillar B cells are positive for DC-SIGN, and activation of peripheral blood B cells with IL-4 and CD40L results in 3 to 3.5 fold increase in DC-SIGN and CD23 expression (Rappocciolo et al., 2006). Activated B cells are more susceptible to KSHV infection and KSHV infected B cells show increased DC-SIGN levels compared to uninfected cells (Rappocciolo et al., 2008). Interestingly, B cells expressing DC-SIGN can bind and transfer HIV-1 virions to T cells (Rappocciolo et al., 2006). Taken together, these observations suggest that KSHV and HIV infections act synergistically. KSHV infection of B cells can facilitate the dissemination of HIV-1 to CD4+ T cells *via* upregulation of B cell DC-SIGN expression, and HIV, in turn, depletes the CD4+ T cell pool creating an immunological milieu in which KSHV benefits from the lack of immune surveillance.

KSHV encodes a variety of glycoproteins which facilitate virion attachment, fusion, and viral entry into the host cell. Among the various KSHV glycoproteins, gH/gL complex is proved to be the major antigenic determinant of KSHV-specific nAbs in the plasma of KS patients regardless of their disease status (Mortazavi et al., 2020), suggesting that this complex is critical for virus entry. Binding of gH/gL glycoprotein complex to the surface is not well characterized, but it is not HSPG-dependent (Hahn et al., 2009). KSHV entry into the BJAB cell line has been linked to gH/gL binding to EphA7 (Großkopf et al., 2019). Eph4 also binds to gH/gL, and is expressed in B cells, endothelial, fibroblast, and epithelial cells (Chen et al., 2019). In HEK293T cells, Eph4 binds more tightly with gH/gL than Eph2 (Chen et al., 2019). RNA sequencing data shows that B cells express Eph4 on their cell surface, albeit not as abundant as endothelial cells but higher than epithelial cells (Chen et al., 2019). Thus, it is possible that gH/gL complex can establish interaction with Eph4 in B cells, since B cells may have almost the same level of Eph4 as HEK293 (epithelial cells) on their surface. However, use of Eph4 as a KSHV entry receptor for B cells has not been studied specifically. Interestingly, the MC116 lymphoma cell line expresses both EphA7 and Eph4, and is susceptible to KSHV infection, but studies with a KSHV mutant lacking gH demonstrated that KSHV entry into MC116 cells is not dependent upon gH/gL (Muniraju et al., 2019). This study, in particular, highlights the significant gaps in our understanding of the molecular virology of KSHV entry into B cells.

Another study showed that K8.1A is required for KSHV infection of both MC116 and CD20+CD3− B cells from tonsil. The cellular receptor interacting with K8.1A in this context is not known, but it is independent of HS binding (Dollery et al., 2019). Finally, the KSHV glycoprotein gB, which is presumed to be the KSHV fusion protein, binds to DC-SIGN *in situ* in dose dependent manner (Hensler et al., 2014), but whether this interaction is essential for KSHV entry into B cells has not been formally studied. xCT, the light chain has been shown to be involved in KSHV fusion and entry in several cell lines. Although, its mRNA expression is undetectable in CD19+ PBMCs (Kaleeba and Berger, 2006a; Kaleeba and Berger, 2006b) xCT is highly expressed on the surface of PEL cell lines and targeting it by xCT selective inhibitor, induces apoptosis in caspase dependent manner. Selective inhibition of xCT in immune deficient mouse xenograft model proves that it plays key role in tumor progression, survival, and growth of PEL cells (Dai et al., 2014). The expression of xCT can be induced by KSHV miRNAs conferring permissiveness to KSHV in murine microphages and HUVEC cells. Additionally, the expression of xCT within the KS lesion is correlated with the tumor stage (Qin et al., 2010).Whether KSHV miRNAs and change in redox balance contribute to upregulation of xCT in primary B cells to increase the KSHV permissiveness, remains to be answered.

To date, no comprehensive studies been done on primary human B cells samples to elucidate the cellular receptors involved in KSHV entry into B lymphocytes or the individual and collective contributions of KSHV glycoproteins to this process. Further studies are needed to determine these important interactions to facilitate the rational design of vaccine strategies that will effectively limit the establishment of infection in the lymphocyte compartment.

## Manipulation of the Cell Cycle

KSHV can establish latent infection in many adherent cell lines, including human and non-human cells of epithelial, endothelial, and mesenchymal origin (Bechtel et al., 2003). Previous studies in primary human B cells report that infection is lytic, particularly in the absence of T cells, but what controls the lytic switch in these cells remains to be established (Myoung and Ganem, 2011a). In addition to T cell control of latency, B cell immunophenotype and activation state have been implicated as factors influencing the lytic/latent balance in B cells (Rappocciolo et al., 2008; Hassman et al., 2011; Myoung and Ganem, 2011a), as well as the immunological status of the individual and the presence of other pathogens (Gregory et al., 2009).

Although the latent phase of infection allows viral persistence and immune-evasion, the production of viral progeny and viral transmission and spread between the cells, depends on the lytic phase. *De novo* infected PBMCs exhibit simultaneous expression of numerous latent and lytic markers at the very beginning of the infection (Purushothaman et al., 2015). This short lytic replication seems to be a prerequisite for the establishment of the latent phase in PBMCs infected with EBV (Halder et al., 2009). Nevertheless, the lytic gene expression is not required for KSHV infection of PBMCs before or after EBV infection or mitogenic activation (Faure et al., 2019). Do B cells represent a significant source of KSHV virions during human infection? The early lytic gene K8 (K-bZIP), a cell cycle regulator showing homology to EBV' Zta, is required for viral lytic DNA replication and virion production in PEL cell lines (Wu et al., 2002; Lefort and Flamand, 2009). Its expression concurs with augmented C/EBPα, p21 and p27 in the nucleus, causing the cell arrest in G1

phase (Wu et al., 2002; Izumiya et al., 2003a). This prolonged G1 arrest is as a result of K8 binding to CKD2, interfering its kinase activity, giving ample time for viral early gene transcription and translation (Izumiya et al., 2003a). K8 also interacts with p53 inhibiting its transcription, preventing apoptosis (Park et al., 2000). However, in another study by Hollingworth et al., lytic replication in PEL cells was shown to require S phase entry (Hollingworth et al., 2020). Replication and transcription activator (RTA) is a protein encoded by ORF8 has been shown to co-localize with K8 within the nucleus of the PEL cells, and its association with the K8 (Izumiya et al., 2003b) can initiate lytic reactivation from latency by binding to a particular sequence on the host and viral DNA further modulating the transcription of viral and host regulatory genes throughout KSHV lytic reactivation (Kaul et al., 2019). Viral DNA replication is controlled by both transcriptional coactivator p300 and CBP. P300 was shown to be involved in the oncogenesis of PEL by driving B cell proliferation and inhibiting KSHV lytic replication. Knockout of p300 in PEL cells decreased KSHV genome copy number and virion production by suppressing lytic gene expression, possibly maintaining the latency of KSHV *via* binding with ATF3 (Sun et al., 2020). Nonsense-mediated mRNA decay (NMD) is an RNA quality control implemented by the cells to restrict the action of the RNA viruses and serve cellular quality control. Interestingly, viral RTA' mRNA is targeted by NMD, impeding KSHV lytic reactivation in PEL cells (Zhao et al., 2020). However, KSHV has evolved to overcome some of these quality controls and exonuclease activities by circularizing its structural and regulatory RNAs incorporated into the virions (Abere et al., 2020). Taken together, the current literature demonstrates that multiple layers of both viral and cellular regulation influence KSHV latency and lytic reactivation in B cells. It is notable that most of this work has been done in PEL cells, and future studies investigating how KSHV manipulates the cell cycle and cell type specific control of latency and reactivation in primary B lymphocytes will be critical for understanding early events in KSHV infection and pathogenesis of KSHV-associated lymphoproliferative diseases.

## Kaposi's Sarcoma-Associated Herpesvirus Immune Evasion

KSHV infection persists for the lifetime of the host and, like all herpesviruses, KSHV must have an arsenal of mechanisms for evading host immunity in order to accomplish this. Lymphotropic gamma-herpesviruses are particularly interesting in this regard because they can manipulate and evade the host immune system *via* mechanisms that require direct infection of immune cells. Moreover, the inflammatory nature of KSHV-associated malignancies indicates that KSHV immune-evasion mechanisms may also directly contribute to pathogenesis in KSHV-associated diseases. Indeed, this immune-evasion is manifested at the transcriptional level within the first few hours of infection, by hampering the expression of immune response genes and inducing the proapoptotic regulators in BJAB cells (Naranatt et al., 2004). KSHV can infect both B and T cells in tonsil primary cell culture, however evidence suggests that infection of T cells is abortive (Myoung and Ganem, 2011b). Moreover, there is reciprocal activation of T cells by KSHV-infected B cells and contact-dependent control of KSHV lytic reactivation by T cells in *ex vivo* tonsil cultures, and in this system the activation of T cells is independent of both KSHV antigen and MHC restriction (Myoung and Ganem, 2011a).

Activated, KSHV infected B lymphocytes from PBMC and tonsils show downregulation of MHC class I (HLA-A, HLA-B, and HLA-C) within 24 h of infection as well as decreased expression of CD20 (Rappocciolo et al., 2008). Modulation of MHC class I expression is also observed in PEL derived B cell-lines and is thought to be partially due to reduced expression of the TAP-1 gene. Importantly, this MHC-I modulation can disrupt cytotoxic T lymphocyte surveillance of KSHV infected cells (Brander et al., 2000), aiding in KSHV persistence and tumorigenesis in B cells. The CD20 low phenotype of KSHV infected cells is also present in MCD and may limit B cell-targeted treatment options for MCD patients. However, these patients still show clinical benefit from rituximab (an anti-CD20 monoclonal antibody) treatment (Naresh et al., 2009).

KSHV encodes four viral interferon regulatory factors (vIRFs). These proteins have minimal homology to human IRFs, but vIRF1, vIRF2, and vIRF3 are known to bind DNA elements similar to their human IRF counterparts (Lubyova and Pitha, 2000; Park et al., 2007; Hu et al., 2016) vIRFs exert their regulatory role at varying levels ranging from hampering the antiviral interferon response to inhibition of signaling pathways to control the function of cellular proteins, thereby interfering with the cellular processes such as apoptosis (Rivas et al., 2001; Nakamura et al., 2001; Lee et al., 2009) proliferation and angiogenesis (Wies et al., 2008; Li et al., 2019; Li et al., 2020) vIRF3 (LANA-2) expression is detected in nearly all virus infected cells in PEL and MCD tumors, and vIRF3 is a bona fide oncogene which can inhibit the function of p53. Moreover, among the KSHV vIRFs, the function of vIRF3 is thought to be B cell-specific. Interestingly, the expression level of vIRF3 does not fluctuate even after lytic reactivation (Rivas et al., 2001), suggesting that there is an alternative level of regulation driving vIRF3 expression in B cells. In latently infected PEL cell lines, vIRF3 is linked to decreased MHC-II expression, and vIRF3 also modulates both type II (Schmidt et al., 2011) and type I interferon responses (Lubyova et al., 2004). vIRF3-mediated inhibiton of IFN$\gamma$results in inhibition of both PIII and PIV promoter of class II transactivator (CIITA) transcription (Schmidt et al., 2011). Importantly, vIRF3 is required for the survival of both EBV co-infected and EBV negative B cell lymphomas *in vitro* (Wies et al., 2008).

## Kaposi's Sarcoma-Associated Herpesvirus Modulation of B Cell Phenotypes

### The Proliferation and Plasmablast Differentiation

PEL is an immunoblastic tumor affecting the pericardial or pleural area of the body cavities. PEL tumor cells are negative for most B cell surface markers except CD138/syndecan, a marker of terminal plasma cell differentiation (Jenner et al., 2003). These terminally differentiated CD138+CD20+ and CD20− plasma cells are highly

targeted by KSHV infection in primary B cells of tonsillar sample, gaining greater survival rate for CD20− cells over 3 days post infection. This indirect survival effect is as a result of differentiation of other B cell lineages into the CD138+ cells (Aalam et al., 2020). Interestingly, more than 60% of the KSHV infected B cells from PBMCs of KS positive patients are positive for CD138 (Bella et al., 2010). In MCD, the pathological cells are monotypic/polyclonal plasmablasts located in the mantle zone of spleen and lymph nodes (Du et al., 2001). These cells express PRDM1 / BLIMP1 marking them as pre-plasma or terminal plasma stage of B-cell differentiation (Chadburn et al., 2008). Most of KSHV infected B cells in MCD patients express IL-6 (Du et al., 2001), and the importance of IL-6 signaling in MCD is illustrated by the finding that tocilizumab (an IL-6R blocking monoclonal antibody) can ameliorate the symptoms or even lead to prolonged remission in some MCD cases (Song et al., 2010; Galeotti et al., 2012; Ramaswami et al., 2020). In *ex vivo* infection models, particularly those performed in tonsillar B lymphocytes, the immunophenotype of infected cells closely resembles the pathological cells present in MCD (Du et al., 2001; Chadburn et al., 2008; Totonchy et al., 2018). Latently KSHV infected B cells from the tonsil (characterized by LANA dots), proliferate, and express a high level of IL-6R and CD27 on their surface exhibiting plasma blast morphology at 72 h post-infection (Hassman et al., 2011). Similarly, KSHV infection of naïve B lymphocytes from human tonsil upregulates IL-6 secretion as well as CD27 expression (Totonchy et al., 2018). *Ex vivo* infection of activated peripheral blood B cells expressing DC-SIGN results in infection of primarily naive and IgM memory B cells at early times post-infection (Rappocciolo et al., 2008). Remarkably, a similar expansion of MZ-like memory and naive B cells is seen in PBMC from HIV negative KS patients (Bella et al., 2010). Taken together, the concordance between pre-disease immunophenotypes, *ex vivo* infection immunophenotypes and the phenotypes seen in KSHV lymphoproliferative diseases suggests that KSHV infection manipulates the B cell compartment toward particular immunophenotypes even in the absence of overt KSHV-associated lymphoproliferation.

### Induction of Immunoglobulin Light Chain Revision

One of the more puzzling characteristics of MCD is the fact that KSHV infection is restricted to Igλ positive B lymphocytes in patient samples (Du et al., 2001). The same restriction is observed in KSHV infected B lymphocytes derived from tonsil samples (Hassman et al., 2011). Moreover, in PEL, most infected B cells are lg negative with occasional Igλ positive B cells (Matolcsy et al., 1998). Our group was able to show that KSHV infection in Igκ tonsil lymphocytes induces Igλ expression *via* re-induction of V(D)J recombination driving BCR revision. These cells express LANA, K8.1 and ORF59 markers, indicating a mixed population of lymphocytes in latent and lytic stages of infection (Totonchy et al., 2018). The same study also detects the Igλ+ KSHV infected cells in biopsies of HIV positive patients with AIDS-related lymphadenopathy (ARL) having no histologically similar characteristics of MCD, again supporting the conclusion that KSHV manipulates B cell physiology even in the absence of KSHV-associated lymphoproliferative disease and establishing that the Igλ+ phenotype in MCD is driven directly by KSHV infection. Further study is needed to characterize the intervening events that drive KSHV infected B cells from these early manipulations of B cell phenotype and physiology to overt pathological lymphoproliferation.

## DISCUSSION

KSHV has been co-evolving within the human immune system for thousands of years, and it has developed a plethora of mechanisms for manipulating both B cell physiology and overall immunology which are just beginning to be understood. In recent years, progress on this has been accelerated by new models allowing efficient infection of tonsil-derived primary B cells (Kang and Myoung, 2017). Some of these primary human samples can last up to 10 days, giving ample time to explore the early infection events. One of the hurdle of studying human primary B cell is their limited survival and difficulty of immortalizing them. In the study by Faure et al. (2019), they could achieve up to 20 fold increase in KSHV infection of peripheral B cells co-infected with EBV. These cells were best infected when exposed to KSHV within 24 h of EBV infection and could survive for months under culture conditions. In our recent paper (Aalam et al., 2020), we have generated a library of 40 tonsil specimen and included detailed B cell subtype analysis and *de novo* infection model. The samples exhibited diverse range of susceptibilities and determined varieties of B cells are susceptible to KSHV infection with CD138+ cells being highly targeted population. However, detailed infection analysis on what drives the susceptibility on these samples are missing as is information about the contributions of cellular and viral genes as well as the immunological milieu to the emergence of pathological lymphoproliferation. While, adherent cells and B cell lines are extensively used for their convenience and ease of manipulation, confirming the same findings within the human primary cells should not be overlooked. Therefore, more systematic and detailed studies are required to evaluate KSHV molecular virology in primary B cells to decode the dynamics of KSHV pathology in the lymphocyte compartment.

## AUTHOR CONTRIBUTIONS

FA was responsible for content curation and writing of the manuscript. JT was responsible for editing the manuscript, supervision, and obtaining funding. All authors contributed to the article and approved the submitted version.

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# Interplay Between KSHV and the Host DNA Damage Response

*Eriko Ohsaki* and Keiji Ueda*

*Division of Virology, Department of Microbiology and Immunology, Osaka University Graduate School of Medicine, Suita, Japan*

Interactions between viruses and cellular factors are essential for viral replication or host defense. The DNA damage response (DDR) orchestrates a molecular network of cellular mechanisms that integrates cell cycle regulation and DNA repair or apoptosis. Numerous studies have revealed that the DDR is activated by virus infection, aberrant DNA structures generated by viral DNA replication, or the integration of retroviruses. Although the DDR is an essential function for maintaining the genomic integrity of cells, viruses may utilize this mechanism to build a convenient environment for themselves, and the resulting perturbation of the DDR has been shown to increase the risk of tumorigenesis. There have been many studies investigating the roles of the DDR in oncogenic viruses such as Epstein-Barr virus (EBV), human papillomavirus (HPV), hepatitis B virus (HBV), human T-cell leukemia virus type 1 (HTLV-1), and Kaposi's sarcoma-associated herpesvirus (KSHV). This review summarizes current knowledge on the roles of DDR in the KSHV lifecycle.

**Keywords: Kaposi's sarcoma-associated herpesvirus, DNA damage response, DNA repair, cell cycle, latency, lytic replication, KSHV, DDR**

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*Asuka Nanbo,*
*Nagasaki University, Japan*

****Correspondence:***
*Eriko Ohsaki*
*eohsaki@virus.med.osaka-u.ac.jp*

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## INTRODUCTION

For the survival of organisms, the faithful transmission of genetic information from a parent cell to its daughter cells is essential. Such accurate transmission requires not only mechanisms for the faithful replication of DNA and segregation of chromosomes, but also mechanisms to prevent spontaneous and/or exogenously induced DNA damages. To accomplish all these goals, cells have monitoring systems that survey aberrant chromosomal structures. After sensing DNA damage, a DNA damage checkpoint coordinates with the cell-cycle regulation and repair systems. In response to DNA damage, the DNA damage response (DDR) controls cell cycle arrest to allow enough time for repair. When DNA damage is too severe to rescue, the apoptosis pathway is activated by the DDR.

KSHV is classified as a member of the Gammaherpesvirinae subfamily, which also includes Kaposi's sarcoma and lymphoproliferative disorders such as primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD), and KSHV is often associated with HIV infection (Cesarman and Knowles, 1995; Soulier et al., 1995). KSHV has two distinct lifecycles, a latent phase and lytic replication phase. During latency, the viral genome is maintained with limited gene expression in host cells (Sarid et al., 1998; Fakhari and Dittmer, 2002; Lieberman, 2013; Campbell et al., 2020). When latency is disrupted, the virus shifts to a lytic phase in which infectious progeny virions are produced (Sun et al., 1998; Purushothaman et al., 2015; Aneja and Yuan, 2017).

Affinity purification of DNA-binding proteins first demonstrated that several DDR proteins such as poly (ADP-ribose) polymerase 1 (PARP1) and MutS homolog 2/3/6 (MSH2/3/6) bind to the terminal repeat (TR) region (Ohsaki et al., 2004), and that MSH2/6, PARP1, DNA-dependent protein kinase (DNA-PK), and Ku70/80 bind to lytic DNA replication origins (ori-Lyt) (Wang et al., 2008). Recently, many studies have reported that DDR proteins are upregulated by viral replication and involved in the KSHV lifecycle, as described in a later section.

Viral infection causes global disruption of nuclear architecture and chromosomal aberration, and DDR is activated as a potent antiviral defense (Fortunato and Spector, 2003). In addition, structures of the viral genome, including linear double stranded DNA (dsDNA) (herpesviruses, adenoviruses), circular dsDNA (polyomaviruses, papillomaviruses), and RNA genomes which are reverse transcribed to linear dsDNA (retroviruses), can be recognized by DDR sensor proteins (Everett, 2006; Kerur et al., 2011; Lilley et al., 2011; Barber, 2014; Hau and Tsao, 2017; Kleinberger, 2020). On the other hand, viruses take advantage of the DDR pathway to modulate the cell cycle and hijack cellular proteins to support viral replication (Everett, 2006; Lilley et al., 2010; Weitzman and Fradet-Turcotte, 2018). Since DDR signaling pathways induce cell cycle arrest or apoptosis, which are negative effects on virus production, viruses have developed suppressive strategies against the DDR. Over the past two decades, numerous studies have reported the interactions between DDR signaling pathways and human tumor viruses including HPV, HTLV-1, HBV, HCV, EBV, and KSHV (Weitzman and Fradet-Turcotte, 2018).

Deregulation of DDRs because of the competition among such virus-host defense systems increases the risk of tumorigenesis. This is because various kinds of cell signaling networks maintaining homeostasis are perturbed. Therefore, an improved understanding of these relationships between viruses and DDR systems will help us to develop strategies for anti-tumorigenic and anti-viral therapies. In the first section of this review we summarize DDR signaling pathways, and in the second section we focus on the relationships between KSHV and DDR.

## DNA DAMAGE RESPONSE

DDR consists of DNA damage sensors followed by transducers, and effectors (**Figure 1**). All the DDR pathways including cell cycle checkpoints and DNA repair pathways are completed by proper signaling from sensors to transducers and to effectors.

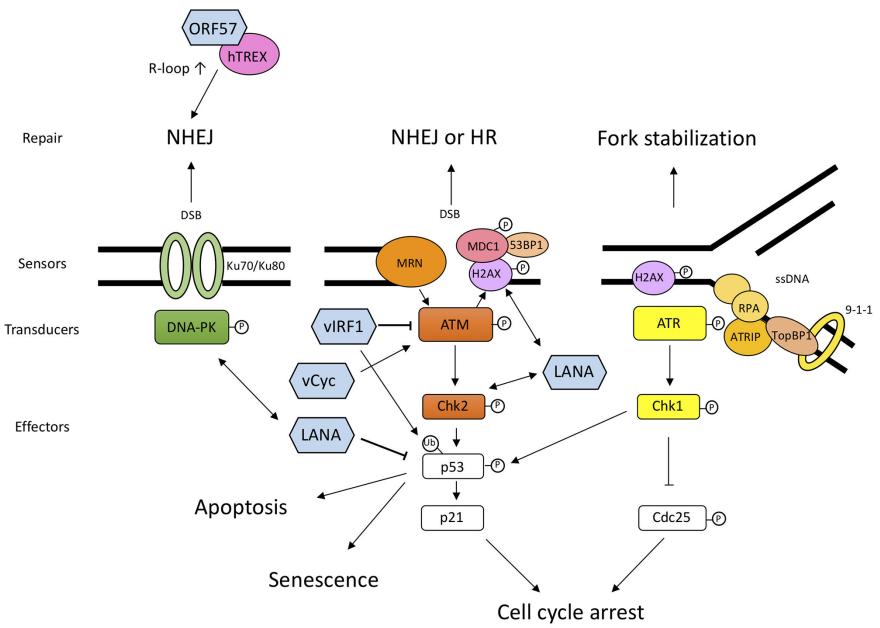


**FIGURE 1** | Schematic diagram of DDR pathways and the interplay between DDR and KSHV proteins. DNA damage sensors recognize the aberrant DNA structure and activate transducers, such as DNA-PK, ATM, and ATR. These transducers activate downstream effectors related to cell cycle arrest and DNA repair. When the DNA damage is too severe, cells undergo permanent cell-cycle arrest, senescence, or cell death. The viral proteins shown in this figure are not exhaustive but include the proteins mentioned in this article. LANA and v-cyclin lead to the activation of sensors and transducers *via* direct or indirect interaction. vIRF1 interacts with ATM and downregulates its kinase activity and ubiquitinates p53 to degrade it. ORF57 expression induces several NHEJ proteins and interacts with hTREX, and consequently transcribed mRNA becomes unstable and forms R-loops, which leads to genomic instability. The double-headed arrow shows the interaction between viral protein and DDR protein. DNA-PK, DNA-dependent protein kinase; ATM, Ataxia telangiectasia mutated; ATR, ATM and Rad3-related; MRN, Mre11-Rad50-NBS1 complex; Chk1, Checkpoint kinase 1; Chk2, Checkpoint kinase 2; MDC1, mediator of DNA damage checkpoint protein 1; ATRIP, ATR-interacting protein; RPA (replication protein A); TopBP1, DNA topoisomerase II binding protein 1.

## DNA Damage Sensors and Transducers

### PIKKs: Transducers of DNA Damage Response

The cellular responses to DNA damage are mainly controlled by three phosphatidylinositol 3-kinase-like kinases (PIKKs): ATM (Ataxia telangiectasia mutated), ATR (ATM and Rad3-related), and DNA-PK (Cimprich and Cortez, 2008; Davis et al., 2014; Blackford and Jackson, 2017; Menolfi and Zha, 2020), which act as DNA damage transducers. ATM and DNA-PK are principally activated in response to double-strand breaks (DSBs). In contrast, ATR is activated by single-stranded DNA (ssDNA) during the S-phase to regulate the firing of replication origins and the stalled replication forks.

### DNA Damage Sensing and Signaling

DSBs are recognized by the MRN complex, which is composed of Mre11, Rad50, and NBS1 and activates ATM (Lavin et al., 2015). MRN mediates cross-talk among the repair and checkpoint machinery. ATM phosphorylates downstream molecules, such as H2AX, a variant of the histone H2A protein family, and Chk2 (Checkpoint kinase 2) (Burma et al., 2001; Bartek and Lukas, 2003). Phosphorylated H2AX (γH2AX) is generated in chromatin near DSBs and recruits critical adaptor proteins such as MDC1 (mediator of DNA damage checkpoint protein 1) and 53BP1 (p53 binding protein 1) (Stewart et al., 2003; Xie et al., 2008; Kilic et al., 2019). Activated ATR and ATM phosphorylate the downstream targets Chk1 (Checkpoint kinase 1) and Chk2, which are key effectors in DDR (Bartek and Lukas, 2003; Smith et al., 2020).

Ku70/Ku80 are other sensor proteins recognizing DSBs and recruit the DNA-PK, and their main role is to facilitate non-homologous end joining (NHEJ), which is one of the DNA repair systems, as discussed in a later section (Jette and Lees-Miller, 2015; Chang et al., 2017). Following the sensing of DNA lesions, phosphorylation of transducer and effector molecules by PIKKs induces cell cycle arrest, DNA repair, and/or apoptosis or senescence.

## Cell Cycle Checkpoints and DNA Repair Pathways

Activated Chk1 and Chk2 phosphorylate downstream targets such as Cdc25 and p53 followed by degradation of Cdc25 and cell cycle arrest (Donzelli and Draetta, 2003; Liu et al., 2020) or by activation of the p53-mediated signaling pathway for DNA repair, cell cycle arrest, and apoptosis (Bartek and Lukas, 2003; Williams and Schumacher, 2016). p21, the downstream target of p53, induces cell cycle arrest through inhibition of the cyclin E/Cdk2 complex (Waldman et al., 1995; Planas-Silva and Weinberg, 1997; Gire and Dulić, 2015).

PIKKs have roles in the recruitment of repair machineries *via* the phosphorylation of downstream elements. For DSB repair, two major pathways are used: NHEJ and homologous recombination (HR). The NHEJ pathway has largely four steps: recognition, resection, polymerization, and ligation of the DNA ends (Chang et al., 2017). In the NHEJ, Ku70/80 primarily recognizes DSBs, and DNA-PK is recruited and activated by Ku-bound DSB ends to promote NHEJ.

DSBs are also recognized by the MRN complex (Carson, 2003), which promotes ATM activation and resection of DSB ends to generate ssDNA overhangs. While NHEJ is active throughout interphase, HR is active only in the S and G2 phases, because a homologous chromosome is available in the S and G2 phases as a template for DNA repair. The relationships between HR proteins and viruses have been reported in EBV (Kudoh et al., 2009), HPV (Gillespie et al., 2012; Park et al., 2014), and HTLV-1 (Belgnaoui et al., 2010), but not in KSHV. Additional investigations will be needed to expand our knowledge of the relationships between KSHV and HR.

The mismatch repair system improves DNA replication fidelity by degrading an error-containing region of the newly synthesized strand and providing a chance for the DNA polymerase to correct errors (Li, 2008; Fishel, 2015; Gupta, 2019). MSH2, MSH3, and MSH6 participate in mismatch repair as heterodimers, i.e., MSH2/MSH6 or MSH2/MSH3. While MSH2/6 recognizes base pair mismatches and small insertion/deletions, MSH2/MSH3 recognizes various DNA mismatches, including DNA loops ranging from 1 to 14 nucleotides as well as longer insertion/deletion mismatches (Jiricny, 2006). Several studies have suggested that the mismatch repair pathway is involved in viral replication not only in KSHV (Ohsaki et al., 2004; Wang et al., 2008; Cha et al., 2010), but also in EBV (Daikoku et al., 2006), as described in a later section.

Poly (ADP-ribose) polymerase 1 (PARP1) is an ADP-ribosylating enzyme and a multifunctional nuclear enzyme that affects various aspects of cellular homeostasis, such as DNA repair, cell proliferation, apoptosis, and inflammation. PARP1 is another important SSB- and DSB-signaling protein and modifies both target proteins and PARP1 itself by poly (ADP) ribosylation (Ray Chaudhuri and Nussenzweig, 2017). PARP1 has a pivotal role in DNA repair and is involved in various repair pathways, such as single-strand break repair (SSBR) (Leppard et al., 2003; Fisher et al., 2007), base excision repair (BER) (Masson et al., 1998; Dantzer et al., 2000; Lavrik et al., 2001; El-Khamisy, 2003; Ronson et al., 2018), nucleotide excision repair (NER) (Pines et al., 2012; Robu et al., 2017), NHEJ (Wang et al., 2006; Mansour et al., 2010; Cheng et al., 2011; Spagnolo et al., 2012; Luijsterburg et al., 2016), and HR (Hochegger et al., 2006; Hu et al., 2014).

# INTERPLAY BETWEEN KSHV AND DDR

## Roles of the DDR in *De Novo* Infection and Latent Infection

During the latent phase, the KSHV genome persists in the host nucleus as a double-stranded circular DNA—i.e., as an extra-chromosomal viral genome (episome)—and a very limited set of viral genes such as LANA (ORF73), vFLIP (ORF71), v-cyclin (ORF72), Kaposin (ORF K12), vIRF3 (LANA2), and 12 miRNAs are expressed (Dittmer et al., 1998; Sun et al., 1999; Parravicini et al., 2000; Cai et al., 2005; Pfeffer et al., 2005; Samols et al., 2005; Lieberman, 2013; Qin et al., 2017).

Like the proteins of other viruses, the KSHV proteins interact with DDR components and activate or prevent the signaling response (**Figure 1** and **Table 1**). *De novo* infection of KSHV in human PBMCs or primary endothelial cells upregulates the level of γH2AX, which is the phosphorylated form of H2AX (Jha et al., 2013; Singh et al., 2014). γH2AX interacts with LANA and contributes to LANA-mediated episome maintenance (Jha et al., 2013). It has been reported that H2AX knockdown reduces the expression of LANA and viral genome copies, suggesting that γH2AX has a role in latent gene expression and establishment of KSHV latency (Singh et al., 2014). In the same manner as phosphorylated H2AX, phosphorylated ATM was induced within 30 min post infection, and the inhibition of ATM activity caused a reduction of LANA expression, while knockdown of Chk1 and Chk2 did not affect LANA expression (Singh et al., 2014). These results suggest that selective activation of the DDR pathway is critical for the initial stages of KSHV infection and establishment of viral latency.

DDR proteins are equally distributed in the nucleus, but recent works have suggested that cytoplasmic DDR proteins sensor the cytoplasmic exogenous DNA and activate the innate immune signaling (Roth et al., 2014). Mariggio et al. demonstrated that the cytoplasmic LANA recruits the MRN complex in the cytoplasm of KSHV-infected B cells to inhibit NF-κB activation and blocks the role of innate immune sensors of cytoplasmic DNA (Mariggiò et al., 2017).

The cell cycle profiles of KSHV-positive cells suggest that LANA inhibits nocodazole-induced G2/M arrest (Kumar et al., 2014). In the same study, Kumar et al. (2014) reported that LANA interacts with Chk2 through the serine rich N-terminal domain of Chk2, and that downregulation of Chk2 expression promotes G2/M arrest in KSHV-positive BC3 cells. These results suggested that LANA interacts with Chk2 to escape from the G2/M cell cycle arrest due to the ATM/ATR signaling pathway.

Some of the DDR proteins play negative roles in latent DNA replication (Koopal et al., 2007; Cha et al., 2010). Using a proteomics approach, Cha et al. (2010) identified DNA-PK, Ku70, and Ku80 as LANA-binding proteins. They further showed that LANA is phosphorylated by DNA-PK/Ku and reduces transient DNA replication. Finally, they reported that overexpression of Ku70 downregulates transient DNA replication, suggesting that the DNA-PK/Ku complex binds with LANA and negatively regulates latent replication (Cha et al., 2010).

KSHV v-cyclin induces replicative stress in EA.hy926—which is a HUVEC-epithelial A549 hybrid cell line and has been used as an endothelial cell model—and also induces DDR and senescence by activating γH2AX, ATM, Chk2, p53, and p21 (Koopal et al., 2007). This v-cyclin-induced DDR is dependent on CDK6, a catalytic subunit of the v-cyclin. From this study, the DDR response appears to be activated by host defense mechanisms.

In addition, a recent study suggested that KSHV miRNAs target GADD45B to protect infected cells from cell cycle arrest and apoptosis (Liu et al., 2017). KSHV infection in primary endothelial cells causes repression of growth arrest DNA damage-inducible gene 45 (GADD45B). This study also demonstrated that KSHV miRNA-K9 inhibits the expression of GADD45B induced by a p53 activator, Nutlin-3, and suggested that KSHV miRNAs play essential roles in protecting cells from the DDR-induced cell cycle arrest and apoptosis.

## Roles of the DDR in Lytic Replication

Murine γ-herpesvirus 68 (MHV68) ORF36, which is a conserved serine/threonine protein kinase in Herpesviridae and is similar to the cellular kinase cdk2 (Romaker et al., 2006), phosphorylates H2AX (Tarakanova et al., 2007) during lytic replication. This ORF36-mediated H2AX phosphorylation is dependent on ATM activity, and is critical for viral replication, suggesting that the association between viral kinase and cellular DDR proteins synergistically supports viral replication. Another study demonstrated that ORF36 phosphorylates histone

**TABLE 1** | List of DDR-KSHV interaction during *de novo* infection, latency, and lytic reactivation and its effects.

| | Cellular DDR proteins | Viral components | Effects | Refs. |
|---|---|---|---|---|
| **De novo infection** | γH2AX | LANA | Episome persistence | Jha et al., 2013 |
| | γH2AX, ATM | ? | Establishment of latency | Singh et al., 2014 |
| **Latent** | PARP1 | TR, LANA | Negative for virus maintenance | Ohsaki et al., 2004 |
| | ATM, Chk2, γH2AX, p53 | v-cyclin | Oncogenic | Koopal et al., 2007 |
| | DNA-PK, Ku70/80 | LANA | Negative for virus maintenance | Cha et al., 2010 |
| | Chk2 | LANA | Protection from G2/M cell cycle arrest | Kumar et al., 2014 |
| | GADD45B | miRNA-K9 | Anti-apoptotic, Protection from cell cycle arrest | Liu et al., 2017 |
| | MRN | cytoplasmic LANA (LANA⊿N) | Modulation of an innate immune signaling pathway | Mariggiò et al., 2017 |
| **Lytic** | PARP1 | RTA | Abortive lytic replication | Gwack et al., 2003 |
| | ATM, p53 | vIRF1 | Downregulation of DDR for viral replication | Shin et al., 2006 |
| | PARP1 | ori-Lyt | Positive for lytic DNA replication | Wang et al., 2008 |
| | Ku70/80, DNA-PK, MSH2/6 | ori-Lyt | Supportive for lytic DNA replication | Wang et al., 2008 |
| | TIP60 | ORF36 | Positive for lytic gene expression | Li et al., 2011 |
| | γH2AX, Mre11, Rad50, Ku70/80, DNA-PK, PARP1, XRCC1, DNA ligase 3 | ORF57 | Genomic instability | Jackson et al., 2014 |
| | RPA, Mre11 | lytic replication foci | Positive for viral DNA synthesis | Hollingworth et al., 2015 |
| | DNA-PK, Ku80 | lytic replication compartments | Negative for viral DNA replication | Hollingworth et al., 2017 |
| | MRN | lytic replication compartments | Positive for viral DNA replication | Hollingworth et al., 2017 |

acetyltransferase TIP60, an upstream regulator of the DDR pathway, and promotes lytic gene expression (Li et al., 2011).

MDM2, an E3 ubiquitin ligase and a repressor of p53, has a negative role in viral reactivation (Balistreri et al., 2016). Lytic reactivation induces a p53 response in PEL cell lines and arrests the cells at G2 phase, which enables efficient lytic replication, as a positive effect of DDR on viral replication.

A proteomics analysis based on SILAC (stable isotope labelling by amino acids in cell culture) identified a large number of NHEJ proteins, including Rad50, Mre11, DNA-PK, Ku70, Ku80, PARP1, XRCC1 (X-ray repair cross-complementing protein 1), and DNA ligase3, that are enriched upon the expression of KSHV ORF57, a viral early protein and post-transcriptional regulator of gene expression (Jackson et al., 2014). In addition, as a consequence of the interaction between ORF57 and hTREX (human transcription and export complex), which is an mRNA export complex, the newly transcribed mRNA becomes unstable and forms R-loops, leading to genomic instability.

Affinity purification and mass spectrometry assays have identified MSH2, MSH3, MSH6, PARP1, DNA-PK, and Ku70/Ku80 as TR-binding proteins (Ohsaki et al., 2004), as ori-Lyt-binding proteins (Wang et al., 2008), and as LANA-binding proteins (Cha et al., 2010). Lytic reactivation in RTA-inducible BCBL1 and KSHV-infected endothelial cells causes DDR activation through the phosphorylation of H2AX, ATM, and DNA-PK and modulates cell cycle progression (Hollingworth et al., 2015). RPA (replication protein A), which is a single-stranded DNA-binding protein, and Mre11 accumulate at viral replication foci, suggesting that DDR proteins contribute to viral DNA replication. These studies suggested that NHEJ proteins such as the MSH2/6 heterodimer and DNA-PK/Ku70/Ku80 heterotrimer are recruited to latent/lytic replication origins and have some roles in the formation of a replication initiation complex.

Another study also demonstrated that the Ku70/Ku80 heterodimer and the MRN complex are recruited to viral replication compartments (RCs) during lytic replication, and the activation of ATM kinase promotes viral replication (Hollingworth et al., 2017). On the other hand, the other DDR proteins, such as γH2AX, MDC1, and 53BP1, localize on the periphery of viral RCs. In addition, knockdown or inhibition of NHEJ proteins such as Ku80 and DNA-PK enhances viral replication, suggesting a negative effect of the NHEJ pathway on viral replication (Hollingworth et al., 2017). Thus, it still remains to be clarified whether such DNA repair components have positive or negative roles in KSHV viral replication. Further work is necessary to elucidate how DNA repair components function in viral lytic replication.

PARP1 has essential roles in posttranslational modification of a large number of target proteins related to various kinds of cellular events and acts as a multifunctional enzyme. A previous study reported that PARP1 inhibits viral transcription through RTA ribosylation and leads to abortive lytic replication (Gwack et al., 2003). PARP1 directly binds to the TR and ribosylates LANA to modulate viral replication in latency (Ohsaki et al., 2004;

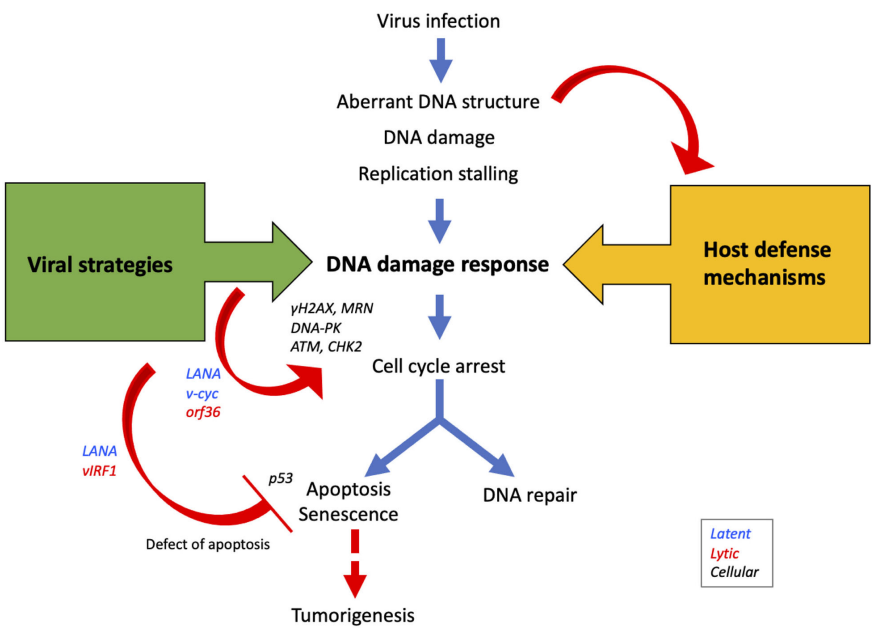


**FIGURE 2** | Viral proteins manipulate DDR signaling pathways to promote viral propagation. During infection, aberrant DNA structures or DNA damages caused by virus replication are recognized by DDR sensors/transducers as host defense mechanisms. On the other hand, viral proteins such as LANA, v-cyc, and ORF36 activate DDR proteins to modulate the cell cycle in the S-phase to promote viral replication. Since apoptosis decreases the opportunity for viral propagation, many viral proteins such as LANA, vIRF1, and vFLIP inhibit apoptosis and promote cell survival. The activation of the apoptotic pathway following the DDR occurs *via* p53, but because viral proteins deregulate this pathway, the risk of tumorigenesis is increased.

Cha et al., 2010). From these studies, PARP1 has negative roles in latent viral DNA replication and in lytic gene expression. On the other hand, another study reported that PARP1 plays a positive role in the early stage of viral DNA replication in lytic reactivation (Wang et al., 2008).

Although the biological significance of DDR activation in KSHV lytic/latent replication is not clear, the activation of upstream signaling of DDR seems to benefit the viral replication. In contrast, some viral proteins directly interact with the downstream signaling components to prevent effectors from suppressing virus replication. Downstream of ATM pathway are inactivated in the late stage of lytic reactivation through the vIRF1 (viral interferon regulatory factor 1)-mediated pathway (Shin et al., 2006). vIRF1 interacts with ATM and downregulates ATM kinase activity and the p53 protein level (Shin et al., 2006).

## CONCLUSIONS

Over the past decades, many studies have elucidated the relationships between KSHV and the DDR signaling pathway. The roles of the DDR in viral replication depend on the type of infection, the structure of the viral genome formed by replication, the cell type, and the cell cycle stage. Aberrant DNA structures and signaling in the course of viral genome replication lead to the DDR pathway as a host defense response. On the other hand, viruses have developed strategies to hijack the DDR signaling pathway for their survival (**Figure 2**). A number of studies introduced in this article suggest that activation of the upstream pathway of the DDR—which includes DNA damage sensors and transducers—contributes to the modulation of both cell cycle progression and viral replication, whereas the downstream signaling pathways, such as the apoptosis pathway, are unfavorable for viruses. Accordingly, KSHV probably has developed strategies to negate a part of the DDR pathway.

Deregulation of the DDR pathway caused by such viral strategies increases the risk of tumorigenesis. More specifically, viruses affect cell cycle regulation to drive viral replication and manipulate the DDR pathway, and the resulting damage to the cellular repair system, increase in mutations, and resistance to apoptosis causes genomic instability and finally promotes tumorigenesis. An improved understanding of the battles between viruses and the DDR will lead to new therapeutic options for controlling viral replication and oncogenesis.

## AUTHOR CONTRIBUTIONS

EO contributed to the design of the manuscript. EO and KU contributed to the concept of the manuscript. KU proofread and modified the manuscript. All authors contributed to the article and approved the submitted version.

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BRIEF RESEARCH REPORT
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# Profiling the Blood Compartment of Hematopoietic Stem Cell Transplant Patients During Human Cytomegalovirus Reactivation

*Biana Bernshtein[1†], Aharon Nachshon[1], Miri Shnayder[1], Lauren Stern[2], Selmir Avdic[3], Emily Blyth[3,4,5,6], David Gottlieb[4,5,6], Allison Abendroth[2], Barry Slobedman[2], Noam Stern-Ginossar[1*] and Michal Schwartz[1*]*

[1] Department of Molecular Genetics, Weizmann Institute of Science, Rehovot, Israel, [2] Discipline of Infectious Diseases and Immunology, Faculty of Medicine and Health, Charles Perkins Centre, University of Sydney, Camperdown, NSW, Australia, [3] Sydney Cellular Therapies Laboratory, Westmead Institute for Medical Research, Westmead, NSW, Australia, [4] BMT and Cell Therapies Unit, Westmead Hospital, Sydney, NSW, Australia, [5] Westmead Institute for Medical Research at the University of Sydney, Westmead, NSW, Australia, [6] Sydney Medical School, Faculty of Medicine and Health, University of Sydney, Camperdown, NSW, Australia

Human cytomegalovirus (HCMV) is a widespread pathogen establishing a latent infection in its host. HCMV reactivation is a major health burden in immunocompromised individuals, and is a major cause of morbidity and mortality following hematopoietic stem cell transplantation (HSCT). Here we determined HCMV genomic levels using droplet digital PCR in different peripheral blood mononuclear cell (PBMC) populations in HCMV reactivating HSCT patients. This high sensitivity approach revealed that all PBMC populations harbored extremely low levels of viral DNA at the peak of HCMV DNAemia. Transcriptomic analysis of PBMCs from high-DNAemia samples revealed elevated expression of genes typical of HCMV specific T cells, while regulatory T cell enhancers as well as additional genes related to immune response were downregulated. Viral transcript levels in these samples were extremely low, but remarkably, the detected transcripts were mainly immediate early viral genes. Overall, our data indicate that HCMV DNAemia is associated with distinct signatures of immune response in the blood compartment, however it is not necessarily accompanied by substantial infection of PBMCs and the residual infected PBMCs are not productively infected.

**Keywords: human cytomegalovirus, blood compartment, hematopoietic stem cell transplantation, reactivation, peripheral blood mononuclear cell**

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****Correspondence:***
*Noam Stern-Ginossar*
*noam.stern-ginossar@weizmann.ac.il*
*Michal Schwartz*
*michalsc@weizmann.ac.il*

***[†]Present address:***
*Biana Bernshtein, Ragon Institute of MGH, MIT and Harvard, Cambridge, MA, United States*

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## INTRODUCTION

Human cytomegalovirus (HCMV) is a widespread pathogen infecting most of the population worldwide. Like other herpesviruses, following primary infection, HCMV establishes a latent infection that persists for the lifetime of the host. Although HCMV infection in healthy individuals is mostly asymptomatic, reactivation from latency in immunocompromised individuals constitutes a serious health burden. For hematopoietic stem cell transplantation (HSCT) patients HCMV

reactivation is a major risk factor (Stern et al., 2019). Reactivation in these patients can lead to HCMV disease that manifests in diverse symptoms, from gastroenteritis to respiratory symptoms, hepatitis and retinitis, and is also associated with graft versus host disease (Cantoni et al., 2010; Ljungman et al., 2017). Overall, HCMV reactivation is a leading infectious cause of morbidity and mortality in HSCT patients (Broers et al., 2000). Since HCMV reactivation is associated with DNAemia, i.e. the detection of viral DNA in the blood, HSCT patients undergo routine surveillance of HCMV DNA levels in the blood during the post-transplant period; most commonly by qPCR analysis of blood samples (Emery et al., 2000). Pre-transplant HCMV serostatus of the donor and recipient is the major risk factor for HCMV reactivation and disease following HSCT, with HCMV seropositivity of the recipient conferring the highest risk (George et al., 2010; Webb et al., 2018). HCMV reactivation develops in more than 50% of the cases where the recipient was HCMV seropositive and the donor was seronegative (R+/D-), while in cases where the recipient was HCMV seronegative and the donor was HCMV seropositive (R-/D+) there is a ~10% risk.

Hematopoietic progenitor cells and monocytes are considered major reservoirs of HCMV latency in humans (Hahn et al., 1998; Slobedman and Mocarski, 1999; Goodrum, 2016) however, the specific source of HCMV reactivation in these patients remains elusive. Understanding the blood cell subsets that are infected with HCMV during reactivation, as well as the effect of HCMV reactivation on the blood compartment, is important in order to elucidate the role of the blood compartment in progression and control of infection and in dissemination. Previous analyses of viral load in primary blood mononuclear cells (PBMCs) relied on relative measurements using quantitative PCR or *in-situ* hybridization and provided a wide range of results (Saltzman et al., 1988; Boivin et al., 1999; Hassan-Walker et al., 2001), and detailed transcriptomic analyses were not performed in such samples.

In order to systematically and accurately characterize the infection of the blood compartment during HCMV reactivation in HSCT recipients, we analyzed PBMCs from HSCT patients that exhibited HCMV DNAemia. We used digital droplet PCR (ddPCR), which allows specific and highly sensitive absolute quantification of DNA even at low amounts, to determine infection of specific cell types in blood samples from patients exhibiting HCMV DNAemia. RNA sequencing was further applied to study the host transcriptome in PBMCs as well as to characterize the viral expression pattern in PBMCs from HCMV reactivating HSCT patients. We found that although HCMV DNA was detected in the plasma at high levels, PBMCs harbored extremely low levels of viral DNA, with monocytes generally exhibiting the highest viral loads. Analysis of the host transcriptome suggested the development of HCMV-specific T-cells and the involvement of regulatory T cells (Tregs) and additional immune pathways during HCMV DNAemia. Interestingly, viral transcript levels were very low, in line with low viral loads found in the different PBMC subsets, however the gene expression pattern that was detected resembled that of early stages of productive infection, indicating that these cells do not go through a full productive cycle. Taken together, our findings indicate that DNAemia in HCMV reactivating HSCT patients is not necessarily accompanied by substantial infection of PBMCs, but is nevertheless associated with evident immune response signatures.

# MATERIALS AND METHODS

## Cells and Virus Stocks

Peripheral Blood Monouclear Cells (PBMC) were isolated from fresh venous blood, obtained from healthy donors, using Lymphoprep (Stemcell Technologies) density gradient. The cells were cultured in RPMI media (Beit-Haemek, Israel) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 100 units/ml penicillin and streptomycin (Beit-Haemek, Israel) at 37°C in 5% CO2. Primary human foreskin fibroblasts (ATCC CRL-1634) were maintained in DMEM with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 100 units/ml penicillin and streptomycin (Beit-Haemek, Israel).

The TB40/E virus containing an SV40-GFP tag (TB40/E-GFP) was described previously (Sinzger et al., 2008; O'Connor and Murphy, 2012). Virus was propagated by electroporation of infectious bacterial artificial chromosome (BAC) DNA into fibroblasts using the Amaxa P2 4D-Nucleofector kit (Lonza) according to the manufacturer's instructions. Viral stocks were concentrated by centrifugation at 26000xg, 4°C for 120 min. Infectious virus yields were assayed on THP-1 cells (ATCC TIB-202).

## Infection Procedures

For experimental infection, PBMCs were infected at a multiplicity of infection (MOI) of 5 and fibroblasts were infected at an MOI of 1. Infection was carried out by incubation with the virus for 2 h followed by two washes to clear out viral particles.

## Cell Staining for Flow Cytometry and Sorting

Cells were counted, and stained in cold MACS buffer (PBS, 5% BSA, 2 mM EDTA). Cell staining was done using the following antibodies:

Anti-human-CD45 (Clone: HI-30, Biolegend), anti-human-HLA-DR, DP, DQ (clone: REA332, Miltenyi Biotec), anti-human-CD14 (Clone: M5E2, Biolegend), anti-human-CD16 (Clone:3G8, Biolegend), anti-human-CD19 (Clone: SJ25C1, Biolegend), anti-human-CD3 (Clone: OKT3, Biolegend), according to manufacturer's instructions. Cells were analyzed and sorted on a BD FACSAriaIII.

## Detection of Viral Genomes by Digital PCR

Detection of viral DNA was done using the QX200 droplet digital PCR system (Bio-Rad), using FAM labeled HCMV primer and probe (Human CMV HHV5 kit for qPCR using a glycoprotein B target (PrimerDesign) and HEX labeled RPP30 copy number

assay for ddPCR (Bio-Rad), as previously described (Shnayder et al., 2020). Cells were counted, dry pelleted, and stored at −80°C prior to DNA extraction. DNA was extracted from the cell pellet in a 1:1 mixture of PCR solutions A (100 mM KCl, 10 mM Tris–HCl pH 8.3, and 2.5 mM MgCl2) and B (10 mM Tris–HCl pH 8.3, 2.5 mM MgCl2, 0.25% Tween 20, 0.25% Non-idet P-40, and 0.4 mg/ml Proteinase K), for 60 min at 60°C followed by a 10 min 95°C incubation, according to the description in (Roback et al., 2001). To avoid biases due to small cell numbers, samples with cell number <1,500 were excluded.

## RNA Library Construction

RNA libraries were generated from samples of ~100,000 cells according to the MARS-seq protocol (Jaitin et al., 2014; Keren-Shaul et al., 2019).

## Sequencing and Data Analysis

RNA-Seq libraries (pooled at equimolar concentration) were sequenced using NextSeq 500 (Illumina), with read parameters: Read1: 72 cycles and Read2: 15 cycles.

Analysis of bulk MARS-seq was done as described previously (Shnayder et al., 2018). The number of Unique Molecular Identifiers (UMIs) were:

| | Low DNAemia | High DNAemia |
|---|---|---|
| patient 6 | 1890938 | 5301580 |
| patient 7 | 1324199 | 2925446 |
| patient 8 | 4780993 | 5068593 |

## Correlation Analysis

Pearson correlation between viral gene expression profiles was calculated using Morpheus (https://software.broadinstitute.org/morpheus/).

## Differential Expression and Enrichment Analysis

The differential expression analysis was done with DESeq2 (version 1.22.2) (Love et al., 2014) using default parameters, with the number of reads in each of the samples as an input.

# RESULTS

## PBMC From Healthy Individuals Harbor HCMV Genomes Following Experimental Infection

Following HSCT, patients are monitored for HCMV reactivation by means of measuring viral DNA loads either in whole blood or in plasma, however it is unclear what is the source of the detected viral DNA and which cells in the blood carry HCMV. To unbiasedly assess the ability of PBMCs to be efficiently infected with HCMV, we first purified PBMCs from the blood of a healthy donor and infected them at high MOI with an HCMV strain TB40/E-GFP, which expresses GFP from an SV40 promoter. Twenty-four hours post infection, cells were FACS sorted based on standard cell markers to distinct blood cell types: CD14+CD16- cells which are mainly classical monocytes, CD16+CD14- cells which include non-classical monocytes (Guilliams et al., 2018), a subset of NK cells (Lanier et al., 1989), and dendritic cells (Fromm et al., 2020), and T and B cells according to cell surface markers CD3 and CD19, respectively (**Figure 1A**). The remaining CD14-CD16-CD3-CD19- cells that were not sorted are most likely subsets of NK cells and dendritic cells. Flow cytometry analysis revealed that HCMV infects all cell types, as evident by appearance of GFP positive cells; however, CD14+ monocytes exhibited significantly higher percentage of GFP positive cells as well as much higher GFP intensity (**Figure 1B**), suggesting that they are most efficiently infected. It is noteworthy that none of these cell types are considered to support productive infection, and specifically monocytes, despite having a clear GFP signal, are latently infected (Shnayder et al., 2018).

Droplet digital PCR (ddPCR) is a relatively recent technology in which PCR amplification of a specific amplicon is partitioned into a large number of discrete reactions, allowing greater precision and reproducibility compared to conventional PCR based methodologies, and highly sensitive absolute quantification of nucleic acids (Hindson et al., 2013; Taylor et al., 2017). We used ddPCR to directly assess the viral load in these different cell populations. In agreement with GFP levels, all cell types harbored some HCMV genomes, while CD14+ monocytes exhibited the highest levels of HCMV genomes per cell (**Figure 1C**), indicating that CD14+ monocytes are much more permissive to HCMV experimental infection compared to the other blood cell types tested, which exhibited low level of infection.

## Low Levels of Viral DNA in Peripheral Blood Mononuclear Cells During Human Cytomegalovirus DNaemia

To define HCMV infection of different cell types in the blood during DNAemia, we analyzed PBMCs from HSCT recipients that exhibited HCMV reactivation, as defined by detectable levels of HCMV genomic DNA in the blood. These patients were periodically monitored for HCMV DNAemia following HSCT and none exhibited HCMV organ disease. PBMCs were purified from blood samples of five HSCT recipients taken at the peak of DNAemia, as measured by HCMV DNA levels in the plasma (**Figure 2A**). PBMCs were sorted to distinct cell populations as described in **Figure 1A** and viral load in these cells was measured by ddPCR. We set a cut-off of at least two positive events, which was determined according to analysis of samples from healthy sero-negative donors (**Supplementary Figure 1**). PBMCs from four of the patients exhibited extremely low (<15 genomes/10,000 cells) to undetectable HCMV genomic levels in all cell types, indicating an extremely low level of infected PBMCs in their blood (**Figure 2B**, patients 2–5). In one DNAemic sample

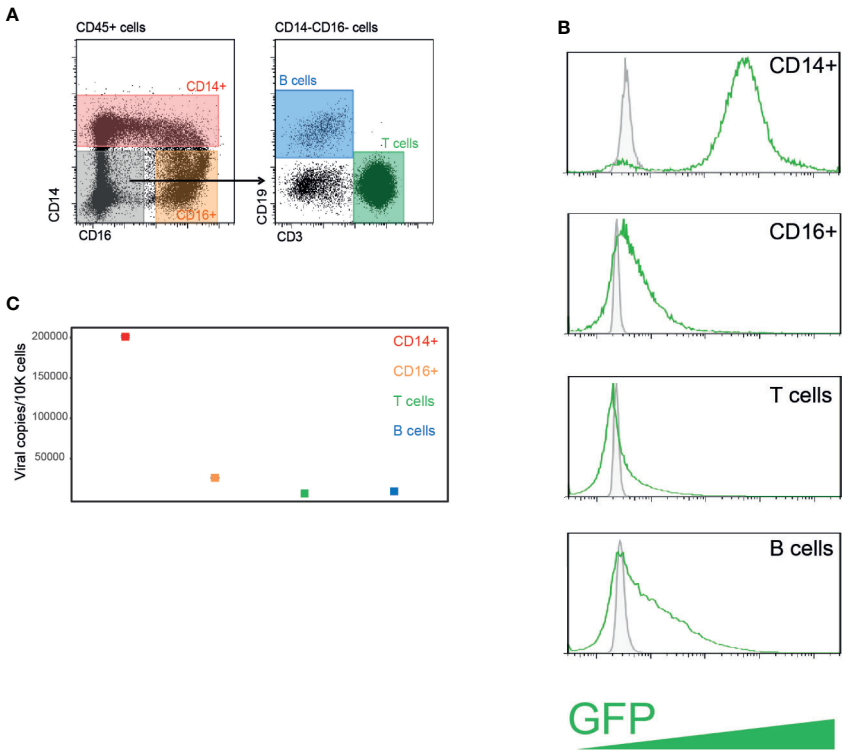


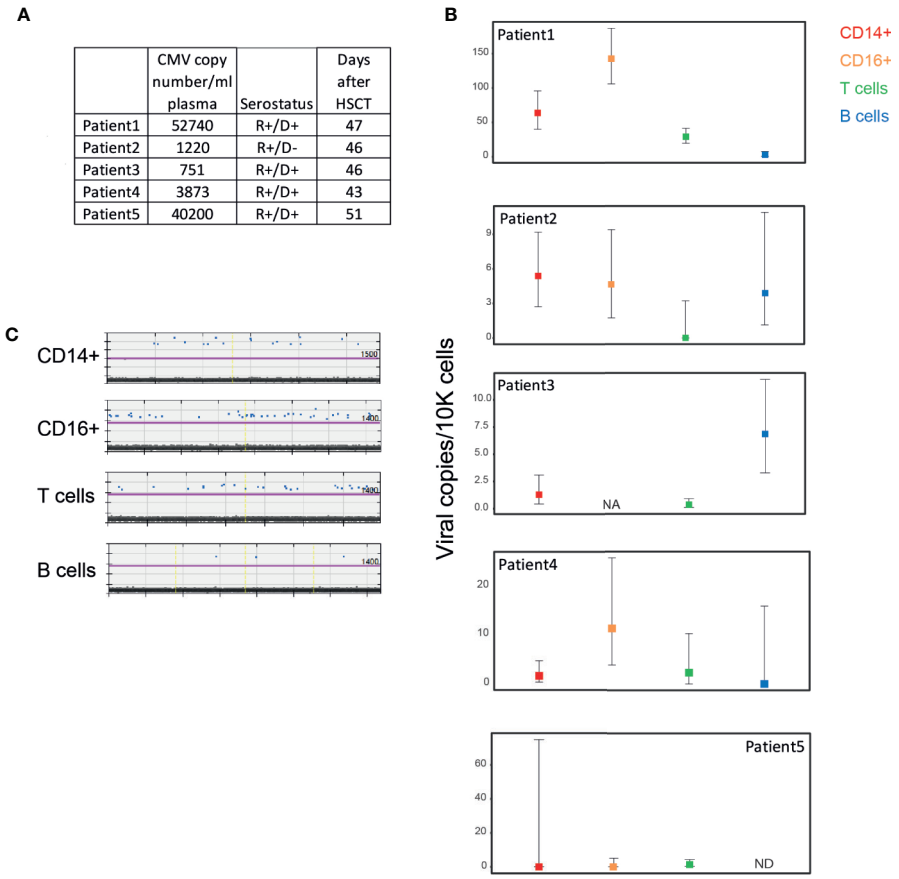

**FIGURE 1** | PBMCs from healthy individuals are efficiently infected with HCMV following experimental infection. **(A)** HCMV infected PBMCs were FACS sorted to four distinct cell populations. **(B)** Flow cytometry analysis of GFP expression levels in PBMCs experimentally infected with HCMV strain TB40/E-GFP at 22 h post infection. Uninfected control cells and infected cells are shown in grey and green lines, respectively. **(C)** Quantification of viral genomes by ddPCR in indicated PBMCs populations presented as copies per 10,000 cells. Graph reflects mean and 95% CV of Poisson distribution, calculated from two technical replicates per sample.

(patient 1) there were relatively higher levels of HCMV genomic DNA, yet still very low, that reached ~50 genomes/10,000 CD14+ monocytes, ~150 genomes/10,000 CD16+ cells, ~35 genomes/10,000 T cells and undetected levels in B cells (**Figures 2B, C**). Although higher viral loads were detected in this patient compared to the other four patients, and there was a preference towards the infection of CD16+ cells and CD14+ which include most of the monocytes, the viral loads were still very low and far from the viral load that could be achieved in experimental infection (**Figure 1C**). These results suggest that although PBMCs are permissive to experimental infection, infection levels are extremely low in the context of reactivation *in-vivo* following HSCT, and cannot explain the high HCMV DNA levels detected in the plasma.

## Transcriptional Changes During Human Cytomegalovirus DNAemia

To characterize the changes in PBMCs from HCMV reactivating HSCT patients during DNAemia and to examine the viral gene expression profile, we analyzed the transcriptome of PBMCs from three patients following HSCT by RNA-seq, at two time points: no detection or very low level of viral DNA in the blood and during measurable DNAemia (**Figure 3A**). These patients are at the stage of reconstitution of their immune system, which likely has a substantial impact on the transcriptional profile of their blood cells. Nevertheless, Principle Component Analysis (PCA) of RNA-seq data indicated that the high-DNAemic samples clustered separately from low-DNAemic samples of the same patient (**Figure 3B**).

Differential expression analysis revealed differences between high-DNAemic and low-DNAemic PBMCs that were shared between all three patients. 25 genes were significantly differentially expressed between the two sample types (adjusted p-value<0.05, fold change>2, **Figures 3C, D**, **Table S1**); two were upregulated and 23 were downregulated in high-DNAemic samples. The two upregulated genes in high-DNAemia were CX3CR1 and EGR1, both of which are expressed by HCMV specific T cells following HSCT (Hertoghs et al., 2010; Hardy et al., 2018). Among downregulated genes were several genes related to inflammatory responses, including two genes belonging to the TNF-receptor superfamily (RELT, TNFRSF12a), as well as genes related to innate immune responses (LCN2, ITGA2B). Interestingly, two downregulated genes, HIC1 and ID1, are known as transcription factors that enhance Treg function and differentiation (Liu et al., 2014; Ullah et al., 2018). Thus, we were able to readily detect a response of blood cells to HCMV DNAemia, which was captured by reproducible transcriptional changes.




| | CMV copy number/ml plasma | Serostatus | Days after HSCT |
|---|---|---|---|
| Patient1 | 52740 | R+/D+ | 47 |
| Patient2 | 1220 | R+/D- | 46 |
| Patient3 | 751 | R+/D+ | 46 |
| Patient4 | 3873 | R+/D+ | 43 |
| Patient5 | 40200 | R+/D+ | 51 |

**FIGURE 2** | Low levels of HCMV DNA detected in PBMCs from HCMV reactivating HSCT patients during HCMV DNAemia. **(A)** Description of samples from five HCMV reactivating HSCT patients. HCMV copy number was measured by RT-qPCR. PBMCs from HSCT patients were sorted to four distinct cell populations as described in **Figure 1A**. **(B)** Quantification of viral genomes in the indicated PBMC populations from individual HSCT patients, presented as copies per 10,000 cells. Graph reflects mean and 95% CV of Poisson distribution, calculated from at least 2 technical replicates per sample. NA, not available; ND, not detected; i.e. positive event number in the sample is <=2. **(C)** ddPCR results of PBMC populations from patient 1, technical replicates separated by yellow vertical lines. The magenta line marks the threshold.

Although these genes are potentially related to HCMV reactivation, they may also be related to immune reconstitution or other processes in the immune system of these patients.

In line with the extremely low viral DNA level found in these cells, analysis of viral gene expression indicated very low to undetectable viral transcript levels even in the high DNAemic samples (**Figure 3E**, **Table S2**). In two high-DNAemia samples, we found 55 and 66 unique viral reads in ~ 5 million total reads, while in the third patient there were only two viral reads (**Figure 3E**, **Table S2**). Surprisingly, the viral transcripts detected in these samples were mainly of genes that are expressed at an immediate early time point such as UL123 and UL36, while the level of transcripts that are abundant at the late stages of productive infection was much lower (**Figure 3F**). Indeed, although the number of viral reads we obtained is low, comparison of viral gene expression pattern of these samples to the expression pattern of early and late stages of productive infection, based on RNA-seq analysis of infected fibroblasts at 8 and 72 hpi, revealed high correlation with early stage of infection (R=0.71, **Figure 3G**). The absence of late transcripts indicates that the cells from which these viral transcripts originate are not productively infected and thus are unlikely to produce infectious virus. Overall, the transcriptome analysis of PBMCs from HSCT patients reveals host response to HCMV infection and low viral gene expression, which resembles early stages of productive infection without clear evidence of a full replicative cycle.

## DISCUSSION

HCMV reactivation constitutes a major clinical burden following bone-marrow as well as solid organ transplantation. Reactivation is detected in patients by measurement of HCMV DNA in the blood. Despite the importance of understanding the role of the blood compartment in HCMV infection and dissemination following reactivation of the virus, the nature of this infection

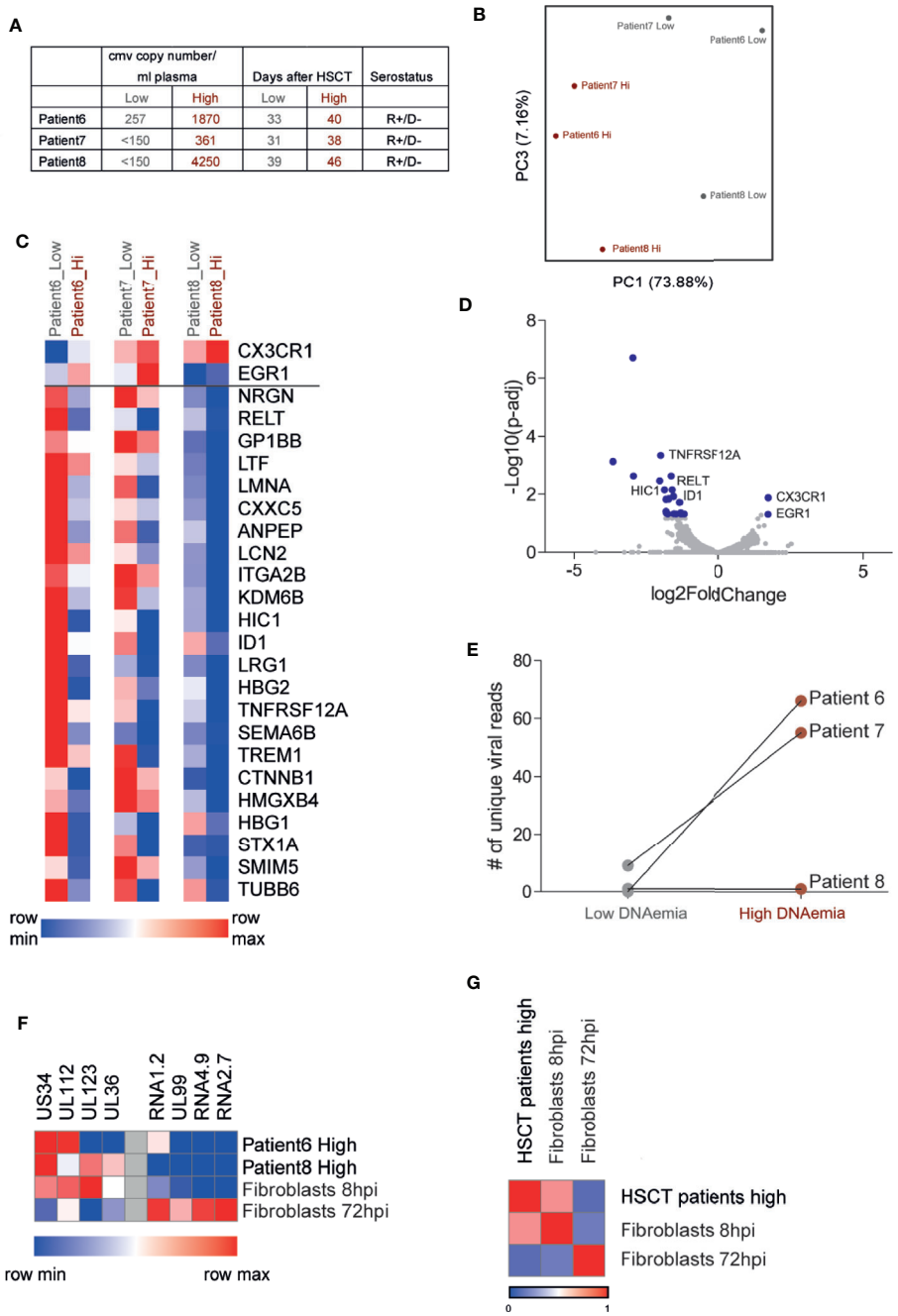



**FIGURE 3** | RNA sequencing analysis of PBMCs from HCMV reactivating HSCT patients reveals low viral transcript levels and a discernible host response to active HCMV infection. **(A)** Description of samples from three HCMV reactivating HSCT patients. HCMV copy number was measured by RT-qPCR. **(B)** Principal component analysis of the transcriptional profile of PBMCs from three HSCT patients at two time points. **(C)** Heat map of 25 host genes significantly differentially expressed (fold change>2 and FDR<0.05) in all three HSCT patients comparing samples of low and high DNAemia. **(D)** Volcano plot of statistical significance (-log10 p-value) against log 2 ratio of host transcript levels between low and high DNAemic samples of PBMCs from three HCMV reactivating HSCT patients, based on RNA-seq data. Blue dots mark significantly up or down regulated genes (fold change>2, adj-p value<0.05). **(E)** Total number of viral reads in PBMCs from low and high DNAemic samples of three HCMV reactivating HSCT patients. **(F)** Heat map showing the expression level of representative immediate early stage and late stage viral genes in PBMCs of HSCT patients or experimental lytically infected fibroblasts at 8 or 72 h post infection. **(G)** Heat map showing Pearson's correlation between viral gene expression program in PBMCs from HSCT patients and experimental lytically infected fibroblasts at 8 or 72 h post infection.

is not well characterized. Depicting the blood cell types that are infected, portraying the nature of viral infection in these cells and its impact on the host will provide insight on these issues.

Quantitative assessments of viral load during HCMV reactivation in different blood cell types were done in the past but relied on less quantitative methodologies than the ones currently available (Saltzman et al., 1988; Boivin et al., 1999; Hassan-Walker et al., 2001). ddPCR is a relatively recent technique allowing absolute measurements of nucleic acids with superb precision and reproducibility (Hindson et al., 2013; Taylor et al., 2017). To delineate the levels of HCMV infection in different PBMC populations, we used this highly sensitive tool to measure the absolute level of HCMV genomes in the different cell types. We show that CD14+ monocytes are markedly the preferential target of HCMV infection following experimental infection of PBMCs. CD14+ monocytes are considered sites of HCMV latency, which may reactivate in response to differentiation (Goodrum, 2016). These cells were indeed very efficiently infected as apparent from the level of the GFP reporter as well as from the amount of viral genomes that were detected in these cells, however this infection is not productive as viral gene expression is repressed and infectious virus is not produced (Shnayder et al., 2018). In comparison, CD16+ cells, B cells, and T cells, are much less efficiently infected. This is in line with the prevalent view of monocytes as the main cell type in the blood to be infected by HCMV.

ddPCR measurements in samples from HSCT recipients with HCMV reactivation, at the peak of DNAemia, also supported the notion that monocytes are generally the most efficiently infected cell type among PBMCs, although as CD16+ cells showed higher viral DNA levels in some of the patients, this may implicate additional cell types. However the levels of infection in all blood cell types tested are extremely low in these settings. This suggests that much of the DNA measured in the plasma does not originate from mononuclear cells in the blood, and perhaps does not reflect infectious virus in the blood. The difference in viral load between experimental infection and in the context of reactivation *in-vivo* after HSCT probably stems from several factors that greatly differ between natural and experimental systems including the high MOI that is used in experimental settings, the viral strain and changes in the environment of the cells.

In light of previous studies, showing much higher levels of HCMV DNA in mononuclear blood cells, the extremely low infection levels we find are surprising. This difference may represent variability between patients or may be related to the more precise measurement method. It is noteworthy that our analysis includes patients with very high DNAemia, negating the possibility that we screened only patients with low levels of HCMV reactivation. We cannot rule out the possibility that in other patients there may be higher levels of HCMV genomes in PBMCs during DNAemia, however in a previous study we found extremely low levels of HCMV genomes in monocytes of additional HCMV reactivating HSCT patients (Shnayder et al., 2020). Further research will be required to delineate the source of HCMV genomes in the blood of patients with low PBMC infection during DNAemia. A possible target is polymorphonuclear cells which were shown to be infected during HCMV reactivation (Saltzman et al., 1988; Hassan-Walker et al., 2001). Interestingly, circulating cytomegalic endothelial cells have been identified in the blood of solid organ transplant patients, AIDS patients, and HSCT patients (Salzberger et al.; Grefte et al., 1993; Percivalle et al., 1993; Gerna et al., 1998).

Analysis of viral transcripts in PBMCs from DNAemic samples supports the notion that these cells are hardly infected with HCMV, as the levels of viral mRNAs were extremely low. Intriguingly, the dominant viral genes that were expressed were immediate early (IE) genes. We previously examined HCMV gene expression in diverse human tissues by analyzing RNA-seq samples from the Genotype-Tissue Expression (GTEx) Consortium. Interestingly, this analysis also uncovered samples with a restrictive gene expression pattern that includes mainly IE transcripts and these were specifically found in blood samples (Shnayder et al., 2018). This appearance of the same pattern implies that there are blood cells with limited viral gene expression that might reflect a threshold that needs to be crossed before the virus can accomplish the complete infection cycle. This specific pattern also suggests that the PBMCs that are infected in these DNAemic samples are not productively infected.

The viral genome levels we find in PBMCs from DNAemic samples are very similar to the levels that were estimated for PBMCs during latency (Slobedman and Mocarski, 1999; Jackson et al., 2017), which may suggest that these could be latent cells which are not related to reactivation. However, several lines of evidence suggest that they are related to reactivation. First, some of the samples used in the study are from R+/D- cases, where latent blood cells are not expected. Second, although the transcript levels are extremely low, the transcription profile is very distinct from what was described for latent monocytes (Cheng et al., 2017; Shnayder et al., 2018). Third, beside patient 5, there is an association between the levels of DNAemia in the patient and the levels of viral genome copies that were measures in PBMCs by ddPCR. Thus although the infection is low, we suspect that it is related to the reactivation of HCMV.

Despite the extreme changes in the blood compartment during reconstitution of the immune system following HSCT, the gene expression profile of high-DNAemic samples clustered away from low-DNAemic samples from the same patient and there were reproducible transcriptional changes. This suggests that these genes are associated with HCMV reactivation although it possible that they are related to other immune processes, e.g. immune reconstitution. HCMV is known to elicit a robust CD8+ T cell response. One of the upregulated genes in high-DNAemic samples, CX3CR1, is upregulated in HCMV specific T-cells following HCMV infection (Hertoghs et al., 2010). The expression of EGR1, which was also upregulated in our data, was characteristic of CMV-specific T-cells in non-immune reactive HSCT patients, which are associated with poor CMV control (Hardy et al., 2018). These results suggest that an immune cellular response to HCMV reactivation has developed in these patients. Regulatory T cells (Tregs) are essential for regulating the function of effector T cells. The proportion of Tregs within CD4+ T-cell population was found to decrease during HCMV reactivation in HSCT patients (Velaga et al., 2013). Notably, two out of the 23 down-regulated genes in samples with HCMV DNAemia, Hic1 and ID1, were shown to promote Treg differentiation, expansion and suppression functions, supporting decrease of Treg functions during HCMV reactivation (Liu et al., 2014; Ullah et al., 2018). In addition, several genes related

to TNF signaling and NF-kappa-b activation, or associated with innate immune response, as well as antiviral processes were also downregulated. Further studies will need to establish the importance and function of these changes during HCMV reactivation in HSCT patients.

Overall, our data suggest that DNAemia in HCMV reactivating HSCT patients is not necessarily accompanied by substantial infection of PBMCs, and that the infected PBMCs are not productively infected. Nevertheless, high DNAemia in these patients is associated with transcriptional changes that indicate an active immune response. Our findings elucidate the nature of HCMV infection in PBMCs during HCMV reactivation in HSCT patients and shed light on the role of the blood compartment in progression and control of HCMV infection.

## DATA AVAILABILITY STATEMENT

All next-generation sequencing data files were deposited in Gene Expression Omnibus under accession number GSE161752.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Weizmann Institutional Review Board (IRB application 92–1) and Human Research Ethics Committee of the University of Sydney and the Western Sydney Local Health District. Informed consent was obtained from all study participants prior to enrollment in accordance with the Declaration of Helsinki. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

BB, BS, NS-G, and MSc designed the research. BB and MSh performed the research. LS, SA, EB, DG, AA, and BS provided critical reagents and advice. BB, AN, NS-G, and MSc analyzed the data, and BB and MSc wrote the paper. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.607470/full#supplementary-material

**SUPPLEMENTARY FIGURE 1 |** ddPCR Analysis of Samples From Healthy Sero-Negative Donors. ddPCR results of PBMC populations from two healthy HCMV sero-negative donors, technical replicates separated by yellow vertical line. The magenta line marks the threshold.

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REVIEW
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# Burkitt Lymphomas Evolve to Escape Dependencies on Epstein-Barr Virus

*Rebecca L. Hutcheson, Adityarup Chakravorty and Bill Sugden* *

*McArdle Laboratory for Cancer Research, University of Wisconsin–Madison, Madison, WI, United States*

Epstein–Barr Virus (EBV) can transform B cells and contributes to the development of Burkitt lymphoma and other cancers. Through decades of study, we now recognize that many of the viral genes required to transform cells are not expressed in EBV-positive Burkitt lymphoma (BL) tumors, likely due to the immune pressure exerted on infected cells. This recognition has led to the hypothesis that the loss of expression of these viral genes must be compensated through some mechanisms. Recent progress in genome-wide mutational analysis of tumors provides a wealth of data about the cellular mutations found in EBV-positive BLs. Here, we review common cellular mutations found in these tumors and consider how they may compensate for the viral genes that are no longer expressed. Understanding these mutations and how they may substitute for EBV's genes and contribute to lymphomagenesis can serve as a launchpad for more mechanistic studies, which will help us navigate the sea of genomic data available today, and direct the discoveries necessary to improve the treatment of EBV-positive BLs.

**Keywords: Burkitt lymphoma, B cell, tumor evolution, cellular mutations, Epstein–Barr virus, next-generation sequencing, compensation**

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***Correspondence:**
*Bill Sugden*
*sugden@oncology.wisc.edu*

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## INTRODUCTION

Infectious agents cause approximately 2.2 million cases of cancer each year (de Martel et al., 2020), about 15% of all human cancers worldwide (Parkin, 2006; de Martel et al., 2012; Plummer et al., 2016; de Martel et al., 2020). Most of these cancers are caused by tumor viruses. In particular, some cases of Burkitt lymphoma (BL) are caused by Epstein-Barr Virus (EBV), the first human tumor virus to be discovered. In the decades since the discovery of EBV, we have learned that contributions that EBV makes to the formation and maintenance of Burkitt lymphomas are complex. In this review, we consider how Burkitt lymphomas evolve to lose some of their dependencies on this oncogenic virus.

In the 1950s, Denis Burkitt observed a childhood tumor common in Uganda characterized by malignant growths on the jaw and within the abdominal cavity (Burkitt, 1958). Burkitt pursued study of the tumor, recognizing it to be its own clinical entity. He soon appreciated that the large areas of Africa in which the tumor was found overlapped with regions in which rainfall was favorable for holoendemic malaria. Burkitt hypothesized an infectious agent as the cause and collaborated with Anthony Epstein, Yvonne Barr, and Bert Achong, who identified a herpesvirus within tumor cells (Epstein et al., 1964). More than 55 years later, we know that EBV not only causes BL in some populations, but also other cancers of lymphoid and epithelial origin. The World Health Organization classifies BL into three subtypes, endemic BL, sporadic BL, and HIV-associated BL (Swerdlow et al., 2016). All BL are characterized by similar histology, hypermutated

immunoglobulin gene sequences, and almost all carry chromosomal translocations in which the c-Myc proto-oncogene is brought under control of one of three immunoglobulin loci, resulting in its constitutive expression and fostering proliferation. While EBV can be found in all three subtypes of BL, each subtype varies in the frequency with which the virus is found. The "high incidence" endemic BL (eBL) found in equatorial Africa and Papua New Guinea is about 95% EBV-positive, whereas in other parts of the world, sporadic BL (sBL) can be anywhere from 20%–80% EBV-positive depending on the geographical region (Magrath, 2012). The endemic and sporadic classification was begun initially to define the two geographical groups of the disease, but more recent analyses of the mutational burden in primary tumors from both eBL and sBL has revealed that EBV presence distinguishes a specific BL phenotype regardless of geographic origin (Kaymaz et al., 2017; Grande et al., 2019).

Though initially controversial, it is now clear that EBV's presence in Burkitt lymphomas does not merely reflect its being a passenger in these B cell tumors. There are several compelling pieces of evidence supporting EBV's causal role in the development of BL. First, in a prospective study in Uganda, antibodies against EBV were found to be exceptionally high in children who eventually develop eBL (de-Thé et al., 1978). Second, molecular analysis found that EBV infection precedes the malignant B cell outgrowth (Raab-Traub and Flynn, 1986; Neri et al., 1991). When EBV circularizes its genome on infecting cells, its terminal repeats are joined, with different numbers of repeats characterizing each circularized DNA (Kintner and Sugden, 1979). BL tumor cells harbor multiple EBV genomes, but within individual BL, cells contained EBV DNAs with the same number of terminal repeats, indicating the tumor likely arose clonally following infection with a single virus (Raab-Traub and Flynn, 1986). Third, we have found that only 84% of EBV genomes—which exist as extrachromosomal plasmids in infected cells—in a population are synthesized each S-phase, indicating that if that population of cells is to maintain the virus, EBV must provide a selective advantage to those cells or its DNA will be lost as the cells proliferate (Nanbo et al., 2007). EBV-positive BL cells maintain the viral genome as plasmids over many generations, and thus, EBV must provide a selective advantage to these tumors (Adams and Lindahl, 1975). Consistent with this prediction, for example, when EBV is evicted from EBV-positive BL cell lines, they die by apoptosis (Kennedy et al., 2003; Vereide and Sugden, 2011).

EBV is a unique pathogen in that it not only infects primary B cells but also induces them to proliferate, (a process hereafter called "transformation"). For EBV to transform a cell it infects, the virus must express certain viral genes. These "transforming" viral genes have been experimentally identified under most conditions as being necessary for transformation in cell culture and include EBNA1 (Yates et al., 1985), EBNA2 (Hammerschmidt and Sugden, 1989), EBNA3A and EBNA3C (Tomkinson et al., 1993), and LMP1 (Wang et al., 1985). EBV lacking any one of these genes cannot transform B cells in culture, with the exception of LMP1, which can be substituted for by growing infected cells on a human fibroblast feeder layer (Dirmeier et al., 2005). In addition to these necessary genes, the virus expresses additional genes that increase the efficiency of transforming B cells, namely LMP2, the viral miRNAs, the small, non-coding RNA EBERs, and the transiently expressed, immediate early genes BZLF1 and BRLF1 (reviewed in Young et al., 2016). EBV also encodes circular RNAs that are derived from multiple transcripts and are candidates for regulating viral gene function, at least by serving as sponges for viral miRNAs (reviewed in Ungerleider et al., 2019).

Strikingly, EBV-positive BL tumors often do not express many of these transforming genes including EBNA2, EBNA3A, EBNA3C, LMP1, and LMP2. (Rowe et al., 1986; Niedobitek et al., 1995; Tao et al., 1998). Thus, on the journey from infected B cell to malignancy, EBV-positive tumor cells evolve to reduce viral gene expression to a limited set of viral genes. We propose that cellular mutations are acquired in these tumors that compensate for the lost viral genes (**Figure 1**). Since it is unlikely that EBV drives the occurrence of these cellular mutations, we suspect that as cellular mutations arise by chance, those cells with mutations that provide selective advantages are able to lose the expression of viral genes with similar functions. The loss of viral gene expression in EBV infected cells *in vivo* may be driven by the immune response. This hypothesis is supported by an observation found in another malignancy caused by EBV, post-transplant lymphoproliferative disorder (PTLD), where therapeutically immunosuppressed patients develop a rapid outgrowth of EBV infected cells after organ transplantation. When PTLDs arise quickly (a median of less than 1 year following transplantation) in these immunosuppressed patients, the malignant cells responsible are commonly infected with EBV and express the full set of viral latent proteins. However, in late-onset PTLD, cells express fewer numbers of EBV's genes (Timms et al., 2003). This observation serves as an example of how EBV-positive cells can evolve over time to express fewer viral genes. In this review, we consider how EBV-transformed B cells evolve as BLs to shut off the expression of some viral genes necessary for transformation and substitute for their functions through cellular mutations.

## PATHWAYS LEADING TO THE LOSS OF EXPRESSION OF VIRAL GENES IN BURKITT LYMPHOMA

Multiple EBV genes are necessary to transform B cells but are no longer expressed in many EBV-positive BL tumor cells. These genes have been found to be downregulated, deleted, or otherwise transcriptionally silenced. The loss of their expression likely reflects the constant pressure of the host's immune system on viral gene products. While people remain infected for their lives with EBV, the virus typically remains latent in memory B cells (Babcock et al., 1998) and only occasionally is found to be reactivated in the saliva (Niederman et al., 1976). These latently infected memory B

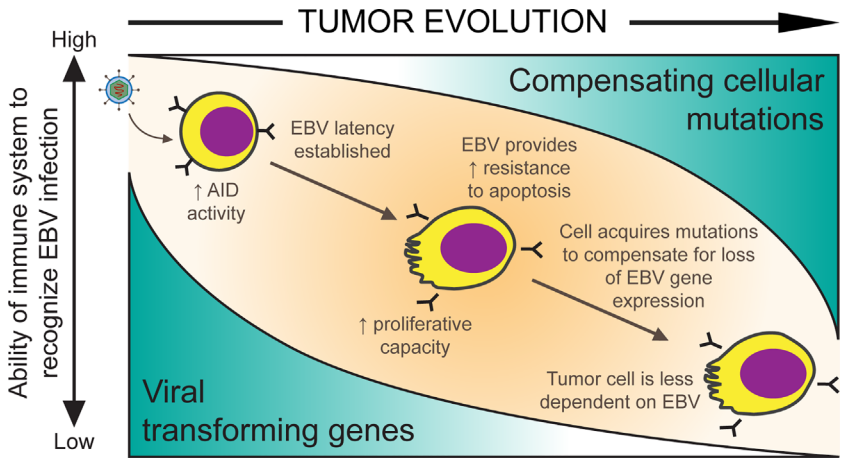


**FIGURE 1** | A model for EBV-positive Burkitt lymphomagenesis. Cells transformed with EBV are provided with oncogenic advantages including the ability to proliferate and the ability to avoid apoptosis through the expression of viral transforming genes. However, EBV infected cells are targeted by the host's immune system. As mutations randomly accumulate in cells, they allow expression of some of EBV's genes to be turned off if compensated by such cellular mutations and reduce immune-mediated tumor cell killing.

cells express few or no viral proteins, so they are unrecognized by the immune response. An observation consistent with this likelihood is that when BL biopsies that express few viral genes are explanted into cell culture, they often re-express additional viral genes, presumably reflecting a lack of immune selection (Rowe et al., 1986; Rowe et al., 1987; Tao et al., 1998). This is also echoed by the earlier onset PTLD in which cells responsible for the disease typically express the full set of EBV latency genes due to the lack of immune surveillance from the host. Consistent with the idea that immune pressure influences EBV gene expression, B cells infected with EBV *in vitro* continue to express the full repertoire of latent viral proteins, known as latency III. In contrast, BL cells usually express a smaller subset of the viral latency genes, known as latency I. Here, we focus on BL specifically, its distinctive pattern of viral gene expression (see **Table 1**) and consider the evidence that supports viral genes being downregulated in these tumors.

Early studies of EBV-positive BL biopsies detected limited viral gene expression, often only EBNA1, among the known transforming genes (Rowe et al., 1987). When the biopsies were grown in cell culture they were found eventually to express the additional transforming genes, EBNA2 and LMP1 (Rowe et al., 1987). In addition to EBNA1, BL biopsies are known to also express viral miRNAs and the EBERs (Niedobitek et al., 1995; Tao et al., 1998). The viral miRNAs and EBERs are thought to be non-immunogenic. EBNA1, on the other hand, apparently avoids immune detection in BL patients. While it is recognized by a $CD4^+$ T cell response, this response is inhibited in BL patients (Moormann et al., 2009). Other EBV transcripts have also been detected in some BL tumors but only in a subset of cells (Niedobitek et al., 1995; Xue et al., 2002).

About 15% of African BLs have deletions of the EBNA2 transforming gene, are termed Wp-restricted BL (Kelly et al., 2002), and as the name implies, express genes from the W

**TABLE 1** | EBV viral gene expression.

| Latency Type | | Viral mRNAs | Viral noncoding RNAs | Immunogenicity* |
|---|---|---|---|---|
| **Latency I** | Canonical BL | EBNA1 | BART miRNAs<br>EBERs | Limited antigen expression |
| **Wp Latency** | Wp-restricted BL | EBNA1<br>EBNA3A, 3B, 3C<br>EBNA-LP (truncated)<br>BHRF1 | BART miRNAs<br>EBERs<br>BHRF1 miRNAs | Increased antigen expression |
| **Latency III** | LCLs | EBNA1<br>EBNA2<br>EBNA3A, 3B, 3C<br>EBNA-LP<br>BHRF1<br>LMP1<br>LMP2A, 2B | BART miRNAs<br>EBERs<br>BHRF1 miRNAs | Highest antigen expression |

**Rowe et al., 1986; Rooney et al., 1986; Rowe et al., 1987.*
*Annotations in acronym or abbreviated form are: LCL, lymphoblastoid cell line.*

promoter (**Figure 2**). This subset of BLs expresses several additional transforming genes, including EBNA3A, EBNA3B, EBNA3C, truncated EBNA-LP, and BHRF1. It is unclear how these EBNA2 deletions occur mechanistically, but they likely reflect a selection against EBNA2 during the evolution of these BLs. Early genetic analyses of EBV's transforming genes identified EBNA2 as essential for transformation (Hammerschmidt and Sugden, 1989), but $CD8^+$ T cells targeting EBNA2 efficiently recognize EBV infected B cells at very early time points post-infection (Brooks et al., 2016), potentially indicating an early selection against EBNA2. EBNA2 was found to suppress expression of IgH, leading to decreased Myc expression in BL cell lines (Jochner et al., 1996). Thus, infected cells that can shut down expression from the C promoter or delete EBNA2 entirely can benefit from higher Myc levels. Although viral transforming genes expressed in Wp-restricted BLs can be immunogenic, they can also provide BLs a selective advantage. For example, EBNA3A and EBNA3C block apoptosis by cooperating to inhibit transcription of the pro-apoptotic gene Bim (*BCL2L11*) (Anderton et al., 2008; Price et al., 2017). Additionally, BHRF1, a viral Bcl2 homologue, can block apoptosis in BL cell lines as can some viral miRNAs (Kelly et al., 2009; Watanabe et al., 2010; Vereide et al., 2014). Evicting EBV from various BL tumor cell lines has shown that these tumor cells rely on EBV for survival depending on the number of viral transforming genes they express (Vereide and Sugden, 2011). These observations indicate that while some BLs escape dependence on some EBV genes, others continue to employ other viral genes to inhibit apoptosis.

Importantly, the overall mutational landscape in Wp-restricted BLs is not significantly different from that in canonical EBV-positive BLs (**Figure 3**), although there are subtle differences in the cellular expression pattern of Wp-restricted versus canonical BL cell lines. For example, Kelly *et al.* find that Wp-restricted BLs consistently display a downregulation of BCL6 and upregulation of IRF4 and Blimp1 (Kelly et al., 2013). Though our analysis of previously available data found no significant differences in the frequency of mutations in the examined genes, one limitation of interpreting these findings is that there were relatively few samples analyzed. Thus, it is likely that Wp-restricted and canonical BLs result from the expansion of similar progenitor cells, perhaps with subtle variations in mutational patterns reflecting differences in selection pressure due to differences in EBV gene expression.

Yet, the question remains: how does an EBV infected B cell evolve to lose expression of some or all of the viral genes initially required for its transformation? Clearly for Wp-restricted BLs,

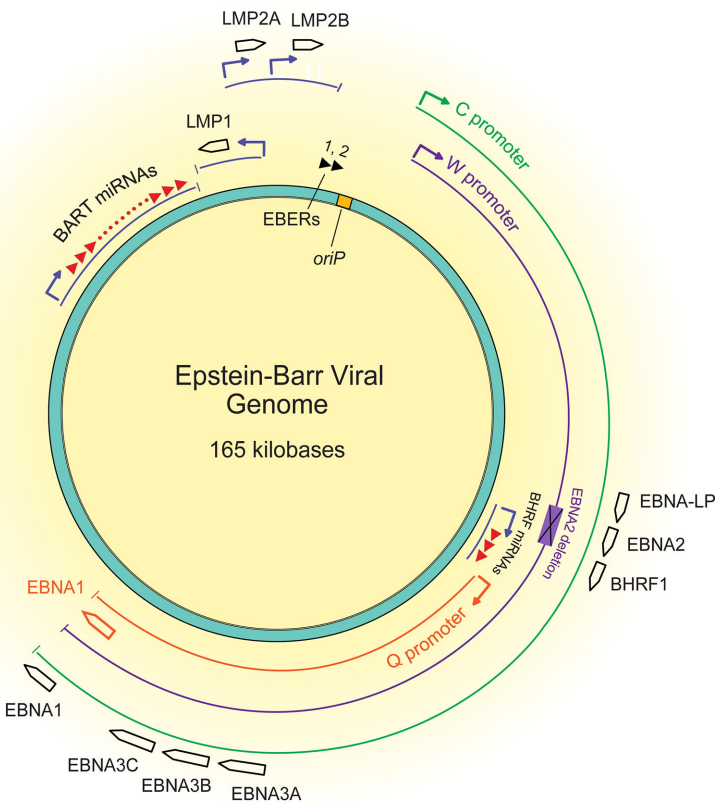


**FIGURE 2** | A map of the latent EBV genome. The 165 kilobase pair double-stranded DNA genome exists as a plasmid maintained within the nucleus of infected cells. The origin of plasmid replication (oriP) is shown in yellow. The thin lines represent transcripts, each color representing the promoter that drives their transcription, C (green), W (purple), and Q (orange). The black boxed arrows represent the approximate locations of exons encoding latent proteins. The black triangles at the top represent the highly transcribed non-coding RNA EBERs, and the red triangles represent the two sets of miRNAs.

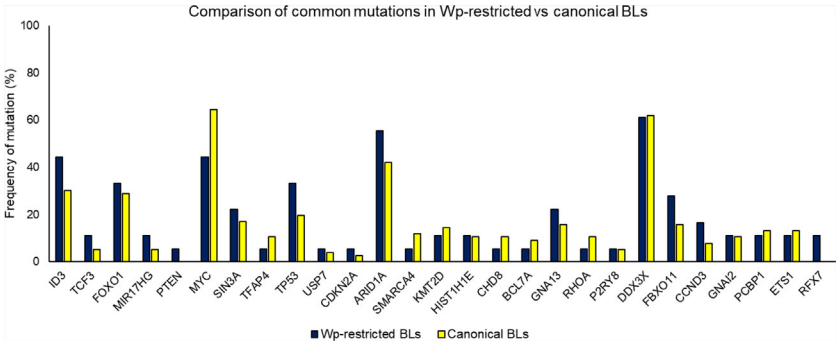


**FIGURE 3** | Comparing mutation loads for select genes in Wp-restricted (blue bars) and canonical (yellow bars) Burkitt lymphoma tumors. Data are derived from publicly available datasets (Grande et al., 2019). Tumors were designated EBV-positive based on detectable levels of EBNA1. EBV-positive tumors were categorized as Wp-restricted if EBNA2 expression was absent and EBNA3A/EBNA3C expression detected.

this loss occurs *via* deletions, at least in part. A second route to this loss is likely transcriptional. EBNA1 is expressed from different viral promoters in most BLs than in B cells transformed *in vitro* (Tao et al., 1998; Altmann et al., 2006). The C promoter drives expression of EBNA1 and the immunogenic EBNA proteins in lymphoblastoid cell lines. Wp-restricted BLs use the W promoter to express multiple transforming genes, and most BLs use the Q promoter from which only EBNA1 is expressed. These changes in viral promoters being used illustrate the evolution of BLs and underlie one mechanism by which BLs downregulate viral genes to escape dependence on them.

The evolution of these EBV infected cells is probably a complex process. For example, it is particularly intriguing to consider the hurdles EBV must overcome for its genomes to acquire mutations and subsequently express them successfully. BL cells maintain multiple copies of viral genomes in a single cell. Mutations arise rarely and surely in single viral genomes such that any one cell would have multiple copies of the wild-type viral gene in the presence of a lone mutant allele. Because EBV genomes are partitioned faithfully 88% of the time (Nanbo et al., 2007), a newly acquired viral mutation is likely to take over in daughter cells only if it provides a robust selective advantage.

In addition to several viral proteins, EBV's miRNAs also contribute to the survival of BL cells (Vereide et al., 2014), presumably without eliciting an immune response. The levels of these miRNAs differ across EBV-positive BLs (Cai et al., 2006; Xia et al., 2008) and across BL cell lines (Pratt et al., 2009). These different levels may also reflect BLs evolving to become independent of EBV. For example, the EBV-positive BL cell lines Daudi, Mutu I, and Akata can all lose EBV DNA spontaneously in culture. Their EBV-positive parents express fewer viral miRNAs than do other cell lines (Pratt et al., 2009), demonstrating, perhaps, that they have evolved to reduce their dependence on these viral miRNAs.

Though there is much evidence for loss of expression of viral genes in BL, either by promoter switching or deletion events, it is not clear yet how BLs compensate for this loss.

## FACTORS CONTRIBUTING TO LOSS OF EXPRESSION OF VIRAL GENES IN BURKITT LYMPHOMA

The viral genes whose expressions are often lost in BL tumor cells are not random; BL viral gene expression is similar across tumor biopsies. Though we do not fully understand the mechanisms behind this expression pattern, there are pieces of evidence that provide glimpses into the forces that shape the loss of some viral gene expression. For example, in one study in which B cells were transformed by a derivative of EBV in which EBNA2 functioned conditionally and c-Myc was expressed at high levels constitutively, the cells were found to no longer express LMP1 when EBNA2 was inhibited (Polack et al., 1996), but they continued to proliferate. This study shows that c-Myc, if expressed at high enough levels, can compensate for the loss of both EBNA2 and LMP1. Another set of cellular genes that may compensate for the loss of expression of viral genes in BL belongs to the Notch signaling pathway, which is known to be upregulated in BL (He et al., 2009). Notch-2 signaling *via* Delta-like ligand 1 was shown to inhibit EBNA2-mediated initiation of LMP1 transcription (Rowe et al., 2014). In addition, a high level of expression of introduced intracellular Notch in cells in which EBNA2 functions conditionally inhibited the expression of LMP1 (Gordadze et al., 2001). These observations indicate that increased levels of Notch signaling may compensate for the loss of expression of LMP1.

Some BL cell lines are less dependent on the virus for proliferation and survival than others (Vereide and Sugden, 2011). When EBV was evicted from BL cell lines, those BLs that formerly expressed more viral genes underwent apoptosis more rapidly than those that expressed fewer viral genes. One latency I BL which expressed the fewest viral genes continued to proliferate slowly even in the absence of EBV. Several of these lost viral latency genes are essential to transform B cells, which indicates that there must be some sort of functional compensation when these viral genes are lost. This compensation is likely to be through cellular mutations that

potentially can be identified through sequencing of primary BL tumors. In the next section, we discuss common cellular mutations found in BL, mutations specific to EBV-positive BL, and how these mutations might compensate for loss of expression of viral genes.

# MECHANISMS FOR CELLULAR COMPENSATION FOR THE LOSS OF EXPRESSION OF EPSTEIN–BARR VIRUS GENES IN BURKITT LYMPHOMA

## Regulation of Apoptosis

With the recent increase in availability and affordability of genome-wide sequencing, there is now a plethora of information about the cellular mutational burden of tumors, including BLs. Studies comparing EBV-positive BLs to tumors that lack the virus have confirmed parallel findings in cells in culture. EBV-positive tumors have significantly fewer mutations in genes affecting apoptosis compared to EBV-negative tumors (Grande et al., 2019), supporting findings that EBV inhibits apoptosis in BL cell lines. Indeed, many of EBV's latency genes target apoptotic pathways, and this was hypothesized early when eviction of the virus from BL cell lines caused death by apoptosis (Kennedy et al., 2003). Later experiments with Wp-restricted BL cell lines found that these tumor cell lines were much more resistant to cell death triggers than either EBV-negative or latency I BL lines (Kelly et al., 2005; Kelly et al., 2006). EBNA3A and EBNA3C—which are expressed in Wp-restricted BLs—were shown to downregulate Bim (Anderton et al., 2008), providing a mechanism for the increased resistance to apoptosis observed in this subset of EBV-positive BL tumors. Wp-restricted BLs can also express BHRF1, a viral gene that interferes with Bim and other pro-apoptotic proteins (PUMA, BID, BAK) to prevent apoptosis in BL cells (Desbien et al., 2009; Fitzsimmons et al., 2018; Fitzsimmons et al., 2020). The finding that BLs that lack EBV contain more cellular mutations in apoptotic genes, including significantly more mutations in *TP53* (Grande et al., 2019), further supports the hypothesis that mutations in cellular genes linked to apoptosis can compensate for the loss of EBV's gene expression in BLs.

## Mutations in Genes Typically Targeted by Epstein–Barr Virus' miRNAs

In addition to encoding several viral proteins, EBV encodes at least 40 miRNAs that can target cellular genes. In fact, few viral genes have been found to be targets of EBV's miRNAs (Pfeffer et al., 2004; Riley et al., 2012; Skalsky et al., 2012; Jung et al., 2014), leading to the hypothesis that they are more likely to target cellular genes by translational inhibition or mRNA degradation. Cellular genes targeted by EBV miRNAs may be yet another avenue by which the loss of expression of EBV genes can be compensated for by mutations in cellular genes. These cellular genes, when appropriately mutated, could provide functions akin to EBV's transforming genes, and thus may be found to also be mutated in EBV-negative BLs. Much is known about how EBV's miRNAs manage the host immune response to EBV infection (reviewed in Albanese et al., 2017); here we will focus on confirmed cellular targets of EBV's miRNAs (reviewed in Kuzembayeva et al., 2014) that when mutated may compensate for viral transforming genes that are downregulated in BL tumors. Collectively, when EBV's BART miRNAs were expressed in cells induced to lose the virus, their presence protected cells from apoptosis in BL cell lines. Further investigation showed the cluster of BART miRNAs targeted Caspase 3 to inhibit apoptosis in these BL cells (Vereide et al., 2014). The BL cell lines used for these experiments only expressed one viral protein, EBNA1, indicating that inhibition of Caspase 3 by EBV's miRNAs compensated for the loss of other EBV genes that block apoptosis. Another example of cellular functional compensation is likely through miRNA BART6-3p inhibiting PTEN (Cai et al., 2015). Interestingly, Ambrosio *et al.* found that protein levels of PTEN are significantly lower in EBV-positive BLs compared with EBV-negative BLs (Ambrosio et al., 2014). This finding supports a mechanism in which a viral miRNA inhibits translation of PTEN. This idea is also supported by the finding that there are more *PTEN* mutations identified in EBV-negative BLs than EBV-positive BLs, although the overall frequency with which *PTEN* mutations occur in BL is only 4% (Grande et al., 2019). Still, EBV benefits enormously from encoding miRNAs given that they take up relatively little genomic space, are thought to be non-immunogenic, and clearly regulate cellular genes to support transformation. If as emerging evidence suggests, extracellular vesicles and/or exosomes containing viral miRNAs, EBERs, and other small viral RNAs are functional, they may support lymphomagenesis (reviewed in Zhao et al., 2019). Through the secretion of these vesicles to other cells or through expression of viral miRNAs in EBV infected cells, mutations in the cellular targets of EBV's miRNAs can overcome the requirement for these miRNAs and reduce BLs dependence on EBV.

## Transcriptional Regulation

Multiple studies show that EBV affects the expression of cellular genes transcriptionally, including through epigenetic mechanisms. For example, Hernandez-Vargas *et al.* found that the presence of the virus was the most significant variable defining variation in DNA methylation in EBV-positive versus EBV-negative BL cell lines (Hernandez-Vargas et al., 2017). The same study also found that two cellular genes commonly mutated in BL, *TCF3* and its negative regulator *ID3*, had different methylation patterns depending on EBV's presence or absence. In multiple genome-wide analyses of BL primary tumors, mutations in cellular genes involved in epigenetic regulation were significantly more frequent in EBV-positive biopsies (Kaymaz et al., 2017; Grande et al., 2019; Panea et al., 2019). These include *KMT2D*, *HIST1H1E*, *CHD8*, and *BCL7A*, with some additional genes identified that involve epigenetic regulatory pathways including *DNMT1*. Both LMP1 and LMP2A have been found to increase expression of DNA methyltransferases including DMNT1 in carcinomas, leading

to inhibition of E-cadherin (*CDH1*) and *PTEN* expression (Tsai et al., 2006; Hino et al., 2009).

EBNA2 and EBNA3A, 3B, and 3C regulate transcription by interacting with cellular transcriptional machinery. EBNA2 was found to bind the members of the SWI/SNF complex involved in enhancer function (Wu et al., 1996; Alver et al., 2017) while all four EBV proteins can associate with the cellular transcription factor, RBPJ, to different extents to foster transcription from partially overlapping but distinct subsets of genes (Wang et al., 2016). This virus-mediated transcriptional regulation must be overcome by alterations in the cellular machinery to allow the loss of expression of EBNA2 and the EBNA3 transforming genes as BLs evolve.

A different mechanism has been uncovered for EBNA1 to contribute to the transcription of cellular genes. Coppotelli *et al.* have emphasized the similarity of EBNA1's AT-hook domain to High Mobility Group A (HMGA) proteins (Coppotelli et al., 2013). They have found that EBNA1, similarly to HMGA proteins, can aid in de-condensation of chromatin and increase the mobility of histone H1. This role for EBNA1 is presumably mediated by its specific binding to sites in cellular DNA (Dresang et al., 2009). While it is possible to envision mechanisms in which EBNA1's mimicry of HMGA proteins is abrogated, its role in maintaining EBV genomes as plasmids appears essential.

A recent approach has identified cellular genes that regulate expression of EBV genes and revealed that both DNMT1 and UHRF1 are instrumental in turning off the expression of multiple viral transforming genes expressed from the C, W, and LMP promoters in BL cell lines (Guo et al., 2020). So long as there are cellular functions, wild-type or mutant, that can compensate for the loss of EBV's transforming genes, DNMT1 and UHRF1 would foster the escape of BLs from immune recognition of viral antigens.

## Activation-Induced Cytidine Deaminase Off-Target Mutations

The surge in genome-wide mutational data across BL tumors is valuable; however, it can be difficult to determine which mutations may be important in the pathogenesis of BL, and which are merely "passenger" mutations. While combing through the hundreds of mutations identified across multiple studies, it has become clear that some of the reported mutations result from off-target modifications by activation-induced cytidine deaminase (AID), where off-target is defined as any non-Ig loci. AID is an enzyme responsible for class-switch recombination and affinity maturation of antibodies by somatic hypermutation (SHM) and is specifically expressed in germinal center B cells (Muramatsu et al., 1999). AID acts by deaminating cytidine residues in DNA, thus introducing U:G mismatches (Di Noia and Neuberger, 2007). If regulated properly, AID functions to increase Ig gene diversification in developing B cells and is primarily restricted to Ig genes. The enzyme preferentially deaminates cytosines located within WRCY/RGYW regions (Dorner et al., 1998); however, these "hotspot motifs" are also found elsewhere across the genome. Studies show that AID has off-target activity for non-Ig loci, resulting in off-target lesions and chromosomal translocations (reviewed in Chandra et al., 2015). Thus, the regulation of this enzyme is critical to maintain genomic integrity.

Because almost all BL tumors contain c-Myc translocations to one of three Ig loci, researchers have hypothesized for years that AID and EBV are somehow intertwined. The evidence for this hypothesis was slowly built over time; for example, Ramiro *et al.* showed that AID is required for Myc/IgH translocations *in vivo* (Ramiro et al., 2004), and later found that AID causes these same translocations in B cells in culture (Ramiro et al., 2006). Studies done using primary B cells infected with EBV showed an increase in AID expression (Epeldegui et al., 2007), leading researchers to probe further for the link between AID and tumorigenesis of BL. The cellular miR-155 suppresses AID expression (Dorsett et al., 2008; Teng et al., 2008), but miR-155 does not appear to be expressed in primary BL tissue (Kluiver et al., 2006). More recently, the EBV gene EBNA3C has been shown to directly induce AID in B cells (Kalchschmidt et al., 2016), and further studies have shown that some common mutations found in BL are likely to be caused by AID and reflect EBV's dysregulation of AID.

Across studies investigating the mutational spectrum of EBV-positive versus EBV-negative BLs, researchers have found fewer cellular "driver" mutations but more non-coding mutations in EBV-positive BLs (Abate et al., 2015; Grande et al., 2019; Panea et al., 2019). Two of these studies reported that EBV-positive BLs were associated with a higher proportion of AID-associated mutations compared with EBV-negative BLs (Grande et al., 2019; Panea et al., 2019) (see **Table 2** for a list of AID-associated mutations found in EBV-positive BL). In particular, Grande *et al.* found AID recognition sites were mutated at higher than expected rates. These and other AID-associated mutations can be found in certain genes where the DNA contains G-quadruplex (G4)-containing substrates mimicking the mammalian immunoglobulin switch regions (Qiao et al., 2017) along with the known AID recognition motifs. In fact, many genes identified to have a high frequency of GGG in G-rich regions by Qiao *et al.* were found to be mutated in more than 20% of EBV-positive BL tumors. From these data, it appears that EBV-enhanced expression of AID increases the number of somatic mutations found in BL. While EBNA3C, which was found to induce AID in B cells, is not expressed in many EBV-positive BL, other factors may foster expression of AID in BLs. The cellular protein Blimp1 has been found to inhibit AID through multiple mechanisms (Xu et al., 2007). Intriguingly, Blimp1 was found to have decreased expression in latency I BL cell lines compared to BL lines that had reverted to a latency III phenotype (Kelly et al., 2013). Downregulation of Blimp1 in latency I BLs may serve as one mechanism to increase AID activity without the need for expression of EBNA3C, which is typically not expressed in latency I. This observation could indicate that BLs can continue to accumulate AID off-target mutations indirectly through manipulation of other cellular processes.

Although many of these mutations induced by AID may not contribute to the pathogenesis of BL, some mutations clearly do. *MYC* has long been identified as a target of AID, and the

**TABLE 2 |** Frequency of some AID off-target genes[1] mutated in EBV-positive BL.

| Gene | Frequency (sample size) | Name | Description † | Compensation for EBV † |
|---|---|---|---|---|
| ***BACH2*** | 97% (n = 39)[2] | BTB Domain And CNC Homolog 2 | – | – |
| ***MYC**** | 50% (n = 20)[3]<br>60% (n = 30)[4]<br>61% (n = 94)[5]<br>97% (n = 39)[2] | v-myc myelocytomatosis viral oncogene homolog | TF that drives cell-cycle progression and transformation. Translocation of *MYC* to one of three immunoglobulin loci causes constitutive expression. | Mutations in *MYC* could compensate for EBV's genes that sustain proliferation |
| ***BCL6*** [a] | 56% (n = 39)[2] | BCL6 Transcription Repressor | Transcriptional repressor. In GC B-cells, represses genes that function in differentiation, inflammation, apoptosis and cell cycle control, also autoregulates its transcriptional expression and up-regulates, indirectly, the expression of some genes important for GC reactions, such as AID, through the repression of microRNAs expression, like miR155. | Mutations in *BCL6* could compensate for EBNA3C through its direct interaction with this protein (Pei et al., 2017) |
| ***BTG2*** | 51% (n = 39)[2] | BTG Anti-Proliferation Factor 2 | – | – |
| ***TCL1A*** | 41% (n = 39)[2] | T Cell Leukemia/Lymphoma 1A | – | – |
| ***ID3*** | 30% (n = 20)[3]<br>33% (n = 30)[4]<br>33% (n = 94)[5]<br>62% (n = 39)[2] | Inhibitor of DNA binding 3 | HLH protein lacking DNA-binding domain. Functions as a negative regulator of *TCF3*, inhibiting transcription. Mutations are inactivating which decrease *TCF3* interaction. | See *TCF3* |
| ***DNMT1*** | 36% (n = 39)[2] | DNA Methyltransferase 1 | Methylates CpG residues. Preferentially methylates hemimethylated DNA. | Mutations in *DMNT1* could compensate for its interaction with LMP1 and LMP2A (Tsai et al., 2006; Hino et al., 2009). |
| ***MCL1*** | 33% (n = 39)[2] | MCL1 Apoptosis Regulator, BCL2 Family Member | – | – |
| ***ARID1A*** | 25% (n = 20)[3]<br>13% (n = 30)[4]<br>45% (n = 94)[5]<br>46% (n = 39)[2] | AT rich interactive domain 1A | – | – |
| ***ETS1*** | 13% (n = 94)[5]<br>51% (n = 39)[2] | ETS Proto-Oncogene 1, Transcription Factor | – | – |
| ***DTX1*** | 31% (n = 39)[2] | Deltex E3 Ubiquitin Ligase 1 | – | – |
| ***PVT1*** [b] | 28% (n = 39)[2] | Pvt1 Oncogene | – | – |
| ***GNA13*** | 17% (n = 30)[4]<br>17% (n = 94)[5]<br>46% (n = 39)[2] | Guanine nucleotide binding protein, alpha 13 | – | – |
| ***BCL7A*** | 13% (n = 30)[4]<br>9% (n = 94)[5]<br>56% (n = 39)[2] | B-cell CLL/lymphoma 7A | – | – |
| ***ZFP36L1*** | 26% (n = 39)[2] | ZFP36 Ring Finger Protein Like 1 | – | – |
| ***TMSB4X*** | 23% (n = 39)[2] | Thymosin Beta 4 X-Linked | – | – |
| ***CXCR4*** | 21% (n = 39)[2] | C-X-C Motif Chemokine Receptor 4 | – | – |
| ***TFAP4*** | 17% (n = 30)[4]<br>10% (n = 94)[5]<br>18% (n = 39)[2] | Transcription Factor AP-4 (activating enhancer binding protein 4) | – | – |
| ***TCF3*** | 15% (n = 20)[3]<br>10% (n = 30)[4]<br>6% (n = 94)[5]<br>28% (n = 39)[2] | Transcription Factor 3 (E2A immunoglobulin enhancer binding factors E12/E47) | TF that plays a critical role in lymphocyte development. It has also been shown to directly enhance Notch signaling targets. Mutations lead to gain of function. | Gain of function mutations in *TCF3* could compensate for EBNA2, 3A, 3B, 3C and/or LMP2A (Robertson et al., 1996; Gordadze et al., 2001; Portis and Longnecker, 2004; Wang et al., 2016). |
| ***PTEN*** | 1% (n = 9)[5] | Phosphatase And Tensin Homolog | Functions as a tumor suppressor by negatively regulating AKT/PKB signaling pathway. | Mutations in *PTEN* could compensate for the inhibition of PTEN by EBV's miRNA BART6-3p (Cai et al., 2015) |

**This frequency does not include MYC translocations.*

[a] *Identified dependency factor for BL cell line P3HR1 (Ma et al., 2017).*

[b] *Significantly more frequently mutated in EBV-positive BL (Grande et al., 2019).*

† *The "Description" and "Compensation for EBV" columns in genes marked with (-) have been intentionally left blank since there is not enough information to interpret their contributions to these tumors.*

*Annotations in acronym or abbreviated form are: TF, transcription factor; AID, activation-induced cytidine deaminase; HLH, helix-loop-helix; GC, germinal center.*

*1. Álvarez-Prado et al., 2018; 2. Panea et al., 2019; 3. Abate et al., 2015; 4. Kaymaz et al., 2017; 5. Grande et al., 2019.*

resulting translocation drives the increased proliferation of these tumors. Additionally, genes encoding the transcription factor TCF3 and its negative regulator ID3 are targets of aberrant SHM (Rohde et al., 2017; Álvarez-Prado et al., 2018). Both have been recurrently identified as playing a role in BL pathogenesis, although these mutations have been found significantly more frequently in EBV-negative BL (Grande et al., 2019). Molecular analyses of tumors by Schmitz *et al.* found that BLs are dependent on TCF3 in part because it enhances pro-survival PI3K signaling (Schmitz et al., 2012). The activation of PI3K signaling is a hallmark of BLs (Sander et al., 2012). This upregulation of TCF3 might be seen more frequently in EBV-negative tumors because Notch signaling is mimicked by EBNA2, 3A, 3B, and 3C in EBV-positive tumor cells (Henkel et al., 1994; Waltzer et al., 1994; Zimber-Strobl et al., 1994; Grossman et al., 1994; Robertson et al., 1996; Zhao et al., 1996; Hsieh et al., 1997; Gordadze et al., 2001; Wang et al., 2016). Given that the mutations in *TCF3* are usually gain of function (Schmitz et al., 2012), these mutations could promote cells becoming independent of EBV. Another potential cellular compensation through gain of function *TCF3* mutations is the loss of expression of LMP2A, which is known to activate the PI3K pathway to mediate B cell survival (Portis and Longnecker, 2004). TCF3 increases expression of the BCR, leading to increased PI3K signaling (Schmitz et al., 2012). Thus, a mutation that removes the need to stimulate the PI3K pathway might allow for the loss of expression of LMP2A. It is even possible that these mutations are found in EBV-negative BLs because they lost their dependence on EBV—and therefore no longer maintain EBV DNAs—following their acquisition of these mutations in *TCF3* along with other compensatory cellular mutations.

*BCL6* mutations are another example of an AID off-target site that has significance for the tumorigenesis of BL. BCL6 is a transcriptional repressor required for germinal center formation and antibody affinity maturation. It has been linked to upregulating AID expression because it represses miR-155, which suppresses AID (Basso et al., 2012). BCL6 expression has been found to be increased in latency I BL cell lines compared to latency III lines (Kelly et al., 2013). In accordance with this observation, BCL6 has also been found to mediate degradation of EBNA3C (Pei et al., 2017). Perhaps in latency type I BL cells which do not express EBNA3C and thus are no longer dependent on it, the elevated expression of BCL6 is compensating for the loss of EBNA3C. The loss of *BCL6* from a BL cell line was found to inhibit growth or survival (Ma et al., 2017), indicating that BCL6 can provide BLs a selective advantage.

## Burkitt Lymphoma Cellular Compensatory Mutations

While AID does give rise to many mutations in BL, some commonly found mutations are not known to be off targets of AID activity. These mutations also have the potential to compensate for EBV's transforming genes allowing their expression to be lost in BL tumors. **Table 3** includes a list of some of the cellular mutations frequently found in EBV-positive BL tumors. The mutations listed have been selected based on their frequency, their known phenotypes making them likely to compensate for viral gene functions, and their potential effects on protein function. We have chosen to highlight these specific mutations, yet the complexity in trying to understand all mutations found in BL tumors becomes apparent when considering information that is lacking. For example, many studies do not report whether the mutations identified in BL tumors affect one or both alleles or how these mutations affect levels of gene expression for many of the mutations reported (Schmitz et al., 2012; Abate et al., 2015; Kaymaz et al., 2017; Panea et al., 2019; Grande et al., 2019). Without examining individual mutations in single tumors, examining functional consequences of that mutation, and considering how that mutation affects other mutations, it is difficult to attribute mechanistic significance to mutations in single genes. Stringently examining each mutation reported across genome-wide mutational studies is beyond the scope of this review.

One challenge in identifying "driver" versus "passenger" mutations in BLs is connecting the mutations to functional effects. Of the common mutations listed in **Table 3**, many were also identified as dependency factors of a BL cell line, P3HR1, using a CRISPR knockout screen (Ma et al., 2017). Deletions in these genes led to decreases in growth or survival of this BL cell line. The examined P3HR1 cell line expresses only EBNA1 among EBV's latent proteins (an unexpected finding given that other P3HR1 cell lines are typically Wp-restricted), which implies that required functions of the other transforming genes have already been compensated by cellular mutations. The mutated genes found as dependency factors include *IGLL5*, *IRF8*, *CCND3*, *RHOA*, *KMT2D*, and *BCL6*. Of these identified candidates, *IGLL5* (along with another frequently mutated gene, *IKZF3*) is regulated by EBV (Zhou et al., 2015). It is certain that some of the cellular genes regulated by EBV can foster B cell transformation. In EBV-negative cells, mutations in *IGLL5* that increase its activity could compensate for the loss of its typical upregulation by EBV. The CRISPR knockout screen also identified *IRF8*, a transcription factor that is thought to function as a regulator of apoptosis and potentially acts as a tumor suppressor. IRF8 is the only known transcription factor shown to be involved in *BCL6* transcriptional induction (Lee et al., 2006). EBNA3C is thought to regulate IRF8 indirectly through its stabilization of IRF4, which leads to the downregulation of IRF8 by enhancing its proteasome-mediated degradation (Banerjee et al., 2013). In the context of the development of BL, an acquired activating mutation in *IRF8* may compensate for the loss of EBNA3C in tumor cells.

Other mutations identified across mutational analyses are in cellular proteins known to interact with EBV latency proteins. For example, SIN3A is a transcriptional repressor that was found to be recruited by EBNA3C to BATF/IRF4 or SPI1/IRF4 composite sites to repress *CDKN2A* transcription (Jiang et al., 2014). *CDKN2A* codes for both p16 and p14arf, which activate the p53 and pRB pathways, respectively. Loss of EBNA3C expression from an EBV-positive BL would therefore likely require compensatory inhibitions of the p53 and pRB.

**TABLE 3 |** Frequency of some mutated genes in EBV-positive BL.

| Gene | Frequency (sample size) | Name | Description | Compensation for EBV |
|---|---|---|---|---|
| ***IGLL5*** [a] | 97% (n = 39)[1] | Immunoglobulin lambda-like polypeptide 5 | Located within the immunoglobulin lambda locus but does not require somatic rearrangement for expression. | Mutations in *IGLL5* could compensate since *IGLL5* is regulated by EBV (Zhou et al., 2015) |
| ***DDX3X*** | 35% (n = 20)[2]<br>40% (n = 30)[3]<br>62% (n = 94)[4]<br>82% (n = 39)[1] | DEAD (Asp–Glu–Ala–Asp) box polypeptide 3, X-linked | ATP-dependent RNA helicase. | Unknown |
| ***IKZF3*** | 44% (n = 39)[1] | IKAROS Family Zinc Finger 3 | TF that plays an essential role in regulation of B-cell differentiation, proliferation, and maturation to an effector state. | Mutations in *IKZF3* could compensate since *IKZF3* is regulated by EBV (Zhou et al., 2015) |
| ***FOXO1*** | 7% (n = 30)[3]<br>30% (n = 94)[4]<br>56% (n = 39)[1] | Forkhead box O1 | Key TF regulated by the PI3K/AKT pathway. Loss of function mutations may have a role in cell growth or escape from apoptosis. | Unknown |
| ***SIN3A*** | 18% (n = 94)[4]<br>41% (n = 39)[1] | SIN3 transcription regulator family member A | Acts as a transcriptional repressor. Corepressor for REST. Interacts with MXI1 to repress *MYC* responsive genes and antagonize *MYC* oncogenic activities. | Mutations in *SIN3A* could compensate for EBNA3C through interaction with Sin3A (Jiang et al., 2014) |
| ***S1PR2*** | 28% (n = 39)[1] | Sphingosine-1-Phosphate Receptor 2 | Receptor for the lysosphingolipid sphingosine 1-phosphate (S1P). S1P elicits diverse physiological effects on most types of cells and tissues. | Mutations in *S1PR2* could compensate for LMP1 which regulates S1PR2 (Vockerodt et al., 2019) |
| ***FBXO11*** | 13% (n = 30)[3]<br>18% (n = 94)[4]<br>36% (n = 39)[1] | F-box protein 11 | Substrate recognition component of a SCF (SKP1-CUL1-F-box protein) E3 ubiquitin-protein ligase complex. Major target is *BCL6*. Loss of function mutations. | Mutations in *FBXO11* could compensate for EBNA3C through *BCL6* interaction (Pei et al., 2017) |
| ***CREBBP*** | 21% (n = 39)[1] | CREB Binding Protein | Acetylates histones, giving a specific tag for transcriptional activation. Also acetylates non-histone proteins, including DDX21, FBL, IRF2, MAFG, NCOA3, POLR1E/PAF53, and FOXO1. | Mutations in *CREBBP* could compensate for EBNA2 through direct interaction with CBP to activate the LMP1 promoter (L. Wang et al., 2000) |
| ***TP53*** [b] | 15% (n = 20)[2]<br>17% (n = 30)[3]<br>22% (n = 94)[4]<br>33% (n = 12)[5]<br>15% (n = 39)[1] | Tumor protein p53 | Encodes p53, a tumor suppressor that binds to DNA and regulates gene expression. Can activate DNA repair proteins when DNA is damaged and arrest the cell cycle at the G1/S checkpoint, or initiate apoptosis if DNA damage is irreversible. Mutations are loss of function. | Mutations in *TP53* could compensate for EBV's genes that block apoptosis |
| ***IRF8*** [a] | 18% (n = 39)[1] | Interferon Regulatory Factor 8 | Plays a role as a transcriptional activator or repressor. Plays a negative regulatory role in immune cells. | Mutations in *IRF8* could compensate for EBNA3C through its interaction with IRF8 (Banerjee et al., 2013) |
| ***CCND3*** [ab] | 5% (n = 20)[2]<br>13% (n = 30)[3]<br>10% (n = 94)[4]<br>31% (n = 39)[1] | Cyclin D3 | A regulator of progression through G1 phase during cell cycle. Loss of C terminal domain leads to constitutive activation. | Mutations in *CCND3* could compensate for unregulated cell cycle progression |
| ***RHOA*** [a] | 20% (n = 20)[2]<br>13% (n = 30)[3]<br>10% (n = 94)[4]<br>15% (n = 39)[1] | Ras homolog family member A | Small GTpase protein in the Rho family. Regulation of signal transduction between membrane receptors and focal adhesion molecules. Likely loss of function mutations. | Unknown |
| ***USP7*** [b] | 4% (n = 94)[4] | Ubiquitin Specific Peptidase 7 | USP7 deubiquitinates target proteins such as p53 and regulates their activities by counteracting opposing ubiquitin ligase activity. | Mutations in *USP7* could compensate for EBNA1 through its interaction (Holowaty and Frappier, 2004) |
| **Mutations involved in epigenetic regulation** [b] | | | | |
| ***HIST1H2BK*** | 49% (n = 39)[1] | Histone H2B | | Unknown |
| ***HIST1H3H*** | 44% (n = 39)[1] | Histone H3.1 | | Unknown |
| ***HIST1H1E*** | 11% (n = 94)[4]<br>64% (n = 39)[1] | Histone H1.4 | | Unknown |
| ***HIST1H1C*** | 28% (n = 39)[1] | Histone H1.2 | | Unknown |

*(Continued)*

**TABLE 3** | Continued

| Gene | Frequency (sample size) | Name | Description | Compensation for EBV |
|---|---|---|---|---|
| ***CHD8*** | 10% (n = 94)[4]<br>26% (n = 39)[1] | Chromodomain Helicase DNA Binding Protein 8 | DNA helicase that acts as a chromatin remodeling factor and regulates transcription. | Unknown |
| ***KMT2D*** [a] | 14% (n = 94)[4]<br>5% (n = 39)[1] | Histone-lysine N-methyltransferase 2D | Colocalizes with TFs on transcriptional enhancers. Plays critical roles in regulating cell fate transition, metabolism, and tumor suppression. | Unknown |

[a]*Identified dependency factor for BL cell line P3HR1 (Ma et al., 2017).*
[b]*Significantly more frequently mutated in EBV-positive BL (Grande et al., 2019).*
[c]*Significantly more frequently mutated in EBV-negative BL (Grande et al., 2019).*
*Annotations in acronym or abbreviated form are: TF, transcription factor.*
*1. Panea et al., 2019; 2. Abate et al., 2015; 3. Kaymaz et al., 2017; 4. Grande et al., 2019; 5. Giulino-Roth et al., 2012.*

Mutations in another gene, *FBXO11*, could also be linked to EBNA3C function. *FBXO11* mutations are thought to be loss of function mutations (Duan et al., 2012). One of the major targets of FBXO11-mediated degradation is BCL6 (Duan et al., 2012), which targets EBNA3C for degradation (Pei et al., 2017). The increased expression of BCL6 can provide a growth advantage to BLs. Similarly, mutations in the gene *S1PR2*, which encodes the receptor for the lysosphingolipid sphingosine 1-phosphate, could promote cell proliferation and suppress apoptosis. S1PR2 was shown to be regulated by LMP1 in diffuse-large B cell lymphoma (Vockerodt et al., 2019). In turn, LMP1 transcription is regulated by EBNA2, which was shown to interact with the co-activator CBP (CREB binding protein), encoded by the gene *CREBBP* (Wang et al., 2000). *CREBBP* mutations were detected in more than 20% of BLs.

Other genes frequently mutated in BLs and playing a role in proliferation include *CCND3*, which was significantly more frequently mutated in EBV-negative BL (Grande et al., 2019). This gene encodes cyclin D3, which regulates cell cycle progression through G1. Mutations identified across studies found truncating lesions, and it is understood that the loss of the C terminal domain of *CCND3* leads to constitutive activation (Schmitz et al., 2012). Unregulated cell cycle progression might compensate for EBV's contributions to BL proliferation were the virus to be lost from BL cells. These mutations found frequently in EBV-positive BL all serve as examples of genes that when mutated could compensate for the loss of EBV's transforming genes and provide a selective advantage to the cells containing these mutations.

## CONCLUDING REMARKS

We hypothesize that EBV-positive tumor cells must have acquired cellular mutations to compensate for the loss of expression of viral genes. This hypothesis leaves multiple questions unanswered but is a starting point for investigating these commonly found mutations in EBV-positive versus negative BLs. One controversial question is: Are some tumors found to be EBV-negative formerly EBV-positive having evolved to be independent of the virus? It has been observed that some BLs spontaneously lose EBV in culture (Akata, MutuI, and Daudi) (Shimizu et al., 1994; Kitagawa et al., 2000; Nanbo et al., 2002). In addition, Grande *et al.* identify *USP7* as being mutated in BL, although relatively rarely. *USP7* encodes a deubiquitinase that counteracts ubiquitination and degradation of proteins such as p53 (Li et al., 2002) and has the mutational pattern of a tumor suppressor in BL. The interaction between p53 and USP7 can be disrupted by EBNA1 (Holowaty and Frappier, 2004). Interestingly, the mutations found in *USP7* were more common in EBV-negative tumors, which obviously do not express EBNA1. *USP7* may serve as one example of a gene that when mutated compensates for EBNA1 and may lead to tumors that have lost EBV genomes, and therefore also lost the expression of EBV latency proteins entirely.

Another question to consider is whether the detection of EBV in tumors is inaccurate. The standard method for assaying for EBV in tumors is through the detection of the EBERs. However, analyses of biopsies have found multiple cases of BL that were EBER-negative and harbored partial viral miRNAs and some genomes (Mundo et al., 2017; Mundo et al., 2020). This hypothesis and the findings consistent with it could indicate that the contribution of EBV to the pathogenesis of BL is greater than previously thought. One technical concern with this notion is that biopsies are typically contaminated with cells other than those of tumor origin, making it difficult to discern if partial or few viral genomes reflect few EBV-positive cells either in the tumor or in contaminating, non-tumor cells. The most compelling evidence for the entire loss of EBV in tumors would be detection of an EBV-positive BL case that subsequently evolves to lose the virus.

We favor the hypothesis that EBV-positive tumors evolve to become less dependent on EBV illustrating the ever-changing selective pressures favoring tumor growth. The surge in data for genome-wide mutational burden in these tumors has tremendous value; however, as more sequencing data becomes available for EBV-associated BL and other EBV-positive malignancies, it will be important to attribute mechanisms to the identified mutations so we might further understand these EBV-associated cancers and their evolution to treat them more effectively.

## AUTHOR CONTRIBUTIONS

RLH wrote the manuscript with contributions from AC and BS. All authors made a substantial and intellectual contribution to

the work, contributed to manuscript revision, read, and approved it for publication. All authors contributed to the article and approved the submitted version.

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REVIEW
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# Herpes Simplex Virus Cell Entry Mechanisms: An Update

*Krishnaraju Madavaraju*[1], *Raghuram Koganti*[1], *Ipsita Volety*[1], *Tejabhiram Yadavalli*[1] *and Deepak Shukla*[1,2]*

[1] Shukla Lab, Department of Ophthalmology and Visual Sciences, University of Illinois at Chicago, Chicago, IL, United States, [2] Department of Microbiology and Immunology, University of Illinois at Chicago, Chicago, IL, United States

Herpes simplex virus (HSV) can infect a broad host range and cause mild to life threating infections in humans. The surface glycoproteins of HSV are evolutionarily conserved and show an extraordinary ability to bind more than one receptor on the host cell surface. Following attachment, the virus fuses its lipid envelope with the host cell membrane and releases its nucleocapsid along with tegument proteins into the cytosol. With the help of tegument proteins and host cell factors, the nucleocapsid is then docked into the nuclear pore. The viral double stranded DNA is then released into the host cell's nucleus. Released viral DNA either replicates rapidly (more commonly in non-neuronal cells) or stays latent inside the nucleus (in sensory neurons). The fusion of the viral envelope with host cell membrane is a key step. Blocking this step can prevent entry of HSV into the host cell and the subsequent interactions that ultimately lead to production of viral progeny and cell death or latency. In this review, we have discussed viral entry mechanisms including the pH-independent as well as pH-dependent endocytic entry, cell to cell spread of HSV and use of viral glycoproteins as an antiviral target.

**Keywords: herpesvirus, HSV, entry, mechanism, endocytosis**

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***Correspondence:**
*Deepak Shukla*
*dshukla@uic.edu*

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## INTRODUCTION

Millions of people worldwide are exposed to herpes simplex virus (HSV) (Looker et al., 2015), following the exposure the virus may remain asymptomatic or may cause mild to life threatening complications (Ramchandani et al., 2016). HSV can be broadly divided into two serotypes: HSV-1 and HSV-2 (Kelly et al., 2009; Fontana et al., 2017) HSV-1 infections are primarily associated with mild to severe symptoms including blisters and inflammation of oral and ocular cells but in some cases, they can progress to more serious illnesses including blindness, hearing impairment, and fatal encephalitis (Koujah et al., 2019; Lobo et al., 2019). Similarly, HSV-2 infections may cause mild genital lesions but can also increase the risk of acquiring and transmitting fatal human immunodeficiency virus (HIV) infections (Looker et al., 2017). Additionally, both HSV-1 and HSV-2 can interchangeably infect oral or genital sites (Agelidis and Shukla, 2015).

HSV-1 and HSV-2 belong to the family Herpesviridae, all of which have unique four layers: the central double stranded DNA, enclosed by an icosapentahedral capsid, which is surrounded by a group of tegument proteins, which in turn, are encapsulated in a lipid bilayer envelope containing membraneproteins and glycoproteins (Karasneh and Shukla, 2011). The Herpesviridae family is classified into three subfamilies: alpha-herpesviruses, beta-herpesviruses, and gamma-herpesviruses

subfamilies. All members of the *Herpesviridae* family establish latency (the ability of a virus to remain dormant within the host cell), but the cells in which they establish latency vary. Most Alpha-herpesviruses establish latency in neurons, beta-herpesviruses establish latency in non-neuronal cells, and gamma-herpesviruses establish latency in B and T lymphocytes (Kelly et al., 2009). But there are few exceptions, for example Marek's disease virus is an Alpha-herpesvirus but establish it's latency in chicken CD4+ T-cells (Parcells et al., 2003).

HSV-1 and HSV-2 belong to the alpha-herpesvirus subfamily, generally have a short replicative cycle and are capable of infecting broad host range. The mature HSV consists of the following: 1) a linear double stranded DNA of ~152 kb encoding at least 74 genes, 2) encased in an icosapentahedral capsid composed of 162 capsomeres made of six different viral proteins, 3) surrounded by 20-23 different viral tegument proteins that have structural and regulatory roles (Albecka et al., 2017), and 4) covered by an envelope that has at least 12 different glycoproteins: gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, gM, and gN on their surface, in distinct shapes and sizes. Some exist as heterodimers (gH/gL and gE/gI), while most exist as monomers. Upon exposure to a suitable host, viral glycoproteins attach to the host cell surface receptors (viral attachment). Later they interact with each other (glycoproteins) and fuse the viral envelope with the host cell membrane, thereby delivering the viral content into the host cell. The presence of four glycoproteins: gB, gD, gH, and gL and their host cell receptors has been reported to be sufficient to deliver viral content into the host cell (Karasneh and Shukla, 2011).

This review gathers and details the experimental evidence and pioneering research on the direct membrane fusion mechanism of the HSV and its essential components. As a central mechanism, the binding of four viral glycoproteins gD, gH/gL and gB to its specific receptors releases the viral contents into the cell (Karasneh and Shukla, 2011). First, the virus attaches to the host's cell surface receptor, heparan sulfate proteoglycans (HSPGs) via its viral glycoproteins gB and/or gC (WuDunn and Spear, 1989; Herold et al., 1991; Shukla et al., 1999). The virus then slides on the cell surface and reach the cell body, a movement termed as viral surfing (Dixit et al., 2008; Oh et al., 2010; Thakkar et al., 2017). It then binds with cell membrane receptors using gD, gH/gL, and gB glycoproteins which triggers direct membrane fusion. In this review, the process of membrane fusion, structural and functional details of these four essential viral glycoproteins, and their host cell surface receptors are discussed in detail. Also, this review briefly discusses the low pH-dependent endocytic entry, the cell to cell spread of HSV and about viral glycoproteins as an antiviral target.

## PLASMA MEMBRANE FUSION

During HSV-1 or HSV-2 infection, the virus fuses its envelope with host cell membrane with the help of fusogens. Fusogens are viral encoded transmembrane fusion proteins usually present over the surface of viral envelope. In case of HSV, gB acts as a viral fusogen. A multi-protein complex involving gB, gD, gH/gL and their cognate receptors is known as the "core fusion machinery", and together they perform the fusion reaction (Eisenberg et al., 2012; Fontana et al., 2017; Sathiyamoorthy et al., 2017; Weed et al., 2017). The fusion reaction delivers the viral nucleocapsid and tegument proteins into the host cell (**Figure 4**).

According to the widely accepted model, the binding of gD to one of its cellular receptors initiates the fusion reaction (Gianni et al., 2013b). Binding causes conformational changes in the gD that changes its auto-inhibitory closed state to its active state and transmits a signal to gH/gL. These series of events activate gB by an unknown mechanism (Cooper and Heldwein, 2015; Atanasiu et al., 2016; Fontana et al., 2017); (**Figure 1**). More precisely, the gH-gL activation model proposed by the Gianni et al. in 2015 postulates gH/gL requires two signals: the first one from receptor-bound gD and the second one from the integrin (gH/gL receptor). Upon receiving these signals, gL disassociates from the complex. This allows gH to bind with its receptor and activate gB. Possibly, gL may act as an inhibitor of gH and help maintain gH in an inhibited form until it receives the appropriate signals. Thus, the disassociation of gL favors the binding of gH to its receptor and activates gH. Activated gH then transmits signals to gB (Gianni et al., 2015). Upon receiving the signal, gB undergo series of conformational changes. One study claimed that HSV gH/gL can regulate a hemifusion state of gB (Subramanian et al., 2007). However, others could not detect gH/gL mediated hemifusion (Jackson and Longnecker, 2010). In any case, the merging of membranes forms a fusion pore through which virus delivers its content into the host cell (Cooper and Heldwein, 2015; Fontana et al., 2017). In the absence of gD, gD receptors, gB, integrin or in the presence of neutralizing monoclonal antibodies to gH and gL, the dissociation of gH and gL does not take place which blocks viral fusion (Gianni et al., 2015).

Combining crystallographic structural analysis with previously published data, "the clamp and wedge model" of fusion mechanism in HSV was proposed (Cooper and Heldwein, 2015; Rogalin and Heldwein, 2015; Cooper et al., 2018). According to this model, the membrane bound cytoplasmic domain (CTD) of gB acts as a clamp and restrains the fusogenic activity of gB by stabilizing the ectodomain in a pre-fusion conformation. Upon binding to its receptor, gD undergoes the conformational change and transmits the signal to gH/gL dimer (Cooper et al., 2018). Viral gH's ectodomain receives this signal *via* its H1 domain and transmits the signal through H2 to membrane proximal H3 domain, which then translates the signal to the cytoplasmic tail of gH. Upon receiving the message gH's cytoplasmic tail acts as a wedge and splits the gB's CTD clamp restrain in the cytoplasmic tail (Rogalin and Heldwein, 2015). This action releases the gB and favors the attachment of gB's fusion loop onto the host's surface which promotes membrane fusion. It is proposed that membrane proximal region (MPR) of the gB may contribute to lipid fusion process since this region is rich in hydrophobic amino acids. Also, the transmembrane domain (TMD) of gB may act as

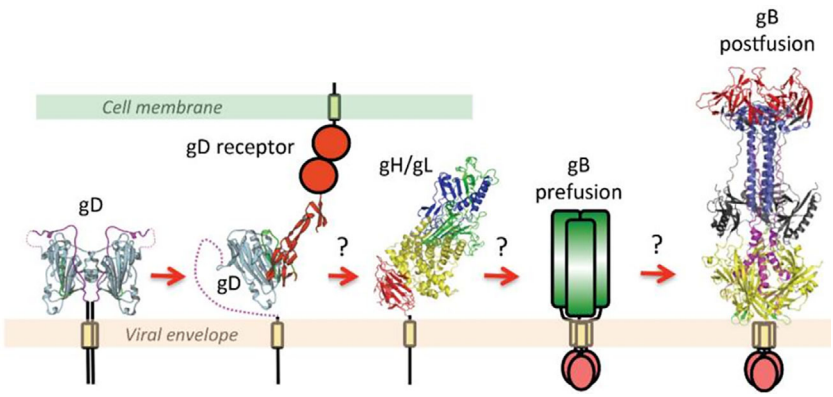


**FIGURE 1** | This figure is taken from the following paper published by Cooper and Heldwein in *Viruses* in 2015, "Herpesvirus gB: A Finely Tuned Fusion Machine" under the Creative Commons Attribution 4.0 International license. The original figure caption is provided as follows: "A schematic diagram of essential steps in HSV glycoprotein-mediated fusion. Crystal structures of apo gD (PDB ID 2C36), gD/HVEM complex (PDB ID 1JMA), gH/gL (PDB ID 3M1C), and the postfusion form of gB (PDB ID 2GUM) are shown. The prefusion form of gB has not yet been characterized and is shown schematically. Conformational changes in gD upon receptor binding are well documented. The order of subsequent steps has been proposed but not yet confirmed. Figure was made with Pymol." Please note that the receptors shown are generalized and not depicting a specific protein.

a conduit facilitate lipid mixing and formation of the fusion pore, ultimately leading to the release of viral content into the host cell (Cooper and Heldwein, 2015; Cooper et al., 2018).

## VIRAL GLYCOPROTEIN B (gB)

HSV gB is a class III fusion glycoprotein highly conserved among herpesviruses (Ruel et al., 2006; Weed et al., 2017) and least characterized (Roche et al., 2006; Roche et al., 2007; Cooper and Heldwein, 2015) gB is 904 amino-acid residues long and consists of an extended rod or spike-like ectodomain (Liu et al., 2006; Fontana et al., 2017), a hydrophobic MPR, a TMD, and a CTD. Initially, it was thought that only the gB's ectodomain actively participates in the fusion reaction. However, recent research confirms the adjacent MPR, TMD, and CTD regions also play a key role (amino acids 730 to 904) in regulating the fusion reaction (Ruel et al., 2006; Cooper and Heldwein, 2015; Fontana et al., 2017).

Crystallographic structure analysis of full-length gBΔ71 (a hybrid of a post-fusion ectodomain and the pre-fusion CTD) from HSV-1 reveals that the ectodomain in its post-fusion conformation rests upon a uniquely folded trimeric pedestal. This pedestal is composed of the MPR, TMD, and CTD, and it interacts extensively with the viral membrane (Cooper et al., 2018); (**Figure 2**). Disturbing this pedestal confirmation and it's interaction with the membrane might be the reason why gB is always extracted in post-fusion conformation during extraction (Cooper and Heldwein, 2015). The gB's ectodomain structure and function are greatly controlled by MPR, TMD and the CTD regions. Thus, minor changes made in these regions affect its normal structure and function. However, the mechanism by which these regions control the ectodomain is still unknown (Cooper et al., 2018).

HSV's gB exists in two forms: pre-fusion and post-fusion form. Much of the details regarding gB's post-fusion ectodomain is obtained from crystallographic studies while the structure and function of pre-fusion ectodomain is still unknown (Cooper and Heldwein, 2015).

### Structure of the Post-Fusion Form of gB

The X-ray crystallography structure reveals that the post-fusion form of the HSV-1 gB ectodomain appears as a trimeric (three protomers combined) spike or rod-like structure (Heldwein et al., 2006). Each protomer is organized into five distinct domains and two linker regions that forms a hairpin shape (Cooper and Heldwein, 2015; Fontana et al., 2017; Arii and Kawaguchi, 2018). These domains interact with their equivalent counterpart domain on the other protomers and form a stable trimeric structure (Cooper and Heldwein, 2015); (**Figure 2**). Domain I houses the fusion loop and is referred to as the fusion domain. Domain II mediates interactions with gH-gL, earning it the description - gH-gL binding domain (Atanasiu et al., 2010b; Cairns et al., 2013). Domain III consists of α-helices, and it forms the characteristic trimeric coiled-coil central core of the protein. Domain IV sits on top of the post-fusion form known as the crown domain and is thought to bind with cellular receptors (Karasneh and Shukla, 2011). This is supported by studies that show that the binding of antibodies to the crown domain prevents gB from binding to its cell receptor (Bender et al., 2005; Hannah et al., 2009). Lastly, domain V, known as the arm domain, consists of a long extension and connects the protomers together (Karasneh and Shukla, 2011). The flexible linker region facilitates the gB conformational change during fusion reactions (Liu et al., 2006; Lin and Spear, 2007; Karasneh and Shukla, 2011; Cooper and Heldwein, 2015). The X-ray crystallographic studies normally exclude post-fusion gB's N terminus (amino acids 31 to 102) due to its flexibility (Fontana et al., 2017). Even though

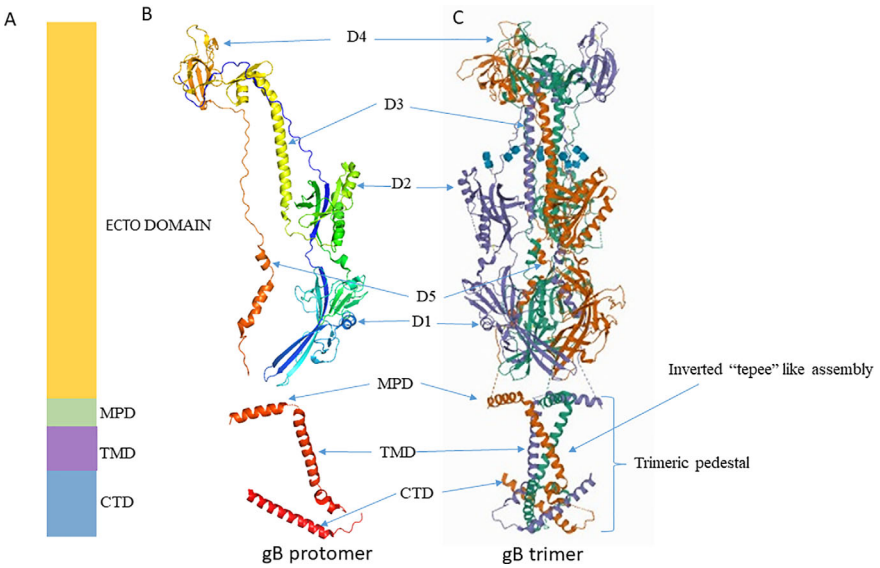


**FIGURE 2** | Crystal structure of gB. **(A)** Schematic representation of gB domains **(B)** Ribbon diagram of a single gB protomer (UniProtKB - P10211) **(C)** Ribbon diagram of gB trimer (PDB ID 5V2S). Figures were made with Pymol. MPD, Membrane Proximal Domain; TMD, Trans Membrane Domain; CTD, Cytoplasmic Tail Domain; D, Domain.

studies rationalize that gB's N terminus does not have any unique function, it is important to note that the significance of gB's flexible region has not been precisely identified yet.

## Structure of the Pre-Fusion Form of gB

Crystallographic studies have expanded on known information related to gB's post-fusion form. In contrast, gB's pre-fusion form remains relatively unclear. During extraction, the gB ectodomain directly adopts its post-fusion form, making the initial form difficult to study and examine (Vitu et al., 2013; Cooper and Heldwein, 2015). One recent study emphasizes the interaction of gB anchoring segments with its lipid bilayer in maintaining gB in its pre-fusion form (Cooper et al., 2018). Disturbing the bilayer scaffold destabilizes the pre-fusion conformation, enabling the gB to refold readily into its post-fusion conformation (Patrone et al., 2014; Cooper et al., 2018). Studies that have attempted to capture the pre-fusion form of gB by modifying it through point mutations, deletions and truncations have not been able to document the pre-fusion form (Vitu et al., 2013).

However, some have captured the 3D image of gB in its pre-fusion form by expressing full-length gB embedded in microvesicles (Zeev-Ben-Mordehai et al., 2016; Fontana et al., 2017). One of the studies have captured the image of gB's pre-fusion form using cryo-electron tomography (cryo-ET) and subtomogram averaging with the help of a series of fusion protein-modified forms of gB and anti-gB antibodies. They propose the pre-fusion form of gB has a globular structure adjacent to the membrane and that it is approximately 8 nm tall and 7 nm wide (Fontana et al., 2017). Based on the observations, the authors suggest that conversion of gB to its post-fusion form requires series of changes to take place in its pre-fusion form. The first change occurs at domain V or at the MPR that allows fusion loops to point away from the viral membrane and toward the host membrane. This results in compacting intermediate conformation 1 which does not attach the fusion loops to the membrane surface. The second change occurs at domain III which allows gB to adopt its extended intermediate conformation 2 that attaches the fusion loop onto the surface of the host membrane. Finally, changes in domain V convert gB to its post-fusion conformation. This conformation brings the viral and host cell membranes close to each other and favors the membrane fusion (Fontana et al., 2017). However, direct experimental evidence on how gB undergoes transition from pre-fusion to post-fusion states is lacking, and the factors that contribute to these changes remain unclear.

## Membrane Proximal Region of gB

Crystallographic studies have revealed that the MPR is approximately 43 amino acid residue long and helical in structure. This region is highly hydrophobic in nature, and it is not part of the post-fusion hairpin (Shelly et al., 2012; Maurer et al., 2013). The MPR lies in between the ectodomain and the TMD, and it is seen in parallel to the membrane bilayer (Cooper and Heldwein, 2015).

The specific function of MPR region is still unknown, but studies have shown alterations like point mutations, deletions, or insertions in this region have a negative impact on viral infectivity (Rasile et al., 1993; Zheng et al., 1996; Lin and Spear, 2007; Shelly et al., 2012; Cooper and Heldwein, 2015; Efler et al., 2015). Experts believe this region determines the lipid mixing during fusion reaction. When conditions are favorable, specific amino acid residues in this region facilitate the

attachment of the virus to the host cell membrane. Similarly, when conditions are unfavorable, another set of amino acids in this region shields and isolates the fusion loops, thereby preventing the fusion reaction (Shelly et al., 2012; Cooper and Heldwein, 2015). In some viruses, this region is involved in the formation of fusion pores (Li and Blissard, 2009) and is essential for cell to cell fusion (Jeetendra et al., 2003). Yet, direct experimental evidence proving the function of this region in HSV is lacking.

## Transmembrane Region of gB

The membrane-bound single pass TMD of gB is approximately 20–22 residues long (Rasile et al., 1993; Gilbert et al., 1994). TMD lies between MPR and CTD (**Figure 2**). It is helical in structure and perpendicularly positioned to the MPR helix and membrane bilayer (Engelman et al., 1986; Arkin and Brunger, 1998). Recent crystallographic study reveals single-pass TMD helices of each promoter cross one another at a 46° angle to form a unique "inverted tepee" like assembly (Cooper and Heldwein, 2015). The N terminals (proximal to MPR) are splayed, and the C terminals (proximal to CTD) are converged thus uniquely forms the inverted tepee structure (**Figure 2**). Amino acid residues in TMD are highly conserved among α-herpesviruses, implying a structural and functional importance of this region in α-herpesviruses. Experiments that replace the TMD with a lipid anchor demonstrate that HSV-1 does not proceed beyond hemi-fusion stage, which denotes that gB TMD is not just a membrane anchor but has essential roles in the later stages of fusion (Engelman et al., 1986). The MPR and TMD may not initiate fusion reaction, but once fusion is initiated by other factors, they facilitate lipid mixing and formation of fusion pore (Markosyan et al., 2000; Bissonnette et al., 2009; Cooper and Heldwein, 2015).

## Cytoplasmic Tail

Structural analyses have revealed that the 109 residue long cytoplasmic tail of HSV-1 gB is organized into domains (H1, H2 and H3) and linkers. The H1 domain further contains subdomains H1a, H1b, and a linker. The H1a and H2 domains forms α-helices, H1b forms a 310 helix, and the structure of H3 is unresolved. Similarly, the structure of the linker that connects H1b, H2, and H2, H3 is unresolved. Long H2 α-helices form the central trimeric core beneath the membrane, and they are angled such that one end faces the membrane while the other end forms a triangular base below the zigzag protrusion. The zigzag protrusion around the central core is formed by H1a and H1b along with TMD. Conserved proline residues (P805 at TMD/H1a and P811 at H1a/H1b junction) create this zigzag protrusion, and they are essential for overall structural stability (Cooper et al., 2018).

Studies that alter cytoplasmic tail domain have shown the importance of this region, as mutations, truncations and insertions in this region affect viral infectivity, especially by enhancing cell fusion (Weed et al., 2017). In cell culture, wild-type HSV does not normally form syncytia (individual cells fused to multi-nucleated cells), but an alteration in the CTD region of gB favors syncytia formation during infection (Cooper and Heldwein, 2015). This suggests that this region of gB is thought to negatively regulate fusion reactions and maintains gB in its pre-fusion form, preventing cell fusion.

As discussed earlier, experts believe the CTD of gB act as a clamp and controls the pre-mature formation of gB's post-fusion. Upon receiving proper signal, cytoplasmic tail of gH/gL releases the clamp which frees gB to unfold to its post-fusion conformation and promotes cell fusion. The role of the CTD clamp may be unique to HSV since other herpesviruses have their own ways of controlling the pre-mature formation of gB's post-fusion conformation (Cooper and Heldwein, 2015).

# VIRAL GLYCOPROTEIN gD

HSV gD is a 369 amino acid residue long type I membrane glycoprotein that is approximately 8-10 nm long and irregularly clustered on the viral membrane surface. HSV gD is organized into an ectodomain, a TMD and a short cytoplasmic tail. Though the gD of all alpha-herpesviruses serves a similar function – binding to host cell receptor and initiating fusion reactions (Cocchi et al., 2004) – it is not replaceable. Experiments that tried replacing it have reported a complete loss of function (Fan et al., 2014; Fan et al., 2017).

According to crystallographic studies, the gD ectodomain has an immunoglobulin-like core, edged by N and C terminal extensions on either ends (Krummenacher et al., 2005; Karasneh and Shukla, 2011). The N-terminus domain is termed as the receptor binding domain (RBD), and this part of the gD binds with specific host receptors. The C-terminus domain is termed as pro-fusion domain (PFD) which interact with gH/gL and gB (Fan et al., 2017). The PFD also binds with the N-terminus region and forms an auto-inhibitory closed conformation. This self-inhibitory conformation is essential to prevent the binding of gD to gH/gL or gB before it binds to its specific receptor. The binding of gD to its specific receptor causes conformational changes in the gD that favor the release of the PFD domain from its N-terminus interaction. This release then allows PFD to bind with gH/gL or gB. Thus, self-inhibitory confirmation prevents the premature binding of gD to gH/gL and or gB. The interaction of PFD with gH/gL and gB is essential for the formation of core fusion machinery (Karasneh and Shukla, 2011). Studies have shown the binding of antibodies to this specific region, blocks the interaction of gH/gL and gB to gD. The PFD region is rich in proline residues (P261-P305) and located proximal to the transmembrane segment. Unfortunately, crystallographic studies could not resolve the structure of this region (Cocchi et al., 2004).

RBD and PFD are both essential for the fusion reaction. The infectivity of a HSV-1 lacking gD can be restored upon addition of exogenous soluble gD but only if the PFD and RBD regions of the gD ectodomain are present in that soluble form as these regions cannot function independently (Gianni et al., 2009; Fan et al., 2014; Fan et al., 2017). The RBD of gD is essential for it to recognize its receptors as well as binding of gB to its receptors, especially to PILRα (Satoh et al., 2008). A recent study that investigated the plasticity of gD by analyzing several gD mutation constructs supports this idea. PQF170 is one such

mutation construct, in which residues 1–32 were deleted. When authors performed the quantitative cell fusion assay to assess the cell fusion activity of the mutants, they observed the mutant lost its binding efficiency with HVEM but retained its binding efficiency with nectin-1. Interestingly, the mutant also lost its binding efficiency with PILRα (gB receptor). This suggests the first 22 amino acids of gD may be essential for gD to recognize its receptors as well as the binding of gB to its receptor (PILRα). This result supports the idea PILRα requires gB as well as gD to induce cell fusion (Fan et al., 2009). These studies illustrate both PRD and RBD both have a crucial role in cell fusion.

Though exogenous soluble gD is enough to restore cell fusion in the presence of other glycoproteins, the MPR, TMR, and CTD of gD might play a unique role during or after the cell fusion reaction. Studies have shown that the membrane-proximal basic residues of gD induce the formation of microvillus-like projections from the plasma membrane of transfected cells (Arii et al., 2016; Carmichael et al., 2019). A mutant in the membrane-proximal basic residues prevents this formation and reduces the viral spread (Nicola, 2016). Also, an arginine/lysine cluster located at the transmembrane-cytoplasm interface of gD critically contributes to viral spread and cell to cell fusion (Nicola, 2016; Carmichael et al., 2019).

## VIRAL GLYCOPROTEIN gH/gL

The HSV heterodimer gH/gL is vital for the fusion reaction, but its precise role is not understood yet (Weed et al., 2017). Previous studies predicted gH may have fusogenic properties (Kinzler and Compton, 2005; Subramanian et al., 2007). In contrast, crystallographic studies conclude that gH/gL is not a viral fusogen as it does not possess any of the reported structural features (trimeric hairpin bundle or internal fusion peptides) of other fusogens (Harrison, 2008). Studies predict gH/gL may interact with gB and gD and thus regulate the fusion reaction. Supporting this concept, neutralizing antibody study shows that gB–gH–gL complex occurs prior to fusion reaction (Muggeridge, 2000). Sequence and structural data have revealed gH/gL heterodimer of both HSV-1 and HSV-2 are similar in sequence and structure. Even the antibodies specific for HSV-1 gH/gL can bind with HSV-2 gH/gL and vice versa (Jha et al., 2016). The gH of both HSV serotypes is a type I membrane glycoprotein consisting of 838 amino acids, with a large ectodomain, a single-pass transmembrane segment and a short cytoplasmic tail of 14 amino acids (Peng et al., 1998; Weed et al., 2017). gL contains 224 amino acids, lacks a transmembrane domain, and is non-covalently bound to the N-terminus of gH (Peng et al., 1998; Chowdary et al., 2010). The gH/gL heterodimer is smaller than gB but larger than gD. The gH/gL heterodimer need each other for their proper folding and structural stability (Jha et al., 2016).

### The gH/gL Ectodomain

Crystallographic studies reveal the binding of gH with gL forms a boot-shaped ectodomain that is approximately 80 Å tall and 70 Å long (Chowdary et al., 2010). The gH ectodomain is organized into three distinct domains: H1, H2, and H3 (N-terminal H1 and H2 and C- terminal H3). Sequence data suggest membrane distal domain H1 is the least conserved, H2 moderately conserved and H3 is highly conserved among herpesviruses. Domain H1 consists of subdomains H1A and H1B and a 20 amino acid residue linker that connects them (Jha et al., 2016); (**Figure 3**). gL is always seen in association H1 domain. Sequences of domain H1 and gL vary among herpesviruses and cannot be interchanged except between HSV-1 and HSV-2 (Muggeridge, 2000; Cairns et al., 2005). Subdomain H1A and H1B, hold gL like "tongs". The interacting surfaces of H1A and gL are highly complementary as the two proteins need each other to fold properly and function normally (Hutchinson et al., 1992). The highly diverse H1 domain may receive a variety of activating signals (Cooper and Heldwein, 2015). The conserved H2 and highly conserved H3 domains then translate these diverse inputs into a common message and transmit it to gB. The conservation of domain H2 and H3 is essential for message transmission during gB activation (Cooper and Heldwein, 2015). Using monoclonal antibodies, it has been shown that gB and gD bind to gH/gL at different sites. The binding of monoclonal antibodies in that specific region prevents the interaction of these glycoproteins and formation of fusion reaction (Atanasiu et al., 2013).

### The gH Transmembrane and Cytoplasmic Tail

Studies support that full-length gH is essential to form the core fusion machinery (Browne et al., 1996; Harman et al., 2002). Full-length gH activates gB more efficiently than its soluble form (Atanasiu et al., 2010a). Replacing its cytoplasmic tail with analogous domains (Jones and Geraghty, 2004) or amino acid substitutions made within transmembrane or cytoplasmic tail did not promote cell fusion (Cooper et al., 2018) or cell to cell fusion (Harman et al., 2002). Mutational studies prove that the gH cytotail regulates and activates core fusion machinery. Insertions or truncations of the gH tail directly affect the fusogenic property of the virus. Changes either reduce or completely disturb viral infectivity or cell to cell fusion (Jackson et al., 2010; Rogalin and Heldwein, 2015; Cooper et al., 2018). The cytoplasmic tail of gH has been proposed to influence gB *via* "inside-out signaling" on the CTD region of gB (Rogalin and Heldwein, 2015). As discussed earlier, the gH cytoplasmic tail may act as a wedge which disrupts the gB clamp and promotes the fusion reaction. Thus, truncation or insertion in the tail region affect the ability of gH to reach the gB CTD and inhibits fusion efficiency (Rogalin and Heldwein, 2015).

## HERPES SIMPLEX VIRUS RECEPTORS

### gD Receptors

#### Herpesvirus Entry Mediator

HVEM is the first identified gD receptor (Montgomery et al., 1996). HVEM belongs to the tumor necrosis factor (TNF) receptor superfamily and regulates host's immune responses (Croft, 2003). It is expressed in a wide variety of immune cells

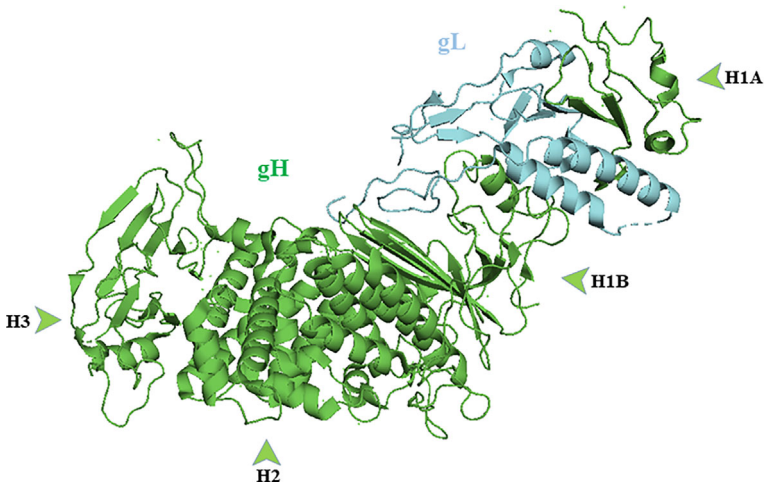


**FIGURE 3** | Crystal structure of gH/gL complex (PDB ID 3M1C). gH domains are in green and gL is in blue. Figure was made with Pymol.

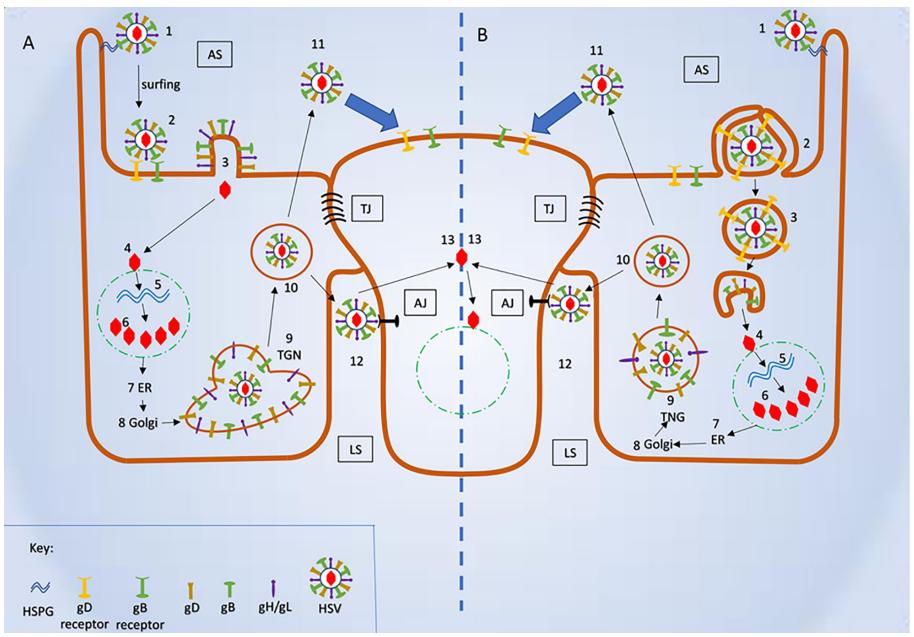


**FIGURE 4** | Schematic representation of herpes simplex virus (HSV) mode of entry and cell to cell spread. **(A)** Plasma membrane fusion and cell to cell spread (1) Virus attached to HSPG. (2) Attachment of viral glycoproteins with host cell receptors. (3) Fusion of viral envelope and release of viral content into the host cell's cytosol. (4) Fusion of viral capsid and release of viral DNA into nucleus. (5-6) Replication of viral DNA and assembly of nucleocapsid. (7-9) Release of newly synthesized nucleocapsid into the Trans-Golgi network (TGN) *via* ER and Golgi. (10) Mature herpes simplex virus (HSV) inside the secretory vesicle. (11) Mature HSV released into apical surface and it is ready to infect uninfected host cell. (12) Mature HSV released into lateral surface and it infects the adjacent uninfected cell *via* binding to adhesion transmembrane proteins and release the capsid into the cytosol. **(B)** Viral entry endocytic pathway and cell- cell spread (1) Virus attached to HSPG. (2) Pseudopodia like projection engulf viral particles along with the host cell receptors. (3) Formation of endosome and fusion of viral envelope with the endosomal membrane and release of viral content into the host's cytosol. (4) Fusion of viral capsid and release of viral DNA into nucleus. (5-6) Replication of viral DNA and assembly of nucleocapsid (7-9) Release of newly synthesized nucleocapsid into the TGN *via* ER and Golgi. (10) Mature HSV inside the secretory vesicle. (11) Mature HSV released into apical surface and it is ready to infect uninfected host cell. (12) Mature HSV released into lateral surface and it infects the adjacent uninfected cell *via* binding to adhesion transmembrane proteins and release the capsid into the cytosol. AS, Apical surface; LS, lateral surface; TJ, tight junction; AJ, Adhere junction; HSPHG, heparan sulfate proteoglycan; ER, endoplasmic reticulum; TGN, Trans Golgi network.

including T cells, B cells, dendritic cells, natural killer cells, macrophages, polymorphonuclear cells, and in other cell types like neurons, epithelial cells and fibroblasts (Edwards and Longnecker, 2017). T cells do not express nectin-1 since HVEM acts as the primary receptor that aids HSV entry into these cells (Spear, 2004; Agelidis and Shukla, 2015). HVEM usually binds with Ig-like ligands (CD160 and BTLA) and TNF ligands (LIGHT and LT) (Edwards and Longnecker, 2017) and regulates immune function in the host. However, certain viruses including HSV use this receptor to enter the host cell. Expressing HVEM in HSV-resistant Chinese hamster ovary (CHO) cells makes them susceptible to HSV infection (Montgomery et al., 1996). Experimental evidence suggest that HVEM along with HSV, facilitate the entry of HIV-1 into HIV-entry resistant cells (Hu et al., 2017).

A study with HSV-1 gDΔ7-15 (a HSV-1 mutant that enter host cell through nectin-1 but not HVEM) concluded that HVEM is not the primary entry receptor in the cornea (Edwards and Longnecker, 2017), suggesting nectin-1 must facilitate the initial entry of HSV on the ocular surface. The same study suggests that HVEM gets overexpressed in the corneal tissue only after the HSV infection, and HVEM-dependent induced cytokines released by macrophages are responsible for the inflammation and loss of corneal sensitivity (Edwards and Longnecker, 2017). Supporting this concept, a recent study suggests that binding of HSV-1 gD to HVEM-monocyte receptor activates NF-κB (Venuti et al., 2019). It is also known that NF-κB induces an inflammatory response in the host during viral or bacterial infection, especially by activating innate immune cells (Dorrington and Fraser, 2019).

It was proposed that gD cannot bind with both nectin-1 and HVEM simultaneously since they share a common set of binding residues (Zhang et al., 2011). The N-terminal of gD binds with HVEM (Spear, 2004) in their cysteine-rich domain 1 (CRD1) (Edwards and Longnecker, 2017). Thus, deletion of 1–32 residues from gD's N-terminal completely or partially prevents the binding of gD with HVEM but not with nectin-1 (Spear, 2004; Fan et al., 2017). Studies suggest both HVEM and nectin-1 are vital for HSV-1 corneal infection (Krummenacher et al., 2004; Arii and Kawaguchi, 2018).

During HSV-1 infection, HVEM plays significant roles in latency and reactivation (Wang et al., 2018) (Allen et al., 2014). The Latency Associated Transcript (LAT) upregulates HVEM expression which in turn downregulates host immune responses (Allen et al., 2014). Additionally, the absence of gD and the presence of the HVEM ligands BTLA, LIGHT, or CD160 enhance viral reactivation from latency (Wang et al., 2018). Thus, blocking HVEM, LIGHT, BTLA, and CD160 impede the viral latency and reactivation (Wang et al., 2018). The role of HVEM during entry is secondary to its interaction with the HSV-1 LAT during viral latency and reactivation.

## Nectin-1 and Nectin-2

Nectin-1 and nectin-2 are type I transmembrane glycoproteins, belonging to the immunoglobulin superfamily. Both nectin-1 and nectin-2 are expressed in a wide variety of human tissues and cell lines (Campadelli-Fiume et al., 2000). They mediate cell to cell adhesion by interacting with nectin on neighboring cells (Kwon et al., 2005). During HSV entry, gD binds the nectin-1 V-domain (Holmes et al., 2019), and this binding disturbs nectin's neighboring cell to cell adhesion function (Zhang et al., 2011). Both HSV-1 and HSV-2 can bind to nectin-1 for entry, but HSV-2 binds with nectin-2 more efficiently (Warner et al., 1998).

The N-terminus of the gD binds with nectin, and alterations made in this region affect its receptor-binding property. Deletion of N-terminus 1-32 amino acid residues from gD does not affect its nectin-1 binding efficiency (Spear, 2004; Fan et al., 2017). However, two or more point mutations at positions 215, 222, and 223 reduce the nectin-1 binding efficiency (Manoj et al., 2004). Similarly, when insertions were made at the N-terminus of gD, the length of the insertion influenced the nectin-1 binding property of the gD (Jogger et al., 2004; Fan et al., 2017). (HSV prefers binding with nectin-1 rather than HVEM or nectin -2 (Manoj et al., 2004). HSV infection and spread were seized in neural and epithelial cells in the absence of nectin-1 (Spear, 2004; Arii and Kawaguchi, 2018). When compared with nectin-2, both HSV-1 and HSV-2 binds efficiently with HVEM (Montgomery et al., 1996). However, experimental evidence has shown that amino acid substitution made at gD's N-terminal conserved region, can enhance the gD binding efficiency towards nectin-2 (Spear, 2004).

## 3-O-Sulfated Heparan Sulfate Proteoglycan

HSV-1 gD, but not HSV-2 gD, binds with 3-O-sulfated heparan sulfate proteoglycan (3-OS-HS) (Shukla et al., 1999). 3-OS HS is a highly sulfated form of heparan sulfate (HS): a long linear polysaccharide (glycosaminoglycan class) chain made of disaccharides (glucosamine and glucuronic acid) which when bound to sulfate-rich, highly negative charged protein (syndecan and/or glypican) forms HSPGs (Thakkar et al., 2017; Masola et al., 2018). Sulfation of glucosamine at the 3-O position by 3-O-sulfotransferases generates 3-OS-HS. Each isoform of these enzyme generates unique 3-OS HS (Karasneh and Shukla, 2011). This adds structural diversity and structural integrity (Thakkar et al., 2017). More importantly, this makes them serve as an attachment receptor for several host proteins that regulate body functions (growth factors, chemokines, cytokines, antithrombin). The unique charge distribution on HS allows it to serve as an attachment receptor to many pathogenic viruses, including HSV, especially in the neural cells (Shukla et al., 1999; Campadelli-Fiume et al., 2000; Shukla and Spear, 2001). HSV gB and/or gC initial binding with HS (Shukla et al., 1999), is not essential for membrane fusion but promotes viral adsorption on the cell surface (Banfield et al., 1995; Laquerre et al., 1998). After the initial attachment, the virus slides down the filopodia and reaches the cell body. It then uses glycoprotein gD to bind with 3-OS HS (or other receptors) and initiates a cell fusion reaction that favors viral entry into the cell (Akhtar and Shukla, 2009; Thakkar et al., 2017). The absence of HS on cell surface reduces the HSV infection by about 100 fold (Gruenheid et al., 1993).

Addition of soluble 3-OS-HS or extrinsic expression in HSV infection-resistant cells makes them susceptible to HSV-1 infection (O'Donnell and Shukla, 2008). 3-OS-HS plays a major role in mediating HSV-1 entry in primary cultures of

human corneal fibroblasts and in zebrafish (Tiwari et al., 2007a). 3-OS-HS can also regulate polykaryocyte formation (Tiwari et al., 2007b). A recent study demonstrated the presence of 3-OS-HS on mouse-derived dorsal root ganglia explants and in a single cell neuronal model. The study also captured the interaction of 3-OS-HS with HSV-1 glycoprotein B (gB) and glycoprotein D (gD) during cell entry. Furthermore, treatment of these cells with heparanase, an endoglycosidase that cleaves HS chains (Masola et al., 2018) inhibited HSV-1 entry considerably and enhanced the expression of chemokines that regulates HS (Sharthiya et al., 2017). These factors highlight the significance of HS and 3-OS-HS during attachment and entry of HSV into the host cell.

Downregulation of 3-OS-HS or competitive inhibitors of 3-OS HS significantly reduce the HSV-1 entry into the host cell (O'Donnell et al., 2010). Since cationic viral glycoprotein bind with negatively charged HS, a series of small cationic peptides (anti-HS peptides) were designed as antiviral agents. The efficiency of these peptides was tested in mouse corneal model (as prophylactic eye drops) and in human cell cultures. The test results concluded that cationic peptides could prevent the viral attachment and block the viral spread in both the models. Also this experiment emphasizes the importance of binding of viral glycoprotein to HS and 3-O-HS during HSV infection (Tiwari et al., 2011).

## gB Receptors

### Paired Immunoglobulin-Like Type 2 Receptor-α

The paired immunoglobulin-like receptor α (PILRα) family is mainly expressed in immune cells, especially in myeloid cells: monocytes, macrophages, and dendritic cells (Satoh et al., 2008; Arii and Kawaguchi, 2018). PILRα members are important surface molecules, binding with ligands to modulate the host immune response (Lu et al., 2014). Expression of the PILRα in HSV resistant cells makes them susceptible to almost all alpha-herpesviruses, except HSV-2 (Arii et al., 2009). PILRα is the first identified gB receptor. PILRα is a paired heterodimer receptor composed of an activator and inhibitor. The inhibitory receptor binds with self-antigens like MHC molecules, and the activating receptors does not bind or recognize self-antigens. HSV binds with inhibitory receptor and delivers inhibitory signals to the host cell (Karasneh and Shukla, 2011). The presence of anti-PILRα antibodies block HSV-1 infection (Satoh et al., 2008). The binding of the gB to PILRα diverts the HSV entry route from endocytosis to direct fusion (Arii and Kawaguchi, 2018).

The binding of gB with PILRα requires several ancillary factors. Studies have shown PILRα to function as a gB receptor it requires gD and its receptor (Satoh et al., 2008; Fan et al., 2009). Also, the same study suggests that the PFD region of gD is required to facilitate the gB-PILRα mediated cell fusion reaction (Fan et al., 2017). These data suggest that the gB-PILRα mediated cell fusion reaction requires gD and it's receptors. Mutation of O-glycosylation sites on gB (threonine-53 and threonine-480) decreases PILRα-dependent viral binding and pathogenesis (Wang et al., 2009; Arii et al., 2010b). Similarly, the presence of tryptophan-139 in PILRα is essential for it to bind with gB (Fan and Longnecker, 2010). Physiological relevance of this receptor is yet to be demonstrated in an animal model.

### Myelin-Associated Glycoprotein

HSV-1-gB binds with myelin-associated glycoprotein (MAG) or sialic-acid-binding Ig-like lectin, present over the surface of glial cells (Arii and Kawaguchi, 2018). MAG is also a paired receptor family like PILRα, and they share 5-12% homology (Suenaga et al., 2010). In glial cells, MAG regulates myelin-axon interactions and inhibits axonal regeneration. The regulation includes myelination, initiation, and myelin integrity maintenance (Karasneh and Shukla, 2011). Both HSV-1 and varicella-zoster virus (VZV) gB bind with MAG and promote viral entry (Suenaga et al., 2010). Fortunately, MAG is not expressed in epithelial cells. Thus, MAG is not the primary receptor for the HSV. Unfortunately, during acute phase infection, HSV may utilize MAG and causes neurological disorders (Karasneh and Shukla, 2011). Future studies using gene knockout animal models will demonstrate the actual use of the receptor during infection.

### Non-Muscle Myosin Heavy Chain IIA

HSV-1 gB also binds with non-muscle myosin heavy chain (NMHC)-IIA (Arii et al., 2010a) and can mediate viral entry into cells that express it (Karasneh and Shukla, 2011). NMHC-IIA is a subunit of non-muscle myosin IIA (NM-IIA). NM-IIA is an isoform of the NM II protein (Vicente-Manzanares et al., 2009). NM-II proteins consisting of two heavy chains, two regulatory light chains, and two essential light chains. In the host cell, NM II binds with actin and regulates normal cellular functions like cell division, adhesion, movement, migration, and contraction (Karasneh and Shukla, 2011; Agelidis and Shukla, 2015). HSV-1 gB binds with heavy chain peptides of NM II molecules and triggers viral entry into the host cell.

NMHC-IIA is ubiquitously expressed in human tissues (Agelidis and Shukla, 2015). Infectivity of HSV-1 in human promyelocytic HL60 cells is directly proportional to the expression levels of NMHC-IIA; The higher the expressions level, higher the cell susceptibility to the HSV-1 infection and vice versa (Karasneh and Shukla, 2011). Similarly, anti-NMHC-IIA antibodies have been shown to inhibit HSV-1 infection in cell lines that express NMHC-IIA. These factors support the important role of NMHC-IIA as a functional gB receptor (Arii and Kawaguchi, 2018). The research data suggest that HSV-1 gB only in the absence of PILRα, binds with NMHC-IIA. The importance of this preference is not clearly understood yet. Unlike PILRα, NMHC-IIA does not influence the HSV's mode of entry in the cell (Arii et al., 2010a).

Studies suggest that HSV infection might take over the host system and use it to support infection and spread. For example, normally NMHC-IIA is seen only in the cytoplasm but HSV infected cells rapidly express NMHC-IIA on their surface (Arii et al., 2010a). Similarly, HSV infection can reorganize the actin cytoskeleton (Clement et al., 2006) and induce filopodia formation. While the observations are intriguing a more clear understanding of the receptor's function in HSV entry will only

be obtained *via* the use of animal models that lack NMHC-IIA gene.

## gH/gL Receptors

### αvβ6 and αvβ8 Integrins

The αV group of integrins includes αVβ3, αVβ5, αVβ6, and αVβ8. Studies indicate that αvβ6 and αvβ8 bind gH with high affinity but at different locations (Gianni et al., 2013b). The binding of αVβ6 with gH requires the presence of the gH integrin-binding motif, Arg-Gly-Asp (RGD), while αVβ8 does not (Gianni et al., 2013b). Both αVβ6 and αVβ8 are expressed in epithelial cells but only the latter is expressed in glial and dendritic cells (Nishimura et al., 1998; Gianni et al., 2013b). The αVβ5-integrin does not bind gH/gL. Studies have concluded that αVβ3-integrin binds with the gH/gL receptor with a very low affinity and does not lead to fusion reaction. However, the binding may help HSV to enter the cell *via* acidic endosome route (Gianni et al., 2010; Gianni and Campadelli-Fiume, 2011).

Experimental evidence suggests that if integrins were blocked by antibodies or if their expression is silenced, HSV entry is restricted. In contrast, if integrins were expressed in integrin-negative cells, HSV entry is favored (Gianni et al., 2013b). Conditions like tissue remodeling (Thomas et al., 2006) and epithelial malignancies (Nishimura et al., 1998) upregulate the expression of αVβ6 in tissue. These conditions favors HSV infection (Petermann et al., 2009). It is proposed that the interaction of HSV gH/gL with integrins results in gL dissociation and is essential for activation (Gianni et al., 2015). Studies have also found that gH/gL can trigger NF-κB activation and innate immune responses through αvβ3-integrin or toll-like receptor 2 binding (Leoni et al., 2012; Gianni et al., 2013a), but the significance of these findings remains unknown.

# LOW PH-DEPENDENT, ENDOCYTIC ENTRY OF HERPES SIMPLEX VIRUS

While this article's main emphasis is to review pH-independent entry mechanisms, it is also important to note how HSV enters the host cell *via* a low pH-dependent, endocytic pathway. During this process, the virions are internalized and transported into the host cell's early endosomes. The mild acidic pH of the endosome induces favorable conformational changes in the viral fusion proteins to fuse the viral envelope with the vesicular membrane (Nicola, 2016). Fusion releases the nucleocapsid from the vesicle into the cytosol, probably close to the nucleus (**Figure 4**).

Though considerable research has been done in the low pH-dependent, endocytic entry of HSV, it is still unclear what initiates the internalization. It is clear that the process is atypical endocytosis demonstrating many attributes of non-professional phagocytosis (Clement et al., 2006). This mechanism cannot be micropinocytosis since it induced only in the presence of entry receptors. Complement-mediated HSV internalization has also been reported (Van Strijp et al., 1989). Once internalized, the virus reaches the cell's early endosomes. Mild acidic pH in endosome activates most of the viral glycoproteins (Dollery et al., 2011). In any mode of entry, the binding of gD to its host receptor is vital for its activation since mild acidic pH has no detectable effect on gD (Dollery et al., 2011). Electron microscopic and fluorescence microscopy have shown the presence of nectin-1 (or HVEM) receptor in the internalized vesicles and virus attached to the vesicles (Clement et al., 2006). Additionally, a mildly acidic pH is not a barrier to gD binding to its receptors (Dollery et al., 2011). However, gB and gH-gL, in the absence of host receptors, may be dependent on endosomal pH for the conformational changes.

The endosomal pH of approximately 6.2 to 6.4 triggers conformational changes in gB (Dollery et al., 2010; Nicola, 2016). A highly fusogenic form of gB resembles the structure of gB that has undergone low pH-triggered conformation changes (Nicola, 2016). Additionally, low pH has shown to effect the antigenic structure of gH/gL (Cairns et al., 2011). However, these alterations in favor of HSV entry have not been reported yet. In short, it is likely that the receptor-bound activated gD and pH-activated gB and gH/gL associate to form fusogenic complex that leads to fusion reaction. The complex ultimately fuse the viral envelope with the host's vesicle and releases the viral nucleocapsid and tegument protein into the cytosol (Clement et al., 2006). Interestingly, apart from activation, a drop in pH may also serve as a "cue" for the virus to escape the endocytic pathway before it reaches lysosome (Nicola, 2016).

# CELL-TO-CELL VIRAL SPREAD

HSV primarily infects cells that form extensive cell to cell contact. These types of cells are called polarized cells, a group which includes epithelial cells. One of the main advantages of infecting polarized cells is that after replication, virions can move rapidly and effectively from an infected cell to adjacent uninfected cells. During this movement, they use host's adhesion transmembrane proteins as their binding receptors, which exist to bind the host cells together. HSV moves between the cells using cell junctions adhesion proteins to avoid being detected by the host immune system (Johnson and Huber, 2002). This spread can be within the same type of cells, or it can be from the primary site to sensory site. The secondary site mostly refers to neuronal cells where they establish latency or can cause encephalitis (Johnson and Huber, 2002; Carmichael et al., 2018). The entry essential glycoproteins (gB, gD, gH-gL) and gD receptors as well as gK play important roles in cell-to-cell spread (Weed et al., 2017). Interestingly, the most commonly found gD receptor, nectin-1 is expressed at cell junctions (Campbell et al., 2017).

Studies suggest HSV uses cell junctions, specifically tight and adherens junctions, to move from infected to uninfected cells (Mateo et al., 2015). In these junctions, HSV uses the glycoproteins gE/gI to attach to its receptors that favor cell to cell spread. Mutational, ocular model, cell culture and rodent studies support the concept that gE/gI promotes cell to cell spread during HSV infection (Johnson and Huber, 2002). gE/gI is a heterodimer that may function exclusively in keratinocytes, epithelial cells, and neurons cells - the type of cells that are

susceptible to HSV infection. Mutational studies demonstrate that the tail of the gE plays the primary role in delivering virions to the lateral surface of the cell. In its absence, virions are released at the apical surface (Carmichael et al., 2018) (**Figure 4**). During HSV infection, the virus accumulates glycoproteins gE/gI at the host Trans-Golgi network (TGN) and in endosomes. The glycoproteins are transferred to the envelope a process which favors lateral spread from infected to uninfected cells (Johnson and Huber, 2002; Krummenacher et al., 2003).

Apart from gE/gI, several other known and unknown viral and host factors may be involved in cell to cell spread. During cell to cell spread, HSV may induce the formation of canal-like fusion pores at the cell to cell junction which are further stabilized by the host cytoskeleton (Mateo et al., 2015). Similarly, viral glycoproteins gK, gM gN and viral tegument proteins UL11, UL16, VP22, and UL51 seem to participate in the spread mechanism (Nozawa et al., 2005; Kim et al., 2013; Wu et al., 2020). Mice infected with a gK-deleted mutant, show low cell to cell spread efficiency and relatively fewer pathological effects (Akhtar and Shukla, 2009). A recent study suggests PTP1B. a host tyrosine phosphatase, seem to be essential for the cell to cell spread. Though PTP1B modulates a wide variety of cellular functions in the host, how it aids the virus in cell to cell spread is not defined (Carmichael et al., 2018). Extracellular spread of HSV is also a route of viral transmission. Recent studies have shown that the HS cleaving enzyme, heparanse, removes the attachment receptor to facilitate viral release. This suggest that viral glycoproteins lose the ability to bind a virus-producing cell (Hadigal et al., 2015; Agelidis et al., 2017).

## VIRAL GLYCOPROTEINS AS ANTIVIRAL TARGETS

Blocking HSV glycoproteins or their interaction with receptors has the potential to inhibit viral entry into the host cell, and cell to cell spread (Cai et al., 1988; Antoine et al., 2013). The interactions among gB, gD, gH-gL are essential for fusion to occur. Thus, targeting glycoprotein-receptor interactions *via* small molecules will have a significant effect in treating HSV infections. These small molecules include peptides, HS mimetics, monoclonal antibodies, aptamers, nanoparticles, synthetic, or natural compounds. A subset of those that were proven to inhibit viral entry by targeting viral glycoproteins or their receptors are discussed below.

### Peptides

Anti-HSV peptides act by interacting with viral glycoproteins or with their receptors. Most antiviral peptides are generally less toxic compared to small molecule compounds with comparative antiviral activity (Galdiero et al., 2013). However, peptides have several limitations and therapeutically limited only to topical use (Akkarawongsa et al., 2009). These peptides can be synthetic or naturally occurring. Synthetic peptides can mimic the viral glycoprotein itself. For example, synthetic peptides HB168–186, HB491514, and HB632–650 mimic the central helical region of the HSV-1 gB (Galdiero et al., 2008) and inhibit viral entry. Similarly gB122, gB131, and gB94 synthetic peptides derived from gB have been shown to restrict viral entry as well. (Akkarawongsa et al., 2009).

Synthetic peptides may lack structural similarities with viral components yet be effective in treating infections. For example, the synthetic theta defensin retrocyclin-2 inhibits HSV entry. The mechanism of action is not confirmed, but the authors speculate that it binds with gB to prevent entry (Yasin et al., 2004). Similarly, G1 and G2, 12-mer peptides that binds with HS and 3-OS HS respectively (Tiwari et al., 2011) and a 3-O-sulfated octasaccharide (Copeland et al., 2009) block the entry and spread of HSV-1 in cultured human corneal cells and in a mouse cornea model (Tiwari et al., 2011). They compete with HSV-1 for HS during attachment and effectively impede viral infection (Tiwari et al., 2011). Furthermore, the delivery of the G2 peptide through a contact lens has shown to significantly inhibit the HSV-1 entry and spread in human corneal epithelial cells (Jaishankar et al., 2016). Application of these peptides as prophylactic eye drops, have suppressed the entry and spread of HSV in *ex vivo* and *in vivo* models (Tiwari et al., 2011; Jaishankar et al., 2016). Similarly, synthetic anti-lipopolysaccharide peptides block HSV entry by binding with heparan sulfate without being toxic at the effective concentrations (Krepstakies et al., 2012).

Naturally occurring peptides can also effectively inhibit HSV entry. For example lactoferrin or it's N-terminal domain, lactoferricin inhibits HSV entry (Jenssen, 2005). Lactoferrin, a 80 kDa peptide is part of our innate immune system and found in various secretory fluids (Antoine et al., 2013). Interestingly, preincubating human lactoferrin with HSV-1 effectively reduced viral entry and spread. However, preincubating lactoferrin with a gD-mutant HSV-1 resulted in less inhibition. This suggests that this peptide restricts viral entry by binding with viral gD or to one of its receptors (Välimaa et al., 2009). Apart from gD, lactoferrin can also interact with gC and/or HS to inhibit viral entry (Jenssen et al., 2008; Valori et al., 2008). Dermaseptins are natural peptides obtained from skin of Hylid frogs (Bartels et al., 2019). They are reported to have antiviral activity against HSV-1 and HSV-2. These peptides have shown to interact with HS and thereby interrupt the initial viral attachment to the host cell (Hadigal and Shukla, 2013). Pre-incubating HSV-2 with biochemically modified Dermaseptins have been shown to inhibit acyclovir-resistant HSV-2 entry (Bergaoui et al., 2013). Mytilin, another entry inhibitor, is a small natural peptide obtained from the Mediterranean mussel (*mytilus galloprovincialis*). Like many of the other peptides, it inhibits viral entry by interfering with the viral attachment site on host cell receptors (Galdiero et al., 2013).

### Antibodies

Since initial studies done by Dix et al. in 1981, several studies have generated monoclonal antibodies against HSV and demonstrated their efficiency in inhibiting HSV entry into host cell. Most of these antibodies were generated against HSV glycoproteins gB, gC, gD, gH, gL, or their host cell receptor molecules (Dix et al., 1981; Nicola et al., 1998; Cairns et al., 2006; Du et al., 2017; Wang et al., 2017). Though monoclonal

antibodies are effective, they are expensive, have complicated production requirements and have limited practical applications as a therapeutic agent (Chames et al., 2009).

## Aptamers and Nanoparticles

To overcome the limitations of antibodies, aptamers were created. Aptamers are short oligonucleotides that have the advantages of monoclonal antibodies such as binding target molecules with high specificity. However, they lack many of the disadvantages of antibodies. They are easy to synthesize, cost effective, less immunogenic, and 10–100-fold smaller than antibodies which allows them to penetrate tissue effectively. Aptamers may soon replace monoclonal antibodies in diagnostic and therapeutic applications (Lakhin et al., 2013). Anti-HSV aptamers are mostly specific for gD (Moore et al., 2011; Gopinath et al., 2012). Recently, we developed 45-nt-long DNA aptamers that bind to HSV-1 gD with high specificity. These DNA aptamers could significantly restrict viral entry *in vitro*, *ex vivo* and *in vivo* models (Yadavalli et al., 2017).

Our group has shown that zinc oxide tetrapod nanoparticles (ZOTEN) can inhibit viral entry as well. These micrometer-sized particles with characteristic nano-dimensional elongations trap viral particles on their surface and restrict them from entering host cells. ZOTEN, owing to its special manufacturing process, possess large amount of oxygen vacancies on their surface, giving them a strong positive surface charge. Its cationic surface charge accounts for its ability to trap virions (Antoine et al., 2016). We have also shown that ZOTEN can target and restrict both HSV-1 and HSV-2 using *in vitro*, *ex vivo*, and *in vivo* assays. Interestingly, virus-ZOTEN particles may also provide an antigen presentation platform to conceptualize a live virus vaccine (Agelidis et al., 2019).

## Heparan Sulfate Mimetics

As mentioned previously gB, gD, and gC viral glycoproteins binds HS effectively, thus molecules that mimic HS structurally have been found to inhibit HSV entry into the cell. For example sulfated heparin and its chemical derivatives (Lycke et al., 1991; Herold et al., 1995; Feyzi et al., 1997). Lignin derivative including sulfated lignins (Raghuraman et al., 2005; Raghuraman et al., 2007), and carboxylated lignins (Thakkar et al., 2010) were able to restrict HSV entry into host cells (Raghuraman et al., 2007; Thakkar et al., 2010). Several other HS mimic include pentosan polysulfate (Herold et al., 1997), dextran sulfate (Dyer et al., 1997; Herold et al., 1997), sulfated maltoheptaose (Herold et al., 1997), sulfated fucoidans (Preeprame et al., 2001; Ponce et al., 2003; Lee et al., 2004), spirulan (Mader et al., 2016), PI-88 (Nyberg et al., 2004), and nonsaccharide glycosaminoglycan mimetics sulfated galloids (Gangji et al., 2018) were successful in restricting HSV entry.

## Vaccines

Vaccines for HSV infection are still in development (Johnston et al., 2016). HSV vaccines tested in human clinical trials were primarily subunit, live attenuated, replication-defective virus based (Bernard et al., 2015). In these candidates, viral glycoproteins play a major role in stimulating the host immune response which protects the host from HSV infection (Belshe et al., 2012; Çuburu et al., 2015; Burn et al., 2018). Thus, viral glycoprotein-based subunit vaccines are seen predominantly in human clinical trials (Johnston et al., 2016). The most common glycoprotein in these vaccines is gD. Other glycoproteins such as gB, gC, gE, gK were utilized in vaccines as well (Corey et al., 1999; Stanfield et al., 2014; Awasthi et al., 2017; Egan et al., 2020). Unfortunately, some vaccines efficient in animal models failed in human clinical trials and were ceased recently. Herpevac (Belshe et al., 2012), VCL-HB01, GEN-003 (Whitley and Baines, 2018; Truong et al., 2019) were removed from human clinical trials. Fortunately, some vaccines are continuing their clinical trials. The following vaccines are currently in clinical trials or show excellent results in preclinical trials: COR-1 (Dutton et al., 2016; Chandra et al., 2019), NE-HSV2 (Truong et al., 2019), HSV-2 trivalent vaccine (Awasthi et al., 2014; Awasthi et al., 2017; Egan et al., 2020), G103 (Odegard et al., 2016), HSV529 (Bernard et al., 2015; Dropulic et al., 2017; Dropulic et al., 2019), RVX201 (Halford et al., 2011), VC2, R2 (Richards et al., 2017; Bernstein et al., 2020), HSV2 ΔgD2 (Dropulic et al., 2017; Burn et al., 2018), and HSV-2 trivalent mRNA (Egan et al., 2020). Hopefully, one or more of these may be developed into a successful vaccine to control HSV infection in the human population.

# CONCLUSIONS

The advancement of technology and contribution of scientists around the globe have unveiled several mysteries in HSV infection that were once unknown. We now understand how HSV benefits from a temporal regulation of HS whereby an abundance of HS moieties facilitates attachment and a heparanase-mediated decline in cell surface HS expression clears the way for viral egress. While using primarily pH-independent entry mechanisms, HSV has been shown to employ a pH-dependent endocytic form of entry to infiltrate the host cell. The necessity of different domains of gD to promote membrane fusion has been uncovered, and recent work has elucidated the role of cell-cell junctions during viral spread. Furthermore, many experimental antiviral therapies have emerged in recent years such as synthetic peptides, natural peptides, monoclonal antibodies, aptamers, and nanoparticles. While many of these are still being refined and investigated, they have the potential to become non-nucleoside analog therapies to treat HSV, a currently vacant niche.

However, our understanding on HSV infection is not complete yet as there are some areas where our knowledge remains cloudy. For example, the order in which its four essential viral glycoproteins are activated, their interactions, the structure of pre-fusion form of gB, and the factors initiating the internalization of HSV in endocytic pathway are unclear. After the entry of HSV into the cell, the virions neutralize almost all antiviral mechanisms of the host. Even worse, they hijack the host immune system and other signaling pathways to their benefit. Thus, stopping them before or during entry seems to be an efficient option. To do that, understanding their entry

mechanisms is essential and will help us understand the host and viral factors involved in the entry of the virus into the cell and help us to prevent HSV infection in future.

## AUTHOR CONTRIBUTIONS

KM and DS conceptualized the study. DS provided the resources. KM wrote and prepared the original draft. KM, RK, IV, TY, and DS wrote, reviewed, and edited the manuscript.RK conducted the visualization. KM, TY, and DS supervised the study. DS acquired the funding. All authors contributed to the article and approved the submitted version.

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# Metabolomics Exploration of Pseudorabies Virus Reprogramming Metabolic Profiles of PK-15 Cells to Enhance Viral Replication

*Hongchao Gou*[1,2,3,4†], *Zhibiao Bian*[1,2,3,4†], *Yan Li*[1,2,3,4†], *Rujian Cai*[1,2,3,4], *Zhiyong Jiang*[1,2,3,4], *Shuai Song*[1,2,3,4], *Kunli Zhang*[1,2,3,4], *Pinpin Chu*[1,2,3,4], *Dongxia Yang*[1,2,3,4] *and Chunling Li*[1,2,3,4*]

[1] *Institute of Animal Health, Guangdong Academy of Agricultural Sciences, Guangzhou, China,* [2] *Guangdong Provincial Key Laboratory of Livestock Disease Prevention, Guangzhou, China,* [3] *Guangdong Open Laboratory of Veterinary Public Health, Guangzhou, China,* [4] *Scientific Observation and Experiment Station of Veterinary Drugs and Diagnostic Techniques of Guangdong Province, Guangzhou, China*

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**Edited by:**
*Subhash C. Verma, University of Nevada, Reno, United States*

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***Correspondence:**
*Chunling Li*
*lclcare@163.com*

*†These authors have contributed equally to this work*

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For viral replication to occur in host cells, low-molecular-weight metabolites are necessary for virion assembly. Recently, metabolomics has shown great promise in uncovering the highly complex mechanisms associated with virus-host interactions. In this study, the metabolic networks in PK-15 cells infected with a variant virulent or classical attenuated pseudorabies virus (PRV) strains were explored using gas chromatography-mass spectrometry (GC-MS) analysis. Although total numbers of metabolites whose levels were altered by infection with the variant virulent strain or the classical attenuated strain were different at 8 and 16 h post infection (hpi), the predicted levels of differential metabolic components were shown to be associated with specific pathways, including glycolysis as well as amino acid and nucleotide metabolism. The glucose depletion and glycolysis inhibitors 2DG and oxamate could reduce the level of PRV replication in PK-15 cells. In addition, the inhibition of the pentose phosphate pathway (PPP) resulted in an obvious decline of viral titers, but the prevention of oxidative phosphorylation in the tricarboxylic acid (TCA) cycle had a minimal effect on viral replication. Glutamine starvation resulted in the decline of viral titers, which could be restored by supplemental addition in the culture media. However, inhibition of glutaminase (GLS) activity or the supplement of 2-ketoglutarate into glutamine-deleted DMEM did not alter PRV replication in PK-15 cells. The results of the current study indicate that PRV reprograms the metabolic activities of PK-15 cells. The metabolic flux from glycolysis, PPP and glutamine metabolism to nucleotide biosynthesis was essential for PRV to enhance its replication. This study will help to identify the biochemical materials utilized by PRV replication in host cells, and this knowledge can aid in developing new antiviral strategies.

**Keywords: metabolomics, metabolic activity, pseudorabies virus (PRV), variant virulent strain, classical attenuated strain, PK-15 cells**

## INTRODUCTION

Pseudorabies virus (PRV) is a member of the family *Herpesviridae* that is related to herpes simplex virus 1 (HSV-1) (Mettenleiter, 2000; Klupp et al., 2004). PRV is the etiological agent of Aujeszky's disease and can infect variety of mammals, including pigs, ruminants, carnivores, and rodents (Szpara et al., 2011). Interestingly, latent PRV infection can only occur in pigs, which are considered to be the natural host for this virus (Pomeranz et al., 2005; Fonseca et al., 2010). Aujeszky's disease is characterized by abnormal nervous symptoms and death in newborn pigs, whereas older pigs exhibit respiratory disorders or reproductive failure (Sun et al., 2016). Since 2012, PRV variant virulent strains have been reported to be epidemic in China (Yu et al., 2014). PRV variants have been shown to be more virulent than the classical strains toward older pigs (Yang et al., 2016). Recently, PRV variants were reported to be related to acute human encephalitis cases (Liu et al., 2020).

For viral replication to occur in host cells, metabolites and energy are necessary for viral biopolymer synthesis and virion assembly. As low-molecular-weight compounds are the basis of metabolic activity, their levels can accurately reflect the response of host cells to viral infection (Munger et al., 2008; Amador-Noguez et al., 2010). Currently, improved metabolomics has made it possible to analyze the mechanisms through which viruses utilize low molecular weight metabolites in host cells. Metabolomics has shown great promise in uncovering complex virus–host interactions. For example, increased 7-dehydrocholesterol levels were shown to be associated with cholesterol metabolism disorder in host cells infected with hepatitis B virus (Rodgers et al., 2009). HSV-1 was shown to alter normal metabolic homeostasis in quiescent and growing cells and to stimulate aspects of glycolysis, the tricarboxylic acid(TCA) cycle, and pyrimidine biosynthetic metabolic pathways (Vastag et al., 2011). Although human cytomegalovirus (HCMV) belongs to the same family (*Herpesviridae*) as HSV-1, HCMV reorganizes its own metabolic program in host cells (Munger et al., 2006). Lipid metabolism is primarily regulated by HCMV to produce substrates for replication, whereas deoxypyrimidine metabolism is primarily regulated by HSV-1. Notably, the metabolic profiles of different host cell types infected with different HSV-1 strains are quite consistent, and the same phenomenon is observed in HCMV-infected cells (Munger et al., 2006; Vastag et al., 2011). This underlines the suggestion that reorganization of metabolic components might be the basic characteristic of virus replication in host cells, independent of the differences between multiple virus strains.

PRV has been demonstrated to hijack RNA transcription and protein synthesis to enhance its replication in host cells (Paulus et al., 2006; Yang et al., 2017). However, how PRV regulates the production of small molecule metabolites to promote macromolecular synthesis remains unclear. In this study, gas chromatography-mass spectrometry (GC-MS) was utilized to analyze the changes in the metabolic networks of PK-15 cells infected with different PRV strains, including the variant virulent GD-WH strain and the classical attenuated Bartha strain. Although the number of metabolites was different in PK-15 cells infected by the variant virulent strain and the classical attenuated strain at 8 or 16 h post infection (hpi), we found that most components altered in response to PRV infection belonged to consistent metabolic pathways such as glycolysis or amino acid and nucleotide metabolism. Further experiments showed that PRV replication was obviously down-regulated by the glucose depletion and glycolysis inhibitors including 2DG and oxamate. In addition, the prevention of oxidative phosphorylation in the TCA cycle had a minimal effect on viral replication, but the inhibition of the pentose phosphate pathway (PPP) also resulted in sharp declines of viral titers. Glutamine starvation resulted in the decline of viral titers, which could be restored by supplemental addition in the culture media. However, glutaminase activity inhibition or the supplement of 2-ketoglutarate into glutamine-deleted DMEM did not change the PRV replication in PK-15 cells. The results of the current study provide the first evidence that PRV reprograms the metabolic activity of PK-15 cells to benefit its infection. The variant virulent strain and the classical attenuated strain displayed similar metabolic features in PK-15 cells. These results may offer a better understanding of the biochemical materials utilized by PRV replication in host cells. This will be beneficial for exploring small molecular compounds that can be used as PRV replication inhibitors.

## MATERIALS AND METHODS

### Cell Culture and Virus

The swine kidney cell line PK-15 (ATCC, CCL-33) was cultured in Dulbecco's modified Eagle medium (DMEM) (11965, Gibico) supplemented with 10% fetal bovine serum (FBS) (Biological Industries, USA) at 37°C with 5% $CO_2$. The classical attenuated strain (Bartha) was purchased from the China Veterinary Culture Collection Center (CVCC Number: AV249). The currently circulating variant virulent strain (GD-WH) (GenBank No. KT948051) was isolated from the brain of a pig suspected to be infected with PRV in 2015. Several rounds of plaque purification were conducted to obtain the pure virus. Viral titers were determined in PK-15 cells and were calculated as the 50% tissue culture infectious dose (TCID50) per milliliter according to the Spearman–Karber method (Reed and Muench, 1938).

### Growth Kinetics

A PK-15 cell monolayer cultured in a 25-$cm^2$ cell culture flask was infected with the GD-WH or Bartha strain at a multiplicity of infection (MOI) of 10. At 4, 8, 12, 16, 20, 24, 28, 32, 36, and 40 hpi, 100 μl of cell supernatant was absorbed and stored at −80°C. The growth kinetics of each viral strain was analyzed according to viral titers in PK-15 cells.

### Sample Preparation for Metabolomics Assay

Approximately $2\times10^6$ PK-15 cells were seeded into 6-cm dishes 24 h before viral infection. Each dish was marked according to

preparation time, with four dishes prepared for each experimental group (24 total dishes). Cell monolayers were inoculated with the GD-WH or Bartha strain at an MOI of 10, with a monolayer inoculated with an equal volume of DMEM used as the mock infection. After 1 h, the inoculum was aspirated, and cells were washed twice with phosphate buffer saline (PBS). Subsequently, DMEM containing no FBS was added to each dish. All of the dishes were placed in an incubator for the indicated hours post infection.

At 8 or 16 hpi, the cell medium in each dish was discarded, and each dish was washed twice with cold PBS. Subsequently, the cells were quenched with 400 μl of cold methanol and chilled at −80°C for 30 min. Tridecanoic acid (91988, Sigma) and norvaline (53721, Sigma) premixed with methanol at a concentration of 1 μg/ml was used as a quality control (QC) and was maintained at -80°C. Cells were scraped from the dishes in 400 μl of ultrafiltered, cold water, and the cell mixture was used for subsequent analysis.

## Biochemical Intervention and Replenishment

For interfering with glycolysis, the TCA cycle, the PPP or glutamine metabolism, PK-15 cells with 80% confluence in 6-well cell culture plates were pretreated with one of the following inhibitors, e.g., 10 or 20 mM 2-deoxyglucose (2DG) (S4701, Selleck), 50 mM sodium oxamate, 1 μM oligomycin A, 500 μM 6-aminonicotinamide (6-AN), or 5 μM BPTES for 4 h. To accurately analyze the effects of biochemical intervention on virus replication, PK-15 cells were infected with the GD-WH strain at an MOI of 1. Then, cells were cultured in fresh medium containing corresponding inhibitors for 16 h. To analyze the effect of glucose starvation on PRV replication, PK-15 cells cultured in 6-well cell plates with 80% confluence were infected with PRV GD-WH strain at an MOI of 1. Then, cells were cultured in normal DMEM or depleted DMEM with no glucose, glutamine, and phenol red (A1443001, Gibico) supplemented with 2 mM L-glutamine (S1749, Selleck) for 16 h. To analyze the effect of glutamine starvation on PRV replication, PK-15 cells infected with PRV GD-WH strain at an MOI of 1 were cultured in normal DMEM or glutamine-free DMEM (11960077, Gibico) for 16 h. In the replenishment groups, different concentrations of L-glutamine (S1749, Selleck) or 2-ketoglutarate (S6237, Selleck) were added to the glutamine-free DMEM.

## Cell Viability Assay

A Cell Counting Kit-8 (CCK8) assay kit (C0037, Beyotime) was used to analyze the cell viability according to the manufacturer's instructions. Briefly, PK-15 cells were cultured in 96-well culture plates at a seeding density of $1 \times 10^4$ cells per well for 24 h. Then, the medium was replaced with 100 μl of DMEM containing one of the following inhibitors: 10 or 20 mM 2-deoxyglucose (2DG) (S4701, Selleck), 50 mM sodium oxamate, 1 μM oligomycin A, 500 μM 6-aminonicotinamide (6-AN), or 5 μM BPTES. After 16 h, cells were washed twice with PBS and cultured in 100 μl of DMEM containing 10 μl of CCK8 for 1 h at 37°C. The optical density was measured at 570 nm by using a model 680 microplate reader (Bio-Tek).

## Metabolite Extraction and Gas Chromatography-Mass Spectrometry Analysis

After the methanol content of the cell mixture was adjusted to 80%, cells were lysed by five rounds of ultra-sonication (a duration of 1 min) at 1 min intervals. After centrifugation at 14,000 g for 15 min at 4°C, 500 μl of each cell lysate supernatant was combined with 10 μl L-norleucine (50 μg/ml). Subsequently, the miscible liquid was evaporated to dryness under a stream of nitrogen. The residue was then redissolved in 30 μl of pyridine containing 20 mg/ml methoxyamine hydrochloride. Following an incubation at 37°C for 90 min, 30 μl of BSTFA (in 1% TMCS) was added. After the resulting mixture was derivatized at 70°C for 60 min, 2 μl of the mixture was analyzed by GC-MS (Agilent 7890A/5975C, Agilent Technologies). The QCs pooled from all samples were prepared and analyzed using the same procedure as that used for the experimental samples. The GC-MS analysis and data preprocessing methods were performed as described previously by Gou et al. (2017).

## Statistical Analysis of Gas Chromatography-Mass Spectrometry Data

For multivariate statistical analysis, including principal component analysis (PCA) and orthorhombic partial least-squares discriminant analysis (OPLS-DA), the data were normalized prior to being preprocessed by unit variance scaling and mean centering using SIMCA (version 14.1, Umetrics, Sweden). The $R^2X$ or $R^2Y$ and $Q^2$ values were used to evaluate the model quality. $R^2X$ (PCA) or $R^2Y$ (PLS-DA and OPLS-DA) is described as the proportion of variance in the data explained by the model and indicates the goodness of fit, while $Q^2$ is described as the proportion of variance in the data predicted by the model and indicates the predictability of the current model. To avoid model over-fitting, a default seven rounds of cross-validation in the SIMCA software was performed throughout the analysis.

## Identification and Statistical Analysis of Differential Metabolites

For univariate statistical analysis, the normalized data were analyzed in the "muma" software package in the R platform. A parametric test was performed on normally distributed data using Welch's t-test, while a nonparametric test was performed on the non-normally distributed data using the Wilcoxon–Mann–Whitney test.

The variable importance in the projection (VIP) values (>1) in the OPLS-DA model and the *P* values (<0.05) from the univariate statistical analysis were used to identify potential differential metabolites. Fold change was calculated as the binary logarithm of the average normalized peak intensity ratio between Groups 1 and 2. A positive value indicates that the average mass response of Group 1 was higher than that of Group 2, whereas a negative value indicates a lower average mass response of Group 1 compared to Group 2.

# RESULTS

## Growth Kinetics of the Variant Virulent and Classical Attenuated Pseudorabies Virus Strains

Viral titers of the variant virulent (GD-WH) strain were higher than that of the classical attenuated (Bartha) strain at each time point tested (**Figure 1A**). The peak viral titers of the GD-WH and Bartha strains were observed at 24 and 32 hpi, respectively. This result indicated that the GD-WH strain replicated faster than the Bartha strain in PK-15 cells. Both the GD-WH and Bartha PRV strains caused obvious cytopathologic effects in PK-15 cells at 24 hpi (**Figure 1B**). Considering that the GD-WH strain began to cause rounding and floating of almost all of the PK-15 cells by 24 hpi, PK-15 cells infected with the GD-WH and Bartha PRV strains at 8 and 16 hpi were sampled for subsequent GC-MS analysis to ensure that enough adherent cells were obtained.

## Principal Component Analysis of Metabolites Analyzed by Gas Chromatography-Mass Spectrometry

The total ion current (TIC) chromatograms of all the samples in each group showed that the GC-MS method was suitable for the metabolite analyses performed in this study. Representative TIC chromatograms in each group are shown in **Figure S1**. The PCA model typically reflected the differences between the groups. To analyze whether PRV altered the composition of metabolites in PK-15 cells, the PCA model was used to analyze the data produced by the GC-MS analysis. The model cumulative interpretation rate, $R^2X$ (=0.809), demonstrated that PCA was suitable for determining the differences between the groups. In the PCA score map (**Figure 2A**), the QC group was concentrated in the middle region, demonstrating the good reliability and satisfactory reproducibility of the GC-MS method used in this study. The different distributions of the GD-WH and Bartha groups with mock groups at 8 and 16 hpi in the PCA score map indicated that PRV reprogrammed the metabolic composition of PK-15 cells. This result was further supported by different trajectory locations of the GD-WH and Bartha groups with mock groups in the three-dimensional score plots (**Figure 2B**).

## Differential Metabolite Analysis

Because metabolomic analyses are more sensitive than transcriptomic or proteomics analyses, the OPLS-DA model was more successful in eliminating the effects of the genetic background and environmental disturbances compared with the PCA model. To precisely explore the changes in metabolites between the virus-infected and mock groups at different hpi, the OPLS-DA model was subsequently used to evaluate the GC-MS data. Similar to the PCA model, the OPLS-DA model also showed notably different distributions between the virus-

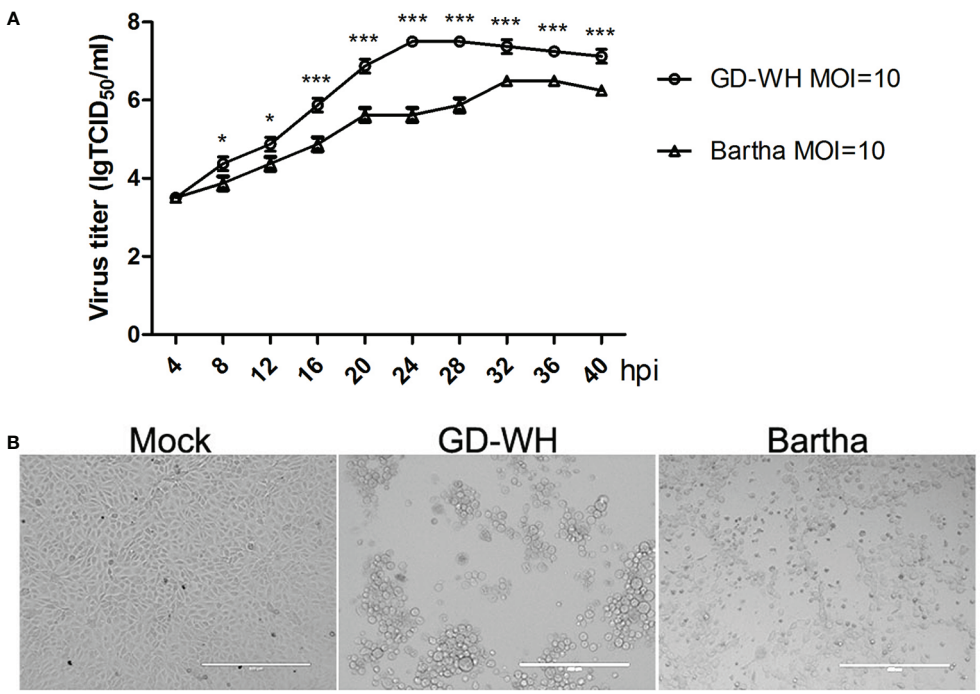


**FIGURE 1** | **(A)** Growth kinetics of the variant virulent and classical attenuated pseudorabies virus (PRV) strains. PK-15 cells were infected with the variant virulent (GD-WH) or classical attenuated (Bartha) PRV strains (MOI = 10), and the growth kinetics of each strain was analyzed as described in the Materials and Methods (mean ± SD; n=3; *$P < 0.5$; ***$P < 0.001$). *P* values were calculated using two-way ANOVA. **(B)** Cytopathic effects of PK-15 cells infected with the variant virulent or classical attenuated PRV strains at 24 hpi. PK-15 cells were infected with the variant virulent (GD-WH) or classical attenuated (Bartha) PRV strains (MOI=10). At 24 hpi, the cytopathic effects of PRV infection of PK-15 cells were observed (scale bars=400 μm).

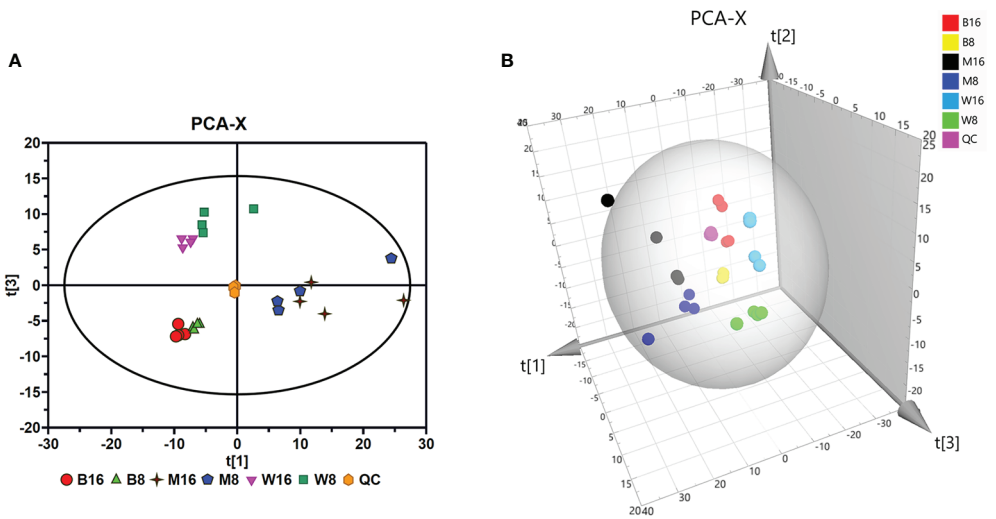


**FIGURE 2** | Principal component analysis (PCA) of gas chromatography-mass spectrometry (GC-MS) spectra. **(A)** Multivariate analysis of GC-MS spectra of metabolites using the PCA model. **(B)** Three-dimensional trajectory analysis of the PCA score plots. In all images, W8 and W16 represent the groups infected with the variant virulent (GD-WH) strain; B8 and B16 represent the groups infected with the classical attenuated (Bartha) strain; M8 and M16 represent the mock groups, and QC represents the quality control group.

infected and mock groups at each hpi. Furthermore, the results of the permutation test confirmed the good fit of this model (**Figure S2**). When the VIP value (>1) of the OPLS-DA model and the *P* value (<0.05) of the univariate statistical analysis were set, the differential metabolites were determined according to their fold changes. The numbers of metabolites altered in response to the GD-WH infection were 45 and 41 at 8 and 16 hpi (**Supplementary Tables S1** and **S2**, respectively). The numbers of differential metabolites in PK-15 cells infected with the Bartha strain were 31 and 43 at different hpi, shown in **Supplementary Tables S3** and **S4**, respectively. Heatmap analysis was performed to display the relationships of differential metabolites in PK-15 cells infected with the GD-WH or Bartha strain at 8 and 16 hpi. Our data indicated that 20 differential metabolites appeared on the heatmaps of both the GD-WH and Bartha strains at 8 hpi (**Figures 3A, C**); these included glyceraldehyde-3P, glycerone-P, lactate, 2-oxoglutarate, thymine, adenine, glutamine, and asparagine. Similarly, 19 differential metabolites appeared on the heatmaps of the GD-WH and Bartha strains at 16 hpi (**Figures 3B, D**). This tells us that different metabolic features may be associated with the GD-WH and Bartha strains infecting PK-15 cells.

## Metabolic Pathway Analysis

Changes in the levels of metabolites at crucial positions in networks are more likely to reflect the flow of pathways than changes at relatively marginal positions. To explore potential pathways in PK-15 cells altered in response to PRV infection, the connections of differential metabolites that appeared on the heatmaps of both the GD-WH and Bartha strains were identified with metabolic pathways according to the KEGG PATHWAY Database. As shown in **Figure 4**, many differential metabolites were related to glycolysis, TCA cycle, amino acid metabolism, and nucleotide metabolism at 8 and 16 hpi in the GD-WH or Bartha strains infecting PK-15 cells.

## Identification of the Effect of the Glycolysis on Pseudorabies Virus Replication

The levels of gluconate-6-phosphate in PK-15 cells infected by the GD-WH strain was continuously down-regulated at 8 and 16 hpi, but decreased levels of gluconate-6-phosphate were only detected in PK-15 cells infected by the Bartha strain at 16 hpi (**Figures 4A, B**). In addition, decreased levels of D-fructose-6p were observed in glycolysis in PK-15 cells infected with the GD-WH strain at 8 and 16 hpi, but not in cells infected by the Bartha strain (**Figures 4A, B**). The different regulation levels of gluconate-6-phosphate and D-fructose-6p might be attributed to the slower replication speed of the Bartha strain than the GD-WH strain, and the basal content of these metabolites in PK-15 cells was sufficient for Bartha strain consumption at 8 hpi. Considering that glyceraldehyde-3P and glycerone-P were both increased in PK-15 cells infected by different PRV strains at 8 and 16 hpi (**Figures 4A, B**), we speculated that glycolysis might have been enhanced for PRV replication. To explore this hypothesis, we inhibited glycolysis of PRV-infected cells by using 2-deoxyglucose (2DG), a glucose analog that blocks glycolysis. PK-15 cells were infected with the GD-WH strain and then treated with different concentrations of 2DG (10 and 20 mM). The results indicated an obvious decrease of viral titers in 2DG-treated groups compared with the control groups, and 2DG treatment did not exhibit cellular cytotoxicity (**Figures 5A, B**). Next, the effect of glucose starvation on PRV replication was analyzed. The results showed that viral titers were clearly decreased in PK-15 cells cultured in DMEM lacking glucose

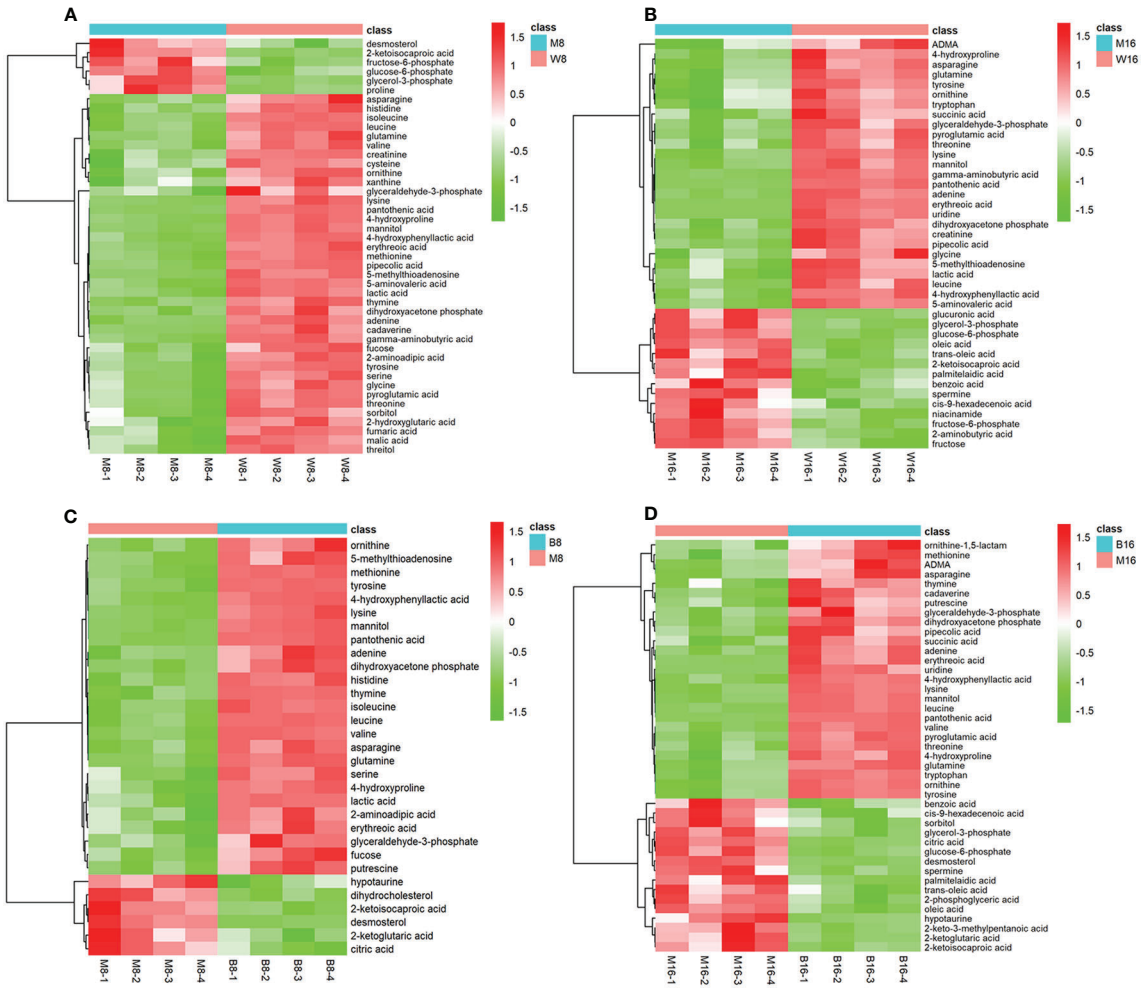


**FIGURE 3** | Heatmap visualization of differential metabolites in PK-15 cells infected with the variant virulent **(A, B)** or classical attenuated **(C, D)** PRV strains at 8 and 16 hpi. Rows: metabolites; columns: samples. The color from red (positive value) to green (negative value) of each rectangle was based on the ratio between pseudorabies virus (PRV)-infected groups vs mock groups. For example, a red color means that the average mass response of the metabolite in PRV-infected groups was greater than that in mock groups. In all images, W8 and W16 represent the groups infected with the variant virulent (GD-WH) strain; B8 and B16 represent the groups infected with the classical attenuated (Bartha) strain, and M8 and M16 represent the mock groups.

(**Figure 5C**). Notably, a tendency toward increased lactate levels was observed in PK-15 cells infected by the GD-WH and Bartha strains at 8 hpi, but the level of lactate was slightly up-regulated in PK-15 cells infected by the GD-WH strain at 16 hpi (**Figures 4A, B**). This indicated that the fold change of lactate content in PRV-infected cells was higher at 8 than 16 hpi, which was not consistent with the time-dependent decreased levels of gluconate-6-phosphate. Therefore, we speculated that the gluconate-6-phosphate in the glycolysis pathway might be converted to other pathways during PRV infection, for example the TCA cycle or the PPP, in addition to being responsible for the production of lactate. When the lactate production was prevented by inhibiting the lactate dehydrogenase (LDH) enzyme using oxamate, a smaller decline of viral titers was observed than that in the glucose depletion or 2DG treatment PK-15 cells (**Figure 5C**). In addition, oxamate did not reduce the cell viability by 16 h (**Figure 5D**). Because lactate is in the downstream segment of glycolysis, the smaller inhibitory effect of oxamate on viral replication than glucose depletion supported our speculation that glycolysis is linked to the TCA cycle or the PPP during PRV infection of PK-15 cells.

## The Effect of the Tricarboxylic Acid Cycle and the Pentose Phosphate Pathway on Pseudorabies Virus Replication

In the TCA cycle, only a few metabolites displayed relatively small changes in PK-15 cells infected by the GD-WH and Bartha strains. Citrate showed a declining tendency in PK-15 cells infected by the Bartha strain at 8 and 16 hpi, but not when infected by the GD-WH strain. However, fumarate and malate in

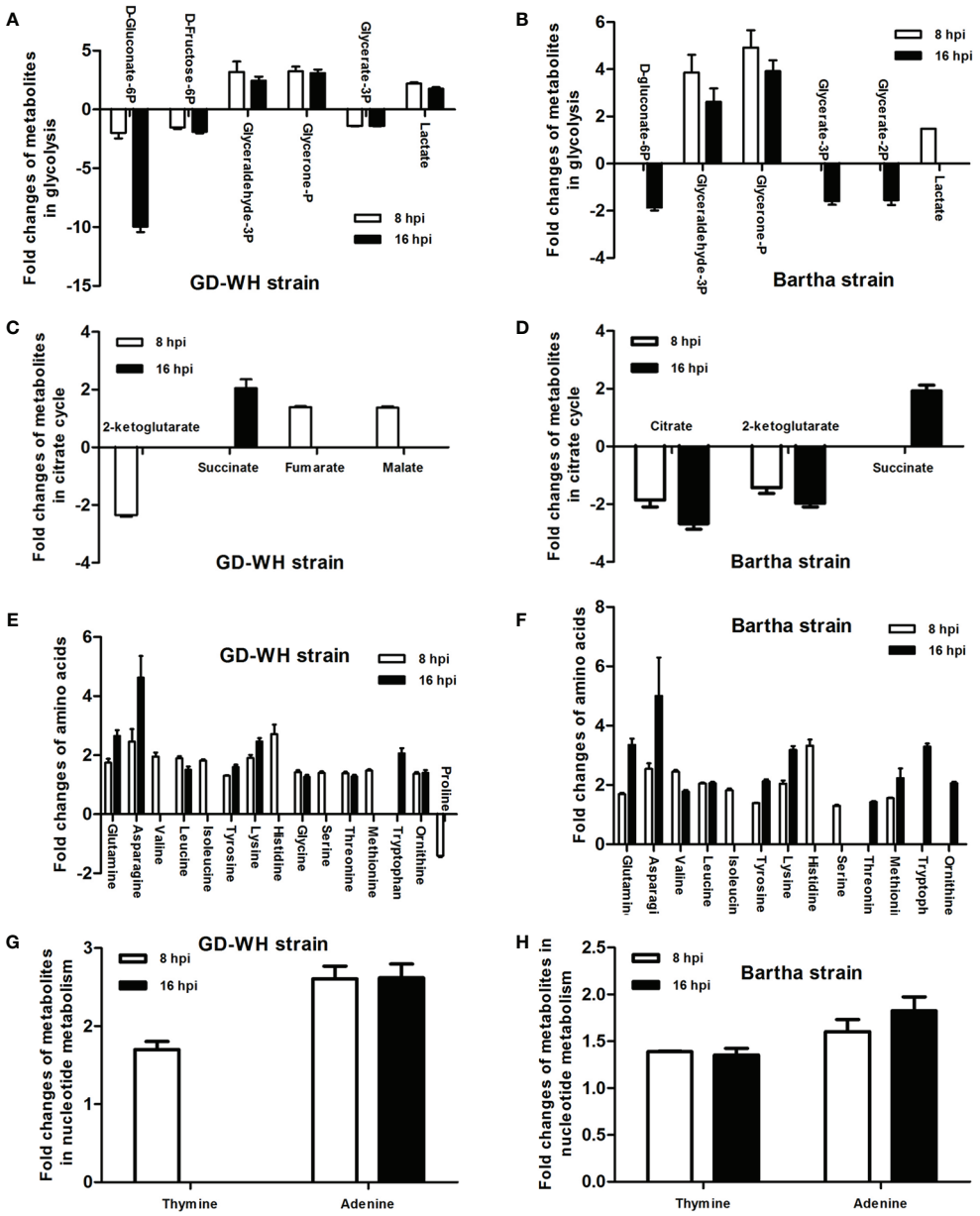


**FIGURE 4** | Statistical analysis of fold change of differential metabolites in PK-15 cells infected with the variant virulent **(A, C, E, G)** or classical attenuated **(B, D, F, H)** pseudorabies virus (PRV) strains at 8 and 16 hpi (mean ± SD; n=4). Fold change was calculated as a binary logarithm of the average mass response (normalized peak area) ratio between PRV-infected groups vs mock groups, where a positive value means that the average mass response of the metabolite in PRV-infected groups was greater than that in mock groups.

the TCA cycle were only increased in PK-15 cells infected by the GD-WH strain at 8 hpi. Decreased levels of 2-ketoglutarate were detected in PK-15 cells infected by different strains of PRV at 8 hpi, but levels of succinate were increased by PRV infection at 16 hpi (**Figures 4C, D**). Given the minimal and independent changes of these metabolites in the TCA cycle, we speculate that the pathway responsible for oxidative phosphorylation and ATP production might not be entirely up-regulated by PRV infection. Possibly only several metabolites were used as carbon sources. To analyze the role of the TCA cycle in PRV replication, oligomycin A was used to prevent oxidative phosphorylation in PK-15 cells infected by PRV. We only observed a minimal decline of viral titer (**Figure 6A**). This indicated that oxidative phosphorylation depending on the TCA cycle is not the key factor for virion production during PRV infection. Meanwhile, it excludes the possibility that the

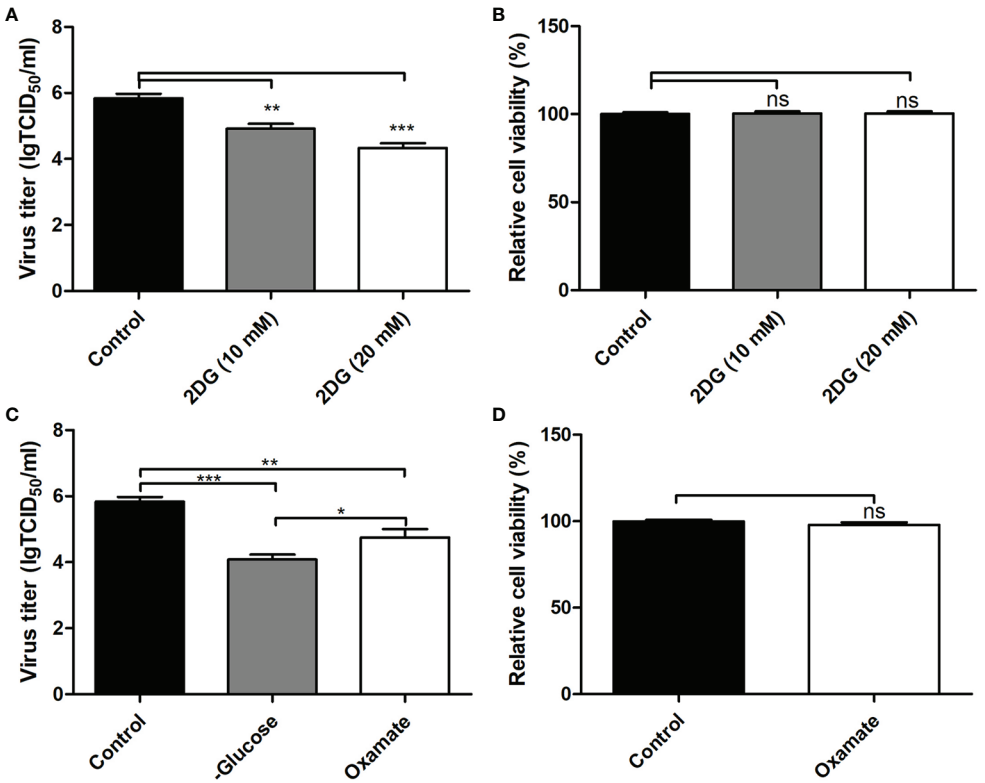


**FIGURE 5** | Inhibition of the glycolysis reduced the replication of pseudorabies virus (PRV) in PK-15 cells. **(A)** The effect of 2-deoxyglucose (2DG) treatment on the virus titers in PK-15 cells infected with the variant virulent PRV strain. PK-15 cells were pretreated with 10 or 20 mM 2DG for 3 h. Then, cells were infected with PRV GD-WH strain at an multiplicity of infection (MOI) of 1 and cultured in Dulbecco's modified Eagle medium (DMEM) containing 10 or 20 mM 2DG. At 16 hpi, the titers of virus were analyzed as described in Materials and Methods (mean ± SD; n=3; $^{**}P<0.01$; $^{***}P < 0.001$). *P* values were calculated by using an unpaired Student's t-test. **(B)** The effect of 2DG treatment on the cell viability of PK-15 cells. The cell viability of PK-15 cells treated with 10 and 20 mM 2DG was analyzed by the CCK8 assay as described in Materials and Methods (mean ± SD; n=3; $^{NS}P>0.05$). **(C)** Glucose depletion or oxamate treatment decreased PRV titers in PK-15 cells. PK-15 cells were starved by be cultured in depletion DMEM repleted with 2 mM L-glutamine or pretreated with 50 mM oxamate for 3 h. Then cells were infected with PRV GD-WH strain at a MOI of 1. After being cultured in depletion DMEM repleted with 2 mM L-glutamine or DMEM containing 50 mM oxamate for 16 h, virus titers were analyzed as described in Materials and Methods (mean ± SD; n = 3; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). *P* values were calculated by using an unpaired Student's t-test. **(D)** The effect of oxamate treatment on the cell viability of PK-15 cells. The cell viability of PK-15cells starved by glucose depletion or treated with oxamate for 16 h were analyzed by the CCK8 assay as described in Materials and Methods (mean ± SD; n = 3; $^{NS}P > 0.05$).

inhibition of glycolysis by the absence of glucose or 2DG reduced PRV replication *via* indirectly affecting the TCA cycle. Furthermore, the PPP in PK-15 cells was inactivated by using 6-aminonicotinamide (6AN), an inhibitor of the PPP enzyme glucose-6-dehydrogenase. The result showed that 6AN had a sharply inhibitory effect on viral titers that was the same as in the glucose starvation and 2DG treatments (**Figure 6A**). This was consistent with our speculation that gluconate-6-phosphate enters the PPP to enhance the viral nucleotide synthesis during PRV infection. Also, neither oligomycin A nor 6-AN treatment reduced the cell viability during 16 h (**Figure 6B**).

## Glutamine Is an Essential Factor for Pseudorabies Virus Replication

The levels of multiple amino acids were changed in PK-15 cells infected by the different PRV strains at 8 and 16 hpi. These included glutamine, asparagine, histidine, leucine, and lysine. These amino acids might be related to protein synthesis or to other metabolic pathways. It has been reported that glutamine can be employed as a material for nucleic acid synthesis during HSV-1 infection (Vastag et al., 2011). Because PRV is a DNA virus with a large genome as in HSV-1, this study focused on the function of glutamine on PRV replication. By cultivating the GD-WH strain with glutamine-deleted DMEM, we found that deletion of glutamine caused a significant reduction of infectious viral particles at 16 hpi. However, viral titers increased in a dose-dependent manner after 2 or 4 mM glutamine was supplemented into glutamine-deleted DMEM (**Figure 7A**). These data suggested that glutamine is essential for PRV replication in PK-15 cells. In addition to being used as a material for nucleotide biosynthesis, it is known that glutamine can also be converted to glutamate by the enzymatic activity of glutaminase (GLS). Subsequently, glutamate can enter the TCA cycle *via* conversion

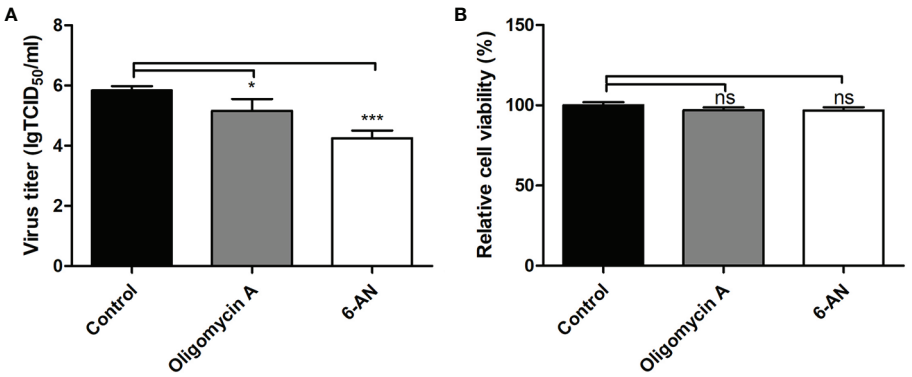


**FIGURE 6** | The effect of the TCA cycle and pentose phosphate pathway (PPP) on pseudorabies virus (PRV) replication in PK-15 cells. **(A)** The effect of oligomycin A or 6-AN treatment on PRV replication. PK-15 cells were pretreated with 1 μM Oligomycin A or 500 μM 6-AN for 3 h. Then cells were infected with PRV GD-WH strain at a MOI of 1 and cultured in DMEM containing 1 μM Oligomycin A or 500 μM 6-AN. At 16 hpi, the titers of virus were analyzed as described in Materials and Methods (mean ± SD; n = 3; $^{*}P < 0.05$; $^{***}P < 0.001$). *P* values were calculated by using an unpaired Student's t-test. **(B)** The effect of Oligomycin A or 6-AN treatment on the cell viability of PK-15 cells. The cell viability of PK-15 cells treated with 1 μM Oligomycin A or 500 μM 6-AN were analyzed by the CCK8 assay as described in Materials and Methods (mean ± SD; n = 3; $^{NS}P > 0.05$).

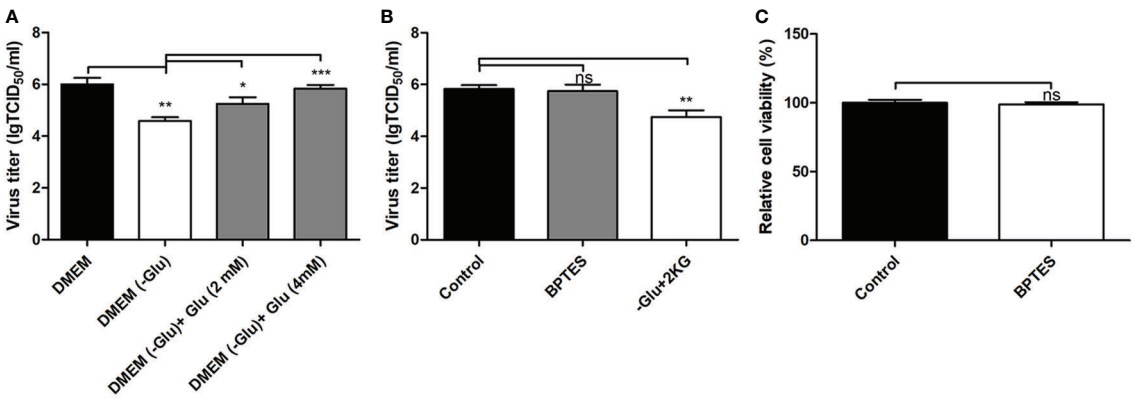


**FIGURE 7** | Depletion of glutamine reduced pseudorabies virus (PRV) replication in PK-15 cells in a manner independent of the TCA cycle. **(A)** Glutamine starvation had a repressive effect on PRV replication in PK-15 cells. PK-15 cells were starved by being cultured in glutamine-free DMEM for 3 h. Then, cells were infected with PRV GD-WH strain at an multiplicity of infection (MOI) of 1 and cultured in normal Dulbecco's modified Eagle medium (DMEM) or no glutamine DMEM. In replenishment groups, 2 or 4 mM L-glutamine was added to glutamine-free DMEM. At 16 h, the titers of virus were analyzed as described in Materials and Methods (mean ± SD; n=3; $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P < 0.001$). *P* values were calculated by using an unpaired Student's t-test. **(B)** BPTES treatment had no effect on PRV replication in PK-15 cells. PK-15 cells were pretreated with 1 μM BPTES for 3 h. Then cells were infected with PRV GD-WH strain at a MOI of 1 and cultured in DMEM containing 1 μM BPTES. At 16 hpi, the titers of virus were analyzed as described in Materials and Methods (mean ± SD; n = 3; $^{NS}P > 0.05$; $^{**}P < 0.01$). *P* values were calculated by using an unpaired Student's t-test. **(C)** The effect of BPTES treatment on the cell viability of PK-15 cells. The cell viability of PK-15cells treated with 1 μM BPTES were analyzed by the CCK8 assay as described in Materials and Methods (mean ± SD; n = 3; $^{NS}P > 0.05$). **(C)** 2-ketoglutarate supplement cannot recover the effect of glutamine starvation on PRV replication in PK-15 cells. PK-15 cells were starved by being cultured in no glutamine DMEM for 3 h. Then cells were infected with PRV GD-WH strain at a MOI of 1 and cultured in normal DMEM or no glutamine DMEM. In 2-ketoglutarate supplement groups, 5 mM 2-ketoglutarate was added in no glutamine DMEM. At 16 h, the titers of virus were analyzed as described in Materials and Methods (mean ± SD; n = 3; $^{NS}P > 0.05$). *P* values were calculated by using an unpaired Student's t-test.

to 2-ketoglutarate by glutamate dehydrogenase (GDH). Hence, we further explored whether PRV utilized glutamine to supply the TCA cycle. When the GLS activity was interfered by using the BPTES in PK-15 cells, PRV replication was unchanged (**Figure 7B**). Moreover, the supplement of 2-ketoglutarate into glutamine-deleted DMEM did not prevent the decline of viral replication in PK-15 cells infected by PRV (**Figure 7B**). In addition, we demonstrated that BPTES is not cytopathic to the cell viability (**Figure 7C**). These results suggested that glutamine was mainly connected to nucleotide biosynthesis in PK-15 cells infected by PRV and was not converted to 2-ketoglutarate in the TCA cycle.

## DISCUSSION

The development of metabolomics makes it possible to perform systematic analyses of complicated cellular metabolic networks, thus offering a new perspective for understanding the basic replication characteristics of viruses in their host cells (Munger et al., 2008). To explore how PRV reprograms metabolic features to benefit its replication in host cells, PK-15 cells were selected to be a viral infection model, a method that is typically used to research PRV growth *in vitro* (Yu et al., 2016). To reveal the basic metabolic characteristics in PRV-infected cells, the variant virulent GD-WH and classical attenuated Bartha strains were used to infect PK-15 cells, and the cells were analyzed by GC-MS in this study. The virulent variant strain was reported to be epidemic in China since 2012, and the Bartha strain was an attenuated vaccine that has been widely administered in the global swine industry since the 1970s (Yu et al., 2014). The GC-MS data showed that many metabolites were increased in glycolysis, the TCA cycle, amino acid metabolism, and nucleic acid metabolism in PK-15 cells infected by the different viral strains. The common metabolic profile of PK-15 cells infected by these different PRV strains demonstrated that metabolite reorganization might be a fundamental biochemical requirement for viral replication, which has also been reported in HSV-1 or HCMV-infected cells (Vastag et al., 2011). Interestingly, although PRV is closely associated with HSV-1, many amino acids in PRV-infected PK-15 cells displayed a reverse tendency compared with HSV-1 infected host cells. We speculate that different viruses might organize their own metabolic networks to serve viral replication. By establishing the relationships between potential pathways, the metabolic networks in PK-15 cells infected by PRV at 8 (**Figure 8A**) and 16 hpi (**Figure 8B**) could be predicted.

In mammalian cells, glycolysis and the TCA cycle are key processes in central carbon metabolism, linking glucose to energy production and amino acid, lipid, and nucleotide biosynthesis. In glycolysis, the levels of glycerol-3-phosphate and glycerone-P were increased at 8 (**Figure 8A**) and 16 (**Figure 8B**) hpi for both viral strains. This indicated that PRV activated the glycolysis pathway in PK-15 cells during the infection stage. The time-dependent consumption of gluconate-6-phosphate in PRV-infected cells further supported this hypothesis. It has been reported that the glycolysis pathway is enhanced by some viruses to benefit their replication. For example, the replication of HSV-1 and norovirus rely on active glycolysis in host cells (Abrantes et al., 2012; Passalacqua et al., 2019). Our results showed that replication of PRV in PK-15 cells could be obviously down-regulated by glucose depletion and the glycolysis pathway inhibitor 2DG or oxamate. However, we only observed a minimal decline of viral titer after oxidative phosphorylation in PK-15 cells was interfered with by using the oligomycin A. Furthermore, we found that the inhibition of the PPP had a clearly inhibitory effect on viral titers in PRV-infected PK-15 cells. These results indicated that glycolysis and PPP is mainly promoted and utilized for PRV replication in PK-15 cells, but not the oxidative phosphorylation in the TCA cycle. It has been shown that the TCA cycle is modulated to promote pyrimidine production by HSV-1 or for fatty acid biosynthesis by HCMV (Munger et al., 2006; Vastag et al., 2011). Considering that citrate and 2-ketoglutarate can be converted to succinate, the opposite change trends of 2-ketoglutarate and succinate in PRV-infected PK-15 cells support the suggestion that succinate in the TCA cycle might be employed as a central carbon source that is subsequently connected with amino acids or nucleotide metabolism but is not involved in ATP production (**Figures 8A, B**). Although PRV belongs to the same family as HSV-1 and HCMV, the flow of the enhanced central carbon metabolism in PK-15 cells needs to be further elucidated in future studies.

Previous reports showed that amino acid metabolism was disturbed during dengue virus infection and that amino acids may be converted into other biomolecules such as pyruvate and acetyl-coA (Birungi et al., 2010). The results of previous proteomic analyses showed that 21 proteins were up-regulated in the protein processing pathway in the endoplasmic reticulum of PK-15 cells infected by PRV (Yang et al., 2017). Whether the increased levels of amino acids observed in this study were associated with enhanced protein synthesis needs to be further explored. It has been demonstrated that glutamine is a key factor for the replication of some viruses (Vastag et al., 2011; Fontaine et al., 2014). Considering this, the role of glutamine in PRV replication was explored in the present study. We found that viral titers of PRV were decreased in the absence of glutamine in the culture medium. Further experiments showed that glutamine supplementation could rescue the replication of PRV. In addition to glucose, glutamine is a primary carbon donor for the TCA cycle. It has been shown that HCMV and vaccinia virus can manipulate glutamine metabolism to compensate for the diversion of glucose *via* the TCA cycle (Chambers et al., 2010; Fontaine et al., 2014). However, inhibition of GLS activity or supplementing 2-ketoglutarate into glutamine-deleted DMEM did not affect PRV replication in PK-15 cells. This indicates that glutamine did not enter the TCA cycle in PRV-infected PK-15 cells, in contrast to host cells infected by HCMV or vaccinia virus. We speculate that PRV utilizes glutamine to serve in purine or thymine synthesis, as has been reported in host cells infected by HSV-1 or gallid alpha herpesvirus 1 (Qiao et al., 2020).

The adenine and thymine contents in PK-15 cells were increased by both the GD-WH and Bartha PRV strain at 8 hpi (**Figures 4G, H**). These results are consistent with previous reports, where the levels of deoxyadenosine triphosphate (dTMP) and deoxythymidine triphosphate (dTTP) were shown to continuously increase in growth-arrested fibroblasts throughout HSV-1 infection (Vastag et al., 2011). Like HSV-1, PRV is a double-stranded DNA virus with an approximately 140-kb genome. Hence, it is essential for PRV to manipulate nucleotide precursors in host cells for viral DNA synthesis. In addition, our study showed that uridine levels increased in PK-15 cells during infection by the GD-WH and Bartha strains; this can occur in DNA as a result of cytosine deamination or through the misincorporation of dUTP (Chen et al., 2002). It has been shown

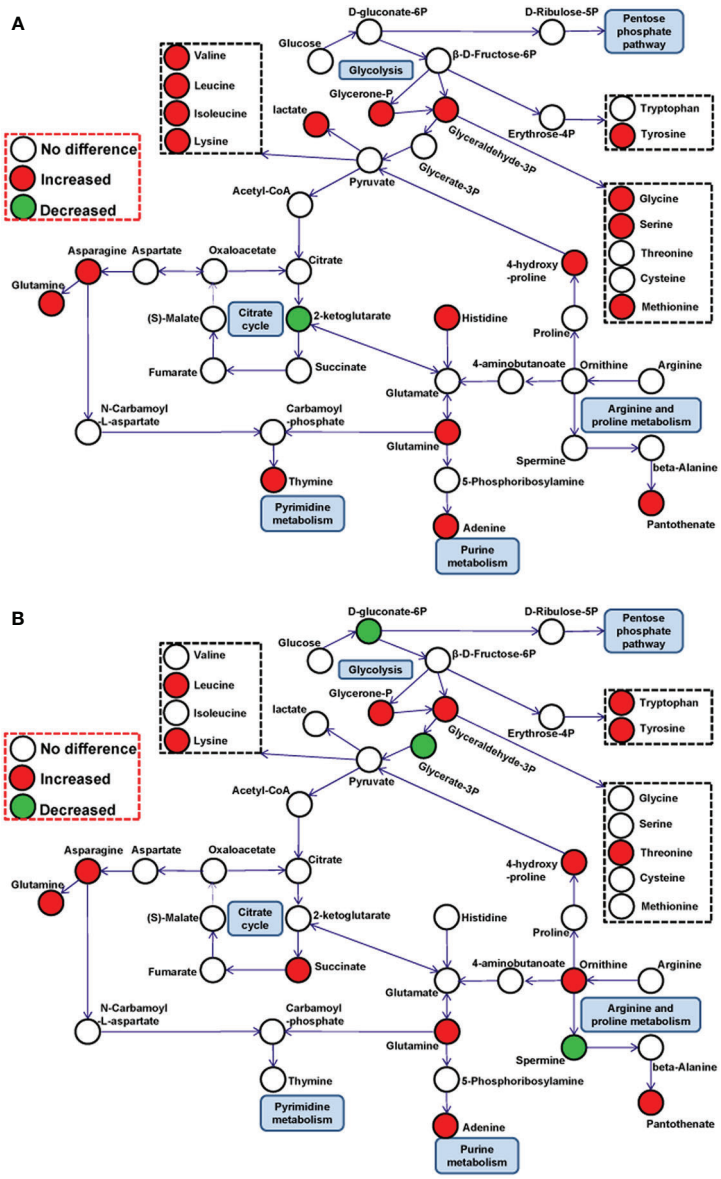


**FIGURE 8** | Schematic overview of metabolic pathways in PK-15 cells infected by different pseudorabies virus (PRV) strains at 8 **(A)** and 16 **(B)** hpi. The metabolites are shown in different colors according to their changes: red indicates increased metabolites; green indicates decreased metabolites, and white indicates no difference metabolites.

that dUTPase (UL50) from HSV-1 can reduce the incorporation of uracil into genomic DNA by increasing the conversion of dUTP to dUMP (Bogani et al., 2009). Furthermore, uracil-DNA glycosylase (UL2) is responsible for the accurate removal of uracil from viral DNA (Bogani et al., 2010). Further studies are needed to ascertain whether the extra uridine content in PK-15 cells infected by the GD-WH or Bartha strain was attributed to the presence of similar viral enzymes as in HSV-1.

In this study, we generated the first metabolic profiles of PK-15 cells infected by the variant virulent or classical attenuated PRV strains. PRV infection primarily affected the metabolic pathways in PK-15 cells, including glycolysis, amino acid metabolism, and nucleotide metabolism, but there was minimal regulation of the TCA cycle. Interestingly, the metabolic profiles were similar for the variant virulent and classical attenuated strains during infection of PK-15 cells. Although PRV belongs to the same family as HSV-1 and HCMV, it leads to different metabolic consequences. The results of this study may clarify the biochemical materials utilized by PRV replication in host cells.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**; further inquiries can be directed to the corresponding author.

## AUTHOR CONTRIBUTIONS

HG carried out the data analysis and drafted the manuscript. ZB, ZJ, and PC participated in the experiments. SS, YL, and KZ participated in the data analysis. RC and CL conceived of the study. DY prepared the materials for the experiments. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.599087/full#supplementary-material

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frontiers in Cellular and Infection Microbiology

REVIEW
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# The Fate of Speckled Protein 100 (Sp100) During Herpesviruses Infection

*Mila Collados Rodríguez* *

*MRC-University of Glasgow Centre for Virus Research, Glasgow, United Kingdom*

The constitutive expression of Speckled-100 (Sp100) is known to restrict the replication of many clinically important DNA viruses. This pre-existing (intrinsic) immune defense to virus infection can be further upregulated upon interferon (IFN) stimulation as a component of the innate immune response. In humans, Sp100 is encoded by a single gene locus, which can produce alternatively spliced isoforms. The widely studied Sp100A, Sp100B, Sp100C and Sp100HMG have functions associated with the transcriptional regulation of viral and cellular chromatin, either directly through their characteristic DNA-binding domains, or indirectly through post-translational modification (PTM) and associated protein interaction networks. Sp100 isoforms are resident component proteins of promyelocytic leukemia-nuclear bodies (PML-NBs), dynamic nuclear sub-structures which regulate host immune defenses against many pathogens. In the case of human herpesviruses, multiple protein antagonists are expressed to relieve viral DNA genome transcriptional silencing imposed by PML-NB and Sp100-derived proteinaceous structures, thereby stimulating viral propagation, pathogenesis, and transmission to new hosts. This review details how different Sp100 isoforms are manipulated during herpesviruses HSV1, VZV, HCMV, EBV, and KSHV infection, identifying gaps in our current knowledge, and highlighting future areas of research.

**Keywords: Sp100, herpesviruses, PML-NB, ISG, epigenetics, immunity**

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***Edited by:***
*Subhash C. Verma,*
*University of Nevada,*
*United States*

***Reviewed by:***
*Maria Kalamvoki,*
*University of Kansas Medical Center,*
*United States*
*Tomohiko Sadaoka,*
*Kobe University, Japan*

***Correspondence:**
*Mila Collados Rodríguez*
*milagros.colladosrodriguez@glasgow.ac.uk*

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## INTRODUCTION

Speckled 100 kDa protein (Sp100) was identified using autoantibodies from patients suffering from primary biliary cirrhosis autoimmune disease (Szostecki et al., 1987; Szostecki et al., 1990). The 'speckled' nuclear distribution of Sp100 predominantly colocalizes with promyelocytic leukemia-nuclear bodies (PML-NBs) (Sternsdorf et al., 1995). Scaffolded by PML (TRIM19), these dynamic nuclear substructures regulate important cellular processes: genome stability, alternative

**Abbreviations:** aa, amino acid residues; ATRX, alpha-thalassemia/mental retardation X-linked; bp, base pairs; Daxx, death domain associated protein; EBNA-LP, Epstein-Barr virus nuclear antigen-leader protein; HIRA, histone cell cycle regulator A; HMG, high mobility group; Kbp, kilobasepairs; MORC3, microrchidia 3; SETDB1, SET domain bifurcated histone lysine methyltransferase 1; STAT, signal transducer and activator of transcription; SUMO, small ubiquitin (Ub) modifier; UBC9, Ub conjugating enzyme 9; UBE2I, Ub conjugating enzyme E2I; VP, viral protein; WHO, World Health Organization.

lengthening of telomeres, epigenetic regulation of chromatin, antiproliferation, senescence, apoptosis and antiviral immunity (Gurrieri et al., 2004; Bernardi and Pandolfi, 2007; Scherer and Stamminger, 2016). This range of functions is accomplished by alternatively spliced PML isoforms (Condemine et al., 2006), and its extensive network of protein interactions, some of which are mediated by PML SUMO modification (Van Damme et al., 2010). The post-translation modification (PTM) of proteins by SUMO is common in proteins that harbor a SUMO consensus motif (SCM) [reviewed in (Celen and Sahin, 2020)]. PML and Sp100 have been found to be mono- and poly-SUMOylated (Sternsdorf et al., 1997; Lang et al., 2010; Maarifi et al., 2015). This SUMO "code" is recognized by SUMO interacting motifs (SIMs) present in a variety of cellular proteins known to associate with PML-NBs (Hecker et al., 2006), with SUMO-SIM interactions playing a key role in PML-NB formation and stability (Zhong et al., 2000; Shen et al., 2006; Bernardi and Pandolfi, 2007).

In the following subsections, the domain composition of Sp100 isoforms is detailed, highlighting a role as epigenetic factor that may be independent of PML and PML-NB, and it is especially evident upon herpesviruses infection (see below).

## Protein Architecture of Sp100 Isoforms

The Sp100 gene spans nearly 130,000 bp and contains 32 exons that can be alternately spliced into 19 variants. Of the 11 protein-coding isoforms[1], only four (Sp100A, B, C, and HMG) have been routinely investigated by the scientific community. These isoforms share the Sp100A domain architecture up to the nuclear localization sequence (NLS) (**Figure 1**), in accordance with their predominant nuclear localization. This N-terminus comprises sequences responsible for Sp100 dimerization and PML-NB localization (Sternsdorf et al., 1999; Negorev et al., 2001), a destruction-box (D-box) required for Sp100 proteasomal degradation (Wang et al., 2011), a SCM (Sternsdorf et al., 1999) and a SIM (Knipscheer et al., 2008; Cuchet et al., 2011) in a histone protein 1 (HP1) interaction site (Seeler et al., 1998), and a trans-activating region (TR) (Szostecki et al., 1990; Szostecki et al., 1992; Xie et al., 1993). Whether these features are shared with the remaining seven coding Sp100 isoforms await to be experimentally determined.

Proteomic studies have shown Sp100 to undergo extensive PTM, including acetylation, phosphorylation, ubiquitination, and SUMOylation ('ProteomicsDB, Sp100', 2014). PML-NBs play an important role in the PTM of Sp100, as depletion of PML significantly abrogates the SUMOylation of Sp100 (Everett et al., 2006; Everett et al., 2008; Tavalai et al., 2011) and potentially, other PTMs as phosphorylation (Sternsdorf et al., 1999). The decreased abundance of these PTMs in the absence of PML is probably due to a defect in Sp100 SUMOylation, as depletion of the human E2 SUMO conjugating enzyme (UBC9/UBE2I) leads to similar Sp100 migration patterns in immunoblots (Boutell et al., 2011). It is likely, therefore, that PML mediates, either directly or indirectly, the PTM of Sp100;

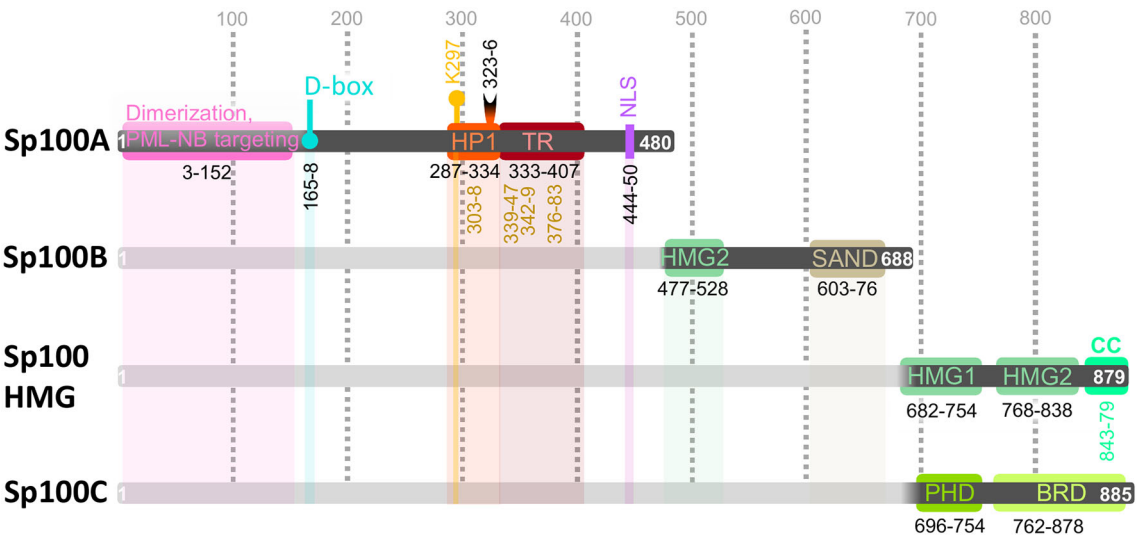


**FIGURE 1** | Sp100 isoform domain composition. Sp100A/B/C/high mobility group (HMG) share domain architecture within their first 477 amino acid (aa) residues: dimerization and promyelocytic leukemia-nuclear body (PML-NB) targeting (aa 3–152, pink), destruction-box (D-box, aa 165–168, teal); HP1 interacting region (aa 287–334, orange) encompassing a SUMO consensus motif (SCM) with Lys297 SUMO modification (K297, yellow pin) and SUMO-interacting motif (SIM, aa 323–326, black half-moon); trans-activating region (TR, aa 333–407, cherry); autoepitopes are indicated with vertical numbers in ochre below HP1 and TR segments (see EBV section); nuclear localization sequence (NLS, aa 440–450, purple). Sp100B/C/HMG are identical up to aa 685, which includes the high mobility group (HMG) 2 (aa 477–528, fern) and Sp100, AIRE-1, NucP41/75, DEAF-1 (SAND, aa 603-676, sand) DNA-binding domains. Sp100HMG contains two additional HMG (HMG1, aa 682–754; HMG2, aa 768–838) domains and a coiled coil (CC, aa 843–879, mint) domain. Sp100C contains a plant homeodomain (PHD, aa 696-754, green) and bromodomain (BRD, aa 762–878, light green) domain. C-terminal domain features are described in **Table 1**. Numbers indicate the positions of aa with each isoform. UniProt IDs: Sp100A, P23497-2; Sp100B, P23497-3; Sp100C, P23497-4; Sp100HMG, P23497-1. Further details on the Sp100 gene locus (ENSG00000067066) can be found at ENSEMBL[1].

indeed, PML has been shown to have SUMO E3 ligase activity and to regulate the SUMOylation of a number of PML-NB proteins, including p53, MDM2, Daxx, and c-jun (Quimby et al., 2006). SUMOylation of Sp100 stabilizes its interaction with the C-terminal chromoshadow domain (CSD) present in HP1 proteins (HP1α/CBX5, HP1β/CBX1 and HP1γ/CBX3) (Seeler et al., 1998), but their intermolecular details remain to be fully defined. HP1 can dimerize through CSD domains, creating a platform for histone methyltransferases (HMTs, "histone writer enzymes", **Figure 2A**) to tri-methylate Lys 9 of histone H3 tails (H3K9me3) where the N-terminal chromo domain (CD) of HP1 binds (Yamamoto and Sonoda, 2003; Larson and Narlikar, 2018), enabling HP1 dimers to bridge consecutive H3 di-nucleosomes (Machida et al., 2018; Kumar and Kono, 2020) (**Figure 2Bi**). HP1 and histones (H2A, H2B, H3, H4, and their respective variants), are examples of the chromatin protein fraction; chromatin is composed of DNA and directly or indirectly associated proteins which compact it in different degrees. Cellular DNA is coiled around nucleosomes formed by histones whose protruding N-terminal tails are subjected to dynamic PTMs (including methylation, acetylation, phosphorylation, ubiquitination and SUMOylation, among others) by histone "writer" and "eraser" enzymes (**Figure 2A**) (Bannister and Kouzarides, 2011); combinations of histone PTMs create chromatin activating or repressive "histone codes" that are interpreted by histone "readers" to regulate transcription (Strahl and Allis, 2000). Such histone modifications are the basis of epigenetics, which lead to cell-type specific and inheritable changes in gene expression without affecting the DNA sequence. The fact that all Sp100 isoforms (Sp100A/B/C/HMG) contain a sequence for PML-NB localization, suggests that some of their functions may be executed at PML-NB; for example, Sp100 crosstalk with chromatin through HP1 interaction may depend on its SUMOylation by PML. Moreover, the impact of homo and heterodimerization of Sp100 (**Figure 2Bii**) on chromatin dynamics is yet to be defined.

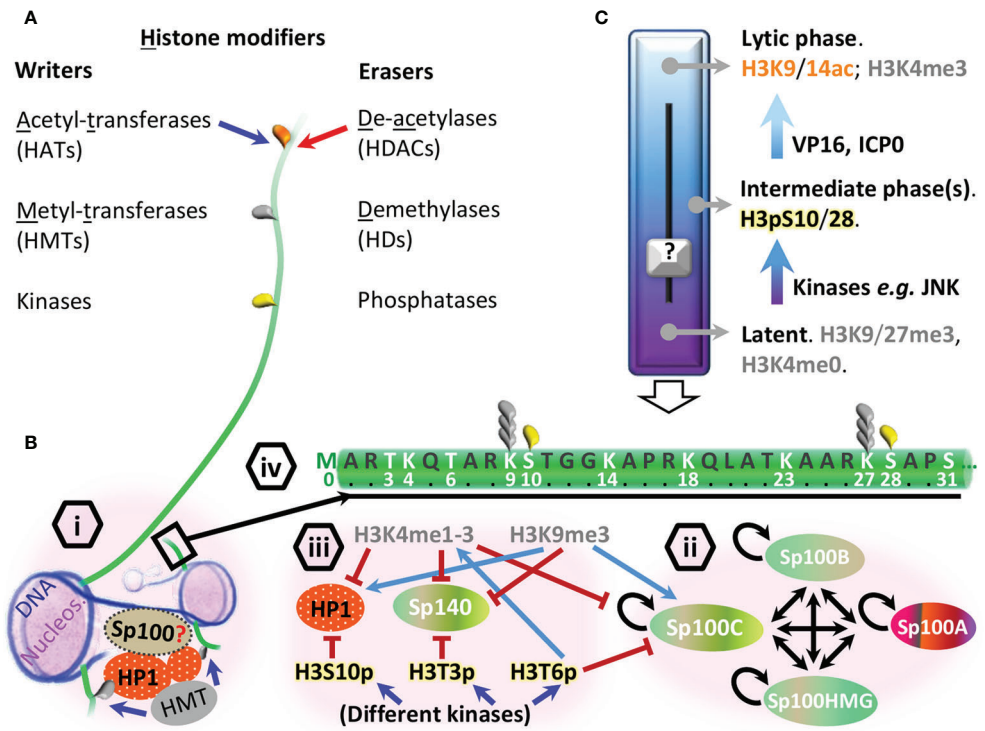


**FIGURE 2** | General epigenetic mechanisms influencing chromatin binding properties of Sp100. **(A)** Histone modifier enzymes sorted as "writers" (blue arrow) or "erasers" (red arrow) that influence the acetylation (orange teardrop), methylation (gray teardrop) or phosphorylation (yellow teardrop) post-translational modification (PTM) status of histones exemplified here through H3 tail (green line, not to scale). **(B)** Models for direct (SAND, HMG) and indirect (HP1, BRD, PHD) DNA binding properties of Sp100 isoforms; (i) dimerized HP1s (orange ovals) bind HMT (gray oval) leading to histone H3K9 trimethylation (gray teardrop) of consecutive nucleosomes (lilac). A red question mark indicates whether Sp100B/C/high mobility group (HMG) binds DNA (dark blue string) and/or HP1 as a monomer or as a dimer; (ii) Sp100 can potentially homo- (circular back arrows) and heterodimerize (double head black arrows) depending on the isoform expression profile and subnuclear localization. Sp100 color code refers to **Figure 1** characteristic features; (iii) examples of histone readers' regulation. H3K4me1-3 inhibits (red flat tip arrows) the binding of Sp100C, Sp140 and HP1 to histone 3, while H3K9me3 promotes (light blue arrows) Sp100C and HP1 binding. Different kinases drive the "phospho-switches" that influence Sp100C, Sp140 and HP1 binding to histone 3 (H3pT3/S10/T6); (iv) histone 3 tail (aa 1–31) highlighting aa and PTMs discussed in the main text associated with the epigenetic silencing of viral DNA and reactivation from latency. **(C)** Viral and epigenetic factors that influence HSV1 transcription; vertical empty arrow pointing down the H3 tail shows an example of post-translational modifications (PTMs) associated with reactivation from latency where the c-Jun N-terminal kinase (JNK) phosphorylates the aa residues next to H3K9/27me3, known as "methyl-phospho switch", a first step in chromatin relaxation. The participation of Sp100 variants as histone readers in each phase remains to be clearly further detailed (black question mark).

While the common N-terminus of Sp100 is mainly involved in protein-protein interactions, features present in its C-terminus directly interact with DNA and histones (**Figure 1**, **Table 1**). Sp100B, C, and HMG contain high mobility group (HMG) and Sp100, AIRE-1, NucP41/75, DEAF-1 (SAND) DNA-binding domains (Guldner et al., 1999; Bottomley et al., 2001). Mechanistically, the Sp100 SAND domain preferentially binds to unmethylated CpG dinucleotides commonly found in foreign DNA (Wilcox et al., 2005; Isaac et al., 2006). HMG binding to DNA opens the minor groove while narrowing the major one, thus bending the DNA to promote nucleosome loading and chromatin remodeling (Thomas, 2001; Malarkey and Churchill, 2012; Lohani and Rajeswari, 2016). Sp100HMG isoform has two additional C-terminal HMG domains (Seeler et al., 1998; Guldner et al., 1999), but their direct participation chromatin modification and assembly warrants additional study. Sp100HMG is also predicted to contain a C-terminal coiled-coil (CC) domain (Kumar et al., 2020), the presence and function of which has yet to be investigated. Sp100C, and Sp100 variant paralogues Sp110, Sp140, and Sp140L which cluster with Sp100 on human chromosome 2, are histone code "readers" since all recognize specific histone tail PTMs through their plant homeodomain (PHD) and bromodomain (BRD) tandem (Dent et al., 1996; Mellor, 2006; Filippakopoulos and Knapp, 2012; Saare et al., 2015; Leu et al., 2018; Zucchelli et al., 2019; Fraschilla and Jeffrey, 2020; Jain et al., 2020). Isothermal titration calorimetry characterization of the Sp100C PHD-BRD tandem peptide ($Sp100C_{PB}$) revealed high affinity for the H3 tails containing the repressive PTMs H3K9me3 and unmethylated H3K4 (H3K4me0), while the chromatin activating marks H3T6p and H3K4me1-3 exclude $Sp100C_{PB}$ binding to H3 tail (**Figure 2Biii**) (Zhang et al., 2016). Overall, this information reveals that Sp100C binding to chromatin can be affected by H3 PTMs. Moreover, *in cellula*, the subnuclear localization of ectopically expressed Sp100C differs from Sp100A and Sp100HMG (Seeler et al., 2001), indicating that the C-terminal domain architecture of each Sp100 isoform also dictates their participation in different chromatin-related processes. Additionally, IFN treatment of epithelial cells has shown to favor the levels of Sp100C mRNA over the other isoforms (Negorev et al., 2009). IFNs are secreted cytokines which activate the assembly of combinatorial STAT complexes that bind to IFN-stimulated response element (ISRE) or to gamma activation site (GAS) at promoters of genes implicated in antiviral defense, known as interferon stimulated genes (ISGs) (Regad and Chelbi-Alix, 2001). Sp100 is an ISG since its promoter contains an ISRE and two GASs binding sites which grant its inducibility by type-I (IFNα, β and κ) and type-II (IFNγ) IFNs in an individual and synergistic way (Grotzinger et al., 1996a; Grotzinger et al., 1996b). Thus, exogenous stimuli can alter the prevailing Sp100 isoform in a given cell type; the molecular details of this shift are still unknown, but splicing driven by Sp100 circular RNA may be involved (Deng et al., 2020).

## Further Details of Sp100C in Crosstalk With Chromatin

Other activating H3 PTMs such as mono- (H3K14/18/27ac) or multi acetylation (H3K14/18/23ac), phosphorylations at H3T3p, H3KS10p, or H3K9me3S10p, do not affect $Sp100C_{PB}$ binding to H3 tail; since the Sp100C BRD could not bind H3Kac *in vitro* either, it was indicated that the BRD molecular function is unknown but it was critical to stabilize the Sp100C PHD fold, given the extensive contacts seen in their crystal structure (Zhang et al., 2016). Of note, the PHDs of other proteins, as Sp140, have shown to facilitate BRD SUMOylation and its association with SETDB1, a HMT of H3K9 that promotes gene silencing (Ivanov et al., 2007; Peng and Wysocka, 2008; Garcia-Dominguez et al., 2008; Zucchelli et al., 2019); interestingly, this PTM also weakens the Sp140 PHD binding to H3 tail (**Figure 2Biii**) (Zhang et al., 2016). Future *in vivo* studies may indicate if Sp100C BRD has affinity for any H3Kac residues (**Figure 2Biv**), whether BRD SUMOylation takes place and affects Sp100C binding to H3, and if Sp100 orthologues cooperate as histone code readers.

Sp100C PHD is singular in tolerating H3T3p, since this PTM acts as a "binary switch" on Sp140 by excluding the binding of Sp140 PHD to H3 (Zhang et al., 2016) (**Figure 2Biii**). H3T3 is phosphorylated upon DNA damage (Salzano et al., 2015) and during prophase (Dai et al., 2005), while it is dephosphorylated during anaphase (Dai et al., 2005). Taken together, this suggests that Sp100C would still be bound to H3T3p during these processes, while Sp140 is not. Moreover, it has been reviewed that H3K4me0 and H3K9me3 represent marks to coordinate and maintain DNA methylation memory through mitosis (Hashimoto et al., 2010); it is tempting to speculate about the participation of Sp100C in ensuring this process.

**TABLE 1** | Alternatively spliced Sp100 C-terminal domains involved in chromatin regulation.

| Domain | Isoform(s) | Function | Reference(s) |
|---|---|---|---|
| HMG2 | Sp100B/C/HMG | DNA binding (see below). | (Lehming et al., 1998) |
| SAND | Sp100B/C/HMG | DNA binding to unmethylated cytosines of CpGs dinucleotides. | (Guldner et al., 1999; Bottomley et al., 2001; Wilcox et al., 2005; Isaac et al., 2006) |
| HMG1/2 | Sp100HMG | Shapes an L composed of three α-helices that bind and open the minor groove of DNA while narrowing the major one thus, bending the DNA and allowing the assembly of nucleosome and other proteins. | (Seeler et al., 1998; Guldner et al., 1999; Thomas, 2001; Malarkey and Churchill, 2012; Newhart et al., 2013; Lohani and Rajeswari, 2016) |
| CC | Sp100HMG | Unknown. | ELM prediction (Kumar et al., 2020) |
| PHD | Sp100C | Binds with most affinity to histone 3 unmethylated in Lys4 (H3K4me0) but tri-methylated in Lys9 (H3K9me3). | (Zhang et al., 2016) |
| BRD | Sp100C | Unclear binding to acetylated Lys (KAc). | (Filippakopoulos and Knapp, 2012; Zhang et al., 2016) |

In post mitotic neurons, H3S10 phosphorylation detaches HP1 from H3K9me3 (**Figure 2Biii**) while retaining ATRX (Noh et al., 2015), an ATP-dependent chromatin remodeller which, as Sp100C, requires both H3K9me3 and H3K4me0 to bind H3; ATRX cooperates with HP1 and the H3.3 chaperone Daxx to keep telomeric and pericentromeric chromatin repressed (Eustermann et al., 2011; Clynes et al., 2013). H3.3 is an histone variant loaded onto chromatin independently of DNA synthesis (Tagami et al., 2004) and then maintained by the PML-NB proteins and H3.3 chaperones Daxx/ATRX (Cabral et al., 2018). Since H3K9me3 and H3K4me0 can be read by multiple molecules, the spatio-temporal dynamics of Sp100C binding to H3 respective to other molecules as Sp140, HP1 and ATRX, remains to be determined. Overall, Sp100C PHD binds to repressive chromatin but tolerates activating epigenetic marks, except H3K4me and H3T6p.

Collectively, Sp100A/B/C/HMG isoforms share the N-terminus which allows their dimerization and location at PML-NB, where they can be modified with SUMO to interact with HP1, a chromatin protein. The Sp100B/C/HMG SAND domain links them to typically exogenous unmethylated DNA CpGs, but functions assigned to additional domains have to be directly established for Sp100 *in vivo*. Detailed Sp100C studies have evidenced its predominant transcription upon IFN treatment and its *in vitro* participation in chromatin compaction when H3 tails lack H3T6p and H3K4me. Further Sp100 characterization is key to understand its participation in cellular and viral chromatin regulation.

## CLINICAL IMPORTANCE OF HERPESVIRUSES AND MOLECULAR BASIS

Herpesviruses are a large family of double stranded DNA viruses that cause a variety of clinically important diseases on a global scale (**Table 2**). Their success lies their ability of reactivating from a latent state of infection whereby viral DNA (vDNA) transcription in the nucleus of terminally differentiated (nerve or white blood) cells is not enough to generate virus progeny, thereby avoiding immune clearance and thus, keeping a host chronically infected for a lifetime. Consequently, understanding the host defenses that limit the reactivation, propagation, and transmission of herpesviruses is of global importance.

Primary infection of herpesviruses starts with their binding to a cell surface, followed by nucleocapsid entering the cytoplasm, transport and attachment to the nuclear pore to release the viral genome into the nucleus; although vDNA is ejected into the nucleoplasm as a naked molecule, viral tegument proteins characteristic of each virus subfamily may access the nucleus with different efficiencies depending on the cell type infected, influencing the subsequent events in diverse ways [reviewed by (Full and Ensser, 2019)]. In parallel, the equilibrium of pro- and antiviral host factors intrinsic (pre-existing) to each cell type, ready to associate with this foreign nucleic acid and viral tegument proteins, dictates whether the infection progresses to lytic replication or remains latent.

When primary infection takes place in epithelial cells (*e.g.* in mucosa), herpesviruses typically initiate a lytic cycle of replication through the coordinated expression of immediate-early (IE), early (E), and late (L) genes, which sequentially contribute to the inactivation of host immune defenses, stimulation of viral gene expression, vDNA replication and virion assembly, respectively (Gruffat et al., 2016). In contrast, infection of terminally differentiated cell types is prone to latency because herpesviruses generally fail to establish this temporal cascade of viral gene expression. This once perceived binary (lytic or latent) behavior has recently been shown to be more heterogeneous, with patterns of viral gene expression dependent on cell type, genome copy number, and degree of pre-immune stimulation prior to infection (Knipe and Cliffe, 2008; Suzich and Cliffe, 2018). One of the intrinsic immunity barriers herpesviruses have to counteract or exploit upon vDNA release into the nucleus is chromatinization and epigenetic modulation (Knipe et al., 2013). Moreover, cellular stress can affect vDNA chromatinization, partially or fully reactivating viral transcription programs with variable production of progeny virions (reviewed by (Weidner-Glunde et al., 2020)). For example,

**TABLE 2** | Clinical relevance of herpesviruses.

| Virus | Clinical manifestations and estimates (references) |
|---|---|
| Herpes simplex virus 1 (HSV1)/ HHV1 | Blindness, dermatitis, gum diseases, sores in the mouth, nose and genitals, newborn fatal encephalitis (Imbronito et al., 2008; Looker et al., 2017; Marcocci et al., 2020). Considered causative factor of sporadic Alzheimer disease (Cairns et al., 2020). In 2016, the WHO (World Health Organization, 2020) estimated that about 67% of the world's population below 49 years old had HSV1 infection. |
| Varicella zoster virus (VZV)/HHV3 | Primary infection causes chicken pox/varicella and infectious shingles upon reactivation; during 2008–2011, VZV mortality rate in the US population was estimated to be 0.05 per million, representing an 87% decrease in comparison to prevaccine years (Johnson and Levin, 2020). |
| Human cytomegalovirus (HCMV)/HVV5 | Worldwide seroprevalence of 66%–90% (Zuhair et al., 2019). It affects transplants' recipients (Schottstedt et al., 2010; Meesing and Razonable, 2018) and is a major cause of congenital disability in children (Davis et al., 2017; Emery and Lazzarotto, 2017). |
| Epstein-Barr virus (EBV)/HVV4 | Mononucleosis and derived lymphomas caused at least 142,000 deaths worldwide in 2010 (Khan and Hashim, 2014; Martinez and Krams, 2017) and a chronic population deterioration resulting from EBV-related autoimmune diseases (Draborg et al., 2016; Balandraud and Roudier, 2018; Trier et al., 2018). |
| Kaposi's sarcoma-associated herpesvirus (KSHV)/ HVV8 | KSHV associated tumorigenesis is responsible for around 55% mortality in South African infected children (Dow et al., 2014), and nearly 20% of deaths in seropositive blood transfusions (Ablashi et al., 2002; Operskalski, 2012). |

neuronal stress promotes the phosphorylation of H3S10/28 at vDNA promoters by c-Jun N-terminal kinase (JNK) (**Figures 2Biv, C**), allowing viral gene expression even when adjacent H3K9/27me3 repressive marks are present (Cliffe et al., 2015). Despite of Sp100 is unaffected by H3pS10 epigenetic switch (see introduction), expression of further herpesviruses proteins neutralize specific antiviral immunity factors as Sp100, triggering periodic reactivation of viral latent pools, leading to *de novo* virus replication, virus progeny production and transmission to new hosts.

## THE SPATIOTEMPORAL JOURNEY OF SP100 AGAINST HERPESVIRUSES

This section focuses on the antiviral properties of the Sp100 variants and how they are hijacked from the outset of infection by herpesviruses factors; **Figure 3** summarizes these viral proteins counteracting Sp100 and indirectly affecting its core network of cellular protein partners. Since different aspects of epigenetic modulation of herpesviruses have been characteristically studied for each family (Knipe et al., 2013), only these known to affect Sp100 fate are highlighted, introduced in the HSV1 section, and subsequently referred.

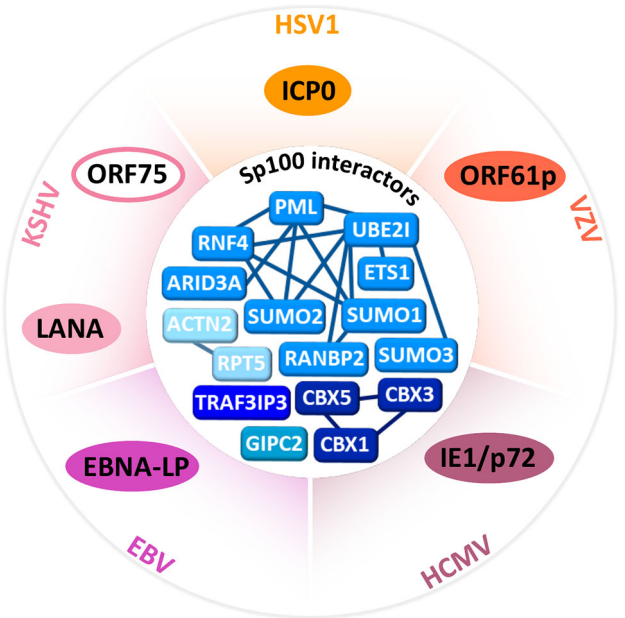


**FIGURE 3** | Sp100 interactors and herpesvirus counteractors. Outer circle, herpesviruses proteins (HSV1, ICP0; VZV, ORF61p; HCMV, IE1/p72; EBV, EBNA-LP; KSHV, LANA, and ORF75) that antagonize Sp100. Inner circle, network of Sp100 related protein interactions likely to be disrupted during herpesvirus infection. Clustered networks of known Sp100 interactors shaded in blue. Interactions retrieved from BioGRID (Stark et al., 2006), minimum experimental evidence from two independent studies.

### Herpes Simplex Virus 1

When HSV1 infection progresses to lytic replication, parental vDNA is not associated to typical nucleosome units but to proteinaceous structures (Muggeridge and Fraser, 1986; Deshmane and Fraser, 1989). This incomplete vDNA chromatinization may result from the blocking of histone loading into naked DNA by VP22, the most abundant tegument protein (van Leeuwen et al., 2003). In fact, H3.3 has been observed to accumulate juxtaposed but unincorporated to vDNA into PML-NB, which could be the aforementioned proteinaceous structures detected at the initial and lytic phases of infection (Conn et al., 2013). Accordingly, PML-NB are known to act as protein depots (Negorev and Maul, 2001), as well as to assemble onto and entrap incoming vDNA (Alandijany et al., 2018). PML-NB entrapped vDNA has been shown to lack H3K4me2 but to be H3.3 decorated with H3K9me3 (Cabral et al., 2018; Cohen et al., 2018), advancing Sp100C presence. Moreover, experiments studying the recruitment of PML-NB proteins to a transgene array as a model for foreign DNA invasion, showed that when UBC9 was depleted, neither Daxx, PML nor H3.3 accumulated onto the array; hence, their accumulation onto invading DNA depends on SUMOylation (Shastrula et al., 2019), an enzymatic activity enriched at PML-NB (see introduction). However, whether SUMOylation is also required for Sp100 accumulation at vDNA is unclear but seems to be isoform-specific, since Sp100 spliced variants have commonalities but also differences (**Figure 1**), which may account for their divergent spatio-temporal behavior: Sp100A has been shown to enhance the expression of the IE infected cell protein-0 (ICP0) promoter, while it is repressed by Sp100 isoforms containing a SAND domain (Sp100B/C/HMG), unless this domain is mutated (Negorev et al., 2006); similarly, Daxx, ATRX and Sp100B/C/HMG repress a CMV promoter reporter while withdrawing Sp100A-driven chromatin decondensation (Seeler et al., 1998; Newhart et al., 2012; Newhart et al., 2013). Taken together, Sp100B/C/HMG cause repression of transcription while Sp100A is activating in a mutually exclusive way.

In turn, this differential transcriptional behavior of Sp100B/C/HMG as opposed to Sp100A is distinctly modulated by ICP0 since when HSV1 ICP0 was included in the CMV promoter reporter, Sp100A presence augmented at this CMV promoter reporter independently of its SUMOylation status, Daxx or ATRX, while Sp100B/C/HMG were degraded (Newhart et al., 2013). It is known that challenging epithelial cells with HSV1 depletes Sp100 SUMOylated isoforms (Everett et al., 2009), as occurs when UBC9 or PML are silenced by shRNA (Everett et al., 2006). Thus, Sp100 degradation could either be a direct target of ICP0 (Perusina Lanfranca et al., 2013), a viral E3 Ub ligase which preferentially targets SUMOylated proteins for proteasomal degradation [(Boutell et al., 2011), reviewed by (Boutell and Everett, 2013; Rodriguez et al., 2020)], or occur as an indirect consequence of PML disposal by ICP0 (Tavalai and Stamminger, 2009). In any case, dismantling of PML-NB by ICP0 or by PML shRNA silencing has no effect on Sp100A (Everett et al., 2006; Everett et al., 2009), advancing that Sp100A transactivating properties may be exploited by HSV1 (see below).

Interestingly, Sp100A SIM deletion has shown that in ΔICP0 HSV1 infected cells, Sp100A can prescind from its SUMOylation

but requires its SIM to appear as nuclear puncta even in the absence of PML (Cuchet et al., 2011; Cuchet-Lourenco et al., 2011); this suggests that Sp100, as PML, may scaffold factors on its own. In order to study the participation of Sp100 in restricting HSV1 infection, mutant viruses lacking ICP0 (ΔICP0) or carrying mutations in its catalytic RING-finger domain responsible of its E3 Ub ligase activity are routinely used to keep PML-NBs intact. Independent or combined depletion of PML and Sp100 shows that their effects in restricting ΔICP0 HSV1 are additive but partial (Everett et al., 2006; Everett et al., 2008; Glass and Everett, 2013). This indicates that both factors have antiviral properties on their own, which are enhanced by their cooperation, but they do not fully restore the ΔICP0 effects to WT HSV1 levels because additional proteins participate in counteracting ICP0. Indeed, the chromatin regulators Daxx, ATRX and MORC3 can be still recruited to incoming viral genomes when both Sp100 and PML are silenced (Everett et al., 2008; Lukashchuk and Everett, 2010; Cuchet et al., 2011; Sloan et al., 2016). MORC3 dimerizes to acts as ATPase when its histone recognition CW domain binds H3K4me3 (Zhang et al., 2019), forming MORC3-NB; speculatively, the ATPase function of MORC3 may fuel chromatin remodeling enzymes (Vignali et al., 2000). Complementarily, silencing MORC3 by shRNA impedes PML, Sp100 and Daxx appearing as puncta before the emergence of replication compartments (RCs) visualized with ICP4; however, once RCs appear, they are PML free but Sp100 is still associated to them (Everett et al., 2006; Everett et al., 2008; Lukashchuk and Everett, 2010; Cuchet et al., 2011; Sloan et al., 2016). This suggests that the recruitment of PML, Sp100 and Daxx to parental HSV1 DNA is orchestrated by MORC3 to form PML-NBs on the one hand, and on the other hand, that a portion of Sp100 associates to ICP4 replication centers independently of MORC3 when PML-NBs have been dismantled by ICP0. Collectively, this indicates that the cell can recruit energy fueling enzymes to ensure subsequent viral chromatin remodeling but, contrary to Sp100A, they are targeted by ICP0.

HSV1 ΔICP0 parental genome have limited gene expression as it remains entrapped by PML-NBs upon infection of the cell nucleus (Everett and Murray, 2005; Alandijany et al., 2018). This is so unless the copy numbers of HSV1 ΔICP0 saturate the intrinsic defenses that PML-NB represent, escaping vDNA entrapment and leading to its replication (Alandijany et al., 2018; McFarlane et al., 2019). Viral replication triggers cytokine signaling which leads to the PML and Sp100-dependent accumulation of the H3.3 chaperone HIRA at pre-existing PML-NB, since shRNA silencing of either PML or Sp100 abrogates HIRA accumulation at PML-NBs (Alandijany et al., 2018; McFarlane et al., 2019). HIRA binds ISGs loci to promote their transcriptional upregulation, further stimulating the innate immune defenses upon HSV1 infection (Alandijany et al., 2018; McFarlane et al., 2019); in this way, undisrupted PML-NB are able to induce an IFN response upon HSV1 ΔICP0 infection.

Another HSV1 factor accessing epithelial cells nucleoplasm is VP16, which transactivates IE genes upon recruiting coactivators to their promoters; one of these coactivators, host cell factor-1 (HCF-1), has been reviewed to associate with H3K4 HMTs to ensure H3K4me3 presence (Kristie et al., 2010; Vogel and Kristie, 2013). This chromatin activation mark would exclude Sp100C, Sp140 and HP1 from H3 (see introduction, **Figure 2Biii**) however, this remains to be assessed. In contrast, HSV1 cannot take advantage of VP16 and VP22 in neurons since these tegument proteins dissociate from the capsid before it reaches the nucleus (Aggarwal et al., 2012), a process favoring latency establishment in these cells over epithelial ones. Nevertheless, literature studying the participation of Sp100 during HSV1 latency establishment and maintenance is scarce (Everett et al., 2007; Cohen et al., 2018), and key questions as whether neurons depleted for Sp100 establish latency are still unanswered.

In conclusion, the chromatin repressive Sp100 SUMOylated isoforms seem to be required to entrap parental vDNA in cooperation with other SUMOylated chromatin-associated factors, but they are (directly or indirectly) targeted by HSV1 ICP0 and possibly, indirectly counteracted by VP16 and VP22. In contrast, the remaining unSUMOylated Sp100A has chromatin activating properties and can form NB independently of PML, which may represent a favorable environment for viral replication. Since Sp100A domain architecture is present in most of the other isoforms, it would be challenging to specifically target it with drugs however, when PML-NB are dismantled by ICP0, Sp100A seems to harbor differential PTM which may make it drug-amenable.

## Varicella Zoster Virus

The above described Sp100 isoforms' dynamics on chromatin also apply to herpesviruses other than HSV1 however, they are affected at a different extent by ICP0 homologs. The ICP0 VZV homolog VICP0/ORF61p only targets Sp100 out of the PML-NB components (Walters et al., 2010). ORF61p also harbors a RING-finger and SIMs to function as E3 Ub ligase on SUMOylated targets however, ORF61p lacks sequences required for binding to the host deubiquitinase USP7, which protects ICP0 from auto-ubiquitination and proteasome-mediated degradation (Kyratsous and Silverstein, 2009). As a result, ORF61p turnover by proteasomal degradation is quicker than for ICP0, making ORF61p more unstable than its HSV1 homolog (Everett et al., 2010). Therefore, IE kinetic studies comparing ORF61p to ICP0 in an HSV1 background evidence that ORF61p incompletely substitutes for ICP0; contrary to ICP0, ORF61p only reduces Sp100 levels without targeting PML (Kyratsous et al., 2009). Consequently, VZV infected cells still harbor PML-NB capable enough of sustaining an IFN response, which further increases Sp100 and PML levels and allows PML to SUMOylate Sp100; in agreement, late kinetic studies by immunoblot show a predominant increase of SUMOylated Sp100A (Kyratsous and Silverstein, 2009). At this late time point, the corresponding immunofluorescence images of cells display a granulated distribution of Sp100 in the nucleoplasm, concomitant with a high abundance of ORF61p (Kyratsous and Silverstein, 2009), but point mutations on its RING domain change the dispersed pattern to nuclear puncta, still colocalizing with Sp100 (Walters et al., 2010); the significance of this observation in the context of VZV life cycle requires further experimental assessment. In summary, Sp100 levels are reduced by VZV ORF61p from the onset of infection but since VZV does not completely disrupt PML-NBs, IFN-response can rise Sp100 protein levels, which are kept dispersed in the nucleoplasm by ORF61p. Whether Sp100

degradation or dispersion also facilitates initiation of VZV lytic replication *per se*, remains to be investigated.

## Human Cytomegalovirus

The role of immunity factors restricting HCMV infection, with Sp100 as PML-NB component, has been reviewed elsewhere (Rossini et al., 2012; Landolfo et al., 2016). As opposed to Sp100 merely being used as a PML-NB marker assumed to behave as PML (Ahn and Hayward, 1997; Sourvinos et al., 2007), this section focuses on Sp100 isoforms during HCMV infection. After parental HCMV genomes enters the nucleus, alternative splicing of a sole HCMV IE transcript results in two proteins: IE1/p72 and IE2/p86 (Stenberg et al., 1985). IE1 initially co-localizes at PML-NBs and then gradually disperses them through the nucleoplasm (Korioth et al., 1996). More in detail, IE1 has been shown to interact with the Sp100 N-terminal dimerization domain, as deletion of the corresponding 3–152 aa abrogates their association, and infection with mutant HCMV lacking IE1 does not cause loss of Sp100 (Kim et al., 2011). In turn, Sp100 depletion favors IE1 expression (Kim et al., 2011; Ashley et al., 2017); individual depletion of PML, Sp100 or Daxx showed that each factor was restrictive on its own (Ashley et al., 2017), combined depletion of Sp100/PML or Sp100/Daxx further enhanced HCMV gene expression initiation (Adler et al., 2011), and combined depletion of all PML/Sp100/Daxx were more permissive to HCMV infection (Ashley et al., 2017), resembling the above discussed intrinsic immunity factor's dynamics against HSV1. Similarly, all PML/Sp100/Daxx depletion also reduces the restriction of HCMV by IFNβ, visualized through IE1 presence and plaque assay upon IFNβ treatment (Ashley et al., 2017), indicating that these PML-NB components mediate the IFN response against HCMV. IE1 co-transfection with each Sp100 isoform was shown to reduce their SUMOylation (Tavalai et al., 2011); this effect is especially evident for Sp100A (Dimitropoulou et al., 2010; Tavalai et al., 2011), but unSUMOylated Sp100A levels also decrease at later times post HCMV infection (Tavalai et al., 2011). Even more, IE1 gets SUMOylated while driving the deSUMOylation of Sp100 and PML (Muller and Dejean, 1999). PML function as E3 SUMO ligase onto IE1 has been evidenced since PML RING domain mutants fail to SUMOylate IE1 (Reuter et al., 2017); this further supports the possibility of PML directly SUMOylating Sp100, indicating that part of the antiviral effect of Sp100 depends on PML. Overall, Sp100 disruption by HCMV IE1 seems to affect all isoforms, although at slower kinetics than HSV1 ICP0.

Furthermore, the promoter of the IE1/2 transcript, the major immediate early promoter (MIEP) has been shown to be repressed by histone deacetylase 3 (HDAC3) and HP1 in peripheral blood monocytes (Murphy et al., 2002), where HCMV establishes latency; this suggests that sustained histone deacetylation may allow histone methyltransferases (HMTs) to lock MIEP chromatin in a repressive state characteristic of a latent state of infection. However, the participation of Sp100 in latency establishment has been excluded using THP-1 derived macrophages partially depleted for either Sp100, Daxx or PML; intriguingly, partial Daxx depletion was enough to increase Sp100A levels similarly to undifferentiated THP-1 monocytes, especially unSUMOylated Sp100A ones, while PML had no effect on Sp100 levels (Wagenknecht et al., 2015), contrasting with previous reports. A more robust cellular KO background may consolidate or rule out the implication of Sp100 isoforms and additional factors in HCMV latency establishment and cell identity.

## Epstein–Barr Virus

Twenty years ago it was stated that latent EBV episomes are tethered to cell chromosomes away from PML-NB (Bell et al., 2000), contrasting with the later observation of the IE EBV protein EBNA-LP colocalizing with Sp100 at PML-NB in EBV immortalized lymphoblastoid cell lines (Ling et al., 2005). Using transfection assays, EBNA-LP was shown to disperse Sp100 from PML-NB by interacting with the Sp100 PML-NB targeting domain (Ling et al., 2005); dispersion by either EBNA-LP or by deleting the Sp100 PML-NB targeting domain, dissociates Sp100 away from PML-NB allowing the viral oncoprotein EBNA2 to act as a transcriptional activator, even in the absence of EBNA-LP (Ling et al., 2005). Interestingly, for this process to occur, the Sp100 HP1 interacting domain was required to be intact, but not the SCM embedded on it, since K297R modified Sp100A still activates EBNA2 even upon IFNβ pretreatment (Ling et al., 2005; Echendu and Ling, 2008). Hence, high resolution microscopy studies looking at whether Sp100 isoforms associate at some point of the viral cycle with EBV episomes await to be accomplished. Moreover, whether Sp100 SIM, which is also embedded in its HP1 interacting domain, is also required for the effective EBV subversion of innate immunity, which culminates in lytic reactivation remains to be addressed. Thus, EBV EBNA-LP is equivalent to HSV1 ICP0 in the sense of dispersing proteins from PML-NB and opens new questions as how different herpesvirus co-infections may affect each other's lytic replication upon reactivation. In fact, EBNA-LP increases HSV1 ΔICP0 replication, depletes Sp100 SUMOylated isoforms and causes a Sp100 mobility shift (Lu et al., 2016) characteristic of lack of localization at PML-NB. To sum up, EBNA-LP selectively binds to the PML-NB targeting domain of Sp100 abrogating its PML-NB localization, but details of the fate of specific Sp100 isoforms are unknown.

The significance of the above described Sp100 overexpression studies establishes parallelisms to clinical pathological cases of Sp100 overexpression and nucleoplasm delocalization which may favor the chance of generating Sp100 autoantibodies by molecular mimicry. EBV has been linked to PML-NB associated autoimmune diseases since two viral proteins share autoepitopes with Sp100 [aa 296–311 and aa 332–351 in ochre, **Figure 1**; (Xie and Snyder, 1995)]. These epitopes partly coincide with the ones described for 20-30% of patient's sera with biliary cirrhosis [aa 303–308 and aa 339–347 in ochre, **Figure 1**; (Bluthner et al., 1999)]. Such Sp100 antigenic regions flank its SIM and fall along the HP1 interacting region and in its TR. Consequently, it can be envisaged that autoantibodies against SIM, HP1 or TR Sp100 regions may sterically impede the interaction of Sp100 SIM with SUMO conjugated to other proteins, the interaction between HP1 proteins and Sp100, and the Sp100 trans-activating capabilities, respectively. Knowing Sp100-derived autoepitopes creates a chance for pharmacological intervention tailored to different Sp100 regions to counteract the EBV targeting of Sp100 at molecular and humoral levels.

### Kaposi's Sarcoma-Associated Herpesvirus

KSHV research comprising Sp100 is mostly related to the establishment of latency, which depends on translating the latency associated nuclear antigen (LANA) (Campbell and Izumiya, 2012), as well as reactivation, which occurs when the tegument protein ORF75 disperses Sp100 from PML-NB (Full et al., 2014). During primary infection, ORF75 has no effect on Sp100 and PML; these PML-NB components restrict KSHV, as their individual silencing allow viral proteins expression (Full et al., 2014). As a result of KSHV infection there is an IFN-mediated increase of Sp100 levels however, the viral encoded E3 SUMO ligase LANA converts the Sp100 soluble pool residing in the nucleoplasm and cellular chromatin into an insoluble one by inducing Sp100 SUMOylation and storage into the insoluble nuclear matrix, presumably corresponding to PML-NB or to another fraction (Gunther et al., 2014). Sp100 insolubilization allows the parental vDNA to establish latency and eventually reactivate by acquiring the H3K27me3 repressive mark (Gunther and Grundhoff, 2010), characteristic of facultative chromatin, which was shown to be favored by silencing Sp100 (Gunther et al., 2014). The Sp100 insolubilization by LANA seems to be unique of KSHV as other γ-herpesviruses as EBV maintain Sp100 soluble during latency (Gunther et al., 2014); nevertheless, similar analysis of the insoluble fractions for other herpesviruses are sparse across the literature. Collectively, undisrupted PML-NB allow IFN induction upon KSHV infection, increasing Sp100 levels but maintaining them in an insoluble form by LANA-mediated SUMOylation; this is concomitant with the acquisition of the repressive H3K27me3 mark, characteristic of latency, by parental vDNA.

## CONCLUSIONS AND FUTURE PERSPECTIVES

Herpesviruses attain a latent state of infection with periodic complete or incomplete reactivation which cause an underestimated quality of life deterioration. Since there is no cure, understanding how the cocktail of Sp100 isoforms are counteracted in each cell type as intrinsic and innate immunity factors by herpesviral proteins may help in its development. Recapitulating, the Sp100B/C/HMG isoforms, and likely other Sp-family members, may sense invading vDNA and coordinate the assembly of H3 and chromatin repressive marks at PML-NB SUMOylation and epigenetic hubs. However, herpesviruses can directly counteract the Sp100 role in viral epigenetics, as well as indirectly by dismantling PML-NB; HSV1 and HCMV IE proteins disrupt PML-NB, while VZV, EBV and KSHV IE proteins selectively target Sp100 thus, PML-NB can still induce an IFN response which leads to vDNA repression, promoting latency establishment. Much remains to be discovered concerning the molecular details leading to latency and the intermediate steps leading to reactivation. This review has highlighted the need to better understand cell-specific dynamics of Sp100 isoforms, characterize further ones, as well as their individual features interplaying with other chromatin factors, framing areas for pharmacological exploration.

## AUTHOR CONTRIBUTIONS

MCR wrote, edited the manuscript, and prepared the figures.

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REVIEW
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# Evasion of Intracellular DNA Sensing by Human Herpesviruses

*Debipreeta Bhowmik and Fanxiu Zhu**

*Department of Biological Science, Florida State University, Tallahassee, FL, United States*

Sensing of viral constituents is the first and critical step in the host innate immune defense against viruses. In mammalian cells, there are a variety of pathogen recognition receptors (PRRs) that detect diverse pathogen-associated molecular patterns (PAMPs) including viral RNA and DNA. In the past decade, a number of host DNA sensors have been discovered and the underlying sensing mechanisms have been elucidated. Herpesviruses belong to a large family of enveloped DNA viruses. They are successful pathogens whose elaborate immune evasion mechanisms contribute to high prevalence of infection among their hosts. The three subfamilies of herpesviruses have all been found to employ diverse and overlapping strategies to interfere with host DNA sensing. These strategies include masking viral DNA or the DNA sensor, degradation of the DNA sensor, and post-transcriptional modification of the DNA sensor or its adaptor protein. In this review, we will discuss the current state of our knowledge on how human herpesviruses use these strategies to evade DNA-induced immune responses. Comprehensive understanding of herpesvirus immune-evasion mechanisms will aid in the development of vaccines and antivirals for herpesvirus-associated diseases.

**Keywords: cGAS, DNA sensing, innate immune response, herpesvirus, viral evasion**

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*Fengwei Bai, University of Southern Mississippi, United States*

***Correspondence:**
*Fanxiu Zhu*
*fzhu@bio.fsu.edu*

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## INTRODUCTION

Herpesviruses belong to a family of large DNA viruses that characteristically cause both latent and lytic infections in a wide range of animals and humans. There are eight types of herpesviruses currently known to infect humans, causing various diseases (Knipe and Howley, 2013). Herpesviruses are subdivided into the α-, β-, and γ-subfamilies, based on biologic properties including cell tropism, genome organization, and sequence homologies of the conserved open-reading frames (ORFs) (Davison et al., 2009) (**Table 1**).

The host innate immune response is the first line of defense against viral infection. It is activated by the host pattern recognition receptors (PRRs) upon detecting conserved molecular moieties from the pathogens, known as Pathogen Associated Molecular Pattern (PAMPs) (Mogensen, 2009). Typical PAMPs include lipopolysaccharides of bacteria that can be recognized by Toll-Like Receptor (TLR)4, and dsRNA of viruses that can be detected by TLR3 in endosome, retinoic acid-inducible gene I (RIG-I), and melanoma differentiation-associated gene 5 (MDA5) in the cytosol. Despite being the genetic blueprint of all life, DNA has long been known to be immune stimulatory (Isaacs et al., 1963). As a potent PAMP, DNA can be recognized by several PRRs (**Table 2**) (Paludan and Bowie, 2013; Unterholzner, 2013; Dempsey and Bowie, 2015; Ma et al., 2018). The first described DNA sensor that detects endosomal CpG-rich DNA to initiate type I

**TABLE 1 |** Classification of human herpesviruses.

| Subfamily | Type | Synonym | Cytopathology | Primary target cells | Site of latency | Pathophysiology |
|---|---|---|---|---|---|---|
| α-herpesvirus | HHV-1 | Herpes simplex virus-1 (HSV-1) | Cytolytic | Mucoepithelial | Neurons | Orofacial infections, Encephalitis |
| | HHV-2 | Herpes simplex virus-2 (HSV-2) | | | | Genital and neonatal infections |
| | HHV-3 | Varicella zoster virus (VZV) | | | | Chickenpox, Shingles |
| β-herpesvirus | HHV-5 | Cytomegalovirus (CMV) | Cytomegalic | Monocytes, lymphocytes, epithelial cells | Macrophages, lymphocytes, epithelial cells | Congenital infection, Sensorineural hearing loss in children, Retinitis, Hepatitis |
| | HHV-6 | Roseolovirus | Lymphotropic | T cells | Monocytes/ macrophages | Exanthem subitum |
| | HHV-7 | Roseolovirus | | T cells | T cells | Roseola infantum |
| γ-herpesvirus | HHV-4 | Epstein–Barr virus (EBV) | Lymphoproliferative | B cells and epithelial cells | B cells | Infectious mononucleosis, lymphoma, carcinoma |
| | HHV-8 | Kaposi's sarcoma-associated herpesvirus (KSHV) | | Lymphocytes and endothelial cells | Lymphocytes | Kaposi's sarcoma, Primary effusion lymphoma, Multicentric Castleman disease |

**TABLE 2 |** Characterization of the DNA sensing pathways.

| Proposed DNA sensor | Ligand | Cite of DNA sensing | Mechanism | Biological Response | References |
|---|---|---|---|---|---|
| **Toll-like receptor** | | | | | |
| TLR9 | CpG DNA | Endosomes | Recognizes unmethylated CpG DNA and recruits the adaptor protein MyD88 to induce activation of NF-κB and IRF7 | Type I IFN | Kawai and Akira, 2011 |
| **PYHIN Family** | | | | | |
| AIM2 | Cytosolic dsDNA | Cytoplasm | Binds to DNA *via* AIM2 HIN200 domain and recruits ASC *via* a pyrin:pyrin homotypic interaction followed by subsequent caspase activation | IL-1β and IL-18 | Hornung et al., 2009 |
| IFI16 | Cytosolic dsDNA Nuclear dsDNA | Cytoplasm, nucleus | 1. Interacts with STING, and subsequently induces IRF-3 phosphorylation. 2. Activates inflammasome responses through ASC and caspase-1 | IFN-β, IL-6, IL-1β | Kerur et al., 2011; Ansari et al., 2013; Johnson et al., 2013 Ansari et al., 2015 |
| **Nucleotidyl Transferase Family** | | | | | |
| cGAS | Cytosolic dsDNA Y-form DNA DNA-RNA hybrid | Cytoplasm, nucleus | Binds to DNA and catalyzes the synthesis of cGAMP that activate STING followed by subsequent activation of IRF3 | IFN-β | Ablasser et al., 2013; Gao et al., 2013; Sun et al., 2013 |
| **Protein Kinase (PK) Family** | | | | | |
| DNA-PK | Cytosolic dsDNA | Cytoplasm | 1. Senses DNA and activates the STING-TBK1-IRF3 axis 2. Induces STING independent DNA sensing pathway by triggering HSPA8 and IRF3 phosphorylation | IFN-λI, IFN-β, IL-6 | Burleigh et al., 2020 |
| Ku70 | Cytosolic dsDNA | Cytoplasm | Senses DNA and induces the production of type III IFN | Type III IFN | Zhang et al., 2011 |
| **DExD/H-Box Helicase Family** | | | | | |
| DHX9 and DHX36 | CpG DNA | Cytoplasm | Detect CpG DNA and activate an MyD88-dependent pathway | TNF-α, IFN-α | Kim et al., 2010 |
| **Other DNA sensors** | | | | | |
| DAI | Cytosolic dsDNA | Cytoplasm | Senses DNA and activates the STING-TBK1-IRF3 axis | IFN-β | Takaoka et al., 2007 |
| Mre11 | Cytosolic dsDNA | Cytoplasm | Senses DNA and activates the STING-TBK1-IRF3 axis | IFN-β | Kondo et al., 2013 |

interferon (IFN) response was TLR9 (Hemmi et al., 2000). Soon after, Absent In Melanoma 2 (AIM2) was found to sense cytosolic DNA and trigger inflammasome response (Fernandes-Alnemri et al., 2009; Hornung et al., 2009). Several other DNA sensors like IFNγ-Inducible protein 16 (IFI16), RNA polymerase III (Pol III), and the Mre11-Rad50-Nbs1 (MRN) complex, have also been reported (Goubau et al., 2013; Paludan, 2015; Volkman et al., 2019). The most recently reported DNA sensor is cyclic

GMP–AMP Synthase (cGAS) which was discovered by Dr. Zhijian "James" Chen's group through elegant biochemical approaches (Sun et al., 2013; Wu et al., 2013). Binding to dsDNA activates cGAS, leading to generation of unique second messenger cGAMP that binds to stimulator of interferon genes (STING) and activates downstream signaling (Ablasser et al., 2013; Gao et al., 2013; Sun et al., 2013). Numerous studies have shown cGAS to be the non-redundant principal cytosolic DNA sensor in most cells. All PRRs share similar modes of action: after the recognition of PAMPs, PRRs induce intracellular signaling pathways through the hierarchical activation of a PRR family-specific adaptor protein. This leads to the expression of genes with pro-inflammatory and microbicidal activities, including cytokines and type I IFNs. Secreted cytokines and chemokines are also critical for shaping effective adaptive immune responses (Akira et al., 2006; Brubaker et al., 2015).

To overcome host defenses, viruses, including herpesviruses, have evolved multiple strategies to evade immune recognition by PRRs (Bowie and Unterholzner, 2008; Beachboard and Horner, 2016; Chan and Gack, 2016; Lee et al., 2019). These strategies include sequestration or modification of viral nucleic acids, sequestration of PRRs, degradation or cleavage of host sensors or their adaptor proteins, interference with specific post-translational modifications of PRRs or their adaptor proteins, and inhibition of the enzymatic activity of PRRs. Evasion of the host innate immune response is crucial for herpesviruses to persist in their hosts. Therefore, understanding immune evasion strategies will advance our knowledge of viral/host interplay and viral pathogenesis in general, and will inform the development of preventive or therapeutic strategies against herpesviruses-associated diseases. In this review, we will discuss how human herpesviruses are sensed by the host innate immune system, with a focus on the DNA sensors, and elaborate the common evasion strategies that target different steps in this signaling pathway.

## DETECTION OF HERPESVIRUSES BY PRRs

Herpesvirus virions contain a large, double-stranded DNA genome encased in a highly ordered icosahedral nucleocapsid. The nucleocapsid is coated with an amorphous layer known as tegument, consisting mostly viral proteins; which layer in turn is encased in a lipid bilayer envelope bearing distinct viral glycoproteins. Upon entry of the virus into host cells, some tegument proteins are released and the capsid is transported to the host nuclear membrane to deliver viral DNA into the nucleus. Transcription, replication of the viral genome, and assembly of the capsid take place in the nucleus (Boehmer and Nimonkar, 2003; Heming et al., 2017). The mature nucleocapsid egresses to the cytoplasm where it acquires tegument and envelope. The mature virions are transported through vesicles and finally released into the extracellular space. Alternatively, herpesviruses can maintain a latent state during which the viral genome is mostly silent and maintained as an episome in the nucleus with no progeny produced. The latent genome can be reactivated to initiate lytic replication upon certain cellular stress cues (Speck and Ganem, 2010; Cohen, 2020). The innate immune system is expected to recognize the components of the herpesvirus viral particles and replication intermediates produced during infection. Besides viral DNA, leaked mtDNA as a result of cellular stress from viral infection can also be sensed by DNA sensors such as cGAS (Paludan et al., 2011; Reinert et al., 2016; Sun et al., 2019).

### TLR9

Toll-like receptors are the first characterized PRRs shown to recognize herpesviral PAMPs, including viral proteins, DNA and RNA. Among the TLRs, TLR2 detects virion components (Boehme et al., 2006; Leoni et al., 2012; Cai et al., 2013), while TLR3 and 9 recognize herpesvirus nucleic acid (Paludan et al., 2011; West et al., 2012). Specifically, TLR3 senses dsRNA (Iwakiri et al., 2009) and TLR9 detects endosomal dsDNA containing un-methylated CpG motifs that are commonly found in the herpesviral genome (Lund et al., 2003; Fiola et al., 2010). After DNA sensing, TLR9 recruits the adaptor protein MyD88 and induces the activation of Nuclear Factor κB (NF-κB) and Interferon Regulatory Factor (IRF)7, leading to the production of IFN-α in plasmacytoid Dendritic Cells (pDCs) (Kawai and Akira, 2011) (**Figure 1**). Infections from HSV-1, HSV-2, KSHV, and EBV have been shown to stimulate TLR9-mediated production of type I IFN in pDCs (Lund et al., 2003; Krug et al., 2004; Fiola et al., 2010; West et al., 2011; Ma and He, 2014). Infection from HSV-2 also induces a TLR9-mediated type III IFN response in DCs that relies more on NF-κB rather than IRFs (Iversen et al., 2010). HSV-induced activation of TLR9-mediated innate immune response is apparently cell-type-specific (Rasmussen et al., 2007). Krug et al. have shown that knockout of TLR9 causes no dramatic enhancement of HSV infection in mice (Krug et al., 2004), whereas another study reported that TLR9 expression in the trigeminal ganglia was required to prevent HSV encephalitis induced by intranasal HSV-1 infection, as TLR9 deficient mice were more susceptible to the virus, with 60% mortality (Lima et al., 2010).

### cGAS

The cyclic GMP–AMP synthase (cGAS) is the most recently discovered DNA sensor (Sun et al., 2013). It senses double-stranded DNA in a length-dependent but sequence-independent manner (Andreeva et al., 2017; Luecke et al., 2017). Binding to dsDNA leads to the formation of phase-separated liquid droplets in which cGAS is activated, and catalyzes the synthesis of cyclic GMP–AMP (cGAMP) from ATP and GTP (Du and Chen, 2018). As a second messenger, cGAMP activates ER-bound adaptor protein STING (STimulator of Interferon Genes) in the same cells or neighboring cells. Activated STING recruits kinase TBK1 and activates transcriptional factor IRF3 to initiate a downstream signaling cascade that culminates the expression of immune and inflammatory genes, such as type I IFNs (Tanaka and Chen, 2012) (**Figure 1**). Previously, it was thought that the cytoplasmic confinement of cGAS enabled it to specifically

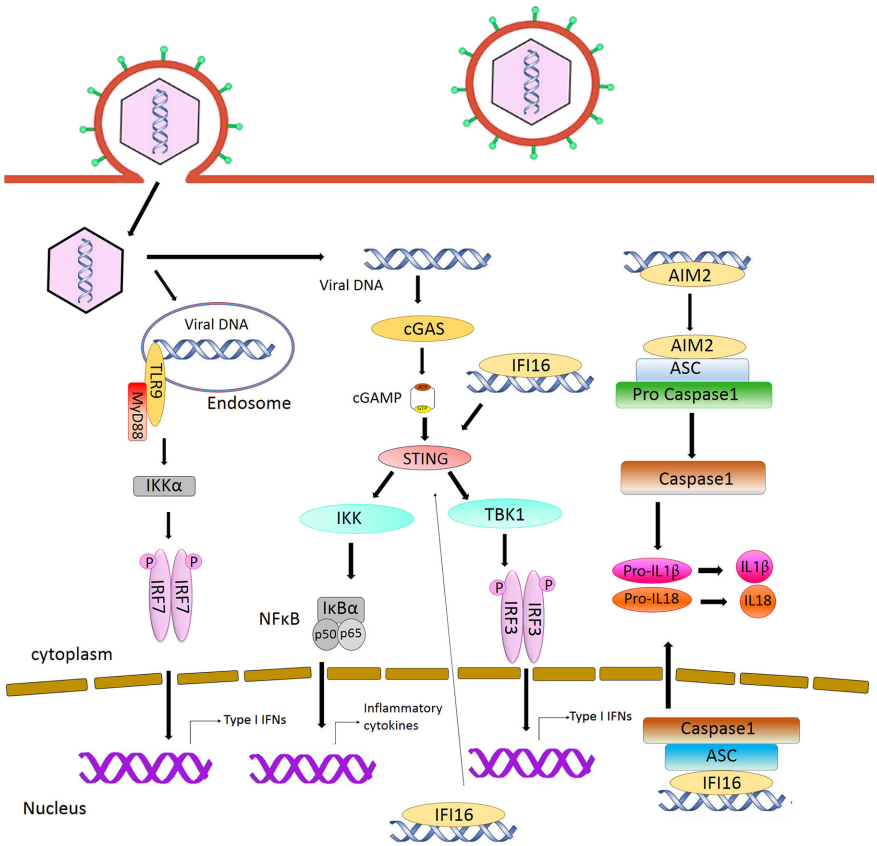


**FIGURE 1** | Intracellular sensing of herpesvirus DNA by DNA sensors and activation of signaling pathway. CpG DNA in the endosome is sensed by TLR9, leading to the production of type I interferon. AIM2 and IFI16 detect DNA in the cytoplasm and nucleus respectively and activate inflammasome pathway. Detection of DNA by cGAS activates STING signaling, resulting in the induction of type I interferon.

recognize pathogen- or damage-associated DNA. However, recent works revealed that cGAS is predominantly nuclear and its tight tethering to the chromatin is important in preventing its auto-reactivity (Volkman et al., 2019; Zierhut et al., 2019; Boyer et al., 2020; Kujirai et al., 2020; Michalski et al., 2020; Pathare et al., 2020; Zhao et al., 2020). The cGAS-cGAMP-STING pathway is crucial for host response to herpesviral infection. Sensing of HSV-1 DNA by microglia in the brain, and in PMA-differentiated macrophage-like THP1, induces type I IFN in a cGAS-STING dependent manner (Reinert et al., 2016), and cGAS deficient mice were found to be more susceptible to HSV-1 infection (Li X. D. et al., 2013; Christensen et al., 2016). Infection with HCMV was reported to activate the cGAS-STING pathway in primary Human Umbilical Vein Endothelial Cells (HUVEC) and in monocytic leukemia cell line THP-1 (Lio et al., 2016; Paijo et al., 2016). Although it has been reported that KSHV infection is also sensed by the cGAS-STING pathway and this pathway regulates the reactivation of KSHV from latency (Ma et al., 2015; Zhang et al., 2016), a recent report has shown that STING signaling is not critical in KSHV latent infection, replication, or in its spread after lytic reactivation in endothelial cells (Vogt et al., 2020).

## AIM2

Absent in melanoma 2 (AIM2) is a cytosolic DNA sensor that belongs to the PYHIN protein family. It contains an N-terminal Pyrin domain and one C-terminal HIN domain. The HIN domain binds to DNA through electrostatic interaction and the Pyrin domain associates with the adaptor protein ASC (apoptosis-associated speck-like protein containing a caspase activation and recruitment domain) through a homotypic PYRIN : PYRIN interaction. ASC further recruits pro caspase-1, leading to the production of active caspase-1. Caspase-1 in turn causes proteolytic maturation of the proinflammatory cytokines interleukin IL1β and IL18 (Hornung et al., 2009) (**Figure 1**). It has been shown that HCMV triggers the assembly of AIM2 inflammasome in THP-1-derived macrophages (Huang et al., 2017a; Botto et al., 2019). HSV-1 also induces AIM2-dependent inflammasome activation and IL-1β secretion in the absence of tegument protein VP22 in macrophages (Maruzuru et al., 2018).

## IFI16

Another member of the PYHIN family, IFI16, recognizes double-stranded DNA both in the cytosol and in the nucleus

(Unterholzner et al., 2010). It contains an N-terminal Pyrin domain and two HIN domains in the C-terminus. The interaction with DNA takes place through the HIN domains. Similar to cGAS and AIM2, IFI16 also recognizes DNA in a length-dependent manner (Stratmann et al., 2015). IFI16 primarily localizes in the nucleus, where the DNA genome of large DNA virus resides and replicates. Kerur et al. showed for the first time that IFI16 acts as a nuclear DNA sensor of herpesvirus infection. Nuclear sensing of KSHV and EBV by IFI16 results in inflammasome responses through ASC and caspase-1, leading to the production of IL1β and IL18 (Kerur et al., 2011; Ansari et al., 2013; Johnson et al., 2013). IFI16 appears to interfere with HSV-1 replication. It has been shown that IFI16 binds to the HSV-1 genome at the transcription start sites of several viral genes, and blocks the recruitment of crucial cellular transcription factors to the promoters (Johnson et al., 2014). IFI16 was also found to be crucial for the maintenance of EBV latency (Pisano et al., 2017). It has been reported that IFI16 forms filamentous structure on DNA in the nucleus to promote the epigenetic silencing of viral DNA (Merkl and Knipe, 2019; Roy et al., 2019). Although IFI16 is predominantly nuclear, it can also detect DNA in the cytosol. During HSV-1 infection, IFI16 has been found to co-localize with viral genomic DNA in the cytoplasm. It is also probable that IFI16 binds to the viral genome in the nucleus and interacts with histone acetyltransferase p300. Acetylation of IFI16 results in its cytoplasmic redistribution, where it interacts with STING and subsequently induces IRF-3 phosphorylation as well as interferon-β production (Ansari et al., 2015). The stability of IFI16 is enhanced in the presence of the principal DNA sensor cGAS, and depletion of cGAS results in reduced expression of IL-6 transcript in a STING-independent manner (Orzalli et al., 2015). Recent studies have shown that IFI16 and cGAS both function cooperatively for the full activation of innate immune response to exogenous DNA (Orzalli et al., 2015; Almine et al., 2017; Jønsson et al., 2017).

## Other DNA Sensors

The first reported cytosolic DNA sensor was DNA-dependent activator of IRFs (DAI) (Takaoka et al., 2007). It detects DNA in the cytosol and activates type I IFNs through NF-κB and IRF3. Type I interferon expression in HCMV-infected fibroblast cells was reported to depend on DAI (Defilippis et al., 2010), which was shown to interact with receptor-interacting protein kinase (RIP)3 to arbitrate virus-induced necrosis during MCHV infection (Upton et al., 2012). DExD/H-box helicases DHX36 and DHX9 are the two sensors that detect CpG DNA and activate an MyD88-dependent pathway in pDCs (Kim et al., 2010). However, the interferon response and inflammatory response only partially depend on DHX36 and DHX9 respectively, presumably because TLR9 is the main sensor for CpG DNA. Several proteins involved in DNA damage repair can also serve as DNA sensors (Mboko et al., 2012). Meiotic recombination 11 homolog A (MRE11) and DNA-dependent protein kinase (DNA-PK) belong to that group. MRE11 also recognizes dsDNA and induces type I interferon (Kondo et al., 2013). DNA-PK is a heterotrimeric protein complex consisting of three subunits: Ku70, Ku80, and the catalytic subunit DNA-PKcs (Ferguson et al., 2012). It can exert antiviral responses in a STING-dependent and a STING-independent manner (Burleigh et al., 2020). The protein was shown to act as a DNA sensor and mediate the induction of type III interferon (Zhang et al., 2011). During HSV infection, cytokine response is impaired both in mice and in individual cells that are deficient in DNA-PK (Ferguson et al., 2012). Interestingly, DNA-PKcs activity has been previously reported to be degraded in an Infected Cell Protein 0 (ICP0) dependent manner in some cell types during HSV infection (Lees-Miller et al., 1996; Parkinson et al., 1999). The role of DNA-PK in sensing other herpesviruses is less clear, although EBV has been shown to trigger a DNA damage response (DDR) during both primary infection and lytic reactivation (Hafez and Luftig, 2017; Hau and Tsao, 2017). It has been recently shown that DNA damage results in the translocation of the principal DNA sensor cGAS to the nucleus, where it suppresses DNA repair and enhances cell proliferation. Thus cGAS appears to exhibit dual functions: as an innate immune sensor in the cytosol and as a negative regulator of DNA repair in the nucleus (Liu et al., 2018; Jiang et al., 2019).

# EVASION OF DNA-STIMULATED IMMUNE RESPONSES BY HERPESVIRUSES

The DNA genome of herpesvirus is shielded within the viral capsid until it reaches the nucleus. The nuclear DNA sensors IFI16 and hnRNP-A2B1 may recognize the viral DNA in the nucleus and translocate to the cytoplasm to elicit the immune response (Diner et al., 2015; Knipe, 2015; Wang et al., 2019). On the other hand, a defective virion could leak DNA into the cytoplasm that could be sensed by the cytosolic DNA sensors. This second notion is supported by observations that mutations altering capsid stability result in leakage of viral DNA in the cytoplasm and robust DNA sensing responses (Horan et al., 2013; Sun et al., 2019). Herpesvirus infection also induces mtDNA stress, which triggers antiviral responses (West et al., 2015). In order to persist in the hosts, viruses must overcome host immune defenses. Herpesviruses have evolved delicate strategies to avoid the host immune recognition. Here we will discuss evasion strategies (**Table 3**) that target either DNA sensors or their adaptor proteins to counteract the nuclear and cytoplasmic DNA sensing.

## Direct Inhibition of the Enzymatic Activity of the DNA Sensor

Viruses encode factors that can directly target the host DNA sensors to block their activation. Unique among the known DNA sensors, cGAS has catalytic activity and transmits signal through its enzymatic product cGAMP. Conceivably, interfering with the production of cGAMP or destroying cGAMP could be effective viral evasion strategies. While poxviruses and baculoviruses encode Poxin, a nuclease that degrades cGAMP, herpesviruses encode factors that directly inhibit cGAS activity. KSHV tegument protein ORF52 (KicGAS) was the first discovered

**TABLE 3 |** Herpesvirus proteins that regulate DNA sensing and DNA activated signaling pathway.

| | PRR | Virus | Viral protein | Experimental system | Proposed Mechanism | References |
|---|---|---|---|---|---|---|
| Inhibition of DNA sensing | IFI16 | HSV-1 | ICP0 | human foreskin fibroblasts (HFF) | Promotes the 7 degradation of IFI16 | Orzalli et al., 2012 |
| | | | UL41 | HFF, Hela | Degrades the *IFI16* mRNA | Orzalli et al., 2016 |
| | | HCMV | pUL83 | HFF, human embryo kidney (HEK) 293T cell | Interacts with the IFI16 pyrin domain and blocks its oligomerization upon DNA sensing | Li T. et al., 2013 |
| | | | pUL97 | HELFs, HEK 293 cells | Binds to IFI16 and relocalizes it to the cytoplasm | Dell'oste et al., 2014 |
| | | KSHV | Lytic Proteins | BCBL-1 and BJAB cells | Promotes the ubiquitination and proteasomal degradation of IFI16 | Roy et al., 2016 |
| | cGAS | HSV-1 | UL37 | human monocyte THP-1 cells | Deamidates human and mouse cGAS | Zhang et al., 2018 |
| | | | UL41 | HEK293T | Selectively degrades cGAS mRNA | Su and Zheng, 2017 |
| | | | VP22 | HEK293T, Hela | Interacts with cGAS | Huang J. et al., 2018 |
| | | HCMV | UL31 | HEK293T | Interacts with cGAS and disassociates DNA from cGAS | Huang Z. F. et al., 2018b |
| | | | pUL83 | HFF, HEK 293 | Binds to cGAS and inhibits its enzymatic activity | Biolatti et al., 2018 |
| | | KSHV | LANA | BCBL-1 PEL cell | Binds to cGAS | Zhang et al., 2016 |
| | | | ORF52 | *In Vitro* enzymatic assay, HEK293T–STING cells | Interacts with cGAS and DNA | Wu et al., 2015 |
| | | EBV | ORF52 | | | |
| | AIM2 | HSV-1 | VP22 | 293FT cells, mouse macrophage cell line | Interacts with AIM2 and prevents its oligomerization | Maruzuru et al., 2018 |
| | | | pUL83 | HEK293T, macrophages | Interacts with AIM2 and inhibits activation of AIM2 inflammasome | Huang et al., 2017b |
| | DNA-pK | HSV-1 | ICP0 | HeLa S3 cells | Degrades the catalytic subunit of DNA-PK | Parkinson et al., 1999 |
| | | EBV | LMP1 | nasopharyngeal carcinoma (NPC) cell line | Inhibits DNA-PK phosphorylation and activity | Lu et al., 2016 |
| **Inhibition of DNA activated signaling pathway** | STING | HSV-1 | ICP0 | in HEp-2 cells | Stabilizes STING in HEp-2 cells, that is necessary for optimal HSV-1 replication | Kalamvoki and Roizman, 2014 |
| | | | ICP27 | THP 1 cells | Interacts with TBK1 and STING | Christensen et al., 2016 |
| | | | UL46 | HEK293T cells | Interacts with STING and TBK1 | Deschamps and Kalamvoki, 2017; You et al., 2019 |
| | | | $\gamma_1$34.5 | HFF | Binds to STING and perturbs its trafficking | Pan et al., 2018 |
| | | HCMV | UL42 | HFF cell, HEK293T STING cell | Interacts with both cGAS and STING, inhibits cGAS-DNA interaction, oligomerization and enzymatic activity of cGAS. Impairs translocation of STING from the ER to perinuclear punctate structures. | Fu et al., 2019 |
| | | | UL82 | HFF | Interacts with and STING and inhibits its translocation from the ER to perinuclear microsomes | Fu et al., 2017 |
| | | | pUL48 | HFF | Deubiquitinase STING | Kumari et al., 2017 |
| | | KSHV | vIRF1 | HEK293T STING cell | Prevents the phosphorylation and activation of STING by disrupting STING -TBK1 interaction | Ma et al., 2015 |

inhibitor of cGAS enzymatic activity. KicGAS is a positively charged small and abundant tegument protein that binds to DNA efficiently. Purified KicGAS protein was shown to inhibit cGAS activity in a dose-dependent manner. This inhibition depends on its ability to bind DNA and cGAS. The inhibition of cGAS by KicGAS can be partially overcome by increasing the amount of DNA in the reaction, suggesting KicGAS uses a mechanism involving competition with DNA (Wu et al., 2015). In addition to KicGAS homologues in gamma herpesviruses, HSV-1 VP22, which exhibits limited structural homology to KicGAS, has also been reported to inhibit cGAS. Although no *in vitro* experimental data were shown, we suspect similar mechanisms were involved (Hew et al., 2015; Huang J. et al., 2018). Cytoplasmic isoforms of KSHV LANA (latency-associated nuclear antigen) interact with cGAS directly and block downstream signaling (Zhang et al., 2016). Two HCMV proteins, UL31 and UL42, interact with cGAS and interfere with cGAS-DNA binding (**Figure 2**) (Huang Z. F. et al., 2018; Fu et al., 2019). Another HCMV tegument protein, pUL83, selectively binds to cGAS and inhibits its enzymatic activity.

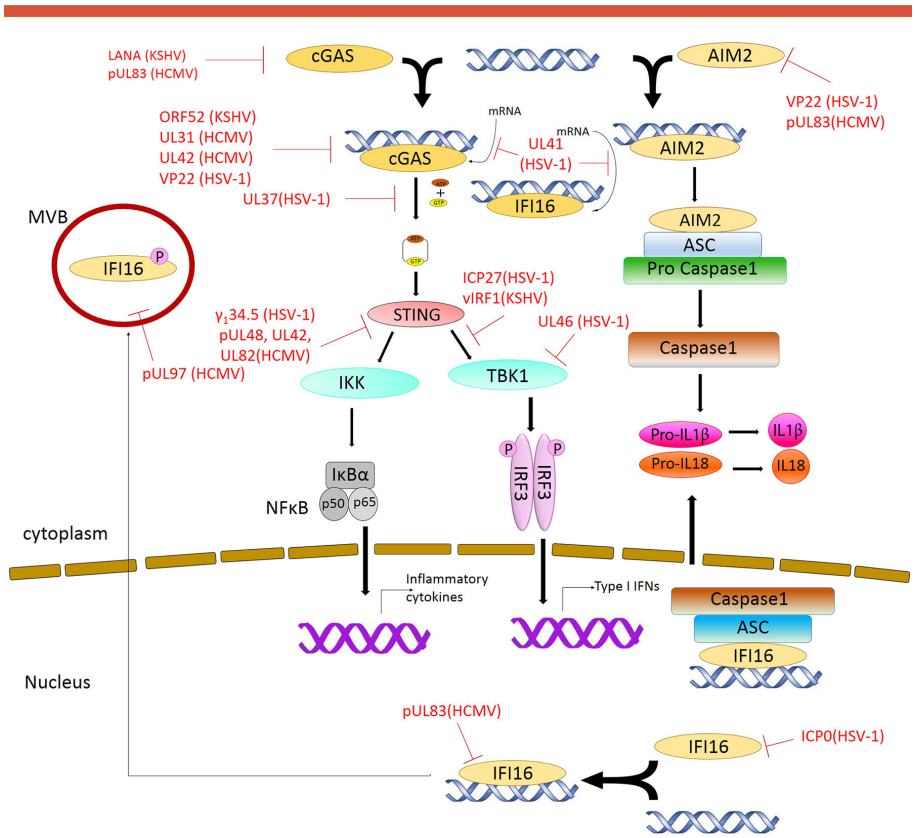


**FIGURE 2** | Modulation of DNA Sensing pathways by herpesvirus. Multiple steps in the signaling pathway are targeted by herpesvirus encoded proteins. Red solid line indicates inhibition of a particular pathway by the respective herpesvirus protein.

The N-terminal domain of pUL83 appears to play an important role in binding, and the interaction is independent of viral DNA (Biolatti et al., 2018).

## Masking of the DNA Sensors

Another strategy used by the herpesvirus is to mask the DNA sensor to prevent downstream signaling. The DNA sensors that were shown to be sequestered by herpesviral proteins are IFI16 and AIM2. In addition to inhibit the enzymatic activity of cGAS, HSV-1 VP22 was also shown to interact with AIM2; the association is mediated by the HIN domain of AIM2. This interaction prevents the oligomerization of AIM2 (Maruzuru et al., 2018), which is the first step of AIM2-dependent inflammasome activation. The HCMV abundant virion protein pUL83 interacts with AIM2 and IFI16 in the cytoplasm and nucleus respectively (**Figure 2**) (Li T et al., 2013; Huang et al., 2017b). Interaction between pUL83 and AIM2 reduces the expression of inflammosome proteins and decreases the cleavage of caspase-1 and maturation of IL-1β. The protein pUL83 contains an N-terminal conserved pyrin *a*ssociation *d*omain (PAD) that interacts with the Pyrin domain of IFI16. It inhibits the nuclear oligomerization of IFI16 by sequestering the pyrin domain through the combined action of its conserved N- and C-terminals. Such oligomerization is essential for IFI16-mediated DNA-dependent immune signaling.

## Degradation of the DNA Sensors

In order to prevent activation of the DNA sensors, herpesviral proteins may target them for degradation. The DNA sensors IFI16, DNA-PK, RNF8, and RNF168 were shown to be degraded during herpesvirus infection. The ring finger domain of HSV-1 ICP0 exhibits E3 ubiquitin ligase activity. The nuclear form of the ICP0 protein was shown to interact with IFI16, relocalize IFI16 to ICP0-containing foci in the nucleus, and promote the proteasomal degradation of IFI16, resulting in impaired sensing of HSV-1 genome in HFFs (Orzalli et al., 2012; Johnson et al., 2013) (**Figure 2**). However, another study showed that ICP0 was neither required nor sufficient to promote the loss of IFI16 in U2OS and HepaRG cells (Cuchet-Lourenço et al., 2013), suggesting that the ICP0-mediated degradation of IFI16 could be cell-type specific. Studies also found IFI16 to be degraded during KSHV lytic replication, likely through the action of late protein(s) (Roy et al., 2016). ICP0 was reported to promote the proteasomal degradation of DNA-PK, RNF8, and RNF168, and modulate the DDR response (Parkinson et al., 1999;

Lilley et al., 2011). The two ubiquitin ligases that anchor the repair factors at sites of damage (Parkinson et al., 1999; Chaurushiya et al., 2012).

In addition, viral proteins can also promote the selective degradation of host mRNA and reduce the level of the DNA sensors cGAS and IFI16. The HSV-1 tegument protein UL41 was shown to selectively degrade cGAS mRNA *via* its mRNA-specific RNase activity, reduce the level of cGAS, and downregulate cGAS STING-mediated signaling (Su and Zheng, 2017). UL41 was also reported to contribute to the reduction of IFI16 levels (Orzalli et al., 2016).

## Post-Translational Modification of the DNA Sensors

DNA sensing is also regulated by post-translational modifications including phosphorylation, ubiquitination, acetylation, sumoylation, and glutamylation of the DNA sensors (Wu and Li, 2020). Acetylation and phosphorylation of IFI16 at different sites have been shown to regulate its subcellular localization (Li et al., 2012). HCMV viral protein kinase pUL97 phosphorylates IFI16 and triggers its relocalization from nucleus to cytoplasm, away from the HCMV genome in the nucleus, thus inhibiting nuclear sensing of viral DNA (Dell'oste et al., 2014). However, ectopic expression of pUL97 alone was insufficient to relocate IFI16, suggesting additional HCMV components may be involved. HSV-1 tegument protein UL37 deamidates a critical Asp in the activation loop of human and mouse cGAS, resulting in impaired cGAMP synthesis (Zhang et al., 2018) (**Figure 2**). It was previously demonstrated that DNA binding leads to conformation change in the activation loop of cGAS that is required for its catalytic activity (Zhang et al., 2014). The deamination of Asp in the activation loop presumably blocks the conformational change. It is to be noted that this Asp is not conserved in non-human primates, thus HSV-1 mediates species specific inactivation of cGAS.

## Interfering With the Downstream Signaling Pathway

Most DNA sensors transmit signals through the adaptor protein STING, which possesses both interferon-dependent and interferon-independent immune responses (Wu et al., 2020; Yamashiro et al., 2020). After activation, STING translocates from the ER to endosomal/lysosomal perinuclear regions where it associates with the kinase TBK1 (Ishikawa and Barber, 2008). This interaction mediates the activation of transcription factors IRF3 and (NF-κB), and promotes the expression of immune and inflammatory genes, such as type I IFNs (Tanaka and Chen, 2012). In order to antagonize the STING-mediated immune response, herpesvirus encodes a number of proteins that directly target STING for deubiquitination, perturb its intracellular trafficking, or block its interaction with other signaling partners (Ahn and Barber, 2019; Liu et al., 2019; Yang et al., 2019). HSV-1 protein pUL36 directly associates with and deubiquitinate STING and perturbs its downstream signaling (Bodda et al., 2020). HCMV protein pUL48 was also reported to deubiquitinate STING (Kumari et al., 2017). The HSV-1 protein $\gamma_1$34.5, and HCMV tegument proteins UL42 and UL82, were shown to impair STING trafficking from the ER to the Golgi apparatus. Protein UL82 interacts with STING and ER-associated protein iRhom2, and disrupts the iRhom2-mediated formation of the STING-TRAPβ translocon complex (Fu et al., 2017). On the other hand, UL42 does not interact with iRhom2. It promotes the degradation of TRAPβ and inhibit STING trafficking (Fu et al., 2019). But how HSV-1 $\gamma_1$34.5 blocks STING signaling is not clearly understood (Pan et al., 2018). The proteins vIRF1 of KSHV and ICP27 and UL46 of HSV-1, were shown to disrupt STING-TBK1 interaction. Interaction between vIRF1 and STING inhibits the STING-mediated phosphorylation of TBK1 (Ma et al., 2015). But ICP27 interacts with the STING-TBK1 signalosome and inhibits the phosphorylation of IRF3 by TBK1 (Christensen et al., 2016). In addition, UL46 interacts with STING and TBK1 *via* its N- and C-termini respectively and impairs the activation of IRF3 by inhibiting the dimerization of TBK1 (Deschamps and Kalamvoki, 2017; You et al., 2019). Interestingly, ICP0 of HSV-1 was found to stabilize STING in certain cell types such as HEp-2 cells, as it is needed for optimal HSV-1 infection in those cells (Kalamvoki and Roizman, 2014). In addition, herpesviral proteins also target STING interaction partners to attenuate innate immune response downstream of STING (Christensen and Paludan, 2017; Liu et al., 2019). Thus, herpesvirus evolved mechanisms targeting various steps of DNA sensing to block immune response.

# CONCLUSION AND PERSPECTIVES

With the discovery of a number of DNA sensors over the past decade, significant progress has been made on understanding the host innate immune response to herpesviruses. Along with this, our understanding on how herpesviruses target different steps of this signaling pathway to establish persistent infection has greatly expanded. However, there are still several key questions that need to be answered. First, if most of the DNA sensors recognize DNA in sequence independent manner, then what is the need of so many DNA sensing pathways, and what are their relative contributions to *in vivo* DNA sensing? One possibility is that the presence of multiple DNA sensing pathways in different cellular compartments may provide multiple opportunities for the innate immune recognition of aberrant DNA (Emming and Schroder, 2019). Different DNA sensors may require different ligand (DNA concentration) thresholds for their activation. So, it may be helpful for the cell to fine-tune the immune response based on the escalating level of danger imposed by viral infection. It remains a major challenge to understand how these pathways act in concert to detect and elicit cell-type–specific or species-specific responses to DNA. Second, nuclear sensors are hypothesized to bind to unchromatinized viral DNA to initiate innate signaling, but viral DNA is chromatinized upon entry into the nucleus. Therefore, the process of nuclear DNA sensing and the time frame between entry and the chromatinizaton of viral DNA is crucial and needs further investigation. Future efforts are necessary to better

understand how nuclear-originating immune signaling is transmitted to the cytosol and back to the nucleus. Recently it has been shown that the dsDNA sensor heterogeneous nuclear ribonucleoprotein A2B1 (hnRNP-A2B1) recognizes viral dsDNA in the nucleus, then dimerizes and translocates to the cytosol to initiate type 1 interferon response *via* TBK1-IRF3 pathway (Wang et al., 2019). Further studies on the molecular mechanism by which the host nuclear DNA sensors such as hnRNP-A2B1 discriminate viral DNA from self genomic DNA will be critical for understanding nuclear DNA sensing. Third, a number of immune evasion strategies have been discovered recently, but the detailed mechanisms remain to be elucidated in most cases. Better understanding of the viral immune evasion mechanisms should aid in the development of vaccines and antivirals against herpesviruses.

In addition, herpesviruses can cause long-lasting infection as a result of the mutualistic equilibrium between the ability of the virus to survive under host antiviral immunity and the ability of the host to tolerate the presence of the virus continuously (Cruz-Muñoz and Fuentes-Pananá, 2018). The mutualistic equilibrium is not always beneficial to the host. It may lead to disease characterized by the abnormality in the immune system. Moreover, there is a significant overlap between the PRRs that sense herpesvirus and the PRRs involved in autoimmune disease. Further investigation in this area is necessary to identify the missing link between herpesvirus and autoimmune disease that could be useful to reduce unwanted inflammation.

## AUTHOR CONTRIBUTIONS

DB and FZ wrote the manuscript and designed the figures. All authors contributed to the article and approved the submitted version.

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REVIEW
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# Targeting Host Cellular Factors as a Strategy of Therapeutic Intervention for Herpesvirus Infections

*Kumari Asha and Neelam Sharma-Walia* *

*H. M. Bligh Cancer Research Laboratories, Department of Microbiology and Immunology, Chicago Medical School, Rosalind Franklin University of Medicine and Science, North Chicago, IL, United States*

Herpesviruses utilize various host factors to establish latent infection, survival, and spread disease in the host. These factors include host cellular machinery, host proteins, gene expression, multiple transcription factors, cellular signal pathways, immune cell activation, transcription factors, cytokines, angiogenesis, invasion, and factors promoting metastasis. The knowledge and understanding of host genes, protein products, and biochemical pathways lead to discovering safe and effective antivirals to prevent viral reactivation and spread infection. Here, we focus on the contribution of pro-inflammatory, anti-inflammatory, and resolution lipid metabolites of the arachidonic acid (AA) pathway in the lifecycle of herpesvirus infections. We discuss how various herpesviruses utilize these lipid pathways to their advantage and how we target them to combat herpesvirus infection. We also summarize recent development in anti-herpesvirus therapeutics and new strategies proposed or under clinical trials. These anti-herpesvirus therapeutics include inhibitors blocking viral life cycle events, engineered anticancer agents, epigenome influencing factors, immunomodulators, and therapeutic compounds from natural extracts.

**Keywords: herpesvirus, latency, lytic, arachidonic acid, antiviral**

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***Correspondence:**
*Neelam Sharma-Walia*
*neelam.sharma-walia@rosalindfranklin.edu*

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## INTRODUCTION

Herpesviruses are divided into three groups: The α-herpesviruses (herpes simplex virus types; HSV-1 and -2 or HHV-1 and HHV-2), and varicella-zoster virus (VZV, or HHV-3), β-herpesviruses as human cytomegalovirus (HCMV, or HHV-5), human herpesviruses 6 and 7 (HHV-6 and HHV-7), and γ-herpesviruses Epstein-Barr virus (EBV, or HHV-4) and Kaposi's sarcoma herpesvirus

**Abbreviations:** AhR, Aryl hydrocarbon receptor; ARNT, AhR nuclear translocator; COX, cyclooxygenase; CCR, chemokine receptors; ChIP, chromatin immune-precipitation; CTL, cytotoxic T lymphocytes; EBV, Epstein-Barr virus; EZH2, enhancer of zeste homolog 2; GMCSF, granulocyte-macrophage colony-stimulating factor; HCMV, human cytomegalovirus; HDM, Histone demethylase; HMT, histone methyltransferase; HSV, herpes simplex virus; ICAM-1, intercellular adhesion molecule 1; KAP1, KRAB-associated protein 1; KICS, KSHV inflammatory cytokines syndrome; KSHV, Kaposi's sarcoma herpesvirus; LO, lipoxygenase; mTOR, mammalian target of rapamycin; NGS, next-generation sequencing; NSAIDs, nonsteroidal anti-inflammatory drugs; PD-1, programmed cell death protein-1; PDL-1, programmed cell death ligand-1; PEL, primary effusion lymphoma PG, prostaglandin; PRC2, polycomb repressive complex 2; RTA, replication and transcription activator; SOCS, suppressor of cytokine signaling; TME, Tumor microenvironment; VZV, Varicella Zoster virus.

(KSHV, or HHV-8). HSV-1 and VZV establish latency in sensory ganglions (Zerboni et al., 2013), and their reactivation from latency results in replication and shedding of infectious virus ready for transmission to the naive population. The α-herpesviruses HSV -1 and -2 infect a broad range of both human and non-human cells including fibroblasts, epithelial cells, and neurons (Spear and Longnecker, 2003). VZV is a both lymphotropic and neurotropic human-specific virus with highly restricted infection in other species (Zerboni et al., 2014; Oliver et al., 2016). VZV infects human neurons, skin cells, T cells, glial cells, and infiltrating macrophages (Zerboni et al., 2014; Oliver et al., 2016). β- (HCMV, HHV-6A, HHV-6B, and HHV-7) and γ- (EBV and KSHV) herpesviruses establish latent infection in cells of the immune system, differentiated lymphoid and myeloid immune cells, epithelial, and fibroblast cell (Cruz-Muñoz and Fuentes-Pananá, 2018). KSHV can infect multiple cell types, such as B-lymphocytes, lymphatic endothelial, vascular endothelial, and epithelial cells (Ganem, 2007). Latent herpesvirus infections and their reactivation during the immune suppression pose a significant risk in developing inflammatory diseases and cancer in humans (White et al., 2012). HSV-1 and HSV-2, and their association with cancer occurrence in humans are controversial (Flaitz et al., 1995; Jain, 2016). β herpesvirus HCMV is a cofactor involved in the etiology of inflammatory bowel diseases (IBDs), psoriatic plaques, rheumatoid arthritis, systemic lupus erythematosus (SLE), and Sjögrens syndrome (SS) (Britt, 2008). HCMV infection is prevalent in cancers of breast, colon, prostate, and brain (Cobbs et al., 2002; Harkins et al., 2002; Rahbar et al., 2003; Soderberg-Naucler, 2006; Herbein and Kumar, 2014). Clinical manifestation of primary infection of HHV-6 includes roseola infantum, a benign febrile exanthem of infancy in approximately 20% of the infected children (Stone et al., 2014). Unlike α- and β-herpesviruses, γ-herpesviruses EBV and KSHV are oncogenic as they can induce cancer in natural (human) or experimental hosts (Damania, 2004). EBV is primarily found in the tumor cells of Burkitt's lymphoma (BL), lymphomas associated with immunosuppression, other non-Hodgkin's lymphomas (NHL), Hodgkin's disease, nasopharyngeal carcinoma (NPC), gastric adenocarcinoma, lymphoepithelioma-like carcinomas, post-transplant lymphoproliferative disorder (PTLD), nasal angiocentric T/NK-cell lymphoma, natural killer (NK)/T-cell lymphoma, and immunodeficiency-related leiomyosarcoma (Hsu and Glaser, 2000; Ambinder, 2003). γ-herpesvirus KSHV is etiologically associated with Kaposi's sarcoma (KS), B cell lymphoproliferative primary effusion lymphoma (PEL), multicentric Castleman's disease (MCD), and KICS (KSHV inflammatory cytokines syndrome) (Cesarman et al., 1995; Soulier et al., 1995; Karass et al., 2017).

Herpesviruses maintain a long-term evolutionary relationship with their host and stay latent in the host for their lifetime. Herpesviruses manipulate their host to hide, survive, replicate, produce new viral particles, and spread the viral infection to the uninfected host cells (Brinkmann et al., 2003; Wang et al., 2003; Feire et al., 2004; Wang et al., 2005; Sharma-Walia et al., 2010; Jung and Münz, 2015; Cavallin et al., 2018; Singh et al., 2018; Ayers et al., 2018; McNamara et al., 2019). Host cells are susceptible to productive or latent infection (Naranatt et al., 2004; Sharma-Walia et al., 2010; Grinde, 2013; Botto et al., 2017; Cavallin et al., 2018; Choi et al., 2018; McNamara et al., 2019). Host provides factors required for each step of herpesvirus complex life cycle that begins with its binding to the host cell and followed by multiple steps such as entry, uncoating, nucleic acid synthesis, gene expression, and viral protein synthesis (Naranatt et al., 2004; Sharma-Walia et al., 2010; Grinde, 2013; Botto et al., 2017; Cavallin et al., 2018; Choi et al., 2018; Singh et al., 2018; McNamara et al., 2019). In order to accomplish its survival and spread to uninfected cells, virus ends up utilizing host cell machinery for viral replication, gene transcription, infected cell division, and proliferation (Brinkmann et al., 2003; Naranatt et al., 2004; Sharma-Walia et al., 2010; Grinde, 2013; Jung and Münz, 2015; Botto et al., 2017; Cavallin et al., 2018; Choi et al., 2018; Singh et al., 2018; McNamara et al., 2019). Host factors include proteins of signaling cascades, transcription factors, cell survival pathways, angiogenic and growth factors, matrix metalloproteases, cell cycle kinases, cell death pathways, autophagy proteins, translation machinery, and inflammatory response pathways, immune evasion factors, chromatin remodeling, and metabolic pathways (Brinkmann et al., 2003; Naranatt et al., 2004; Sharma-Walia et al., 2010; Grinde, 2013; Jung and Münz, 2015; Botto et al., 2017; Ayers et al., 2018; Cavallin et al., 2018; Choi et al., 2018; Singh et al., 2018; McNamara et al., 2019).

Here, we focus only on the role of pro-inflammatory, anti-inflammatory, and resolution lipid metabolites of the arachidonic acid (AA) pathway in the lifecycle of herpesvirus infections. To date, cytotoxic systemic chemotherapies developed for non-virus-associated cancers are widely implemented for the treatment of herpesvirus associated cancers. These lesser effective treatment methods target DNA replication of all dividing cells and thus possess multiple side effects, particularly in immunocompromised patients and neonates. There is significant interest in developing new antiviral drugs targeting viral binding, entry, uncoating, viral nucleic acid synthesis, replication, gene expression, and viral protein synthesis. Here, we discuss recent development in the antiviral therapies targeting multistep lifecycle events of the viral life cycle, gene regulation, latency-lytic switch inducers, immunomodulators, and natural therapeutic compounds (**Figure 1**).

## Strategies to Combat Herpesviral Infection

### Targeting Host Factors Involved in Multistep Herpesvirus Life Cycle

The herpesviruses enter host cells *via* a multistep process, which begins with a coordinated attachment of the virus to the host cell surface followed by interaction with specific binding and entry receptor(s), and subsequent induction of host signaling pathways to facilitate virus entry (Nemerow et al., 1985; Li et al., 1997; Haan et al., 2000; Speck et al., 2000; Spear and Longnecker, 2003; Wang et al., 2003; Feire et al., 2004; Wang et al., 2005; Chandran, 2010; Hahn et al., 2012; Hensler et al., 2014; Chen et al., 2019). Different viral envelope glycoproteins mediate herpesvirus entry

into the host cell through either membrane fusion or receptor-mediated endocytosis (Nemerow et al., 1985; Tanner et al., 1987; Ligas and Johnson, 1988; Miller and Hutt-Fletcher, 1992; Turner et al., 1998; Muggeridge, 2000; Pertel, 2002; Feire et al., 2004; Naranatt et al., 2004; Compton, 2004; Avitabile et al., 2009; Chandran, 2010). Blocking herpesvirus binding, fusion, entry, and host signaling pathways is an attractive antiviral strategy to suppress viral infectivity (**Table 1**).

Herpesvirus binding and entry into the host are followed by uncoating viral DNA from the capsid, delivering viral genome to the nucleus, sequential transcription, and translation of viral immediate-early, early, late genes, and DNA replication. Nucleoside analogs have been tested for antiviral activity as these inhibit herpesviruses replication. Among all known inhibitors are nucleoside analogs, non-nucleoside analogs, helicase-primase inhibitor, and protein-protein interaction inhibitors that stop DNA replication, transcription, and translation (**Table 1**). Acyclovir, a synthetic nucleoside analog, has been efficiently used against HSV-1/2 and VZV-associated disease (**Table 1**). Ganciclovir, cidofovir, foscarnet, and letermovir have also been used successfully in patients with herpesvirus infection (**Table 1**). Inhibitors blocking virion assembly and egress of newly assembled herpesvirus particles from infected cells can be used effectively to treat herpes virus infections (**Table 1**). Cyclin-dependent kinases inhibitors that prevent proliferation in cancer cells are also used against herpesviruses (**Table 2**). These cellular protein kinase inhibitors play a crucial role in managing ER stress and active unfolded protein response (UPR) associated with various cancers and viral malignancies (Asha and Sharma-Walia, 2018).

## Epigenetic Targeted Therapy

Recent studies focusing on herpesvirus infections identifying viral proteins regulating histone-modifying enzymes and regulating the transcriptional machinery have brought revolution in the development of antiviral epigenetic modifiers (Kristie, 2012) (**Table 1**). Histone deacetylation and methylation are associated with condensed chromatin (heterochromatin) and accompany transcription inhibition, whereas histone acetylation and demethylation do the reverse. For many of herpesviruses,

**TABLE 1** | Inhibitors targeting various stages of herpesvirus life cycle.

| Drugs | Virus | Life cycle events |
|---|---|---|
| Compound SP-510-50 | HSV-1 | Virus binding or entry inhibitors |
| **PI3K inhibitor:** | | |
| Idelalisib | HSV-2, HCMV | |
| NVP-BEZ235 | KSHV, EBV | |
| **Dermaseptins** | HSV-1, HSV-2, EBV | |
| **Endocytosis inhibitor:** Chlorpromazine | KSHV, EBV | |
| **anti-gHgL antibodies:** CL40 and CL59 | EBV | |
| **Amantadine analog:** Tromantadine | EBV | |
| **Saturated fatty alcohol:** Docosanol | HSV-1 | |
| **Epigenetic therapy** | | |
| **DNMT inhibitors:** 5-azacytidine, RG108 | HSV-1, HSV-2, HCMV, VZV, | Repression of viral immediate early gene expression. |
| **HDAC inhibitors:** MC1568, Sodium butyrate, | EBV, KSHV | Production of infectious progeny in quiescently infected cells. Latency reversal |
| Trichostatin A (TSA), Vorinostat (SAHA), Valproic Acid | HSV-1, HCMV | Significant activation of the lytic transcriptional program in HCMV |
| **HMT inhibitors:** DZNep (EZH2i) | HSV-1 | Blockage of lytic HSV-1 replication in latently infected ganglion explant model |
| GSK126, GSK343 | HSV-1 | Repression of viral IE gene expression |
| **HDM inhibitors:** ML342, DMOG, OG-L002, TCP | | |
| **Nucleoside analogs** | | |
| Trifluridine, Edoxudine, Brivudine, Cytarabine, | HSV-1, HSV-2, VZV, HCMV, | DNA synthesis inhibitor. These drugs competitively stop the incorporation of |
| Cidofovir, Acyclovir | EBV, HHV-6, HHV-7, KSHV | dNTPs by the viral DNA polymerase and thereby end the elongation of viral DNA |
| Ganciclovir, Penciclovir, Virdarabine, Indoxuridine | | |
| **Non-nucleoside inhibitors:** | | |
| Foscarnet | HSV-1, HSV-2, VZV, HCMV | DNA synthesis inhibitors. |
| PNU-183792 | HSV, VZV, HCMV | |
| NSC 373989 | KSHV | |
| **Helicase-primase inhibitor:** | | |
| BAY 57-1293 | HSV-1, HSV-2 | Inhibit progression of the |
| T-0902611 | HCMV | replication fork |
| BILS 179BS | HSV-1, HSV-2 | |
| **Protein-protein interaction inhibitor** | | |
| BILD1263 | HSV-1 | Inhibits viral replication |
| **Pyrazolo** | | |
| Quinoline, Benzothiophene | HSV-1 | Gene expression |
| **Phosphorothioate oligonucleotides** | HCMV, EBV, KSHV | |
| **Acridones, Thiourea inhibitors** | HSV, EBV | Assembly or egress |
| **Phenylenediamine:** Sulfonamides | HCMV, KSHV | |
| **Ribosylbenzimidazoles** | HCMV | |

**TABLE 2 |** Cellular protein kinase tested for anti-herpesvirus associated cancers.

| Drug | Virus | Primary targets | Ref |
|---|---|---|---|
| Chk2 inhibitor II | HSV | Chk2 | (Alekseev et al., 2015) |
| Roscovitine (seliciclib) | HSV, VZV, HCMV and EBV | CDKs | (Schang et al., 2002; Kudoh et al., 2004; Taylor et al., 2004; Shin et al., 2008) |
| Purvalanol A | HSV, VZV and EBV | | (Schang et al., 2002; Kudoh et al., 2004; Moffat et al., 2004) |
| Olomoucine II | HSV and HCMV | | (Moffat et al., 2004) |
| Indirubin-3'-monoxime | HCMV | GSK3β | (Hertel et al., 2007) |
| Torin1 | HSV and HCMV | mTOR | (Li and Hayward, 2013) |
| Everolimus | HCMV, EBV | | (Poglitsch et al., 2012; Rittà et al., 2015) |
| Letermovir | HCMV | Terminus inhibitor | (Gerna et al., 2019) |
| Maribavir | HCMV | UL97 viral protein kinase | (Papanicolaou et al., 2019) |
| KU55933 | HSV, HCMV and EBV | Ataxia telangiectasia mutated (ATM) | (Hagemeier et al., 2012; Costa et al., 2013; Alekseev et al., 2014) |
| TBB | HSV-1 and EBV | Casein kinase 2 (CK2) | (Medina-Palazon et al., 2007; Smith et al., 2011) |
| DMAT | | | |
| TMCB | | | |
| SP600125 | VZV | c-Jun N-terminal kinase (JNK) | (Zapata et al., 2007) |
| Gleevec (imatinib mesylate) | HCMV, KSHV | Inhibits multiple tyrosine kinases | (Soroceanu et al., 2008; Koon et al., 2014) |
| Sorafenib (BAY 43-9006) | | PDGFR and Raf kinases | (Michaelis et al., 2011) |
| STO-609 | | Calmodulin-dependent kinase kinase (CaMKK) | (McArdle et al., 2011; McArdle et al., 2012) |
| LY294002 | | PI3K | (Johnson et al., 2001a) |
| U-0126 | HCMV and EBV | MEK1/2 | (Johnson et al., 2001b; Goswami et al., 2012) |
| Flavopiridol (alvocidib) | EBV | CDKs | (Goswami et al., 2012) |
| Kenpaullone | | GSK3β, LcK | (Goswami et al., 2012) |
| Dasatinib | | Inhibits multiple tyrosine kinases | (Chakraborty et al., 2012; Goswami et al., 2012; Hahn et al., 2012) |
| K252A | | | |
| BAY 11-7082 | | Inhibitor of κB kinase (IKK) | (Goswami et al., 2012) |
| Wortmannin | | Phosphatidylinositol-3-kinase (PI3K) | (Goswami et al., 2012) |
| PCI-32765 | | Bruton's tyrosine kinase (Btk) | (Zocchi et al., 2018) |
| MLN8237 | | Aurora A kinase | (Iankov et al., 2015) |
| CCI-779 (temsirolimus) | | mTOR | (Kawada et al., 2014) |
| BI-D1870 | | p90 ribosomal S6 kinases (RSKs) | (Kuang et al., 2008) |
| SU5416 | | VEGFR tyrosine kinase | (Aoki and Tosato, 2004) |

viral DNA associates itself with histones as soon as it enters the cell, making it a vulnerable candidate to epigenetic modification (Reeves, 2011). These modifications are basic cellular defense mechanisms against viral gene expression and to prevent abortive infection. Eventually, at the onset of the lytic cycle during stress or treatment with histone modifier, the viral genome opens up from its repressed heterochromatin state to initiate the expression of immediate early lytic genes followed by productive infection (Woodhall et al., 2006). Arsenal of histone modifiers have been identified over the last decade, which play an important role(s) throughout the different viral life cycle stages (Kristie, 2012). Histone modifiers include writers (the enzymes that make the chemical modifications), readers (proteins that detect and respond to the chemical modifications), and erasers (enzymes that remove these chemical groups). DNA methyltransferases (DNMTs), histone methyltransferases (HMTs), and histone acetyltransferases (HATs) are writers. Readers include chromatin remodeler complex SWItch/Sucrose Non-Fermentable (SWI/SNF), the bromodomain (BRD), and extra terminal domain family of adaptor proteins (BET). Erasers are DNA-demethylating enzyme histone demethylase (HDM) and histone deacetylases (HDACs). Epigenetic therapy (inhibitors of DNMTs, HDAC, HMT, chromatin-modifying complex; The polycomb repressive complex 2 or PRC2, and enhancer of zeste homolog 2 or EZH2) has been well studied and demonstrated success in HSV-1, HSV-2, HCMV, EBV, and KSHV latency and viral HCMV-HSV, HIV-HCV, and HIV-HBV viral co infections (**Table 1**) (Schultz et al., 2002; Bain et al., 2003; Gwack et al., 2003; Wright et al., 2005; Rosenfeld et al., 2006; Abraham and Kulesza, 2013; Kumar and Herbein, 2014; Torres and Tang, 2014; Su et al., 2017; Hopcraft et al., 2018). Backed up by favorable results from both cell culture labs and mouse models, epigenetic drug candidates are clinically evaluated as promising antivirals. Despite the roadblocks in designing specific epigenome modifying drugs with few off-target effects, there are ongoing clinical trials to enhance safety using improved preclinical models. The combination of valganciclovir with Tractinostat (VRx-3996) is now being examined in EBV-related lymphoid malignancies. HDAC inhibitors such as benzamide MS275 or sodium butyrate (**Table 1**) might induce lytic gene expression and act as stimulators to antivirals like ganciclovir used for the cure of EBV-associated lymphomas (Ghosh et al., 2012). In another clinical study, DNMT inhibitor azacytidine (**Table 1**) initiated viral gene re-expression in EBV linked tumors (Chan et al., 2004). While these inhibitors are promising candidates as the therapeutics to control of herpesviral infections (**Figure 1**), the possibilities of reactivation and status of co-infection with other

viruses must be meticulously examined (Ritchie et al., 2009). For example, the usage of suberoylanilide hydroxamic acid (SAHA) or trichostatin A (TSA) (**Table 1**) provoked myocarditis through Coxsackievirus B3-induced myocardial apoptosis (Zhou et al., 2015).

An *in vivo* study showed that bortezomib (Btz) usage, a proteasome inhibitor, improved the survival in an immune-compromised xenograft mouse model of PEL that was treated with doxorubicin alone (Sarosiek et al., 2010). A combination of Btz and HDAC inhibitor, SAHA could effectively reactivate KSHV, thereby inducing PEL cell death and increasing survival in PEL-bearing mice, and strongly advocates using the proteasome/HDAC inhibitor combination therapy in PEL (Bhatt et al., 2013).

## Lytic Cycle Induction and Combination Therapies

Latency intervention is one of the well-known strategies to target herpes infection and control herpes virus associated cancers. Among all known herpesviruses, EBV is the only virus for which proteins associated with maintenance of latency have been best characterized and tested for the anti-latency approach. There have been attempts to target EBV nuclear antigen (EBNA1) and latent membrane protein 1 (LMP1) using antisense oligonucleotides or adenovirus vector-delivered ribozymes (Li et al., 2010). Cellular signaling kinase associated with the LMP2A pathway has been targeted to cure EBV infection (Li et al., 2010). However, more effective strategies against the virus could be unmasking of latently infected cells by inducing lytic reactivation and then explicitly targeting viral DNA replication. In recent work Rauwel et al., 2015, suggested that knocking down transcriptional corepressor Krüppel-associated Box-associated protein 1 (KAP1) or induction of KAP1 phosphorylation can force HCMV out of latency, and this process can be made possible by activating NF-kB with TNF-α. These results suggest new approaches both to limit HCMV infection and to eliminate the virus from organ transplants (Rauwel et al., 2015). In a similar study authors suggested that Chloroquine employs ataxia telangiectasia mutated (ATM) to phosphorylate the KAP1/TRIM28 at serine 824 to facilitate repair of double-stranded breaks in heterochromatin and triggers EBV replication (Li et al., 2017). Further studies proved that EBNA1 and LMP1 associated sumoylation plays a crucial role in the maintenance of EBV latency through KAP1 (Bentz et al., 2015). Therefore, $EBNA1^{SIM}$ motif can play a potential drug target against EBV-associated cancers (Wang et al., 2020). KSHV latency-associated nuclear antigen (LANA) interacts with the host protein, KAP1, and represses lytic gene expression to facilitate the establishment of KSHV latency (Sun et al., 2014; Zhang et al., 2014). Further studies proved that the LANA has an exclusive SUMO-interacting motif ($LANA^{SIM}$), which plays an indispensable role and thus can play a potential drug target against KSHV-associated cancers (Cai et al., 2013).

Reactivation from latency is vital for developing therapies to fight or eliminate herpes-associated cancers, and this strategy is successful against herpes infection in HIV-positive patients. One of the most talked-about methods has been "*lytic induction therapy*". This therapy is based on the theory of synergistic usage of antiviral drugs and the stimulation of viral reactivation. This approach was successful against PEL growth in mouse xenograft models (Feng et al., 2004; Fu et al., 2008; Bhatt et al., 2013; Zhou et al., 2017). This method has been extensively investigated for KSHV and EBV. *Ex vivo* studies have established romidepsin as an effective inhibitor that works against lymphoproliferative diseases (Smolewski and Robak, 2017) and is a better agent for viral reactivation (Wei et al., 2014) than other HDAC inhibitors. Likewise, a recent study from our lab has shown anti-inflammatory lipoxin A4 (LXA4) as a promising candidate for lytic induction therapy (Asha et al., 2020). LXA4 treatment regulates KSHV reactivation and life cycle through chromatin modification (Asha et al., 2020) and the host's hedgehog signaling pathway (Asha et al., 2020).

The most recent strategies chosen to treat the herpesvirus is based on the concurrent induction of oncolysis by viral replication and reassertion of an immune response to viral lytic cycle antigens. For example, Oncolytic HSV (G47Δ) has shown its improved efficacy against NPC (Wang et al., 2011), glioma, breast cancer (Liu et al., 2005), and other fatalities and thus can be used in combination with immunotherapy and chemotherapy to treat various malignancies. Such combinational strategies can also be used against EBV-associated lymphoproliferative diseases. Ganciclovir (GCV) is a nucleoside analog antiviral drug that facilitates the killing of EBV-positive cancer cells when given lytic inducers. BGLF4, an EBV lytic protein kinase expressed during viral reactivation, can change GCV into its cytotoxic state (Feng et al., 2002), which gets integrated into viral and host DNA. Cytotoxic GCV induces termination of premature DNA to kill EBV-associated malignancies and causes apoptosis of the host cells (**Table 1**) (Feng et al., 2004; Feng and Kenney, 2006; Wang et al., 2010). Similarly, [125I]2′-fluoro-2′-deoxy-beta-D-5-iodouracilarabinofuranoside ([125I]FIAU) has been used against lytically induced EBV-positive BL cells (Fu et al., 2008). In NPC patients, the usage of valproic acid (**Table 1**) and gemcitabine as lytic inducers has shown effective clinical response generating moderate momentary toxicity (Wildeman et al., 2012). In a recent study, derivatives from biologically active alkaloid tetrahydrocarboline were found to reactivate EBV lytic markers Zta (ZEB replication activator, the product of BZLF1) and Early Diffuse Protein in all EBV-positive cell lines irrespective of their type of latency. Two of these derivatives have EC50 values in the range of 150–170 nM and showed low toxicity to EBV-negative cells. When combined with GCV, these small molecules were selectively cytotoxic to EBV-positive cells (Tikhmyanova et al., 2014).

## Therapeutic Compounds From Natural Extracts

Much research focuses on identifying new therapeutic compounds obtained from natural plant extracts with inhibitory activity against herpesviruses (**Table 3**). These natural plant extracts have proved effective against different

**TABLE 3** | Natural extracts effective against Herpesvirus infection.

| Plant | Active compound | Mode of action |
|---|---|---|
| *Spirulina platensis* | Sulfated polysaccharides Calcium spirulan | Viral entry (HSV-1, HCMV, VZV)<br>Inhibition of cytopathic effect<br>blocking HCMV attachment and penetration into host cells |
| *Allium sativum* | Organosulfur compound | Interferes with HSV adsorption and penetration |
| *Green tea* | *Epigallocatechin-3-gallate (EGCG)* | Anti-HSV-1<br>Inhibits lytic infection of EBV and KSHV |
| *Rhus javanica* | Moronic acid and Betulonic acid | Inhibits lytic infection of EBV |
| *Thelypteris torresiana* | *Protoapigenone*; flavonoid compound | Inhibits lytic infection of EBV |
| *Andrographis paniculata* | andrographolide | Inhibits active EBV virions |
| *Polygonum cuspidatum* | *Emodin* | Inhibits early steps of the EBV replication cycle |
| *Saururus chinensis* | Lignans<br>*Manassantin B* | Inhibits NF-kB<br>against active EBV infection |
| *Angelica archangelica* | *Angelicin* | Anti-HSV-1<br>Interferes with the initial lytic KSHV replication |
| *Marine natural product* | *Cytarabine* | Regression of PEL tumors in a xenograft mouse model. Degrades LANA-1, inhibit latent and lytic replication |
| *Garcinia* | Cambogin | Regression of PEL tumors in a xenograft mouse model |
| *Euphorbia pekinensis* | Euphorbia extracts | Oncolytic effect on EBV-positive gastric carcinoma cells |
| *Euphorbia peplus* | PEP005 | Inhibits PEL tumors in a xenograft model |
| *Allium sativum* | Allicin | Interferes with HCMV replication<br>Inhibits latent KSHV |

stages of viral infection including viral binding/entry, replication, and release. Extracts from Allium sativum has proved effective against viral adsorption and penetration (Rouf et al., 2020). *Epigallocatechin-3-gallate (EGCG)*, the active ingredient in green tea, has potential antiviral activity against HSV-1 (de Oliveira et al., 2013) and lytic infection of EBV (Chang et al., 2003; Liu et al., 2013) and KSHV (Xie et al., 2020). Herbal extract from *Rhus javanica*, a medicinal herb has two major anti-HSV compounds called moronic acid and betulonic acid (Kurokawa et al., 1999). Moronic acid inhibits EBV lytic cycle (Chang et al., 2010). *Protoapigenone*, a naturally occurring group of flavonoid compound obtained from *Thelypteris torresiana*, inhibits EBV lytic replication (Tung et al., 2011). The diterpenoid andrographolide present in *Andrographis paniculata*, a medicinal plant, has proven effective against active EBV virions (Lin et al., 2008). The anti-inflammatory and anti-immunostimulatory activities of the diterpenoid andrographolide were effective against inflammatory diseases, bacterial and viral infections (Wiart et al., 2005; Aromdee et al., 2011; Uttekar et al., 2012). *Emodin*, a significant component from *Polygonum cuspidatum*< intervenes with the early steps of the EBV replication cycle in a dose-dependent manner (Yiu et al., 2014). Emodin inhibits the activation of ERK, MAPK, and JNK signaling, and affect the activation of the promoters stimulated by transcription factor activator protein-1 (AP-1) and activating transcription factor 1 (ATF1) (Lee et al., 2006; Lin et al., 2007; Li et al., 2013). Lignans obtained from *Saururus chinensis* inhibits NF-kB (Hwang et al., 2003) and HIV protease (Lee et al., 2010). *Manassantin B*, another lignans fraction isolated from *Saururus chinensis*, works effectively against active EBV infection at significantly low toxicity (CC50 > 200 μM) dose (Cui et al., 2014). Likewise, *Angelicin*, a natural compound found in the roots of *Angelica archangelica*, inhibits the autoactivation of the EBV and KSHV RTA promoter and consequential interferes with the initial lytic viral replication (Cho et al., 2013) (**Table 3**). Through a high-throughput screening of characterized compounds, Gruffaz et al. showed that *cytarabine*, an FDA-approved compound, induced regression of PEL tumors in a xenograft mouse model. Interestingly, cytarabine degraded KSHV latency protein LANA-1, which is required for PEL cell survival (Gruffaz et al., 2018). Furthermore, cytarabine inhibited KSHV lytic replication program, preventing virion production. These findings suggest cytarabine to be a novel therapeutic agent for treating PEL as well as for eliminating KSHV persistent infection (Gruffaz et al., 2018) (**Table 3**). Cambogin, a bioactive natural product isolated from the *Garcinia genus* when used at nanomolar concentration, could reduce the PEL tumor in xenograft mouse model (Ding et al., 2019). Dong-Eun Kim et al. showed that treatment with *Euphorbia pekinensis* extract led to a selective oncolytic effect on EBV-positive gastric carcinoma cells, SNU-719 (Kim et al., 2018). *E. pekinensis*'s ability to induce lytic activity was mediated by PKC and MEK signaling (Kim et al., 2018) (**Table 3**). Likewise, the combination of FDA-approved drug ingenol-3-angelate (PEP005) with a BRD and BET protein inhibitor (JQ1) induced KSHV lytic replication and reduced IL6 production in the PEL model (Zhou et al., 2017). The combination of drug PEP005 and JQ1 inhibits PEL growth efficiently and interrupts tumor growth in a PEL xenograft tumor model (Zhou et al., 2017). PEP005 activates NF-κB pathway that primes the increased occupancy of RNA polymerase II onto the KSHV genome, thereby reactivating KSHV (Zhou et al., 2017). Allicin, and glycyrrhizic acid (GA) obtained from garlic and licorice respectively, had antiviral effects against latent KSHV infection (Xie et al., 2020) (**Table 3**).

## Immune Modulation

Extensive modification of the host immune system by herpesviruses has opened up new prospects for immunotherapy

as clinical intervention to effectively treat herpesvirus-associated malignancies. Recent studies have identified immune cells and blood cells as target of herpes infection (Hill et al., 2010; Quinn et al., 2016; Jones et al., 2019). Successful cancer immunotherapy involves immune effectors of both the innate (innate lymphoid, NK, T, and B cells) and adaptive immune systems to develop anticancer immunity. Immunotherapy includes cancer vaccines to prime and expand tumor-specific T cells with potent antitumor activity and immune checkpoint blockers to abrogate negative inhibitory signals that diminish T-cell activation (Vanpouille-Box et al., 2017). Inhibitory molecules overexpressed in many tumors that facilitate immune escape include cytotoxic T lymphocyte associated protein 4 (CTLA4), programmed cell death 1 (PDCD1 or PD1), and PD1 ligand CD274 (PD-L1) (Jinesh et al., 2017). Anti-PD1/PD-L1 is the most promising immunotherapy currently (**Figure 1**).

Opportunistic infection is still the main reason for mortality in allogeneic stem cell transplant recipients with active extensive chronic graft-*versus*-host disease. The toxicity of prolonged and recurring antiviral treatment along with occasional drug resistance account for major limitation to therapy. For the effective treatment, successful propagation of virus-specific donor-derived CD8+ CTLs is essential. CTLs are effective against VZV, HCMV, and HHV-6 infections in the immunocompromised patients (Kapp et al., 2007; Hill et al., 2010; Gerdemann et al., 2013; Becerra et al., 2014; Quinn et al., 2016; Jones et al., 2019). EBV-specific CTL immunotherapy has raised hopes of treating NPC effectively (Jain et al., 2016; Cao, 2017). EBV proteins such as LMP1, LMP2, and EBNA1 are used to develop vaccines that can be used as adjuvant therapy to avoid NPC relapse (Jain et al., 2016; Taylor and Steven, 2016). KSHV infected monocytes express high levels of PD-L1 (Host et al., 2017). NK cells obtained from KS patients frequently display higher levels of PD1 (Beldi-Ferchiou et al., 2016). Usage of immune checkpoint blockades such as nivolumab or pembrolizumab showed effective treatment against KS infection in HIV patients (Galanina et al., 2018; Uldrick et al., 2019). Pembrolizumab has successfully made through phase I clinical trial for patients living with HIV and KS and is in its phase II trial against KSHV (Uldrick et al., 2019). Aiming two distinct T cell inhibitory proteins simultaneously may end up in a more significant T cell function. Based on this theory, combination therapy of ipilimumab and nivolumab has been tried on classical KS patients (Lingel and Brunner-Weinzierl, 2019). The FDA approved Pomalidomide, after the phase I/II clinical is used in a new clinical trial in the endemic population of KS (Polizzotto et al., 2016). Pomalidomide treatment could reestablish MHC-I expression during the lytic replication and restore T cell costimulators (B7-2) in PEL cells (Davis et al., 2017). Blocking PD-L1 can effectively enhance virus specific CD8+ T cell effector functions (Jones et al., 2019).

In HSV-1 and HSV-2 infection, both humoral and cellular immune responses are specifically directed towards the surface glycoproteins gB and gD (Cairns et al., 2014). Animal studies on HSV-1 and HSV-2 demonstrated that humanized antibodies focused on these receptor proteins are prophylactically and therapeutically beneficial (Baron et al., 1976; Bravo et al., 1996). Rituximab, an anti-CD20 antibody, provides clinical benefit by inducing B cell death upon binding and is efficient against PEL and KSHV-MCD (Hoffmann et al., 2011; Kim et al., 2014). Ongoing clinical trials also use combinations of lenalidomide, chemotherapy and rituximab. Clinical trial of anti-IL6 antibodies was carried against MCD (Yu et al., 2017).

As an alternative, attempted antivirals can aim at controlling periodic infections by boosting immunity. For instance, resiquimod controls the innate immune response to prevent genital herpes infection (Govindan, 2014). Oncolytic virotherapy using genetically engineered replication competent viruses to destroy cancers is also an emerging treatment modality for various malignancies. The combination of oncolytic virotherapy with other possible immune-based therapies would prove beneficial for malignancies linked to viral infections (Zamarin and Wolchok, 2014).

## Targeting Inflammatory Membrane Lipid Pathways

Herpesvirus infections create a unique inflammatory microenvironment conducive to its latency and survival in the host. A few components of this microenvironment milieu are cytokines, chemokines, growth factors, and lipid pathway metabolites. Recent studies revealed that inflammatory cytokines activate phospholipases to induce the release of polyunsaturated fatty acids (PUFAs) from the cell membrane phospholipids. PUFAs generate bioactive lipids that augment the anti-cancer action of immunotherapy, prevent cytokine storm, and play an essential role in eliminating cancer cells. Herpesviruses utilize a PUFA called Arachidonic acid (AA), which is present in the phospholipids of the cell membrane and its downstream metabolites and receptors to maintain their successful life cycle in the host (**Figures 2A, B**) (Janelle et al., 2002; Konson et al., 2004; Gebhardt et al., 2005; Hooks et al., 2006; Chandrasekharan et al., 2016; Gandhi et al., 2017). AA is transformed into various bioactive lipid mediators to regulate inflammatory networks in the host cell (Samuelsson, 1991). AA is released from the membrane by the action of the enzyme phospholipase A2 (PLA2) (**Figure 2**). AA is metabolized by the pro-inflammatory cyclooxygenase (COX) and lipoxygenase (LO) pathways to produce eicosanoids (all-cis-5, 8, 11, 14-eicosatetraenoic acid) such as prostaglandins (PGs) and leukotrienes (LTs) (Fanning and Boyce, 2013; Chandrasekharan et al., 2016) (**Figure 2A**).

### Cyclooxygenase Pathway

COX1 and COX2 are two distinct isoforms of the COX enzyme COX1 expression, where COX1 is constitutive and generates prostaglandins for normal physiological functions, and COX-2 is inducible in cancers and viral infections (Vane and Botting, 1998). AA converts to the intermediate prostaglandin H2 (PGH2), which then uses specific synthetases to synthesize PGE2, PGF2, PGD2, and PGI2, and thromboxane. Pro-inflammatory PGE2 mediates its actions by its interaction with the rhodopsin superfamily of serpentine receptors (G-protein-

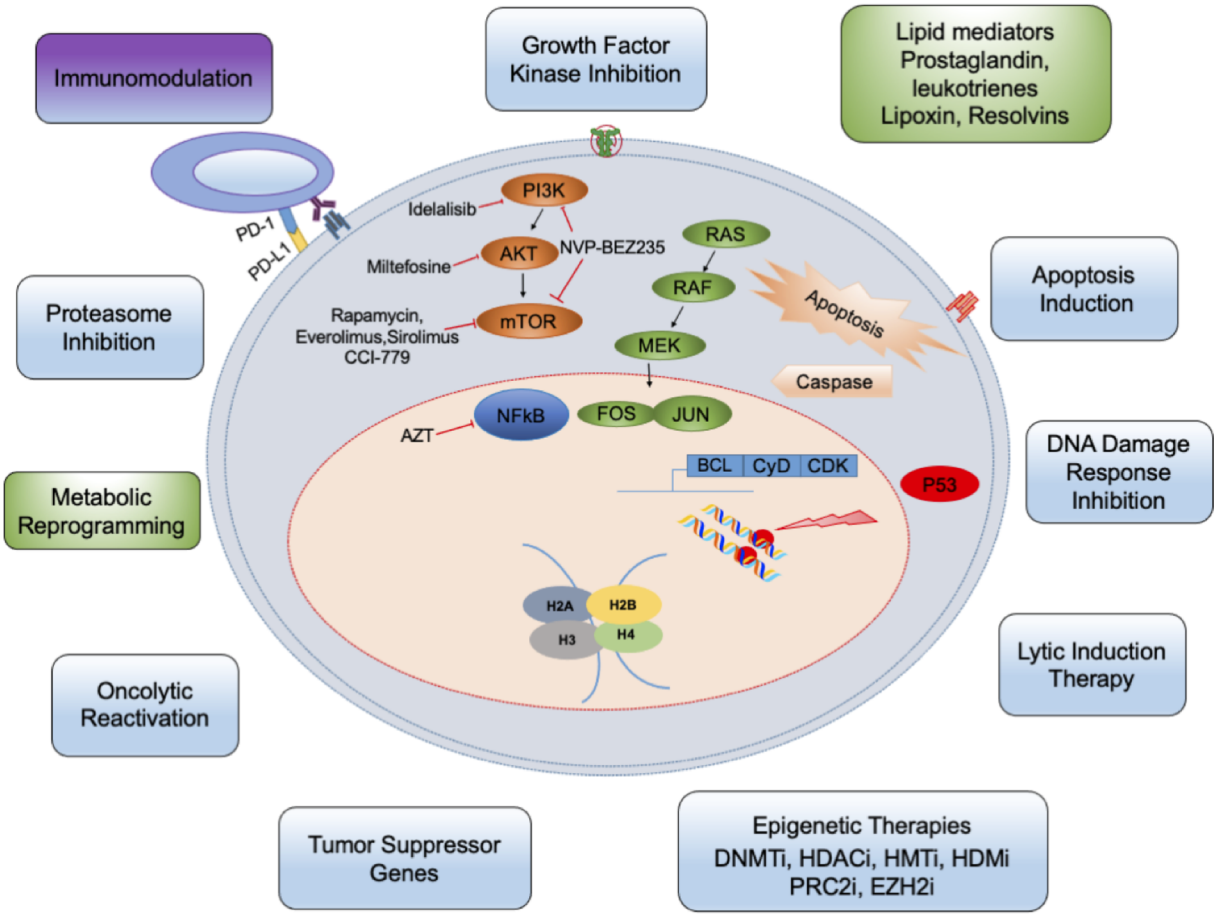


**FIGURE 1** | The targeted therapy principles against herpesvirus: Several metabolic and physiological processes including glycolysis, lipid synthesis, cholesterol metabolism are potentially altered in herpes infected cells (indicated by green boxes). Many cellular/signaling processes are also altered. The infection can be targeted by small-molecule inhibitors (blue) and other drugs. These inhibitors either target cyclin dependent cell cycle, epigenetic factors controlling viral life cycle or DNA damage, etc. Besides, immune cells (purple) and other cells of the tumor microenvironment can be targeted. DNMTi, DNMT inhibitors; HDACi, HDAC inhibitors; HMTi, HMT inhibitor; HDMi, HDM inhibitors; PRC2i, PRC2 inhibitors; EZH2i, EZH2 inhibitors.

coupled seven-transmembrane-domain receptors), also called E-type prostanoids (EP) receptors such as EP1, EP2, EP3, and EP4 (**Figure 2B**). Functional EP receptors have been localized on the plasma membrane and nuclear membranes in the perinuclear region of a variety of cell types and tissues (Bhattacharya et al., 1999). PGE2 has also been shown to induce the expression and activity of a nuclear receptor superfamily member nuclear factor NR4A2 (Nurr1) in colorectal carcinoma cells by PGE2 (Holla et al., 2006).

HSV-1 recurrent infection reactivates the latent virus in the central nervous system. The reactivation of the virus promotes the onset of painful lesions in peripheral tissues and presents a compelling clinical health issue. The reactivation process reprograms the host gene expression and transcription factors (Shimeld et al., 2001), and the inflammatory cyclooxygenase-2 (COX2) pathway is one among those (Gebhardt et al., 2005). HSV thymidine kinase (HSV-tk) gene transduction augments tumor growth rate, COX2 protein, and prostaglandin E2 (PGE2) release in murine colon cancer cells (Konson et al., 2004). Increased level of PGE2 promoted the development of resistance against antiviral GCV treatment in HSV-tk related tumor development (Konson et al., 2004). COX2 inhibitor nimesulide treatment inhibited the growth rate of HSV-tk-transduced murine tumors (Konson et al., 2004). Oral therapy with COX2 selective inhibitor, Celecoxib reduced viral reactivation in the trigeminal ganglia, suppressed virus shedding when given prophylactically by the gastrointestinal route in mouse models (Gebhardt et al., 2005). HSV-1 reactivation and nerve inflammation are involved in vertigo-related vestibular pathogenesis. Non-steroidal anti-inflammatory drugs (NSAIDs), including indomethacin and Celecoxib, are frequently used to suppress reactivation of HSV-1 (Liu et al., 2014). However, whether NSAIDs can affect the reactivation of HSV-1 in vestibular ganglions is not certain. Future studies are required to confirm the effects of NSAIDs on the HSV-1 life cycle (Liu et al., 2014). Celecoxib treatment could significantly suppress HSV-1 reactivation, reduce the numbers of corneas and ganglia containing

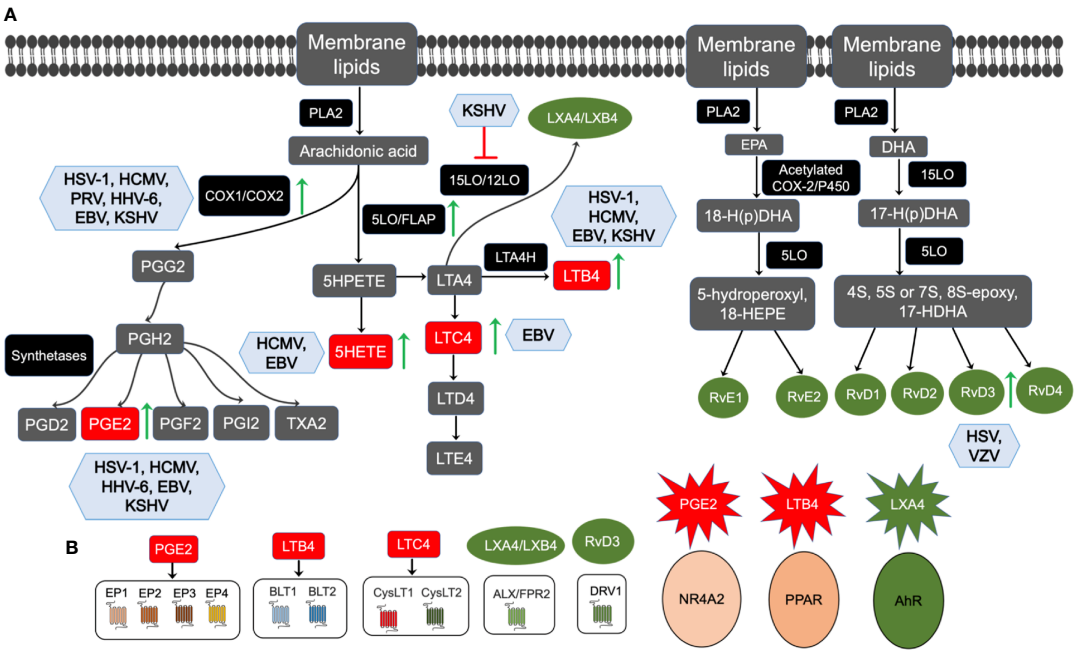


**FIGURE 2** | Overview of membrane lipids metabolism and herpesvirus infections: **(A)** Membrane lipids move to the cytoplasm by the activation of calcium-dependent cytosolic phospholipase A2 (cPLA2) to form Arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). AA's movement to the cytoplasm by cPLA2 liberates free AA, which enzymatically metabolizes to eicosanoids *via* three major pathways, such as the cytochrome P450, cyclooxygenase (COX), and 5-lipoxygenase (5LO). AA is converted to the intermediate prostaglandin H2 (PGH2), which is then metabolized to prostaglandins (PGs), such as PGE2, PGF2, PGD2, and PGI2, and thromboxane TXA2 by specific synthetases. In the 5LO pathway, AA is metabolized by 5LO and 5-lipoxygenase activating protein (FLAP) to 5-HPETE, which then forms leukotriene A4 (LTA4). LTA4 is consequently transformed into LTB4 by LTA4 hydrolase or glutathione to LTC4 by LTC4-synthase and glutathione-S-transferase. LTC4 forms LTD4 and LTE4 *via* ubiquitous enzymes. LTA4 can be catalyzed by 15LO/12LO enzymes to form anti-inflammatory lipoxins as LXA4 and LXB4. The resolvins family is divided primarily into two groups, E-series resolvin, and D-series resolvin, based on their parent omega-3 PUFA. For E-series resolvin biosynthesis, EPA is the substrate for acetylated COX or cytochrome P450 enzymes, giving rise to 18-hydroperoxide (18-H(p)DHA), which then forms either resolvin (Rv) E1 or RvE2 by 5LO. Furthermore, DHA is the substrate for 15LO, giving rise to a 17-hydroperoxide (17-H(p)DHA), which is subsequently converted to D-series resolvins (RvD1, RvD2, RvD3, RvD4). Viral infections regulating the AA pathway enzymes or metabolites are shown in blue boxes, upregulation is shown by green arrows, and inhibition by red. **(B)** PGE2, LTB4, LTC4, LXA4/LXB4. RvD3 governs its downstream functions *via* interaction with its respective receptor, as indicated.

the infectious virus, and placebo treatment in mice. These experiments strengthen the possibility of using COX2 inhibitors to prevent HSV-1 reactivation in high-risk patients by drug prophylaxis (Gebhardt et al., 2005). Intraperitoneal therapeutic and oral prophylactic plus treatments of a non-specific inhibitor of cyclooxygenases such as acetylsalicylic acid (ASA) could inhibit ocular reactivation shedding of HSV-1 in tears in mice model (Gebhardt et al., 2004). These findings implicate that using non-toxic inhibitors of prostaglandin synthesis may be useful in humans suffering from HSV-1 complications (Gebhardt et al., 2004). Pseudorabies virus (PRV), an α-herpesvirus distantly related to HSV-1, increases COX2 mRNA and protein level in PRV infected rat embryonic fibroblast cells (Ray et al., 2004). HCMV establishes lifelong persistence and latent infection following primary exposure and is a common cause of opportunistic infections and subsequent morbidity and mortality in immunocompromised patients. CMV immediate early (IE) protein and COX2 proteins were identified in CMV infected human retinal pigment epithelial (RPE) cells in retinal tissue sections from patients with CMV retinitis. Induction in COX2 mRNA, the protein was also accompanied by increased PGE1 and PGE2 levels in HCMV infected human RPE cell cultures. The induction of COX2 and PGE2 during retinal HCMV infection was suggested to augment virus replication HCMV retinitis (Hooks et al., 2006).

HCMV encodes the constitutively active chemokine receptor US28, which induces an oncogenic phenotype both *in vitro* and *in vivo via* induction of COX2 expression. US28 stimulates COX2 expression *via* activation of NF-kB. Targeting COX2 *in vivo* with Celecoxib significantly delayed the onset of tumor formation in nude mice injected with US28-transfected NIH-3T3 cells. Celecoxib treatment reduced the subsequent growth of the tumor by downregulating the US28-induced angiogenic activity (Maussang et al., 2009). mCMV (mouse cytomegalovirus) induced COX2 has been reported to induce ERK phosphorylation necessary for viral pathogenesis (Melnick et al., 2011).

HHV-6 infection induces COX2 gene expression and PGE2 synthesis within a few hours of infection of monocytes/macrophages. HHV-6 immediate-early protein 2 was discovered as a modulator of COX2 gene expression in monocytes/macrophages, and the addition of PGE2 could increase HHV-6 replication (Janelle et al., 2002).

COX2 pathway has been extensively studied in the EBV mediated tumorigenesis, and EBV induced proliferation of B lymphocytes and COX2, PGE2, and PGE2 receptors EP 1-4 are frequently over-expressed in EBV positive cancer cells in people with chronic inflammatory conditions, Burkitt's lymphoma, and NPC (Gandhi et al., 2015). Upregulated COX2 has been shown to modulate EBV latency through its downstream effector PGE2 (Gandhi et al., 2015). EBV has been shown to suppress the biosynthesis of PGE2 in monocytes *via* inhibition of the inducible COX2 isoform expression both at the transcriptional and translational levels, not altering the gene expression of the constitutive COX1 isoform. This regulation serves as EBV's strategy to evade immune surveillance. The inhibition of PGE2 biosynthesis was relieved in the presence of an inhibitor of herpesviruses DNA polymerase, demonstrating that viral replication and viral proteins were involved in this process (Savard et al., 2000).

COX2, its lipid metabolite PGE2, PGE2 receptors, or eicosanoid receptors (EP1-4) have been widely studied in KSHV associated diseases such as endothelial cell tumor KS and PEL (George Paul et al., 2010; Sharma-Walia et al., 2010; Paul et al., 2011; Paul et al., 2013). Therapeutic potential of non-steroidal anti-inflammatory drugs (NSAIDs) targeting COX-2, 5LO pathways, PGE2 receptor (EP receptor) antagonists blocking LTB4 secretion has been tested in the vascular malignancy KS and B cell lymphoproliferative disease PEL (George Paul et al., 2010; Paul et al., 2011; Paul et al., 2013; Sharma-Walia et al., 2014). Besides herpesviruses, COX2 is one of the critical mediators of inflammation in response to other viral infections such as the dengue virus (DENV), which utilizes it in replication (Lin et al., 2017).

## Lipoxygenase Pathway

The Lipoxygenase pathway consists of 5LO, 8LO, 12LO, and 15LO enzymes and their products, leukotrienes (LTs), including LTA4, an unstable intermediate, LTB4, LTC4, LTD4, and LTE4 (Smith and Fitzpatrick, 1991). Leukotrienes are potent pro-inflammatory lipid mediators that play a central role in cardiovascular diseases, including arteriosclerosis, myocardial infarction, stroke, and have also been tested in viral infections related to inflammation. Leukotriene LTB4 is a highly chemotactic lipid mediator that triggers adherence to the endothelium, activates, and recruit leukocytes to the site of injury, and plays a pathogenic role in inflammatory diseases. BLT1 and BLT2 receptors can recognize LTB4 (**Figure 2B**) (Tager and Luster, 2003). LTB4 is a physiologically relevant endogenous ligand for the peroxisome proliferator-activated receptor alpha (PPARα) (Fiedler et al., 2001; Narala et al., 2010). LTC4 binds G-protein coupled receptors (GPCRs) called CysLT1 and CysLT2, mediating calcium flux, and activate signaling cascades (**Figure 2B**) (Evans, 2002).

Selective lipoxygenase inhibitor, TEI-1338, has been tested to inhibit HSV-1 infection *in vivo* with reduced protein leakage into aqueous humor in the rabbit corneal infection model (Bazan, 1988; Limberg et al., 1988). TEI-1338 has also been recommended to treat human herpes keratitis (Bazan, 1988; Limberg et al., 1988). In the context of the lipoxygenase pathway, the chemotactic leukotriene LTB4, in the presence of Ca2+, could significantly augment the killing of HSV-1 infected cells by enhancing target cell recognition by cytotoxic effector cells and subsequently by expanding their lytic efficiency (Gagnon et al., 1987).

HCMV infection of human vascular smooth muscle cells (SMCs) increases 5LO mRNA levels enabling them to synthesize bioactive LTB4 (Qiu et al., 2008). HCMV-infected vascular SMCs expression of 5LO protein and leukotriene production contributes to local inflammation and pathogenesis (Qiu et al., 2008). HCMV *ex vivo* infection in human placentae and umbilical vein endothelial cells (HUVEC) demonstrated increased 5LO expression and 5-hydroxyeicosatetraenoic acid (5HETE) and LTB4 secretion in their culture supernatant (Benard et al., 2014). Placentae from fetuses with congenital HCMV infection and brain damage showed expression of HCMV-immediate-early-antigen and 5LO when tested by immunohistochemistry (Benard et al., 2014). These findings suggest the lipoxygenase pathway's role in the pathogenesis of congenital HCMV disease (Benard et al., 2014).

EBV interacts with monocytes to actively enhance the activation of the 5LO and the release of the formation of LTB4 and LTC4 (Gosselin and Borgeat, 1997). This activation was dependent on the viral binding as the effect of EBV was abolished by prior treatment of viral particles by heat or by an antibody raised against the glycoprotein gp350 of the viral envelope, but not by UV irradiation of the viral particles (Gosselin and Borgeat, 1997). Exposure of mononuclear cells to EBV was also dependent upon cell stimulation with a second agonist that activates cytosolic phospholipase A2, the enzyme required to form essential lipid mediators of inflammation (Gosselin and Borgeat, 1997).

EBV infection increases the formation of pro-inflammatory leukotrienes in human peripheral mononuclear cells (Belfiore et al., 2007) and triggers the malignant transformation of lymphocytes to Burkitt's lymphoma cells. These Burkitt's lymphoma cells are characterized by increased resistance to apoptosis (Gosselin and Borgeat, 1997). These cells overexpressed 5LO and 15LO, and studies with inhibitors show that 5LO and other LO-isoforms might be involved in EBV-mediated lymphoma progression. LTB4 has been can activate innate immunity and decrease the proliferation of EBV-induced induced B cells (Klein et al., 2008). LTB4 and its receptor BLT1 mediated LTB4-BLT1 lipid chemoattractant pathway can induce T-cell activation by enrichment of activation markers CD38 and HLA-DR and also express effector cytokines (IFN-γ, IL4) and inflammatory chemokine receptors (CCR1, CCR2, CCR6, and CXCR1) and subsequently inhibit the EBV-induced proliferation of B lymphocytes (Lin et al., 2008) is vital in early effector T-cell recruitment in mouse models of inflammation. BLT1 (+) T cells are enriched for (Chu et al., 2008).

EBV has been shown to bind to human neutrophils and stimulate homotypic aggregation, total RNA synthesis, and expression of the chemokines IL8 and macrophage

inflammatory protein 1α (MIP1α) (Roberge et al., 1998). Neutrophils get primed with granulocyte-macrophage colony-stimulating factor (GMCSF), and treatment of neutrophils with GMCSF before EBV activation enhanced the production of LTB4 along with both chemokines MIP1α and IL8 (Roberge et al., 1998). EBV infection-induced neutrophils mediated induction of chemotactic cytokines, and LTB4 may improve its ability to infect B and T lymphocytes *via* increased recruitment to infection (Roberge et al., 1998).

EBV-transformed human B cells have been demonstrated to alter membrane phospholipid metabolism and intracellular calcium levels to modulate another potent mediator of the inflammatory response called platelet-activating factor (PAF) (Schulam et al., 1991). PAF receptors are expressed on Burkitt and non-Burkitt B cell lymphoma cell lines (Travers et al., 1991). PAF binds to B cells and induced arachidonic acid release and 5-hydroxyeicosatetraenoic (HETE) acid production (Schulam et al., 1991). It is interesting to note that apoptosis-resistant EBV-converted Burkitt's lymphoma clones overexpress 5- and 12- lipoxygenases. The resistance to apoptosis increased concurrently with 5LO expression. 5LO inhibition reduced peroxide level indicating that 5LO promotes oxidative stress in EBV+ cells, drives tumorigenesis in multiple cell types, and engages in lymphomagenesis (Belfiore et al., 2007).

Blockade the 5LO enzyme and LTB4 secretion significantly downregulated KSHV latent ORF73, immunomodulatory K5, viral macrophage inflammatory protein 1 (MIP-1), and viral MIP-2 gene expression (Sharma-Walia et al., 2014). Inhibiting 5LO had no consequence on the KSHV lytic genes such as ORF50, immediate early lytic K8, and viral interferon-regulatory factor 2 (Sharma-Walia et al., 2014). Surprisingly, restraining 5LO activation diminished TH2 but elevated TH1-related cytokine secretion (Sharma-Walia et al., 2014). Blocking 5LO abrogated human monocyte recruitment, adhesion, and transendothelial migration of KSHV infected cells (Sharma-Walia et al., 2014). 5LO inhibition decreased fatty acid synthase (FASN) promoter activity and gene expression, much needed in lipogenesis during KSHV latency (Sharma-Walia et al., 2014).

## Boosting Membrane Lipid Anti-inflammatory Pathways

Pieces of evidence from recent studies indicate that lipid mediators play an essential role in pro-inflammation and inflammation resolution. Among these are resolvins, lipoxins, aspirin-triggered lipoxins (ATLs), which are increasingly being used to treat diseases such as metabolic diseases, diabetes, cardiovascular diseases, cancers herpesviruses. Lipoxins (LXs), an endogenously produced lipoxygenase interaction products of arachidonic acid metabolism, actively restore homeostasis by signaling metabolic and cellular action.

Lipoxins gets synthesized transcellular, in a bidirectional enzyme-mediated process. Arachidonic acid (AA) is converted to 15-HEPTE using the coordinated activity of 5LO in neutrophils and a closely allied enzyme, which is either 12LO or 15LO from platelet and endothelial cell, respectively. 15HEPTE, when acted upon by 5LO/12LO, synthesizes either LXA4 or LXB4 (Serhan et al., 1984). Additionally, lipoxin epimers, including aspirin-triggered lipoxin (ATL) are formed under the influence of aspirin treatment, as described by Serhan et al. (Chiang et al., 2004).

Aspirin has been the oldest of all successful non-steroidal anti-inflammatory drug (NSAID), analgesic-antipyretic therapeutic available for human usage. Aspirin acetylates serine residue on the active site of COX1 and COX2 in an irreversible manner. Aspirin mediated inhibition of COX2 halts the formation of pro-inflammatory prostaglandins and helps create 15R-hydroxy eicosatetraenoic acid from AA. 15-(R)-HETE is metabolized by 5LO to endogenous novel carbon 15-epimers of lipoxins, called ATLs (Chiang et al., 1998). Epilipoxins are more stable stereoisomers of lipoxins, and its analogs could help develop drugs that would not possess an adverse effect associated with COX1 inhibition. Owing to their low IC50 value and high potency, lipoxins and epilipoxins serve as a safe alternative to Aspirin, which possesses side effects like ulcer formation, bronchoconstriction, and nephropathy (Isakson et al., 1995).

Studies done on human KS and PEL cells have proved that KSHV strategically promotes its latency and malignant transforming ability by suppressing the production of anti-inflammatory signaling agents such as LXA4. At the same time, KSHV fosters the production of pro-inflammatory cytokines, lipoxygenases, cyclooxygenase, and metabolites of the latter two classes of enzymes to increase further the infectivity of the virus (Schultz et al., 2002; Rosenfeld et al., 2006). Interestingly, the anti-inflammatory lipoxin LXA4 is downregulated during KSHV infection to facilitate infected cell survival. The use of AA pathway inhibitors or supplementing anti-inflammatory lipid mediators has been proposed as an effective alternative therapeutic (Chandrasekharan and Sharma-Walia, 2019). Treatment of the KSHV infected cells with LXA4 or 15-epi-LXA4 reverses this pro-malignancy profile of pro-inflammatory signaling by an ALX/FPR receptor (**Figure 2**) dependent mechanism (Bentz et al., 2015). Lipoxin also interacts with signaling molecules and transcription factors such as NF-κB, AP-1 consisting of a heterodimer between c-Fos and c-Jun, nerve growth factor-regulated factor (NGF) 1A binding protein 1, and PPAR γ (Wang et al., 2020).

At higher concentrations (>30 nmol/liter), Lipoxins have been shown to interact with the Aryl hydrocarbon receptor (AhR); after that, AhR enters the nucleus where it joins the AhR nuclear translocator (ARNT). AHR/ARNT complex binds to xenobiotic response elements to initiate transcription of genes, including SOCS2 (suppressor of cytokine signaling), and thus participates in immunomodulation (Flavell and Murray, 2000; Chu et al., 2008). These findings suggest that the lipoxins or their analogs must be tested in animal models to ascertain if they can be used to treat KSHV associated cancers such as KS and PEL. In a recent study (Cai et al., 2013), it has been postulated that KSHV infected endothelial cells, when treated with a high concentration of LXA4, led to the nuclear translocation of the AhR protein. In the same study, PEL nuclear lysates, when passed through the

LXA4 affinity column, yielded elutes rich in cellular nucleosome complex proteins, including MDMs and SWI/SNF protein, as identified by LC/LC-MS. Further, in the study, it was suggested that the nuclear translocation of AhR was responsible for the affinity interaction of cellular nucleosome complex proteins to LXA4. These nucleosome complex proteins play a significant role in chromatin modulation and the lytic induction of KSHV (Cai et al., 2013). These findings are suggestive of the prospect of lipoxins to be used for lytic induction therapy.

Mass spectrometry analysis from our experiment also suggested LXA4 be interacting with factors associated with metabolic and signaling pathways, immune response, cell proliferation, angiogenesis, transport-related proteins, and nuclear protein related to KSHV replication and life cycle (Asha et al., 2020). In the study, we identified that during the KSHV infection, RTA (replication and transcription activator) protein regulating the latent-lytic switch, recruits SWI/SNF chromatin remodeling complex and other cofactors to the viral promoter to facilitate RTA dependent KSHV gene expression. We also observed overexpression of SMARCB1 in KSHV infected skin and cells. SMARCB1 is expected to be associated with the lytic phase of KSHV. Studies have also proved that inhibitors related to these signaling pathways (mTOR) help control KSHV production (Feng et al., 2004). Keeping all these findings in mind, we speculate lipoxin as a very potent therapeutic agent against KSHV.

Among lipid mediators, many resolvins such as RvD1, epimer aspirin-triggered RvD1 (AT-RvD1), RvE1, neuroprotectin D1, and 11(12)-EET have been reported to serve as a novel antiviral drug for HSV-1 infections (Rajasagi et al., 2017; Zhang et al., 2020). Latency switch from the latent to the lytic cycle not only kills tumor cells but also triggers the immune response in lymphoma associated with EBV. NF-κB, a transcription factor and critical player of inflammation, is often activated in EBV infection. Previous studies have shown that aspirin treatment of EBV-positive lymphoma decreases nuclear translocation of NF-κB and promotes the lytic cycle. Aspirin, along with other anticancer drugs, could effectively treat EBV-positive lymphomas (Li et al., 2010).

## CONCLUSION AND FUTURE PERSPECTIVE

Usage of new therapeutic strategies against herpesvirus infection still needs to meet challenges concerning their specificity, broad-spectrum effects, dosage requirement, toxicity, and in clinical trials. Co-infections with multiple viruses, parasites, malarial vector mosquitoes, and periodontal pathogens and antiviral resistance and the emergence of resistant viruses add to the complexity and severity of disease pathogenesis in immunocompromised patients, children, and neonates. Therefore, the development of newer and safer agents with novel targets with improved potency, lesser off-target, and side-effects is needed. Monotherapy fails in the immunocompromised patients as prolonged therapies are associated with the risk of antiviral resistance and combination antiviral therapy is preferred and more efficacious choice. Proinflammatory pathways regulate multiple aspects of inflammation, production of cytokines/chemokines/interferons, various immune cells [macrophages, dendritic cells, T cells, cytotoxic T lymphocytes (CTLs), Tregs, Th1/Th2 immunity, myeloid-derived suppressor cells], immunometabolism, and immune cell recruitment. COX2/PGE2 axis inhibition using COX inhibitors or PGE2 receptors antagonists along with PD-1 blockade has emerged as a promising adjunct therapy that had additive effects in enhancing CTL function, numbers, could rescue CTL exhaustion in cancer and warrants testing in herpesvirus related malignancies (Chen et al., 2015). LTB4 is often the first responder to infection, and LTB4/BLT1 has implications in immune cell recruitment and activation, chemokine skewing, $CD8^+$ T cell recruitment initiating anti-tumor immunity, and blocking LTB4/BLT1 attenuates neutrophilic inflammation (Jala et al., 2017). Many LTB4 pathway drugs are in Phase 2 trials and offer innovative therapeutic opportunities for viral infections and their associated malignancies.

The unresolved chronic inflammation associated with herpesvirus infection leads to the production of reactive oxygen species (ROS), hypoxia, extracellular acidosis, and remodels microenvironment and reprograms the metabolism and recruitment of immune cells abrogating the efficacy of anticancer drugs and this might be the same scenario during infection (**Figure 1**). Recent advancement in understanding of the resolution-based pharmacology to resolve chronic inflammation has underscored the importance of safer anti-inflammatory drugs (lipoxins, aspirin-triggered lipoxins, resolvins, and their synthetic analogs) with versatile resolution properties. These drugs are immunomodulatory as their receptors are present on immune cells (innate lymphoid, NK, T, and B cells). These anti-inflammatory drugs can control $CD4^+$ T cell differentiation into Th1 and Th17 effectors, decrease production of IL-2, IFN-γ, and TNF-α by $CD8^+$ T cells. Since there is no perfect drug or magic bullet for herpesviral infections, we speculate that anti-inflammatory drugs from lipid targeting pathways would be beneficial as adjuvants to other antivirals, conventional and immune-based therapies (Zhang et al., 2017). Their targetable nuclear (NR4A2, ALXR/FPR, AhR) and plasma membrane (EP1-4, BLT1, BLT2) receptors (**Figure 2B**) may provide a breakthrough in herpesviral infection interventions (Zhang et al., 2017).

## AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version. NS-W apologizes to all the colleagues whose work could not be cited in this manuscript.

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# The Role of Lytic Infection for Lymphomagenesis of Human γ-Herpesviruses

*Christian Münz**

*Viral Immunobiology, Institute of Experimental Immunology, University of Zürich, Zürich, Switzerland*

Epstein Barr virus (EBV) and Kaposi sarcoma associated herpesvirus (KSHV) are two oncogenic human γ-herpesviruses that are each associated with 1-2% of human tumors. They encode bona fide oncogenes that they express during latent infection to amplify their host cells and themselves within these. In contrast, lytic virus particle producing infection has been considered to destroy host cells and might be even induced to therapeutically eliminate EBV and KSHV associated tumors. However, it has become apparent in recent years that early lytic replication supports tumorigenesis by these two human oncogenic viruses. This review will discuss the evidence for this paradigm change and how lytic gene products might condition the microenvironment to facilitate EBV and KSHV associated tumorigenesis.

**Keywords: Epstein Barr virus, Kaposi sarcoma associated herpesvirus, viral IL-10, viral IL-6, viral G-protein coupled receptor, early lytic EBV antigen specific T cells, natural killer cells**

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***Correspondence:**
*Christian Münz*
*christian.muenz@uzh.ch*

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## INTRODUCTION ON EBV AND KSHV INFECTION AND TUMORIGENESIS

Epstein Barr virus (EBV) and Kaposi sarcoma associated herpesvirus (KSHV) are the two human γ-herpesviruses (human herpesvirus 4 and 8, respectively) (Ehlers et al., 2010). Both are quite successful pathogens in the human population and have no other known animal hosts (Münz, 2019; Cesarman et al., 2019). EBV persistently infects more than 95% of the human adult population, and while rare in the Northern hemisphere, persistent KSHV infection is found in more than 50% of individuals in sub-Saharan Africa. Both viruses encode latent and lytic gene products (Mariggio et al., 2017; Frost and Gewurz, 2018). While lytic replication allows through the expression of immediate early, early and late structural γ-herpesvirus genes the production of infectious viral particles, latent gene expression is thought to maintain viral DNA in proliferating lymphocytes, probably primarily B cells, rescue them from apoptosis and drive them into differentiation to long-lived memory cells for persistence (Thorley-Lawson, 2001; Dittmer and Damania, 2016; Frohlich and Grundhoff, 2020). For this purpose, EBV encodes six latent nuclear (EBNA1, 2, 3A, 3B, 3C and LP), two latent membrane (LMP1 and 2) and non-translated RNAs (Epstein–Barr virus-encoded small RNAs or EBERs and miRNAs) (Kempkes and Robertson, 2015). They are grouped in four latency patterns (0, I, II and III). Latency III expresses all latency genes and can be found in naïve B cells of healthy virus carriers, latency II only EBNA1, LMP1 and 2 plus non-translated RNAs in germinal center B cells, latency I only EBNA1 at the protein level in homeostatically proliferating memory B cells and latency 0 only non-translated RNAs in resting memory B cells as the site of EBV persistence (Babcock et al., 2000; Hochberg et al., 2004). KSHV expresses latency-associated nuclear antigen (LANA), vCyclin, viral FADD-like interleukin-1-beta-converting enzyme [FLICE/caspase 8]-inhibitory protein (vFlip), Kaposins and non-translated RNAs

(miRNAs) during latency (Frohlich and Grundhoff, 2020). However, lytic KSHV gene products are often co-expressed even in poorly infectious virus-producing cells, and KSHV latency has not yet been linked to a human B cell differentiation program.

Nevertheless, latent EBV and KSHV gene products have oncogenic abilities and, therefore, both human γ-herpesviruses are designated WHO class I carcinogens (Parkin, 2006; Bouvard et al., 2009). Although EBV encodes the more potent growth transforming gene products (Kulwichit et al., 1998; Sin and Dittmer, 2013; Sin et al., 2015; AlQarni et al., 2018) and can readily immortalize human B cells *in vitro* (Pich et al., 2019), each of the two viruses is associated with around 1-2% of all cancers in humans (Cesarman, 2014; Cesarman et al., 2019; Shannon-Lowe and Rickinson, 2019). These include primarily B cell lymphomas and epithelial cell carcinomas for EBV, and endothelial cell cancers and B cell lymphoproliferations for KSHV. EBV latency I is present in Burkitt's lymphoma, latency II in Hodgkin's lymphoma and nasopharyngeal carcinoma, and latency III in some diffuse large B cell lymphomas (DLBCL) and post-transplant lymphoproliferative disease (PTLD) (Shannon-Lowe and Rickinson, 2019). KSHV is associated with the endothelial cell tumor Kaposi sarcoma and multicentric Castleman's disease (Cesarman, 2014; Cesarman et al., 2019). Finally, primary effusion lymphoma (PEL), a plasmacytoma (Klein et al., 2003), is to 100% associated with KSHV and harbors in addition EBV in 90% of cases (Cesarman et al., 1995; Nador et al., 1996; Cesarman, 2011). Interestingly, it is also the only KSHV associated tumor, from which readily transformed cell lines can be established *in vitro* that maintain KSHV (Frohlich and Grundhoff, 2020). Interestingly, co-infection with EBV allows KSHV persistence in mice with reconstituted human immune system components (humanized mice), and results in PEL-like lymphomagenesis (McHugh et al., 2017). Similarly, the two γ-herpesviruses or their monkey orthologues seem to be also co-transmitted in Cameroonian children and macaques (Bruce et al., 2018; Labo et al., 2019). EBV seropositivity was also found to be the strongest correlate of KSHV seropositivity in a rural Ugandan patient cohort (Sallah et al., 2020). Finally, EBV supports KSHV persistence after primary B cell infection and improves KSHV DNA maintenance after infection of EBV negative PEL *in vitro* (Bigi et al., 2018; Faure et al., 2019). Thus, KSHV might rely on EBV for its persistence, bidirectionally influencing their viral gene expression. I will primarily focus in this review on this interaction of EBV and KSHV in associated lymphomas.

## CONTRIBUTION OF LYTIC γ-HERPESVIRUS INFECTION TO LYMPHOMAGENESIS

One facet of how these two tumor viruses influence each other is that KSHV induces lytic EBV replication (McHugh et al., 2017). This is observed in double-infected B cells of humanized mice and double-infected PELs of patients. Surprisingly, this increased lytic EBV infection contributes also to the more frequent lymphomagenesis that is observed in KSHV and EBV infected humanized mice, because co-infection with lytic replication deficient EBV lacking the immediate early lytic activator BZLF1 (BamH1 Z fragment encoding leftward reading frame 1) does not cause more tumors than EBV single infection (McHugh et al., 2017). Similarly, BZLF1 deficient EBV infection was reported to cause fewer lymphomas than wild-type EBV infection in a smaller percentage of humanized mice (Ma et al., 2011; Antsiferova et al., 2014). This effect seemed more pronounced for lymphoma dissemination to liver and kidney than in spleen (Antsiferova et al., 2014). Vice versa, BZLF1 promotor variants that increase lytic EBV replication promote lymphomagenesis in humanized mice (Ma et al., 2012; Bristol et al., 2018). This was shown for a triple mutant (ZV, ZV' and ZIIR) and the nasopharyngeal carcinoma associated V3 variant of the BZLF1 promotor. Decreased lymphomagenesis in the absence of lytic EBV replication is somewhat counterintuitive because infectious particle production is thought to lead to infected cell death, counteracting tumor cell proliferation.

Increased tumor formation in the presence of lytic EBV replication is, however, not only observed in humanized mice. Viruses that are enriched in EBV associated NK/T cell lymphomas and DLBCLs frequently carry deletions in the BART (BamH1 A fragment encoding rightward transcripts) miRNA encoding region that is thought to suppress lytic EBV replication by targeting expression of BZLF1 and the other lytic transactivator BRLF1 (Okuno et al., 2019). Moreover, plasma rather than cell-associated viral loads seems to be predictive of EBV associated tumorigenesis, such as nasopharyngeal carcinoma, PTLD, DLBCL, NK/T cell lymphoma and Hodgkin's lymphoma, suggesting that lytic EBV replication is associated with these EBV associated malignancies (Kanakry et al., 2016).

How might such lytic EBV replication support tumor formation? It is likely that abortive early lytic EBV infection plays a pro-tumorigenic role (Münz, 2019). Accordingly, B cells transformed with a mutant EBV lacking the late lytic gene product BALF5 were more efficient in establishing lymphomas in immune compromised mice (Okuno et al., 2019). The pro-lymphomagenic effects of early lytic EBV gene products could be in part mediated by shaping the tumor microenvironment. Along these lines EBV transformed B cells with higher spontaneous lytic reactivation produce more tumour necrosis factor (TNF), CCL5 (RANTES) and IL-10 (Arvey et al., 2015) (**Figure 1**). In addition, EBV encodes also viral IL-10 (BCRF1) (Jochum et al., 2012). These could promote an immune suppressive tumor microenvironment through vIL-10 and IL-10 mediated T cell response suppression, as well as CCL5 dependent recruitment of myeloid suppressor cells (Casagrande et al., 2019; Walens et al., 2019). Similarly, early lytic KSHV gene products might promote lymphomagenesis (**Figure 1**). Along these lines viral IL-6 (ORF-K2) supports B cell lymphoma dissemination in immune compromised mice and B cell hyperproliferation in transgenic mice (Suthaus et al., 2012; Fullwood et al., 2018). Together with cMyc overexpression it can also support lymphoma formation in mice (Rosean et al., 2016). Thus, viral IL-6 probably serves as an auto- and paracrine growth factor for KSHV infected B cells. Furthermore, transgenic expression of viral protein kinase of KSHV (ORF36) in mice leads to B cell hyperproliferation and lymphoma development at increased frequency, compared to littermate mice (Anders et al., 2018). Viral protein kinase seems to facilitate B cell activation during KSHV infection. Moreover, also K1 transgenic mice develop lymphoproliferations and lymphomas in half of the animals (Prakash et al., 2000; Prakash et al., 2002; Prakash et al., 2005;

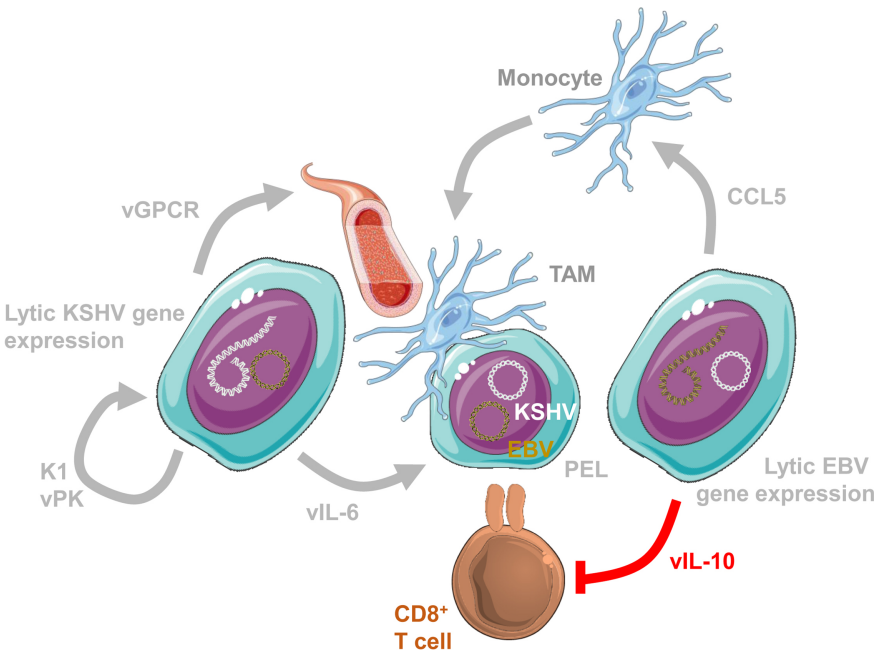


**FIGURE 1** | Lytic EBV and KSHV gene expression condition the tumor microenvironment. Conditioning of the tumor microenvironment by lytic EBV and KSHV gene products occurs most likely at the same time in primary effusion lymphomas (PELs) that are 100% KSHV and 90% EBV infected. Lytic EBV replication attracts monocytes *via* CCL5 to become immune suppressive tumor associated macrophages (TAMs). Viral IL-10 (vIL-10; BCRF1) suppresses $CD8^+$ T cell recognition. In addition, the lytic KSHV product viral G-protein coupled receptor (vGPCR; ORF74) promotes angiogenesis. Furthermore, K1 (ORF-K1) and viral protein kinase (vPK; ORF36) promote proliferation of KSHV infected cells. Finally, viral IL-6 (vIL-6; ORF-K2) promotes KSHV infected B cell proliferation. This figure was created in part with modified Servier Medical Art templates, which are licensed under a Creative Commons Attribution 3.0 unported license: https://smart.servier.com.

Wang et al., 2007; Berkova et al., 2015). K1 encodes an activating receptor that promotes B cell stimulation and apoptosis resistance, including inhibition of Fas mediated extrinsic cell death induction. Finally, inducible expression of viral G protein-coupled receptor (ORF74) promotes angiogenesis and thereby Kaposi sarcoma-like tumorigenesis in mice (Yang et al., 2000; Holst et al., 2001; Guo et al., 2003; Montaner et al., 2003; Montaner et al., 2004; Jensen et al., 2005; Grisotto et al., 2006). Thus, early lytic gene products of both EBV and KSHV might condition the tumor microenvironment for more efficient malignancy development.

## PROTECTION FROM TUMORIGENESIS BY LYTIC γ-HERPESVIRUS REPLICATION SPECIFIC IMMUNE RESPONSES

In addition to this evidence from infections with mutant or variant γ-herpesviruses and early lytic viral gene product overexpression, protection by lytic replication specific immune responses against EBV and KSHV associated tumors points towards the importance of lytic infection in virus-associated malignancies. Uncontrolled lytic EBV replication might cause symptomatic primary EBV infection, called infectious mononucleosis (IM) (Luzuriaga and Sullivan, 2010). While two thirds of Europeans and Northern Americans acquire EBV prior to the age of two, the remaining one third often gets infected in the second decade of life (Dunmire et al., 2018). One third to half of these developed a strong $CD8^+$ T cell lymphocytosis four to six weeks after EBV encounter. They shed infectious virus at increased titers into saliva often for months, develop antibodies against structural proteins like viral capsid antigen (VCA), but not yet EBNA1, and the majority of the expanding $CD8^+$ T cells are directed against early lytic EBV antigens with individual peptide specificities constituting up to 40% of the total $CD8^+$ T cell compartment (Callan et al., 1998). In contrast, latent EBV antigen specific $CD8^+$ T cells emerge at IM convalescence and were therefore proposed to be the protective entity of EBV specific immune responses (Taylor et al., 2015). IM increases the risk to develop EBV associated Hodgkin's lymphoma 4fold, but only for around 5 years after primary EBV infection (Hjalgrim et al., 2003). This was characterized in more than 50'000 adolescents and young adults in Scandinavian countries, as well as several follow-up studies (Hjalgrim et al., 2007; Hjalgrim et al., 2010). Therefore, uncontrolled lytic EBV replication could predispose for some EBV associated lymphomas.

Along with $CD8^+$ T cells, natural killer (NK) cells expand during IM (Williams et al., 2005; Balfour et al., 2013; Azzi et al., 2014). Primarily, early differentiated NK cells expressing inhibitory NKG2A but not killer immunoglobulin-like receptors (KIRs) expand around 4fold (Azzi et al., 2014; Hendricks et al., 2014). These degranulate their cytotoxic molecules preferentially in the presence of lytically EBV replicating B cells (Chijioke et al., 2013; Azzi et al., 2014). Accordingly, NK cells of humanized mice which are also enriched in this early differentiated NK cell phenotype (Strowig et al., 2010) restrict wild-type but not BZLF1 deficient EBV

infection (Chijioke et al., 2013; Landtwing et al., 2016). Restriction of lytic EBV replication by NK cells also reduced lymphoma formation, because NKp46 directed antibody depletion of NK cells leads to higher frequencies of DLBCL-like lymphomas in humanized mice (Chijioke et al., 2013; Landtwing et al., 2016).

Recognition of lytically EBV replicating B cells by NK cells seems to be primarily mediated *via* recognition by natural killer group 2 member D (NKG2D) with DNAX accessory molecule 1 (DNAM-1) as co-receptor (Pappworth et al., 2007). Their ligands are up-regulated on B cells upon induction of lytic EBV infection (Pappworth et al., 2007). NKG2D surface expression on NK and $CD8^+$ T cells is reduced due to inefficient glycosylation by loss-of-function mutations in the $Mg^{2+}$ transporter MAGT1 in patients with X-linked immunodeficiency with magnesium defect, Epstein-Barr virus (EBV) infection, and neoplasia (XMEN) (Chaigne-Delalande et al., 2013; Dhalla et al., 2015; Patiroglu et al., 2015; Brigida et al., 2017; Ravell et al., 2020a). EBV associated lymphomas develop in one third of the affected patients, but interestingly also one patient with Kaposi sarcoma was reported (Brigida et al., 2017; Ravell et al., 2020b). $Mg^{2+}$ supplementation can restore NKG2D surface expression and EBV specific immune control in some of these patients (Chaigne-Delalande et al., 2013), but has not proven to be a successful durable therapy of XMEN (Ravell et al., 2020b). Nevertheless, NKG2D recognition of lytically replicating EBV infected B cells seems to be essential to prevent lymphomas.

In addition, the other primary immunodeficiencies that predispose for EBV associated diseases also seem to point towards cytotoxic lymphocytes, including NK and $CD8^+$ T cells, as main components of EBV specific immune control (Damania and Münz, 2019; Latour and Fischer, 2019; Tangye and Latour, 2020). These affect the cytotoxic machinery (perforin, Munc13-4, Munc18-2), T cell receptor signaling (ITK, PI3K, RasGRP1, ZAP70, CORO1A), co-stimulation (CD27, CD70, 4-1BB, CTLA-4, SAP) as well as cytotoxic lymphocyte development and expansion (GATA2, MCM4, XIAP, STK4, CTPS1). While EBV specific immune control seems to be independent of type I and II interferons (IFNs) and antibodies (Latour and Fischer, 2019; Münz, 2020), type II IFN signaling seems to be required for KSHV specific immune control, and is compromised by mutations in IFNγR1 and STAT4 (Damania and Münz, 2019). Furthermore, the requirements for co-stimulation seem to be different with OX40 being essential for KSHV specific immune control (Byun et al., 2013). Nevertheless, T cells rather than B cells seem to be important for the immune control of both γ-herpesviruses.

Among these, adoptive transfer of early lytic EBV antigen specific $CD8^+$ T cells has been shown to transiently control EBV infection in humanized mice (Antsiferova et al., 2014). Furthermore, late lytic EBV antigen specific $CD4^+$ T cells have been demonstrated to control EBV transformed B cells in immune compromised mice (Linnerbauer et al., 2014). Both of these T cell specificities display cytotoxicity against EBV transformed B cell lines (Heller et al., 2006). Early lytic EBV antigen specific $CD8^+$ T cell responses are also maintained at higher frequency than latent and late lytic antigen specific responses (Abbott et al., 2013). Similarly, KSHV lytic antigens are also more frequently recognized by $CD4^+$ and $CD8^+$ T cells (Robey et al., 2010; Roshan et al., 2017). Their protective functions against KSHV infected cells and in humanized mice need to be characterized in more detail in the future.

## CONCLUSIONS

Recent evidence suggests that most likely abortive early lytic replication in many cells or productive lytic replication in a few cells promotes KSHV and EBV associated lymphoma formation (Münz, 2019). In healthy virus carriers a large proportion of the cytotoxic $CD8^+$ T cell response is dedicated to the recognition of early lytic KSHV and EBV antigens, probably more than to their latent antigens (Long et al., 2019). In contrast, most EBV specific vaccination approaches have so far focused on latent antigens to elicit protective T cells and late lytic antigens to induce antibodies (Taylor et al., 2004; Smith et al., 2006; Moutschen et al., 2007; Gurer et al., 2008; Ruiss et al., 2011; Meixlsperger et al., 2013; Kanekiyo et al., 2015; van Zyl et al., 2018; Rühl et al., 2019; Bu et al., 2019). From these studies the latent EBV antigens EBNA1, LMP1 and LMP2 have emerged as protective antigens (Rühl et al., 2020). In humanized mice, EBNA1 incorporated into an EBV derived virus-like particle (VLP), but not the VLP itself protected from challenge by EBV infection (van Zyl et al., 2018). In contrast to the B cell trophic VLP, EBNA1 targeting to dendritic cells (DCs) with recombinant antibodies and a potent adjuvant to activate classical DCs was not able to elicit sufficient T cell responses for protection (Gurer et al., 2008; Meixlsperger et al., 2013), even so both vaccines elicited primarily cytotoxic $CD4^+$ T cell responses (Meixlsperger et al., 2013; van Zyl et al., 2018). Recombinant viral vectors are more efficient in eliciting $CD8^+$ T cell responses and they can be combined with $CD4^+$ T cell eliciting vaccines in heterologous protective vaccination (Rühl et al., 2019). For such comprehensively $CD4^+$ and $CD8^+$ T cell eliciting vaccines incorporation of early lytic EBV antigens, like BMLF1 (Antsiferova et al., 2014) should be considered. If proving efficient such vaccine formulations could then also be extended to lytic KSHV antigens. Thus, the new appreciation of a contribution of early lytic replication to possibly both EBV and KSHV associated tumorigenesis gives us also additional antigens that could be explored for vaccination against these two human tumor viruses.

## AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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MINI REVIEW
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# Inhibition of Host Gene Expression by KSHV: Sabotaging mRNA Stability and Nuclear Export

*Carissa Ikka Pardamean and Ting-Ting Wu**

*Department of Molecular and Medical Pharmacology, University of California, Los Angeles, CA, United States*

Viruses are known for their ability to alter host gene expression. Kaposi sarcoma-associated herpesvirus has two proteins that obstruct host gene expression. KSHV SOX, encoded by the open reading frame 37 (ORF37), induces a widespread cytoplasmic mRNA degradation and a block on mRNA nuclear export. The other KSHV protein, encoded by the open reading frame 10 (ORF10), was recently identified to inhibit host gene expression through its direct function on the cellular mRNA export pathway. In this review, we summarize the studies on both SOX and ORF10 in efforts to elucidate their mechanisms. We also discuss how the findings based on a closely related rodent virus, murine gammaherpesvirus-68 (MHV-68), complement the KSHV findings to decipher the role of these two proteins in viral pathogenesis.

**Keywords: gammaherpesvirus, Kaposi sarcoma-associated herpesvirus (KSHV), host mRNA nuclear export, mRNA stability, host gene expression inhibition, virus host interaction**

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***Correspondence:**
*Ting-Ting Wu*
*twu@mednet.ucla.edu*

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## INTRODUCTION

Kaposi sarcoma-associated herpesvirus (KSHV), or the human herpesvirus-8 (HHV-8), belongs to the gamma subfamily of herpesviruses. KSHV is associated with malignancies and life-threatening diseases (Chang et al., 1994; Soulier et al., 1995; Brambilla et al., 1996; Cesarman et al., 1996; Corbellino et al., 1996a; Corbellino et al., 1996b; Dittmer and Damania, 2013; Goncalves et al., 2017), including Kaposi sarcoma, primary effusion lymphoma, multicentric Castleman's disease, and KSHV inflammatory cytokine syndrome. KSHV has two distinct phases in its life cycle: latency and lytic replication. Latency is considered to be immunologically silent with few viral genes expressed and no virion production. During lytic replication, over 80 viral genes are expressed in a cascading order, resulting in the assembly and release of infectious particles, generating new infections to replenish the pool of latently infected cells.

Restricted host range limits most KSHV studies to molecular biology experiments. Murine gammaherpesvirus-68 (MHV-68) is closely related to human gammaherpesviruses (Virgin et al., 1997; Barton et al., 2011). Like KSHV, MHV-68 infection in mice leads to acute lytic viral replication followed by the establishment of latency in B-cells. 63 out of the 80 MHV-68 open reading frames (ORFs) share sequence homology with KSHV ORFs with 10-60% shared sequence identity (Virgin et al., 1997). Unlike KSHV, MHV-68 *de novo* infection readily leads to lytic replication. Therefore, MHV-68 provides a valuable model for *in vitro* and *in vivo* functional studies of KSHV viral protein homologs.

Viruses strategically exploit various cellular mechanisms to down-regulate host gene expression for their own benefit. The best studied KSHV protein to inhibit host gene expression is the shutoff and exonuclease (SOX) protein encoded by KSHV ORF37 (Glaunsinger and Ganem, 2004), which accelerates cytoplasmic mRNA degradation. Another common viral strategy, frequently employed by RNA viruses, targets mRNA nuclear export to inhibit host gene expression (Yarbrough et al., 2014). Most RNA viruses synthesize their mRNAs in the cytoplasm and do not depend on cellular mRNA nuclear export mechanism. In contrast, DNA viruses, including herpesviruses, risk hindering the exit of their own mRNAs if they block mRNA nuclear export. Nevertheless, SOX induces mRNA hyperadenylations and their nuclear retention due to global mRNA degradation in the cytoplasm (Kumar and Glaunsinger, 2010). Moreover, we identified KSHV ORF10 as another inhibitor of host gene expression by interacting with a cellular mRNA export factor, Rae1. We review the current knowledge regarding the molecular mechanisms of SOX and ORF10 as well as their roles in the context of viral lytic replication and pathogenesis (**Figure 1**).

## ORF37: A VIRAL SCISSOR FOR MRNA

Herpesviruses undermine mRNA stability through proteins that possess an endoribonuclease activity (Read, 2013). These include the virion host shutoff (vhs) protein encoded by HSV-1 UL41 (Fenwick and McMenamin, 1984; Strom and Frenkel, 1987; Elgadi et al., 1999) and KSHV SOX (Glaunsinger and Ganem, 2004). Unlike vhs, SOX originates from the herpesviral alkaline exonuclease protein family involved in viral DNA genome processing. Only SOX homologs of the gamma subfamily evolved a separate endonuclease activity causing host shutoff (Glaunsinger and Ganem, 2004; Rowe et al., 2007; Covarrubias et al., 2009). The alkaline exonuclease and host shutoff functions of SOX are genetically separable with specific mutations affecting only one of the two functions (Glaunsinger et al., 2005).

SOX causes host shutoff through a sequential action with a cellular 5'-3' exonuclease, Xrn1 (PACMAN), resulting in cytoplasmic transcript degradation (Covarrubias et al., 2011). The current working model is that SOX internally cleaves the target transcripts in a non-random manner with preferred sites defined by a degenerate sequence motif (Gaglia et al., 2015; Mendez et al., 2018), providing Xrn1 with an entry point to complete degradation. In addition to site preferences, co-sedimentation of SOX with 40S ribosomal subunits indicates that SOX targets transcripts early during translation, further corroborated by SOX's inability to initiate degradation of translation-incompetent reporters or RNA Pol I and Pol III transcripts (Covarrubias et al., 2011).

Remarkably, SOX targets both cellular and viral mRNAs (Abernathy et al., 2014). The MHV-68 SOX homolog shares the same host shutoff activity as KSHV SOX (Covarrubias et al., 2009). When the shutoff function of SOX is disabled in MHV-68,

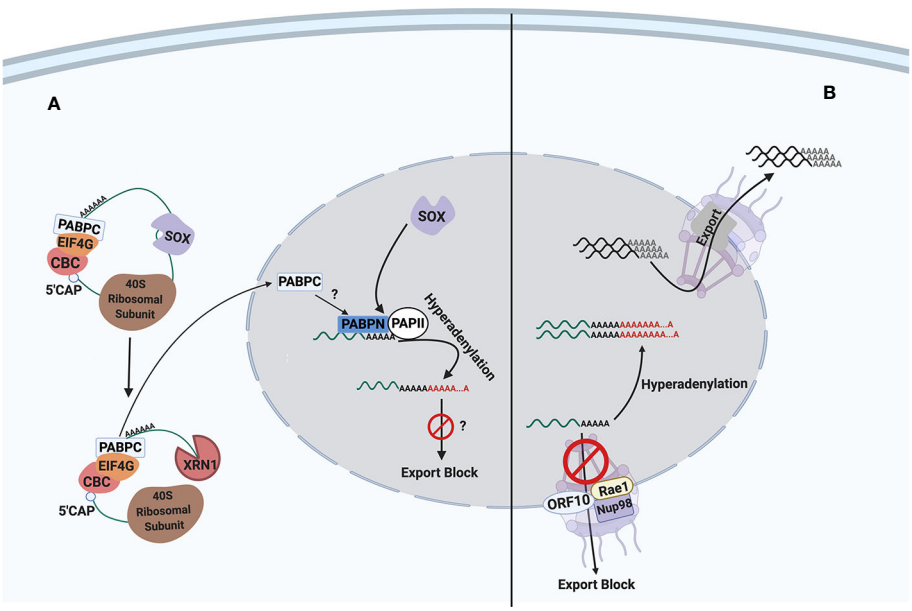


**FIGURE 1** | The inhibitory mechanisms of SOX and ORF10 proteins on host gene expression. SOX protein targets transcripts for degradation, affecting both the host and viral transcripts. On the other hand, ORF10 acts solely on cellular transcripts for export inhibition. Interestingly, both SOX and ORF10 cause the hyperadenylation of transcripts within the nucleus. **(A)** Cytoplasmic SOX protein, as encoded by ORF37, causes host shut off through its endonuclease activity. The viral endonucleolytic action on host transcript then triggers the cellular exonuclease, Xrn1, to complete transcript degradation. **(B)** At the nuclear envelope, ORF10 inhibits only the export of host transcripts by forming a complex with cellular export factor Rae1 and its partnering nucleoporin, Nup98. The hijacking of Rae1-Nup98 leads to nuclear retention of mRNAs. CBC, cap-binding complex; PABPC, cytoplasmic poly(A)-binding protein; PABPN, nuclear poly(A)-binding protein; PAPII, poly (A) polymerase II. Question marks indicate potential or hypothetical mechanisms and effects within the model.

the majority of MHV-68 genes have higher mRNA levels (Richner et al., 2011). Not all MHV-68 genes with increased transcript levels have higher protein expression. However, this altered viral protein expression unexpectedly impacts the composition of virions produced by the SOX mutant without changing the ratios of particles to plaque forming units. In addition, the resulting changed virion composition affected viral entry. Despite enhanced viral gene expression, the SOX MHV-68 mutant is attenuated *in vitro* and *in vivo*. This is a surprising finding since host shutoff is generally thought to help viruses seize cellular resources to maximize their own protein expression for the highest virion production. Instead, SOX controls the viral gene expression to produce virions with a proper composition for optimal viral replication.

## ORF37: A CYTOPLASMIC TERMINATOR WITH A NUCLEAR IMPACT ON MRNA EXPORT

As many RNA-binding proteins are capable of nucleo-cytoplasmic shuttling, a SOX-mediated massive decrease in cytoplasmic mRNA can lead to the nuclear relocation of cytoplasmic RNA-binding proteins (Gilbertson et al., 2018). PABPC, a predominantly cytoplasmic poly(A) binding protein, is one such nucleo-cytoplasmic shuttling protein (Afonina et al., 1998). It interacts with the cap-binding complex, eIF4F, through subunit eIF4G, to form a closed loop structure (Tarun and Sachs, 1996; Wells et al., 1998), which promotes the recruitment of ribosome 40S subunit for translation. Upon RNA binding, the PABPC nuclear import signal is masked, and thus when cytoplasmic polyadenylated transcripts drop substantially due to SOX activity, RNA-free PABPC is imported into the nucleus (Kumar et al., 2011). A SOX mutant that cannot accelerate mRNA turnover also fails in PABPC1 nuclear relocalization (Lee and Glaunsinger, 2009). Moreover, overexpression of a cytoplasmic deadenylase to reduce the abundance of cytoplasmic poly(A) RNA also induces nuclear import of PABPC1 (Lee and Glaunsinger, 2009). PABPC nuclear relocalization by SOX is observed during KSHV lytic replication (Lee and Glaunsinger, 2009). HSV-1 vhs can also cause nuclear accumulation of PABPC1 (Kumar and Glaunsinger, 2010), indicating a common impact in cytoplasmic mRNA drop. The major consequences of nuclear relocalization of PABPC1, by SOX, vhs, or the fusion with a nuclear retention signal (NRS) to PABPC1, are hyperadenylation of mRNAs and inhibition on their nuclear export (Kumar and Glaunsinger, 2010). Notably, nuclear PABPC1-NRS does not destabilize GFP reporter mRNA but diminishes GFP protein expression (Kumar and Glaunsinger, 2010), perhaps by retaining the GFP transcript in the nucleus. This indicates that in addition to cytoplasmic mRNA degradation, the impact of ORF37 on nuclear export of mRNA due to PABPC nuclear translocation is a critical part of its host shutoff function.

Nuclear mRNA hyperadenylation has been observed when RNAs are not exported (Hilleren and Parker, 2001; Jensen et al., 2001; Hammell et al., 2002; Qu et al., 2009; Bresson and Conrad, 2013). Since hyperadenylation and nuclear export are intimately linked, it is difficult to determine whether a block on export or hyperadenylation occurs first upon the PABPC nuclear relocation. SOX-induced hyperadenylation depends on PABPC1 and poly(A) polymerase, PAPII (PAPα) (Lee and Glaunsinger, 2009). The involvement of cleavage/polyadenylation CPA machinery is assumed. CPA is carried out by a large complex consisting of multi-subunit of cleavage and polyadenylation specificity factor (CPSF) and PAP that adds adenosines to the cleavage fragment (Proudfoot, 1996). Subsequently, nuclear poly(A)-binding protein (PABPN) associates with the newly-added poly(A) tail and stimulates PAPII to produce a long poly(A) tail of ~250 nucleotides (Bienroth et al., 1993; Kühn et al., 2009; Kühn et al., 2017). It has been proposed that additional adenosines beyond 250 residues cannot support a productive CPSF-PAPII complex for efficient polyadenylation (Wahle, 1995; Kühn et al., 2009). PABPN and CPSF contribute to the recruitment of nuclear export complex to mRNA (Shi et al., 2017). The loss of PABPN can lead to a shortened poly(A) tail and mRNA nuclear retention (Apponi et al., 2010). It is possible that relocating nuclear PABPC1 replaces PABPN on the poly(A) tail of mRNAs and prevents RNA export factor recruitment, interrupting mRNA nuclear export and causing subsequent hyperadenylation.

Despite a predominant nuclear SOX presence, cytoplasmic SOX largely mediates the effects of accelerated cytoplasmic mRNA decay, relocalization of PABC, and nuclear mRNA hyperadenylation (Covarrubias et al., 2009). SOX degrades cytoplasmic RNA, causing the PABPC1 nuclear import, which causes nuclear mRNA hyperadenylation and nuclear retention. Interestingly, the SOX impact on host gene expression in uninfected cells is less substantial compared to the extent of host shut-off during KSHV lytic replication (Clyde and Glaunsinger, 2011). This suggests that KSHV employs additional mechanisms beyond SOX to inhibit host gene expression, as other herpesviruses do (Rivas et al., 2016).

## ORF10: AN INHIBITOR IN THE MRNA NUCLEAR EXIT

Mature mRNA are associated with a variety of proteins to form export-competent messenger ribonucleoprotein (mRNP) complexes (Carmody and Wente, 2009; Björk and Wieslander, 2017). Export of mRNPs requires the transit of these mRNPs through the nucleopore complex (NPC). An NPC consists of ~30 nucleoporins (Nups), with many containing phenylalanine-glycine (FG) repeats (Strambio-De-Castillia et al., 2010). At the central channel of nuclear pores, the FG-repeats of Nups form a permeability barrier (Terry and Wente, 2009). Majority of mRNA nuclear export through the NPCs involves the TAP-p15 (or NXF1-NXT1) heterodimer (Conti and Izaurralde, 2001; Köhler and Hurt, 2007; Carmody and Wente, 2009). The TAP-p15 heterodimers load mRNPs onto FG-containing Nups (FG-Nups) (Katahira et al., 1999; Bachi et al., 2000). The interaction of TAP with the FG repeats overcomes the permeability

barrier of the nuclear pore, enabling mRNP translocation across the central channel (Grünwald et al., 2011; Powers and Forbes, 2012).

Our work identified a novel KSHV post-transcriptional regulator, encoded by ORF10, as an inhibitor of mRNA nuclear export (Gong et al., 2016). ORF10 expression leads to the reduction of cytoplasmic RNA levels for 24% of cellular genes, indicating a role of ORF10 in suppressing host gene expression during KSHV replication. While both ORF37 and ORF10 cause mRNA nuclear retention, ORF10 directly targets the nuclear export pathway by interacting with Rae1. We and others have independently identified, through mass spectrometry, that KSHV and MHV-68 ORF10 interacts with Rae1 (Davis et al., 2015; Gong et al., 2016), which is a highly conserved eukaryotic cellular export factor (Bharathi et al., 1997; Kraemer and Blobel, 1997; Sabri and Visa, 2000). Rae1 is involved in mRNA export by interacting with Nup98 at NPC (Pritchard et al., 1999; Blevins et al., 2003). We engineered a recombinant MHV-68 expressing FLAG-tagged ORF10 and identified Rae1 and Nup98 as ORF10-interacting proteins in the context of infection (Gong et al., 2016). Unlike TAP, Rae1 does not interact with the FG repeats of Nup98. Instead, the interaction is mediated through an evolutionarily conserved sequence within the Nup116/Nup98 family, referred to as Gle2-binding sequence (GLEBS) (Bailer et al., 1998; Pritchard et al., 1999). Rae1 also interacts with TAP (Bachi et al., 2000; Blevins et al., 2003) and thus, rather than functioning in the transit of mRNPs through NPCs, Rae1 potentially facilitates the docking of export-competent mRNPs onto NPCs. KSHV and MHV-68 ORF10s share 19% amino acid identity, highlighting the functional importance of Rae1 interaction during gammaherpesvirus infection. A structural study determined the MHV-68 ORF10 residues involved in the interactions with the Rae1-Nup98 complex (Feng et al., 2020). Some residues are highly conserved across the gammaherpesvirus ORF10 homologs with their mutations impairing interaction with the Rae1-Nup98 complex, causing the loss of mRNA nuclear export inhibition.

We have shown that KSHV and MHV-68 ORF10 induce nuclear accumulation of poly(A) RNA, which is abolished by mutations that disrupt ORF10-Rae1 interaction or Rae1 knockdown. Additionally, ORF10 is enriched at the nuclear rim. This localization depends on Rae1 and Nup98. Our current working model is that ORF10 interacts with Rae1, which in turn interacts with Nup98, to interfere with the Rae1-Nup98 complex function in mRNA export. While global mRNA export is not impacted by the absence of Rae1 (Babu et al., 2003), expression of the GLEBS domain of Nup98 induces nuclear accumulation of poly(A) RNA (Pritchard et al., 1999). The GLEBS domain sequesters Rae1 from binding to the wild type Nup98 at NPCs. ORF10 does not disrupt the interaction between Rae1 and Nup98; instead, it undermines the function of the complex. Both studies support a role of Rae1 in mRNA export, but the precise mechanism remains unknown. Cell fractionation combined with RNA sequencing indicates that the ORF10 impact on mRNA export is not global. While a strong correlation was found between RNA abundance and SOX-mediated degradation (Gilbertson et al., 2018), such correlation is not seen for ORF10-mediated export inhibition. It is possible that the Rae1-Nup98 complex is utilized by a subset of mRNAs for rapid exit of the nucleus. Without Rae1, this subset of mRNAs can still exit the nucleus albeit less efficiently, which can only be observed with careful kinetics studies.

Unlike SOX, we did not find viral transcript inhibition by ORF10. Herpesviral lytic genes are expressed in a cascade order and classified as immediate early, early and late genes. RNA sequencing showed that in the absence of ORF10, the transcription of late genes during KSHV lytic replication was most impacted, resulting in reduced virion production (Gong et al., 2016). This phenotype was recapitulated by the null function ORF10 mutant lacking Rae1 interaction or by Rae1 expression knockdown during KSHV lytic replication. These results indicate that ORF10 facilitates efficient viral late gene expression through its Rae1 interaction. Nevertheless, how the inhibition on nuclear mRNA export by ORF10 affects viral gene transcription requires further investigations.

## RAE1-NUP98: A POPULAR VIRAL TARGET

Gammaherpesviral ORF10 is not the only viral protein targeting Rae1-Nup98. Vesicular stomatitis virus (VSV) and influenza A virus (IAV) encode proteins that interact with Rae1 and inhibit mRNA export (Faria et al., 2005; Satterly et al., 2007), underscoring the importance of Rae1-Nup98 in viral replication. Recently, ORF6 encoded by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) was found to interact with Rae1 (Gordon et al., 2020; Li et al., 2020), although the functional consequence is unknown (Miorin et al., 2020). A structural study suggests that the matrix (M) protein of VSV prevents Rae1 from binding to the RNA phosphate backbone, blocking Rae1 function in mRNA export (Quan et al., 2014). MHV-68 ORF10 also binds to a similar interface between Rae1-Nup98 as VSV M (Feng et al., 2020), highlighting a vulnerable structural aspect for viral exploitation. The C-terminal tail of MHV-68 ORF10 interacts with the RNA-binding groove of Rae1-Nup98 but on a different side from the M protein target. The binding of VSV M or gammaherpesviral ORF10 is expected to disrupt the RNA-binding ability of Rae1-Nup98. This prediction is true for the M-Rae1-Nup98 complex but not the ORF10-Rae1-Nup98 complex (Feng et al., 2020). It appears that ORF10 provides an alternative RNA-binding surface for the ORF10-Rae1-Nup98 complex. The IAV NS1 protein forms a complex with Rae1 and Tap-p15 heterodimer (Satterly et al., 2007) but currently no structural information is available on its interaction with Rae1. Embryonic fibroblasts from heterozygous knockout mice of Rae1 ($Rae^{-/+}$) or Nup98 ($Nup98^{-/+}$) or both ($Rae^{-/+}Nup98^{-/+}$) are more susceptible to IAV-induced cell death but produced more virions than the wild type cells (Satterly et al., 2007). This differs from our Rae1 knockdown results that show Rae1 requirement for efficient KSHV lytic replication (Gong et al., 2016). The likely

explanation is that nuclear export of cellular genes is impacted differently by the Rae1-Nup98 complex with a subset of genes more dependent than others. mRNAs of several immune-related genes have higher nuclear to cytoplasmic ratios in $Nup98^{-/+}$ and $Rae^{-/+}Nup98^{-/+}$ cells. Moreover, Nup98 is upregulated by interferons, and treatment by interferons can overcome VSV M-mediated inhibition on mRNA export (Enninga et al., 2002). Certain cellular genes may require the Rae1-Nup98 complex for exporting their mRNAs into the cytoplasm. Some of these Rae1-dependent genes may encode antiviral proteins, which accounts for the increased replication of IAV in the absence of fully functional Rae1-Nup98. However, there could also be cellular mRNAs that do not need Rae1 for export but recruit it for rapid export under special conditions, such as stress (Izawa et al., 2004). Through Rae1 interaction, ORF10 gains access to Rae1-regulated mRNAs and inhibits their export to promote viral replication.

## CONCLUDING REMARKS

KSHV encodes SOX and ORF10, known to inhibit host gene expression through distinct molecular mechanisms. SOX targets viral transcript for degradation, resulting in reduced viral protein expression, maintaining virion production with balanced composition (Abernathy et al., 2014). In contrast, ORF10 does not seem to impact nuclear export of viral mRNAs but is required for efficient expression of viral proteins and virion production (Gong et al., 2016). While SOX and ORF10 are capable of inhibiting host gene expression, their functions during gammaherpesvirus infection do not overlap. Moreover, SOX and ORF10 have different timings of expression during KSHV lytic replication; the former is an early gene and the latter is a late gene (Arias et al., 2014). Does ORF37 coordinate with ORF10 to down-regulate host gene expression for optimal viral replication or does their inhibition on mRNA export serve different purposes? Investigating the roles of ORF10 and ORF37 in the context of infection combined with cell fractionation and RNA sequencing will provide insight into their impact on host gene expression. Additionally, the impact of these two viral genes on the host proteome remained to be determined. Due to the functional conservation of ORF10 and ORF37 in KSHV and MHV-68, a combination of *in vitro* molecular biology and *in vivo* infection model with MHV-68 will certainly provide a comprehensive overview of their functions in viral pathogenesis. ORF10 interacts with Rae1 to achieve export inhibition, yet Rae1 functions in RNA export is still largely unclear. Therefore, ORF10 also serves as a valuable tool to understand this cellular pathway that is targeted by multiple viruses.

## AUTHOR CONTRIBUTIONS

CP and T-TW wrote the manuscript and prepared the figure. All authors contributed to the article and approved the submitted version.

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# iTIME.219: An Immortalized KSHV Infected Endothelial Cell Line Inducible by a KSHV-Specific Stimulus to Transition From Latency to Lytic Replication and Infectious Virus Release

*Stephen J. Dollery*[*†], *Tania D. Maldonado*[†], *Eric A. Brenner* and *Edward A. Berger*[*]

*Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, United States*

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***Correspondence:**
*Stephen J. Dollery*
*dollery@bmi-md.com*
*Edward A. Berger*
*edward_berger@nih.gov*

[†]*These authors share first authorship*

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Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8) is the causative agent of Kaposi's sarcoma and two B cell lymphoproliferative disorders: primary effusion lymphoma and KSHV-associated multicentric Castleman's disease. These distinct pathologies involve different infected cell types. In Kaposi's sarcoma, the virus is harbored in spindle-like tumor cells of endothelial origin, in contrast with the two pathologies of B cells. These distinctions highlight the importance of elucidating potential differences in the mechanisms of infection for these alternate target cell types and in the properties of virus generated from each. To date there is no available chronically KSHV-infected cell line of endothelial phenotype that can be activated by the viral lytic switch protein to transition from latency to lytic replication and production of infectious virus. To advance these efforts, we engineered a novel KSHV chronically infected derivative of TIME (telomerase immortalized endothelial) cells harboring a previously reported recombinant virus (rKSHV.219) and the viral replication and transcription activator (RTA) gene under the control of a doxycycline-inducible system. The resulting cells (designated iTIME.219) maintained latent virus as indicated by expression of constitutively expressed (eGFP) but not a lytic phase (RFP) reporter gene and can be sustained under long term selection. When exposed to either sodium butyrate or doxycycline, the cells were activated to lytic replication as evidenced by the expression of RFP and KSHV lytic genes and release of large quantities of infectious virus. The identity of the iTIME.219 cells was confirmed both phenotypically (specific antigen expression) and genetically (short tandem repeat analysis), and cell stability was maintained following repeated serial passage. These results suggest the potential utility of the iTime.219 cells in future studies of the KSHV replication in endothelial cells, properties of virus generated from this biologically relevant cell type and mechanisms underlying KSHV tropism and pathogenesis.

**Keywords: Kaposi's sarcoma-associated herpesvirus/ HHV-8/Kaposi's sarcoma, viral tropism, time, endothelial cell line, inducible, latency, lytic replication**

## INTRODUCTION

Since its initial discovery (Chang and Moore, 2014), Kaposi's sarcoma-associated herpesvirus (KSHV, human herpesvirus 8) has been implicated in distinct malignancies and lymphoproliferative disorders commonly associated with HIV/AIDS (Goncalves et al., 2017; Hussein et al., 2018; Weed and Damania, 2019). The various pathologies of this gammaherpesvirus are associated with different infected cell types. In Kaposi's sarcoma (KS) (Cesarman et al., 2019), the most common tumor in AIDS patients, the virus is harbored latently in spindle-like tumor cells found within KS lesions; these spindle cells are of endothelial origin. By contrast, the two KSHV-associated lymphoproliferative disorders, primary effusion lymphoma (PEL) (Shimada et al., 2018) and the plasmablastic form of multicentric Castleman's disease (MCD) (Koga et al., 2017), involve KSHV-positive B lymphocytes. Moreover, B cells constitute major reservoirs of latent KSHV persisting in infected individuals and likely represent the source of virus oropharyngeal shedding and transmission by saliva (Minhas and Wood, 2014).

Diverse *in vitro* models involving the relevant target cell lineages have been developed in efforts to unravel the mechanistic complexities of KSHV infection and tropism (McAllister and Moses, 2007; Kang and Myoung, 2017; Dollery, 2019). PEL-derived B cell lines have played important roles in KSHV research, including as a source of infectious virus by induction with pleiotropic agents. Whereas extensive KSHV infection studies have been performed with primary human B cells from various tissue sources, transformed B cell lines have proven largely resistant to infection with cell-free virus; some recent exceptions have been reported for specific B cell lines, with the establishment of cells chronically infected with recombinant reporter viruses that can be induced by biologically relevant stimuli to produced infectious virus (Kati et al., 2013; Dollery et al., 2014). Regarding the other major KSHV target cell type, efficient infection with a reporter virus has been reported for primary endothelial cells (Jeffery et al., 2013) as well as an immortalized derivative (Lagunoff et al., 2002). A stable KSHV-infected endothelial cell line capable of robust infectious virus production upon activation of a KSHV-specific trigger would be particularly valuable.

Our starting point for this effort was the stable human endothelial cell line TIME (telomerase-immortalized microvascular endothelial), which is capable of continuous proliferation while retaining the characteristic surface markers of endothelial cells (Venetsanakos et al., 2002). TIME cells have proven to be a valuable *in vitro* model in KSHV research (McAllister and Moses, 2007). Particularly relevant for the present study are the early demonstrations (Lagunoff et al., 2002; Bechtel et al., 2003) that TIME cells are highly susceptible to infection by cell-free KSHV virions; the infected cells can be induced to transition from latent to lytic phase and production of infectious virus by treatment with phorbol ester or by ectopic expression of the KSHV replication and transcriptional activator (K-RTA), the lytic switch protein product of the viral ORF50 gene. We describe herein the development of a novel cell line designated iTIME.219, using an approach similar to that previously reported for a different parental cell line (Myoung and Ganem, 2011). The features of iTIME.219 cells suggest its potential value in studies of KSHV tropism and pathogenesis.

## MATERIALS AND METHODS

### Cells and Reagents

Endothelial (TIME, TIME.219, iTIME.219) cells were cultured in VascuLife® Basal Medium supplemented with the LifeFactors® kit and 12.5 μg/ml of Blasticidin S HCL (Life Technologies). Epithelial (Vero, iSLK.219) cells were grown in DMEM media supplemented with 10% FBS (Sigma) and 5% L-Glutamine and 5% Penicillin Streptomycin (Quality Biological). Maintenance of KSHV infected cell lines (TIME.219, iTIME.219, iSLK.219) involved the addition of 10 μg/ml puromycin (Mirus Bio). A 200 μg/ml Geneticin (G418) (Life Technologies) selection was included for iTIME.219 and iSLK.219.

### Development of the Endothelial Producer Cells

KSHV infections were performed with recombinant strain KSHV.219 (rKSHV.219) (Vieira and O'Hearn, 2004). The virus was generated from iSLK.219 cells that were propagated in the presence of puromycin and induced to produce cell-free KSHV as described previously by others (Myoung and Ganem, 2011) Virus infectious units were established by titration on 293F cells (Dollery et al., 2014).

iTIME cells were generated by infecting subconfluent TIME cells with rKSHV.219 at an MOI 3 and allowing infection to proceed for three days. Following initial infection, cells were selected by culture in media containing 10 μg/ml of puromycin. For transductions, pRetroX-Tet-On Advanced (rtTA plasmid from Clonetech) and pRetroX-Tight-Hyg-RTA (RTA gene cloned into pRetroX-Tight-Hyg from Clonetech) were separately transfected into GP2-293 cells along with an RD114 envelope vector. Culture supernatants containing retroviral particles were harvested after 2 days. TIME.219 were first transduced with retrovirus encoding rtTA in the presence of polybrene (8 μg/ml) for 3 h at 1,500 RPM (Thermo Scientific Heraeus Multifuge X1R with TX-400 rotor). Cells harboring the rtTA construct were selected by G418 at 200 μg/ml. The cells were then transduced again with retrovirus containing the pRetroX-Tight-Hyg-RTA construct. Transductants were selected by hygromycin at 400 μg/ml. Cells were maintained in media with 200 μg/ml G418 and 10 μg/ml puromycin.

### Immunocytochemistry of Cell Type-Specific Markers

The following monoclonal antibodies (mAbs) or isotype control were employed for cell marker detection: for endothelial cells, the anti-CD31 rabbit monoclonal antibody (mAb) EPR17259 (Abcam, 1:50 dilution); for epithelial marker detection, the anti-pan cytokeratin mAb AE1/AE3 (Abcam, 1:10 dilution). Cells were fixed with ice cold methanol (CD31) or 3% paraformaldehyde (cytokeratin). To permeabilize samples for cytokeratin detection, 0.2% Saponin (CalbioChem) in 1% BSA

(Sigma)/PBS was employed. Samples were blocked with 2% goat serum plus 1% BSA in PBS and then incubated for 1 h with primary antibodies. Cells were then washed with 1% BSA in PBS and blocked with previously described serum. For detection, cells were probed with either Alexaflour (AF) 647 goat anti-rabbit IgG (H + L) (Invitrogen) or Alexaflour (AF) 647 goat anti-mouse IgG (H + L) (Invitrogen). After two washes, nuclei were stained with DAPI (1/1,000) (AAT Bioquest) for 5 min. All micrographs were taken with the EVOS FL Auto 2 (Invitrogen) at 10× using the 2.0 Imaging System. Images were enhanced with Adobe Photoshop CC 2015 software.

## Antibody Staining of Viral Proteins

For chronically infected and newly infected cells, iTIME.219, TIME, and infected Vero cells were plated overnight at $8.3 \times 10^4$ cells per well in a 24 well plate. Cells were washed with 1% BSA in PBS. Samples were then fixed with 3% PFA for 10 min and permeabilized with 0.2% Triton X-100 for 25 min. Samples were washed twice and blocked with 5% BSA/PBS for 30 min. Anti-LANA mAb LN53 (EMD Millipore) (1:100 dilution) was added to cells and incubated for 1 h. Cells were then washed in 1% BSA in PBS and blocked with 2% goat serum in 1% BSA/PBS for 30 min. Samples were then incubated with AF 647 Goat anti-Rat (Invitrogen) at a 1:500 dilution. Representative micrographs were taken with an EVOS FL Auto 2 at 20× using the 2.0 Imaging System. For glycoprotein detection by fluorescence microscopy, uninduced and induced iTIME.219 cells were plated at a concentration of $1 \times 10^5$ cells per well, washed with 1% BSA/PBS, and fixed with 3% paraformaldehyde. Samples were blocked with 5% BSA/PBS and incubated with a 1:100 dilution of anti-gH mAb YC15 (Cai and Berger, 2011) or ant-K8.1 mAb 4C3 (Zhu et al., 1999) for 45 min. Cells were again washed with 1% BSA/PBS and blocked with 2% goat serum prepared in 1% BSA/PBS. For detection, samples were incubated for 20 min with Alexaflour (AF) 647 Goat anti-Mouse IgG (H + L) (Invitrogen, 1:2,000). All infected, newly infected, uninduced, and induced samples were stained with DAPI as described before. Micrographs were taken with an EVOS FL Auto 2 and processed using the EVOS 2.0 Imaging System and the Adobe Photoshop CC 2015. For viral glycoprotein detection by flow cytometry, cells were dislodged from monolayers with *CellStripper* Dissociation Reagent (Thermo), fixed with 3% PFA, and $1 \times 10^6$ cells per sample were then stained and as above with the addition of centrifugation to pellet the samples before solution changes. Analysis was performed using a FACSCanto II (BD Biosciences), and data analysis was performed using FlowJo (Treestar). For the detection of eGFP or DS-RED detection alone, induced and/or infected cells were prepared for flow cytometry as above, with antibody probing steps and blocking steps omitted. Analysis was again performed using a FACSCanto II (BD Biosciences), and data analysis was performed using FlowJo (Treestar).

## Induction and Infection of Cell Lines

To test induction of the KSHV lytic cycle and virion production of latently infected cells, engineered cell lines and controls were exposed to doxycycline (DOX) and/or sodium butyrate (SB) in media without selective agents puromycin or G418. The indicated concentrations of DOX (μg/ml) and SB (mM) were added to the cells plated overnight in six well plates ($3.5 \times 10^5$ cells in 2 ml per well). Cells were incubated with reagents for 3 days. Following induction, micrographs of induced cells were captured using an EVOS FL Auto 2 (Texas Red cube) (10×) and processed (EVOS 2.0 Imaging System and the Adobe Photoshop CC 2015). Immediately following microscopy, supernatants were harvested to quantitate progeny virions. 2 ml of the supernatant was collected and clarified by pelleting debris at 3,000 rpm for 10 min at 4°C (Thermo Scientific Heraeus Multifuge X1R with TX-400 rotor). Progeny virions in the supernatant were then concentrated by pelleting at 16,000 ×g at 4°C for 3.5 h. Concentrated virus was then resuspended in 200 ul of Vero media, serially diluted in Vero media and titered on Vero cells. Micrographs of infected Vero cells and controls were taken at 3 days post-exposure to supernatant with an EVOS FL Auto 2 (GFP cube) (10×) and processed (EVOS 2.0 Imaging System and Adobe Photoshop CC 2015). Cells were analyzed by flow cytometry using a BD FACSCanto II cytometer, and the data were processed using DIVA and FloJo version 10.4.2. The data sets presented for all infection and induction experiments are representative of at least three independent repeat experiments.

## Statistical Analysis

Statistical significance was calculated using a two-tailed unpaired student's t-test.

## Short Tandem Repeat Analysis

For STR analysis and identification of the TIME, TIME.219, and iTIME.219 cells, genomic DNA was isolated using the DNeasy Blood & Tissue Kit ® (Qiagen). Isolated DNA was sent on ice to the Genetic Resources Core Facility (GRCF) at the Johns Hopkins University School of Medicine for STR analysis using the Promega PowerPlex 16 HS system.

# RESULTS

## Generation of Chronically Infected Endothelial Cells Based on the TIME Cell Line

In order to generate a stable KSHV-infected endothelial cell line capable of robust production of infectious virus upon KSHV-specific induction, we subjected TIME cells to a modification of the approach reported for production of the iSLK.219 cell line (Myoung and Ganem, 2011) (see *Materials and Methods*). TIME cells were first infected with the recombinant virus rKSHV.219 (Vieira and O'Hearn, 2004), which contains two fluorescent reporter genes: the eGFP gene linked to the constitutive elongation factor 1$\alpha$ cellular promoter plus the RFP gene under control of the KSHV early lytic-phase PAN promoter. The recombinant virus also contains the puromycin-resistance gene linked to the constitutive Rouse sarcoma virus promoter, which enables positive selection of cells harboring the KSHV episome. The KSHV-infected TIME cells were subjected to

puromycin selection to generate a cell population designated TIME.219; these cells retain the recombinant virus genome indefinitely when maintained under selection (tested up to passage 30, data not shown). In order to confer on TIME.219 cells a KSHV-specific system for activating the virus from latent to lytic phase, we introduced genetic components whereby expression of KSHV RTA, the lytic transactivator protein, is induced upon addition of the tetracycline analog doxycycline (Dox). These cells, designated iTIME.219, were expanded and maintained under continuous selection with puromycin plus G418 (see *Materials and Methods*).

Assessment of eGFP expression in uninduced cells by either fluorescence microscopy (**Figure 1A**) or flow cytometry (**Figure 1B**) revealed that for both the TIME.219 and iTIME.219 populations, the vast majority of cells were infected with the KSHV reporter virus. To confirm true KSHV infection and its maintenance during continued selection, staining for the latency-associated nuclear antigen (LANA) was performed; punctate nuclear LANA staining is commonly used to verify the presence of KSHV episomes during the latent stage of infection. As shown in **Figure 1C** for both TIME.219 and iTIME.219, most cells displayed LANA punctate staining in regions that were also positive for the nuclear DAPI stain; by contrast, no LANA staining was observed in the nuclei of the uninfected TIME cells. These results confirm the maintenance of KSHV infection during continuous propagation and selection of the TIME.219 and iTIME.219 cells.

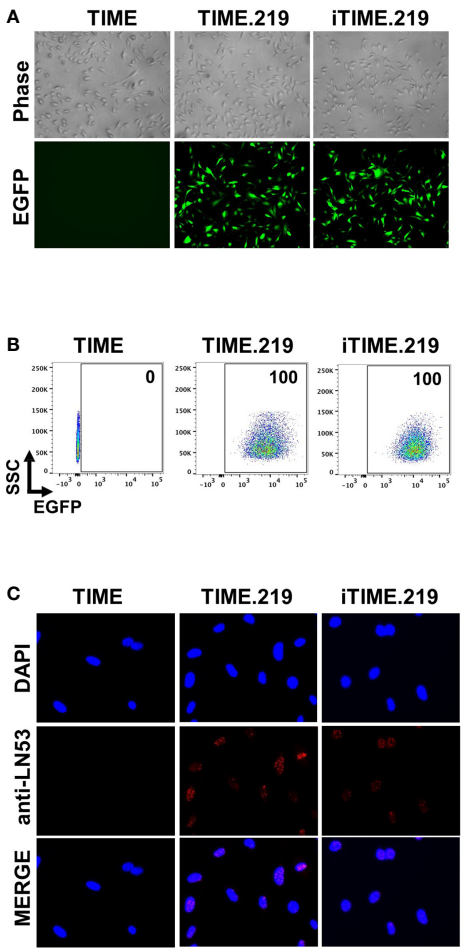


**FIGURE 1** | Analysis of uninduced TIME cells and the KSHV chronically infected derivatives. TIME, TIME.219, and iTIME.219 cells were analyzed for KSHV infection as assessed by expression of eGFP, the latent phase (constitutively expressed) reporter. **(A)** Phase contrast (top) and fluorescence microscopy for eGFP expression (bottom). Magnification 10×. **(B)** Infection by rKSHV.219 analyzed by flow cytometry for eGFP expression. Results are depicted as scatter graphs plotting side scatter (SSC) against log fluorescence intensity. Numbers in the upper right corners indicate the percentage of cells within the eGFP$^+$ gate. **(C)** Cells were fixed, permeabilized and probed with anti-LANA mAb LN53; detection was with an Alexa-647 conjugated anti-rat antibody (orange). Nuclei were stained with DAPI (blue). Images (magnification 20×) are shown for DAPI (top), LANA (middle), and merged (bottom).

## Induction of KSHV Lytic Phase

We next examined these cells for their ability to transition to KSHV lytic phase, comparing uninduced and induced conditions (**Figure 2**). Two potential inducing agents were tested: sodium butyrate (SB), a non-specific compound with pleiotropic effects likely due to histone deacetylase inhibition, and Dox, a selective activator in the engineered iTIME.219 cells. We initially analyzed the expression of the lytic phase RFP reporter. Based on flow cytometry, the TIME.219 cells (**Figure 2A**, left panel) displayed no detectable induction by either agent at the doses tested, alone or in combination. The iTIME.219 cells (**Figure 2A**, right panel) also were refractory to SB alone but were efficiently induced by 1 μg/ml Dox alone (~50% RFP$^+$); combination treatment resulted in even further induction (~90% RFP$^+$). In more extensive dose–response analyses of iTIME.219 cells (**Figure 2B**), Dox alone at concentrations above 1 μg/ml did not further enhance RFP induction significantly; SB alone produced modest induction when increased to 2.5 or 5 mM (15–20% RFP$^+$), with lesser effect at higher concentrations; induction by the combination of 1 μg/ml Dox plus 1 mM SB was not significantly improved by different concentrations of each agent (not shown). **Figure 2C** shows the corresponding data for each cell population analyzed by fluorescence microscopy (left panels) and flow cytometry plots.

We extended the RFP reporter results by testing whether KSHV late lytic phase proteins are expressed upon induction (**Figure 2D**). Immunofluorescence microscopy and flow cytometry revealed that KSHV glycoproteins K8.1 (**Figure 2D** upper panels) and gH (**Figure 2D** lower panels) were minimally detectable in uninduced iTIME.219 cells; upon induction with Dox + SB, the expression of both glycoproteins appeared to increase in the majority of cells, as evidenced by the shifted populations. These results demonstrate inducer-mediated transition of the iTIME.219 cells to the lytic phase of the KSHV infection cycle.

We next assayed infectious virus production from the TIME.219 and iTIME.219 cells treated with the inducing agents employed above (**Figure 3**). Culture supernatants were collected at day 3 post-treatment, virus was concentrated by centrifugation, and the resuspended virus pellets were plated onto Vero cells, a commonly used target for KSHV infection

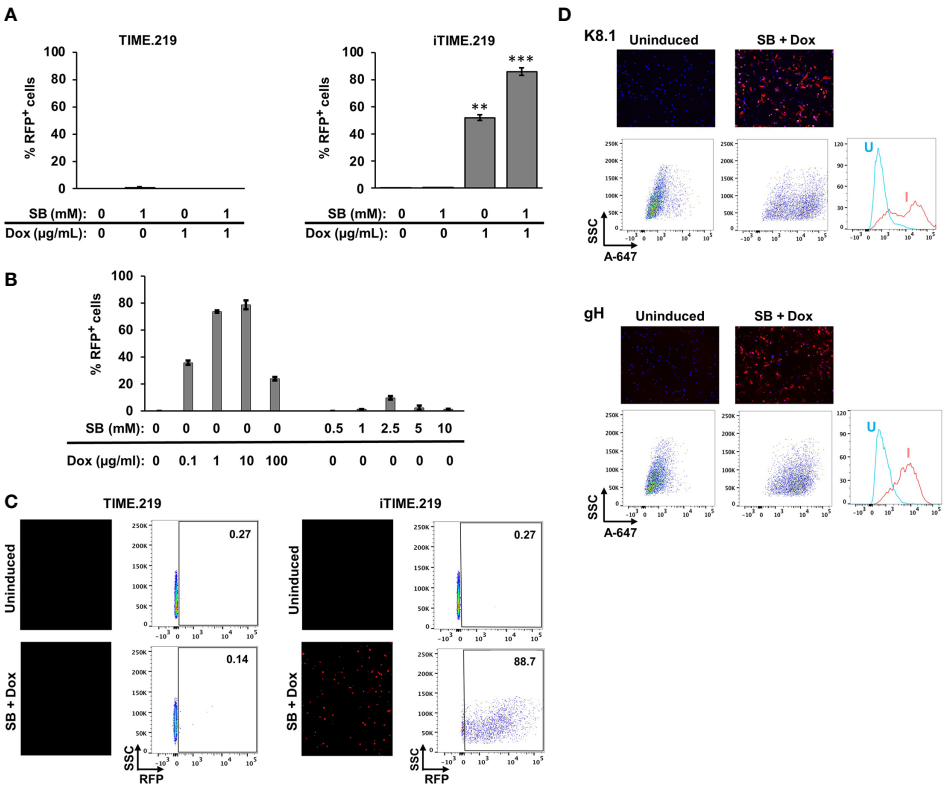


**FIGURE 2** | Induction of TIME.219 and iTIME.219 cells by various combinations of sodium butyrate and doxycycline. In panels **(A–C)**, induction was assessed based on expression of RFP, the lytic phase reporter. Cells were exposed for three days to the indicated concentrations of SB, DOX, or SB plus Dox. TIME.219 (left) and iTIME.219 cells (right). **(A)** Cells were analyzed for RFP expression by flow cytometry. Data are plotted as percent induced cells (% RFP$^+$). **P ≤ 0.01; ***P ≤ 0.001, relative to no treatment. Error bars indicate standard error of the mean (SEM). **(B)** iTIME.219 cells were exposed to the indicated concentrations of DOX or SB for three days. Cells were analyzed for RFP expression by flow cytometry. Data are plotted as percent induced cells (% RFP$^+$). **(C)** Data for a subset of cells from the experiment in panel **(A)** including TIME.219 (left) and iTIME.219 cells (right) either uninduced (upper subpanels) or induced with 1 mM SB + 1 μg/ml Dox (lower subpanels). For each cell type, the data shown are fluorescence photomicrographs (left subpanels, magnification 10×) and scatter plots (right subpanels; numbers in each upper right corner indicate percent cells within the RFP$^+$ gate). **(D)** iTIME-219 induction analysis based on expression of KSHV glycoproteins K8.1 (probed with mAb 4C3, top set of panels) and gH (probed with mAb YC15, bottom set of panels), in each case for cells that were uninduced (left subpanels) and induced (1 mM SB + 1 μg/ml Dox, right subpanels). Detection was performed with an anti-mouse-AF647 conjugate. Cells were analyzed by fluorescence photomicroscopy (top subpanels) and flow cytometry (middle and bottom subpanels). For each glycoprotein, the flow cytometry plot (bottom subpanel) shows comparison of fluorescence intensities for cells that were uninduced (U) *vs*. induced (I).

studies. eGFP expression was used as an initial marker of Vero infection. **Figure 3A** shows that TIME.219 cells failed to generate appreciable detectable infectious KSHV upon treatment with SB or DOX, alone or in combination. By contrast, iTIME.219 cells were induced to produce infectious virus by Dox alone; SB alone was ineffective but enhanced the Dox-mediated induction. **Figure 3B** shows the corresponding fluorescence microscopy and flow cytometry analyses for the cells treated with SB plus Dox. Overall, the response patterns for induction of infectious KSHV production (**Figure 3A**) paralleled those described above for RFP expression in the induced cells (**Figures 2A, B**). Further analyses of dose–response effects on infectious virus production (data not shown) yielded results similar to those noted above for RFP expression. Thus, increasing Dox alone above 1 μg/ml gave minimal improvement of yield; increasing SB alone to 2.5 or 5 mM generated low but detectable amounts of infectious virus, and virus yield from combination treatment with 1 μg/ml Dox plus 1 mM SB was not significantly improved by modifying the doses of either agent.

To verify that the supernatant-exposed Vero cells had truly entered latent phase of infection, LANA staining was performed as described above for the iTIME.219 cells. As shown in **Figure 3C**, LANA puncta were visible in the nuclei of Vero cells exposed to the supernatants from induced iTIME.219 cells, but not the uninduced cells. Taken together, the results in this section demonstrate that iTIME.219 cells are latently infected with recombinant rKSHV.219 and can be triggered to undergo the essential features of the lytic phase by activating a KSHV-specific induction pathway.

To gain perspective on the efficiency of infectious virus production, we compared the iTIME.219 cells with the previously reported iSLK.219 cells (Myoung and Ganem, 2011),

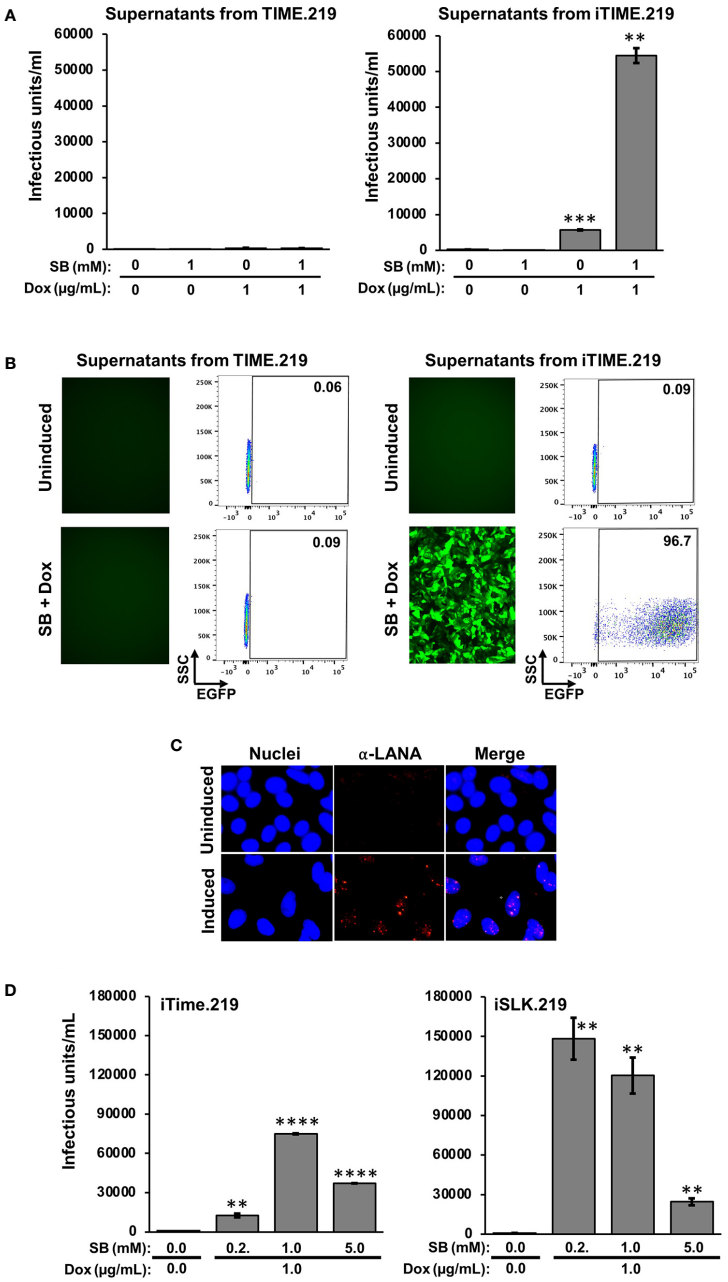


**FIGURE 3** | Comparison of infectious virus induction from TIME.219 and iTIME.219 cells by various combinations of sodium butyrate and doxycycline. Supernatants from the experiment in **Figure 2** were collected, clarified, concentrated, and resuspended in 200 µl of fresh media. The concentrated supernatants were analyzed for infectivity on Vero target cells as assessed by eGFP fluorescence. **(A)** Vero cells were exposed to serial dilutions of supernatants from TIME.219 cells (left panel) and iTIME.219 cells (right panel) and cultured for three days in the presence of the indicated concentrations of SB, DOX, or SB plus Dox. Infected cells were assessed by flow cytometry. Infectious unit was defined as the amount of virus needed to result in a single cell expressing eGFP. Infectious units per ml were quantitated from dilutions within the linear range of detection and are plotted on the y-axis. **(B)** Selected Vero target cells generated as described in panel **(A)**, infection based on eGFP expression was assessed by fluorescence photomicroscopy (left subpanels, magnification 10×) and flow cytometry (right subpanels; numbers in each upper right corner indicate percent cells within the eGFP$^+$ gate). **(C)** LANA staining: Vero cells exposed to supernatants from uninduced (upper panels) or induced (lower panels) iTIME.219 cells were fixed, permeabilized and stained with anti-LANA mAb LN53 followed by an Alexa-647 conjugated anti-rat antibody. Nuclei were stained with DAPI (blue, left panels) LANA (orange, center panels); merged (right panels) Images shown are at 20× magnification and are cropped to further aid visualization of puncta. **(D)** Comparison of virus production from iTIME.219 and iSLK.219 cells. iTIME.219, and iSLK.219 cells were exposed to the indicated concentrations of SB and Dox, and concentrated supernatants containing virus were prepared. Vero target cells were exposed to the preparations, and infectious units were calculated as described for **(A, B)** **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001. Error bars indicate SEM.

which have proven valuable as a reliable source for generating the recombinant rKSHV.219 reporter virus. **Figure 3D** shows that both cell types produce robust amounts of virus, with iSLK.219 cells producing around 40% more at the previously described optimal concentrations of SB plus Dox.

## Cell Line Verification

In light of the concerns associated with potential cell line misidentification (Horbach and Halffman, 2017), we applied two approaches to verify the authenticity of the TIME cells and its derivatives. We first assessed immuno-phenotype, using CD31 expression as a classical endothelial cell marker and human vascular endothelial cells (HUVECs) as an endothelial cell standard; we also analyzed pan-cytokeratin as a classical epithelial cell marker, using the iSLK.219 epithelial cell line (Sturzl et al., 2013) as a standard. Corresponding isotype control antibodies were used for negative controls. As shown in **Figure 4**, the TIME, TIME.219, and iTIME.219, as well as HUVEC cells all stained positive for CD31 and negative for pan-cytokeratin, consistent with endothelial phenotype. The iSLK.219 cells showed the reciprocal immuno-staining pattern, *i.e.* negative for CD31 and positive for pan-cytokeratin, as expected for its epithelial phenotype.

We next applied the favored method for cell authentication, short tandem repeat (STR) profiling (Masters et al., 2001). This procedure was applied to genomic DNA samples prepared from TIME, TIME.219, and iTIME.219 cells and subjected to STR profiling. The profiles were compared to two databases of known STR profiles from the American Type Culture Collection (ATCC) and the Deutche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). The data in **Table 1** indicate that at all nine loci, there was a 100% match between each of the TIME-derived experimental STR profiles and the TIME profiles in both databases.

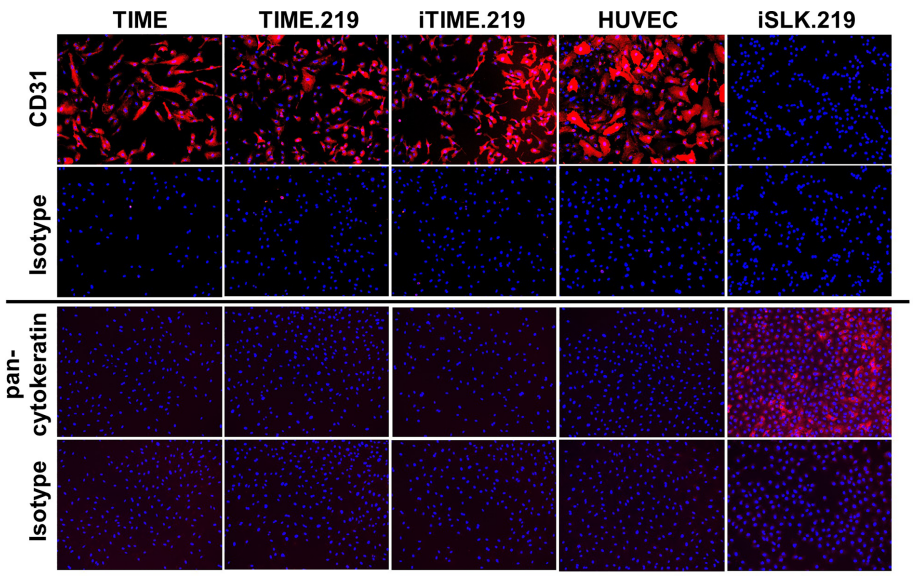


**FIGURE 4** | Phenotypic analysis for cell type-specific markers. The indicated cells were grown to 95% confluence, fixed, and stained with mAbs specific for CD31 (endothelial marker), or PAN cytokeratin (epithelial): or isotype-matched non-specific antibody controls. Detection was performed with an Alexa-Fluor-647-conjugated anti-mouse IgG antibody and is shown in orange. Nuclei were stained with DAPI (blue). Images are shown at 10× magnification.

**TABLE 1** | STR analysis of TIME cells and KSHV chronically infected derivatives.

| Cell Line | Locus names | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | D5S818 | D13S317 | D7S820 | D16S539 | vWA | TH01 | AMEL | TPOX | CSF1PO |
| TIME | 11,11 | 9,11 | 8,9 | 9,12 | 16,18 | 6,7 | X,Y | 8,8 | 11,12 |
| TIME.219 | 11,11 | 9,11 | 8,9 | 9,12 | 16,18 | 6,7 | X,Y | 8,8 | 11,12 |
| iTIME.219 | 11,11 | 9,11 | 8,9 | 9,12 | 16,18 | 6,7 | X,Y | 8,8 | 11,12 |
| TIME (DSMZ) | 11,11 | 9,11 | 8,9 | 9,12 | 16,18 | 6,7 | X,Y | 8,8 | 11,12 |
| TIME (ATTC) | 11 | 9,11 | 8,9 | 9,12 | 16,18 | 6,7 | X,Y | 8 | 11,12 |

*STR analysis was performed on the TIME, TIME.219, and iTIME.219 cells used in this study (upper three lines) and results were compared to data on TIME cells obtained from two biorepositories (lower two lines): the Deutche Sammlung von Mikroorganismen und Zellkulture (DSMZ) and the American Type Culture Collection (ATCC). The table indicates the locus names and the number of STR repeats found at each locus for the indicated cell line.*

Taken together, our data verify the endothelial phenotype of the TIME-derived cells developed in this study and their genetic identity with the TIME cells reported in the established ATCC and DSMZ databases.

## DISCUSSION

The findings presented herein demonstrate that 1) iTIME.219 endothelial cells maintain latent infection with rKSHV.219, 2) the cells can be induced to progress through the complete virus lifecycle, 3) induced cells produce robust amounts of infectious KSHV, and 4) induction can be achieved by activating the natural trigger for the latent to lytic transition, without the need for agents that promote non-specific pleiotropic effects.

To establish the iTIME.219 cells, we employed a modification of the approach previously described by Myoung at Ganem for generating a chronically infected endothelial cell line designated iSLK.219 (Myoung and Ganem, 2011), including use of the rKSHV.219 recombinant virus and incorporation of a doxycycline-inducible RTA gene. In that study, the authors employed SLK as the parental cell line based on its previous description as endothelial-derived (Herndier et al., 1994). However a subsequent study revealed SLK to be a contaminant from a renal carcinoma cell line, and the investigators noted its unsuitability for KSHV/endothelial biology and pathogenesis (Sturzl et al., 2013). Thus, the iTIME.219 cells described herein represent a novel system for investigating the various phases of the KSHV cycle in endothelial cells, for analyzing properties of virus particles generated from this critically relevant cell type, and for comparing these features in endothelial cells with those in B lymphocytes, the other major cell type involved in KSHV pathogenesis.

A particularly interesting question is the possible influence of producer cell type on KSHV tropism. There is precedent for such relationships among both animal and human gammaherpesviruses, whereby cell type-specific variations in viral glycoprotein expression result in complex effects on tropism (Gillet et al., 2015; Hutt-Fletcher, 2017). For bovine herpesvirus-4, virions produced from infected epithelial cells have high levels of the gp180 glycoprotein and are minimally infectious for $CD14^+$ circulating cells, whereas virions generated from myeloid cells express reduced gp180 levels and are readily infectious for these cells; these expression differences are associated with alternative splicing of the corresponding gene (Machiels et al., 2011; Machiels et al., 2013). For the human pathogen Epstein–Barr virus, epithelial cells produce virus expressing glycoprotein complexes containing gp42, whose binding to cell surface HLA II is essential for virus fusion/entry into B lymphocytes. By contrast, B cells generate virus with relatively reduced expression of gp42 owing to its intracellular capture in the HLA II processing pathway, resulting in relative enhancement of epithelial cell infection. Thus each of these important target cell types generates virus with reciprocal relative tropism (Wang et al., 1998; Borza and Hutt-Fletcher, 2002).

Might KSHV also exploit producer cell-dependent variations in tropism in establishing infection of its major target cell populations, B lymphocytes and endothelial cells? Based on studies with a permissive B cell line and primary B cells from tonsil, we have reported (Dollery et al., 2019) that the K8.1A glycoprotein of KSHV is critical for B lymphocyte infection but dispensable for primary endothelial cells (and for other non-B cell targets, as had been well-established in the literature). This finding complemented extensive evidence in the literature demonstrating the tropism involvement of proteins encoded by positionally homologous genes in cell tropism of other gammaherpesviruses, as described above for gp180 of bovineherpesvirus-4 (Machiels et al., 2011; Machiels et al., 2013), as well as for gp350/320 of Epstein–Barr virus (Hutt-Fletcher, 2007). This raises the question of whether variations in expression or processing of K8.1 gene products in different virus producer cells play a role in KSHV tropism. The iTIME.219 endothelial cells provide a framework for investigating such questions, involving comparisons with KSHV chronically infected B cell lines that can be activated for virus production by KSHV-specific pathways (Dollery et al., 2014; Kati et al., 2015).

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

## AUTHOR CONTRIBUTIONS

EBr designed and performed preliminary experiments with oversight from SD and EBe. TM continued experiments and instigated validation studies with oversight from EBe and SD. SD, TM, and EBe prepared the initial draft of the manuscript. All authors edited subsequent drafts. EBe oversaw all aspects of funding. All authors contributed to the article and approved the submitted version.

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frontiers in Cellular and Infection Microbiology

REVIEW
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# KSHV Reprogramming of Host Energy Metabolism for Pathogenesis

*Xiaoqing Liu*[1], *Caixia Zhu*[1], *Yuyan Wang*[1], *Fang Wei*[2] *and Qiliang Cai*[1]*

[1] Ministry of Education (MOE) & National Health Committee (NHC) & Chinese Academy of Medical Science (CAMS), Key Laboratory of Medical Molecular Virology, Department of Medical Microbiology and Parasitology, School of Basic Medicine, Shanghai Medical College, Fudan University, Shanghai, China, [2] Sheng Yushou Center of Cell Biology and Immunology, School of Life Science and Biotechnology, Shanghai Jiao Tong University, Shanghai, China

Reprogramming of energy metabolism is a key for cancer development. Kaposi's sarcoma-associated herpesvirus (KSHV), a human oncogenic herpesvirus, is tightly associated with several human malignancies by infecting B-lymphocyte or endothelial cells. Cancer cell energy metabolism is mainly dominated by three pathways of central carbon metabolism, including aerobic glycolysis, glutaminolysis, and fatty acid synthesis. Increasing evidence has shown that KSHV infection can alter central carbon metabolic pathways to produce biomass for viral replication, as well as the survival and proliferation of infected cells. In this review, we summarize recent studies exploring how KSHV manipulates host cell metabolism to promote viral pathogenesis, which provides the potential therapeutic targets and strategies for KSHV-associated cancers.

**Keywords: KSHV, energy metabolism, viral reprogramming, oncogenesis, herpesvirus**

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***Edited by:***
*Subhash C. Verma,*
*University of Nevada,*
*United States*

***Reviewed by:***
*Arunava Roy,*
*University of South Florida,*
*United States*
*Fanxiu Zhu,*
*State College of Florida,*
*Manatee–Sarasota,*
*United States*

***Correspondence:**
*Qiliang Cai*
*qiliang@fudan.edu.cn*

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## INTRODUCTION

Reprogramming of energy metabolism is a hallmark of cancers. Cancer cells often rewire metabolic pathways to balance ATP production for supporting rapidly tumor proliferation. In normal cells, glucose is converted into pyruvate through glycolysis, and pyruvate is oxidized in mitochondria to produce ATP (**Figure 1**). While in cancer cells, pyruvate is preferentially catabolized into lactate even in the presence of oxygen, this metabolic process is known as "aerobic glycolysis" or "Warburg effect" (Delgado et al., 2010; Courtnay et al., 2015). In Warburg effect, glucose-derived carbon is directed away from the TCA cycle and ATP production is decreased. For producing energy sufficient to maintain cancer cell proliferation, glutaminolysis is also often elevated in most of cancer cells. Glutaminolysis is shown to replenish the TCA cycle *via* converting glutamine to glutamate, which is in turn transaminated to α-ketoglutarate and produces citrate within mitochondria, while a proportion of this citrate is exported into the cytoplasm, and cytoplasmic citrate is then metabolized into acetyl-CoA for fatty acid synthesis and related biosynthetic precursors (Biswas et al., 2012; Dyer et al., 2019). This process is also termed as anaplerosis, and is a dominant metabolism mode in rapidly growing malignant cells besides Warburg effect. Thus, the metabolic plasticity of cancer cells is essential to generate energy required for cell proliferation.

Increasing evidence has shown that virus infection, similar to cancer development, depends on the reprogramming of cellular metabolism to produce biomass for viral replication and virion

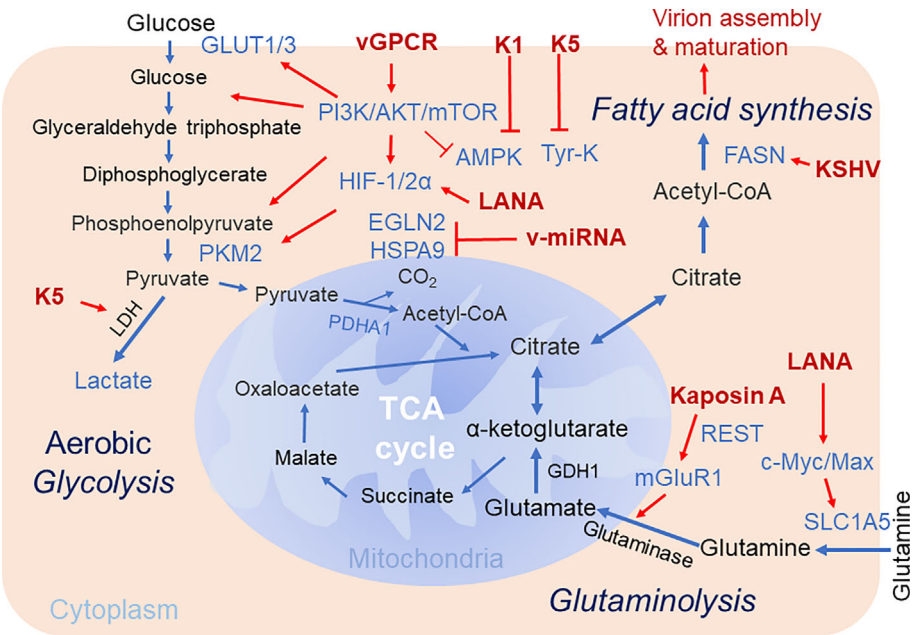


**FIGURE 1** | Brief schematics of KSHV-mediated alteration of host cellular metabolic pathways. The key steps of three cellular metabolic pathways including aerobic glycolysis, glutaminolysis and fatty acid synthesis targeted by KSHV for pathogenesis are highlighted. The increased expression of enzymes and metabolites are shown in blue. The KSHV-encoded proteins are shown in red. Red arrow denotes the regulation by KSHV. TCA Cycle, tricarboxylic acid cycle; GDH, glutamate dehydrogenase; PKM2, pyruvate kinase 2; Tyr-K, Tyrosine kinase; EGLN2, HIF prolyl hydroxylase 2; mGluR1, metabotropic glutamate receptor 1; SLC1A5, glutamine transporter.

production. Virus infection may reprogram host metabolism for viral genome and protein synthesis, as well as lipid envelope generation for virion production. Interestingly, in the oncoviruses-associated cancers, accumulating literatures have also reported that many oncogenic viruses alter the function of pivotal cellular proteins that involve in reprogramming of cell metabolism to facilitate cancer development in addition to supporting virus propagation (Yu et al., 2018; Purdy and Luftig, 2019).

Kaposi's sarcoma-associated herpesvirus (KSHV), a human oncogenic gamma-herpesvirus, can establish life-long latent infection in human. KSHV is tightly associated with Kaposi's sarcoma (KS) and two B-cell lymphomas including primary effusion lymphoma (PEL) and multicentric Castleman disease (MCD) by infecting B-lymphocyte and endothelial cells, respectively. Like other herpesviruses, KSHV contains two phases of life cycle: latency and lytic replication (**Figure 2**). During latency, only a limited number of proteins including LANA (Latency-Associated Nuclear Antigen, ORF73), vCyclin (viral Cyclin, ORF72), vFLIP (viral FLIP, ORF71), Kaposin (K12), and 12 microRNAs (v-miRNA) are expressed, which play critical roles in host cell transformation and growth. In contrast, the majority of the KSHV genome-encoded genes including K1, K5, K-bZIP (K8), and vGPCR (viral G Protein-Coupled Receptor, ORF74) are activated with the expression of the lytic master regulator of RTA (ORF50) during lytic replication. Within the tumor tissue, most of the infected cells are in latency state, only about 5% of the cells undergo lytic replication (Staskus et al., 1997). It has been assumed that both latent and lytic replicative cycles of KSHV contribute to tumor formation and development (Ganem, 2006). Since viruses do not possess a metabolic network but require metabolites for viral survival, it becomes a barrier for virus replication and inducing tumorigenesis. To overcome this barrier, it has been demonstrated that KSHV has evolved multiple strategies to alter metabolic pathways, including aerobic glycolysis, fatty acid synthesis, oxidative phosphorylation, reactive oxygen species (ROS) production, glutaminolysis and amino acid synthesis, which appears to participate in virus production at different stages of the viral life cycle (Delgado et al., 2012; Sanchez et al., 2017). In addition, KSHV also alters the metabolic pathways for sustaining and promoting the proliferation and survival of host cells, particularly under stress conditions (Singh et al., 2019; Münz, 2020). Thus, the metabolic properties of KSHV-infected cells are closely resembled to the metabolic hallmarks of cancer cells.

Given both viral replication and tumorigenesis are energy-consuming processes, it is not surprising that the regulation of host cell metabolism is a barrier for establishment of oncovirus persistent infection and invasion. Considering there are multiple similarities between metabolic reprogramming by oncogenic viruses and cancer cells, the alterations in cancer-associated metabolic reprogramming may play profound effects on viral gene expression, cell transformation, and the response to tumor microenvironment during the life cycle of viral infection

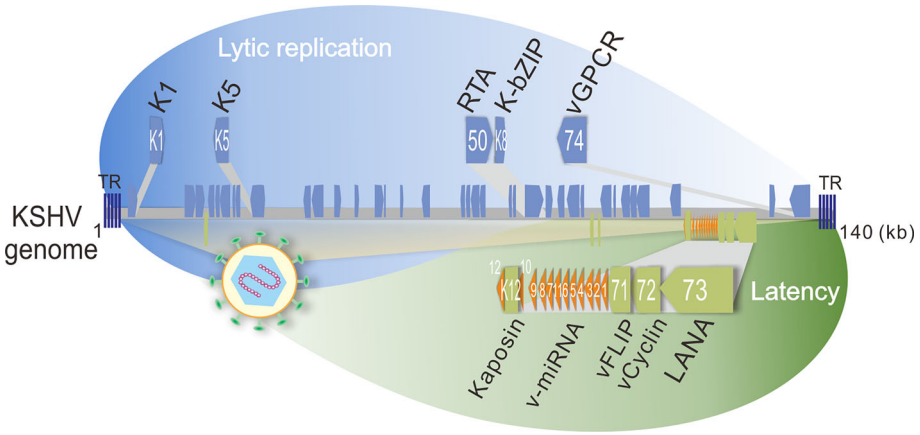


**FIGURE 2** | Schematic representation of KSHV-encoded proteins that involve in host energy metabolism reprogramming during latency and lytic replication. The lytic genes (K1, K5, orf50, K8, and orf74 which encodes K1, K5, RTA, K-bZIP, and vGPCR, respectively) are shown on the top panel, and the latent genes (K12, v-miRNA, orf71, orf72, and orf73 which encodes Kaposin, v-miRNA cluster, vFLIP, vCyclin, and LANA, respectively) are shown at the bottom panel. TR, terminal repeat; kb, kilo base pair.

(Pavlova and Thompson, 2016). In this review, we summarize most recent studies regarding to how KSHV alters host cellular metabolism for viral production and oncogenesis, particularly three major pathways of cellular metabolism including central carbon metabolism, aerobic glycolysis, glutaminolysis and fatty acid synthesis are addressed and discussed below (**Figure 1**).

## KSHV INFECTION ALTERS THE HOST CENTRAL CARBON METABOLISM

It has been shown that KSHV latent infection can induce aerobic glycolysis and lactic acid production with decrease of oxygen consumption, which is consistent with the Warburg effect (Delgado et al., 2010). Inhibitors of glycolysis, including Oxamate and 2-Deoxyglucose, is able to selectively induce apoptosis of the KSHV-infected endothelial cells instead of mock cells (Delgado et al., 2010), indicating that KSHV-transformed endothelial cells depend on glycolysis to establish viral latency infection and survival.

In addition, it has also been demonstrated that KSHV latently-infected endothelial cells are prone to take advantage of glutamine, and glutamine deprivation or glutamine transporter inhibition could induce cell death of KSHV latently-infected cells (Sanchez et al., 2015). Unlike most cancer cells that primarily utilize glutamine and asparagine to complete the TCA cycle, KSHV-infected cells depend on glutamine and asparagine to provide γ-nitrogen for purine and pyrimidine biosynthesis (Zhu et al., 2017). Knockdown of any one of three rate-limiting enzymes in the *de novo* purine biosynthesis pathway, including phosphoribosyl pyrophosphate aminotransferase (PPAT), phosphoribosyl pyrophosphate synthetases 1 and 2 (PRPS1/2), or a rate-limiting enzyme like carbamoylphosphate synthetase 2 (CAD) in the pyrimidine biosynthesis pathway, will dramatically impair the proliferation of KSHV-latently transformed endothelial cells instead of its untransformed cells (Zhu et al., 2017). This indicates that the glutamine-dependent nucleotide biosynthesis is required for the anabolic proliferation of KSHV-infected cells.

A global metabolomics analysis of KSHV-infected cells indicates that metabolites of several pathways, including glycolysis, amino acid metabolism, the pentose phosphate pathway and lipogenesis, are commonly deregulated by KSHV. For example, KSHV infection elevates majority of the detectable metabolite products of *de novo* fatty acid synthesis (FAS), and inhibition of the key enzymes in fatty acid synthesis will lead to apoptosis of KSHV-infected cells but not uninfected cells (Delgado et al., 2012), suggesting that increased level of fatty acid synthesis is also essential for survival of KSHV latently-infected endothelial cells. In addition to endothelial cells, KSHV also induces B-cell lymphoma, including B-cell non-Hodgkin lymphomas (B-NHL) and primary effusion lymphoma (PEL), to maintain high levels of glycolysis and fatty acid synthesis. It has been revealed that aerobic glycolysis and FAS occur in a PI3K-dependent manner, and the fatty acid synthesizing enzyme (FASN) is overexpressed in PEL (Sommermann et al., 2011; Bhatt et al., 2012).

In cancer cells, the induction of glycolysis and fatty acid synthesis is considered to serve as a resource for rapid energy production, or as other specific metabolites necessary for increased cell division or proliferation (DeBerardinis et al., 2008). In addition to deregulation of multiple cellular metabolic pathways during KSHV latent infection, emerging evidence shows that KSHV also modifies host cellular carbon source utilization to ensure optimal environments for viral DNA replication and virion production during lytic replication. For example, Sanchez et al. demonstrated that KSHV lytic replication depends on the host major central carbon metabolic pathways. Inhibition of glycolysis, glutaminolysis, or fatty acid synthesis could efficiently reduce KSHV virion production from both endothelial lytic system and the iSLK cell-inducible KSHV system (Sanchez et al., 2017).

## MECHANISMS BY WHICH KSHV CONTROLS AEROBIC GLYCOLYSIS, GLUTAMINOLYSIS AND FATTY ACID SYNTHESIS

A better understanding of how KSHV switches to cancer-like cell metabolism will be helpful in seeking novel therapies for KSHV infection. Increasing evidence shows that KSHV reprograms host cell metabolism mainly through targeting directly regulation of metabolic enzymes at the transcriptional or translational levels, or indirectly activating metabolic regulators that are commonly occurred in cancer cells, such as HIF-1α, c-MYC, AMPK and so on, to alter host metabolic gene expression (**Table 1**).

### Aerobic Glycolysis

It has been demonstrated that KSHV-encoded microRNAs (v-miRNA) within the latent gene cluster collaborate to induce aerobic glycolysis with decreased oxygen consumption, increased lactate secretion and glucose uptake. Mechanistically, v-miRNA cluster alters host cell energy metabolism through regulating the translation and mRNA stability of their target genes, such as HIF prolyl hydroxylases 2 (EGLN2) and the mitochondrial heat shock protein A9 (HSPA9). Knockdown of any one of these gene, will stabilized HIF1α, decreased oxygen consumption and reduced mitochondria volume (Yogev et al., 2014). It was recently shown that the KSHV v-miRNAs can be transferred to the surrounding uninfected cells to induce a reverse Warburg effect by exosomes. 'Reverse Warburg Effect' is a new term that means cancer cells can induce aerobic glycolysis in adjacent stromal cells except for their intrinsic metabolic alteration. This raises the possibility that viruses could use exosomes to shape the metabolism of host tissue microenvironment during persistent infection (Yogev et al., 2017). In addition, some lytic gene products can also contribute to transform endothelial cells. For examples, KSHV-encoded K5, as the first discovery of viral E3 ubiquitin ligase, is able to increase aerobic glycolysis and lactate production, as well as promote oncogenesis through modulating endocytosis of host cellular growth factor-binding receptor-associated tyrosine kinase, which in turn increases the sensitivity of cells to autocrine and paracrine factors (Karki et al., 2011).

Hypoxia-inducible factor-1α (HIF-1α) is an intracellular key factor that mediates many cellular responses to hypoxia. Intensive studies have demonstrated that HIF-1α is a metabolic regulator which regulates the transcription of genes involved in aerobic glycolysis or fatty acid synthesis during cancer development and oncovirus infection (Singh et al., 2016; Lo et al., 2017). It has been shown that KSHV targets and activates HIF-1α, and several KSHV-encoded genes are in turn activated by HIF-1α, suggesting HIF-1α might play a substantial role in KSHV-induced oncogenesis. Previous studies have shown that LANA encoded by KSHV stabilize HIF-1α by degrading the E3 ubiquitin ligase VHL in the KSHV latently-infected B cells (Cai et al., 2006), and the stabilized HIF-1α in turn upregulates pyruvate kinase 2 (PKM2) (which is the key step enzyme of the glycolytic to maintain aerobic glycolysis in infected cells) and increases lactate production (Ma et al., 2015). Consistence with this observation, HIF-1α knockdown in PEL cell lines results in a reduction of both aerobic/anaerobic glycolysis and lipid biogenesis, indicating that HIF-1α is necessary for maintaining a metabolic state optimal for growth of PEL (Shrestha et al., 2017). Interestingly, some studies have also revealed that the expression of the lytic oncoprotein vGPCR leads to induction of both HIF-1α and HIF-2α in a mTOR-dependent manner (Jham et al., 2011). Given that PI3K-AKT-mTOR pathway is essential for controlling cell proliferation and regulating anabolic activities in B lymphocytes and endothelial cells (Tomlinson and Damania, 2004; Wang et al., 2004), PI3K/AKT signaling is also shown to involve in regulation of aerobic glycolysis by controlling the expression and localization of the glucose transporter 1/3 (GLUT1/3) and other glycolytic enzymes (Manning and Cantley, 2007), or inactivating the negative regulator of aerobic glycolysis AMPK (Wang and Damania, 2008). For example, the KSHV-encoded K1 protein has been shown to inactivate AMPK for deregulation of aerobic glycolysis by binding to its γ- subunit (Anders et al., 2016).

**TABLE 1** | List of KSHV-encoded proteins reprogramming host metabolism.

| Viral gene | Target pathway | Mechanism of metabolism rewiring | Ref |
|---|---|---|---|
| LANA | Aerobic glycolysis | Stabilization of HIF-1α by degrading the E3 ubiquitin ligase VHL, the stabilized HIF-1α upregulates PKM2 | (Cai et al., 2006; Ma et al., 2015) |
| K1 | Aerobic glycolysis | Inactivation of AMPK | (Anders et al., 2016) |
| vGPCR | Aerobic glycolysis | Increased expression of HIF-1α and HIF-2α in a mTOR-dependent manner | (Jham et al., 2011) |
| K5 | Aerobic glycolysis | Modulating endocytosis of cellular growth factor-binding receptor-associated tyrosine kinase | (Karki et al., 2011) |
| v-miRNAs | Aerobic glycolysis | Down-regulation of EGLN2 and HSPA9 to decrease mitochondrial biogenesis | (Yogev et al., 2014) |
| LANA | Glutaminolysis | Upregulation of the glutaminase and glutamate by activating c-Myc | (Valiya Veettil et al., 2014) |
| Kaposin A | Glutaminolysis | Increased expression of the metabotropic glutamate receptor 1 | (Valiya Veettil et al., 2014) |

## Glutaminolysis

Glutamine is the other major substrate that contributes to energy production. It has been shown that glutamine is usually transported into cells through glutamine transporter SLC1A5, and then converted into a-ketoglutarate at the catalysis of glutamate dehydrogenase 1 (GDH1) to replenish TCA cycle (Daye and Wellen, 2012), or shunt a-ketoglutarate into citrate for lipid production, which is called gxlutaminolysis. The profile analysis of high-throughput RNA sequencing has revealed that the expression levels of many enzymes (such as glutaminase 2, GLS2; glutamate dehydrogenase 1, GDH1; and glutamic-oxaloacetic transaminase 2, GOT2) in the glutamine pathway are upregulated by KSHV infection, which supports the notion that KSHV mediates host cellular glutaminolysis for promoting proliferation of KSHV-transformed cells (Sanchez et al., 2015; Zhu et al., 2017).

During latent infection, it is not only KSHV-encoded LANA protein upregulates the glutaminase and glutamate by activating c-Myc, the latent viral protein Kaposin A also upregulates expression of the metabotropic glutamate receptor 1 (mGluR1 receptor) by binding and sequestering host REST (RE-1 Silencing Transcription Factor) in the cell cytoplasm to relieve the REST-mediated suppression of the mGluR1 gene. Binding of glutamate to mGluR1 in turn induces signaling and proliferation of infected cells (Valiya Veettil et al., 2014). In addition, abundant evidence has demonstrated that extended c-Myc network contributes to glutamine addiction in some cancers (Dang, 2013). Consistence with the discovery that MondoA/Mlx signaling pathway is essential for regulation of glutaminolysis in cancer cells, KSHV also induces expression of the heterodimeric transcription factors c-Myc-Max and their related heterodimer MondoA-Mlx to upregulate glutamine transporter SLC1A5, leading to increased glutamine uptake for glutaminolysis in the KSHV-infected endothelial cells (Sanchez et al., 2015).

## Fatty Acid Synthesis

Fatty acid, as the main component of all biological lipid membranes, is another essential substrate for energy metabolism, and appears to play a role in cancer pathogenesis (Dang, 2013). Although the result from metabolomics analysis showing that an increase in fatty acid precursor metabolites is generally associated with increased FAS (i.e. choline and phosphocholine), however, the metabolites of glycerophosphorylcholine and glycerol-3-phosphate, that are associated with fatty acid production, are tend to be reduced due to degradation of phospholipids (Delgado et al., 2012). Despite whether KSHV activates fatty acid synthesis remains undefined, it is still possible that KSHV increases the fatty acid synthesis-related enzymes to promote its synthesis. In addition, the increased acetyl-CoA from glutaminolysis flux may be the material for *de novo* synthesis of fatty acids.

Similar to the observation that KSHV-transformed cells depend on glutamine for their growth, proliferation, and survival (Zhu et al., 2017), the induction of fatty acid synthesis is required for the survival of KSHV latently-infected endothelial cells, and inhibition of FAS will greatly increase apoptotic death of the latently infected cells instead of uninfected counterparts (Sanchez et al., 2017). Therefore, the product of FAS is necessary for the survival of endothelial cells with KSHV latent infection.

Although both glycolysis and glutaminolysis have been shown to involve in viral genome replication, they are also required for regulation of lytic gene expression at different early steps. For examples, glycolysis is necessary for early gene transcription, while glutaminolysis is necessary for early gene translation. Likewise, inhibition of fatty acid synthesis leads to a reduction of KSHV infectious particles production without defects in viral gene expression or replication (Sanchez et al., 2017). Similar phenomenon is also observed that inhibition of FAS results in reduction of infectious particles in other enveloped viruses including EBV (Li et al., 2004), HCMV, influenza virus (Munger et al., 2008), varicella-zoster virus (VZV) and HCV (Yang et al., 2008), supporting the notion that FAS is essential for the viral envelop membrane synthesis (Delgado et al., 2012), albeit the precise mechanism remains to be further investigated. Therefore, inhibition of these specific cellular metabolic pathways could not only eliminate latently infected cells but also block lytic replication (Sanchez et al., 2017), which will provide the potential therapeutic targets and strategies against KSHV-associated cancers.

# CONCLUSION AND PROSPECTIVE

In this review, we focus to address how KSHV infection rewires host cellular metabolism. During KSHV infection, aerobic glycolysis, glutaminolysis and fatty acid synthesis are the major pathways for host cell energy mass production, while other pathways including amino acid metabolism and the pentose phosphate pathway are also altered. EBV, another gamma-herpesvirus with high homologues of KSHV, has also been shown to manipulate many metabolic pathways by encoding different viral proteins (Piccaluga et al., 2018), particularly a recent study showing that EBV can remodel mitochondrial one-carbon (1C) metabolism to drive B-cell transformation (Wang et al., 2019). This indicates that KSHV may also rewire other pathways by encoding various viral proteins, in addition to only few of them related to metabolic pathways are reported so far. It can also be concluded that KSHV alters host cells metabolism mainly through the activation of viral oncogenes or the common host metabolic regulators such as HIF-1α and c-Myc, which is similar as that has been demonstrated in the EBV-transformed B-cell lymphomas (Piccaluga et al., 2018). Thus, the studies about how KSHV infection alters host metabolism for the survival of latently infected cells will provide novel therapeutic strategies for eliminating latent infection of KSHV, and will also shed a light on how a virus targets host cell metabolism for driving tumor development.

Although intensive studies have focused to address how metabolic reprogramming influences the propagation and survival of host cells, how metabolic alteration influences virus replication or production is still unclear. Further studies are required for uncover how metabolic reprogramming impacts the viral life cycles, particularly KSHV. Due to both immune response and immune escape play crucial roles in viral infection, whether metabolic reprogramming involves in those processes is still an open question and need be further investigation in future. Uncovering metabolic factors that contribute to anti-virus or viral

infection will also provide novel therapeutic targets against oncoviruses infection and their associated cancers.

## AUTHOR CONTRIBUTIONS

XL drafted the manuscript. CZ and YW provided critical reading. FW and QC supervised final manuscript. All authors contributed to the article and approved the submitted version.

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# Immunological Identification and Characterization of the Capsid Scaffold Protein Encoded by UL26.5 of Herpes Simplex Virus Type 2

*Xueqi Li[1†], Jianbin Wang[1†], Tangwei Mou[1], Yang Gao[1], Lichun Wang[1], Shengtao Fan[1], Xingli Xu[1], Guorun Jiang[1], Pingfang Cui[1], Xiangxiong Xu[1], Suqin Duan[1], Jingjing Zhang[1], Dandan Li[1], Yun Liao[1], Li Yu[1], Heng Zhao[1], Ming Lu[1], Hailian Zhu[1], Ran Gu[1], Ying Zhang[1*], Wei Dong[2*] and Qihan Li[1*]*

[1] Institute of Medical Biology, Chinese Academy of Medicine Sciences & Peking Union Medical College, Yunnan Key Laboratory of Vaccine Research and Development for Severe Infectious Diseases, Kunming, China, [2] Reproductive & Gynecology Department, The First People's Hospital of Yunnan Province, Kunming, China

Herpes simplex virus type 2 (HSV2), a pathogen that causes genital herpes lesions, interferes with the host immune system *via* various known and unknown mechanisms. This virus has been used to study viral antigenic composition. Convalescent serum from HSV2-infected patients was used to identify viral antigens *via* 2-D protein electrophoresis and immunoblotting. The serum predominantly recognized several capsid scaffold proteins encoded by gene UL26.5, mainly ICP35. This protein has been primarily reported to function temporarily in viral assembly but is not expressed in mature virus particles. Further immunological studies suggested that this protein elicits specific antibody and cytotoxic T lymphocyte (CTL) responses in mice, but these responses do not result in a clinical protective effect in response to HSV2 challenge. The data suggested that immunodominance of ICP35 might be used to design an integrated antigen with other viral glycoproteins.

**Keywords: herpes simplex virus type 2, ICP35, 2-D protein electrophoresis, immunization, viral replication**

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***Edited by:***
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*Agustin Valenzuela-Fernandez,*
*University of La Laguna, Spain*

****Correspondence:***
*Qihan Li*
*imbcams_qhli@163.com*
*Ying Zhang*
*cherryzhang629@126.com*
*Wei Dong*
*weiwei_d@126.com*

*[†]These authors have contributed equally to this work and share first authorship*

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## INTRODUCTION

Herpes simplex virus type 2 (HSV2), a human alpha-herpesviridae virus (Shukla and Spear, 2001), is known to spread in teenage and adult populations and to cause herpetic pathogenic lesions in the skin of the genitals (Looker et al., 2015), which can seriously impact quality of life (Castillo et al., 2020). Epidemic data have recently suggested that more than 400 million people are infected with HSV2 worldwide (James et al., 2020). Approximately 45 million infected patients have been identified in the United States, and this number increases by 1 million patients each year (Mathew and Sapra, 2020). Given this background and the lack of specific treatment methods (Sandgren et al., 2020), the development of HSV2 vaccines has become a major concern for the control of this disease (Spicknall et al., 2019); however, no applicable vaccine for HSV2 has been reported to date, although some clinical trials of a HSV2 vaccine have been initiated (Corey et al., 1999; Belshe et al., 2012). Based on the data from these trials, no protective effect has been observed in the individuals

immunized with these experimental vaccines, although neutralizing antibodies were detected in serum samples (Corey et al., 1999; Belshe et al., 2012). These results suggest that our knowledge about how the HSV2 antigen trains the immune system to recognize and target the invading virus is lacking. Studies of HSV2 pathogenesis have indicated that various proteins expressed during viral infection are capable of interacting with the host immune system and interfering with antiviral immunity (Suazo et al., 2015), although these viral proteins should be recognized by the immune system and elicit specific responses, such as specific antibody responses against each viral protein (Tognarelli et al., 2019). Thus, understanding the mechanism of host immunity against this virus depends upon integrated analyses of the immune recognition of the majority of its proteins and the roles played by these proteins during infection. Based on this hypothesis, antibodies were used in neutralizing assays and enzyme-linked immunosorbent assays (ELISAs) to evaluate the immunological reactions that occur during HSV2 infection; however, these methods were insufficient to show the relationships of various viral proteins and immune serum. Here, we used protein 2-D electrophoresis and immunoblotting associated with immunofluorescent assay to investigate the recognition of viral proteins by convalescent serum collected from HSV2-infected patients. Interestingly, we found that several viral capsid scaffold proteins encoded by genes UL26.5 and UL26, especially ICP35, were predominantly recognized by the convalescent serum compared with other viral structural proteins that elicit neutralizing antibodies (Cairns et al., 2015). Moreover, further immunological characterization of ICP35 suggested the possibility that immunodominance might be used to design an integrated antigen with other viral glycoproteins.

## MATERIALS AND METHODS

### Virus and Cells

The HSV2 HG52 strain was purchased and preserved by the Virus Immunization Room of the Institute of Medical Biology, Chinese Academy of Medical Sciences. The virus was grown in Vero cells (ATCC, Manassas, USA), which were cultured in an incubator at a constant temperature of 37°C using Dulbecco's modified Eagle medium (DMEM; Corning, NY, USA) supplemented with 5% fetal bovine serum (FCS; HyClone, Logan, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin.

### Viral Titration

All experimental procedures were performed under BSL-2 laboratory conditions. Virus samples were serially diluted 10-fold with serum-free DMEM. Different viral dilutions were added to a 96-well plate. Each dilution (100 μl per well) was added to 8 parallel wells. Then, 100 μl of Vero cell suspension was added to each well at a concentration of $2.5 \times 10^5$ cells/ml. After the plate was incubated at 37°C in 5% $CO_2$ for 7 days, the cytopathic effect (CPE) was observed and assessed with an inverted microscope (Nikon, Tokyo, Japan).

### Neutralizing Antibody Test

Heat-inactivated serum was diluted and coincubated with live virus (100 $lgCCID_{50}$/well) for 2 h at 37°C, and then, 100 μl Vero cells ($10^5$/mL) were added. Then, the plates were incubated at 37°C in 5% $CO_2$ for 7 days. The CPEs were observed and assessed with an inverted microscope (Nikon) to determine the neutralizing antibody titer of the serum. The geometric mean titers (GMTs) of the neutralizing antibodies were measured.

### Plaque Assay

HSV2 virus (MOI=1) and serum samples were added to a 6-well plate with a confluent monolayer of cells and coincubated for 2 h. Then, the plate was incubated at 37°C in 5% $CO_2$ for 2-3 days after a mixture of 2× DMED and 2% methylcellulose was added. Plaques could be observed after staining with crystal violet dye.

### Viral Purification

Monolayers of Vero cells were infected with HSV2 at a MOI of 1, and at 48 h postinfection, the virus liquid was harvested. The virus liquid was concentrated by Millipore Centricon Plus-70. The concentrated virus liquid was layered on a 15 to 55% iodixanol gradient and centrifuged at 30,000 rpm in an SW41 rotor for 2.5h. The purified virus samples were observed *via* electron microscopy and were harvested at -80°C.

### Immunofluorescence

When the 293 cells were 70%-90% confluent, they were transfected with the eukaryotic vectors of intact ICP35, VP22a and VP24 according to the Lipofectamine™ 3000 Reagent Protocol in glass bottom cell culture dishes, and the samples were harvested at 18h. In addition, Vero cells were infected with the HSV2 virus at an MOI of 0.2, and the samples were harvested at 4h, 12h, 24h postinfection. These dishes were fixed in 4% paraformaldehyde for 30 min and then blocked using 4% bovine serum albumin (BSA). For detection of the HSV-2 ICP35, VP22a, VP24 antigen and trace ICP35 protein during viral infection, the dishes were sequentially incubated with primary antibody convalescent serum, immune serum of ICP35 and secondary antibody Alexa Fluor® 647 Goat pAb to Rb IgG (Lot: GR33281 42-4, Abcam), Alexa Fluor® 488 Goat anti-human IgG (H+L) (Lot: 2196582, Invitrogen). All cell nuclei were detected with DAPI. Fluorescence was visualized and analyzed using a confocal microscope (TCS SP2, Leica).

### Co-Immunoprecipitation

Vero cells were infected with the HSV2 virus at an MOI of 1. At the indicated time points (8, 14 and 20 h p.i.), the cells were washed with PBS and lysed in 1 ml RIPA lysis buffer for 10 min. The lysates were centrifuged (12,000 rpm, 4°C for 10 min) and precleared with 50 μl of control agarose beads. After centrifugation, the cell lysate supernatants were incubated overnight with the anti-ICP35 antibody or negative control at 4°C with rotation. Pretreated protein A + G beads (50 μl) were added, and the samples were incubated at 4°C for an additional 2 h and then washed five times and eluted with 500 μl of Tris-EDTA buffer containing 1% SDS. The DNA was then extracted and analyzed using real-time PCR. The specific primer sets used are listed in **Table 1**.

**TABLE 1** | The qRT-PCR primers and ICP35 plasmid construction.

| Name | Sequence (5' - 3') |
|---|---|
| HSV2-Gg-F | CGCTCTCGTAAATGCTTCCCT |
| HSV2-Gg-R | TCTACCCACAACAGACCCACG |
| HSV2-Gg-Probe | FAM-CGCGGAGACATTCGAGTACCAGATCG-BHQ1 |
| HSV-2-UL26.5-F | ATAGAATTCATGAACCCCGTTTCGGCATC |
| HSV-2-UL26.5-R | GCGAAGCTTTTTATTTAAAATATATGAAAACACACA |

*F, Forward primer; R, Reverse primer.*

## 2D Protein Electrophoresis and Western Blot Analysis

Purified HSV2 virus samples were lysed with 2D protein lysis buffer (8 M urea, 4% CHAPS, 100 mM DTT, and 2% IPG buffer). The total protein concentration was determined using a bicinchoninic acid (BCA) protein concentration determination kit (Beyotime Biotechnology, Shanghai, China) according to the manufacturer's instructions. Approximately 80 μg of protein in 170 μl of hydration solution was separated with the IPGphor instrument based on their pI values using immobilized linear gradient strips (Immobiline™ DryStrip, GE Healthcare, USA) covering the pH range 4-7. Then, the protein was separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to polyvinylidene fluoride (PVDF) membranes, blocked with 5% skim milk (prepared in TBST), incubated with convalescent serum from clinically diagnosed HSV2 patients, and incubated with peroxidase-conjugated AffiniPure rabbit anti-human IgA + IgG + IgM (H+L) (Jackson, USA). The PVDF membranes were incubated with an ECL ultrasensitive chemiluminescence reagent and placed in a Bio-Rad gel imager for exposure and color development.

## Silver Stain

The reaction system of each glue in the silver dyeing process is 250ml. The dyeing procedure and the preparation of the solution required for each step are as follows: fix (25ml acetic acid, 100ml methanol, 125ml milli-Q water) twice, 15 minutes each time; sensitize (75ml methanol, 0.5g $Na_2S_2O_3$, 17g NaAc, milli-Q water to 250ml) for 30 minutes; rinse (250ml milli-Q water) 3 times, 5 minutes each time; silver dyeing (0.625g $AgNO_3$, milli-Q water to 250ml) 30 minutes; rinse (250ml milli-Q water) 2 times, 1 minute each time; color development (6.25g $Na_2CO_3$, 100μl formaldehyde, milli-Q water to 250ml) 4 minutes; stop solution (3.65g EDTA, milli-Q water to 250ml) for 10 minutes; after rinsing (250ml milli-Q water) for 1 minute, use Image Scanner III to scan and save the pictures, and use ImageMaster 2D platinum 7.0 software to analyze and screen out the required protein gel spots.

## ICP35 Recombinant Protein

The ICP35 recombinant protein was constructed with the prokaryotic expression vector pET-32a (+) (Solarbio, Beijing, China), which was purified with the Beyotime His Tag Protein Product Purification Kit (Beyotime Biotechnology, China). The sequences of the primers used are listed in **Table 1**.

## Animals

Mice: Four-week-old female BALB/c mice (Beijing Vital River Laboratory Animal Technologies Co. Ltd., Beijing, China) were used in this study. The mice were anesthetized by inhalation of 2% isoflurane for all the procedures, and every effort was made to minimize suffering. The mice were bred in strict accordance with the standard procedures for breeding laboratory animals.

## Ethics

Human experiments: Convalescent serum samples were collected from patients diagnosed with HSV2 infection at the First Affiliated Hospital of Kunming Medical University, and the patients provided informed consent. The protocols were reviewed and approved by the Experimental Management Association of the IMB, CAMS (approval number: DWSP 201803018-1).

Animal experiments: The animal experiments were designed and performed according to the principles in the "Guide for the Care and Use of Laboratory Animals" and in the "Guidance for Experimental Animal Welfare and Ethical Treatment". The protocols were reviewed and approved by the Experimental Animal Management Association of the IMB, CAMS (approval number: DWSP 201902030-1). All the animals were completely under the care of veterinarians at the IMB, CAMS. The study was adhered to standard biosecurity and institutional safety procedures and has appropriated equipment and facilities.

## Immunization and Viral Challenge

Mice were randomly divided into three groups and intramuscularly immunized with 20 μg of purified ICP35 (the purified ICP35 protein concentration was determined by BCA) (n=30) or with $Al(OH)_3$ adjuvant alone (n=30) used as a control, and PBS negative control (n=10), on days 0, 28 and 42. The serum samples were obtained at 7 and 28 days after immunization. The mice were infected with HSV2 virus on day 7 after the booster injection except negative control. HSV2 infection ($2x10^4$ $CCID_{50}$/mouse) *via* the vaginal route was performed on the mice immunized with the ICP35 protein or with the adjuvant control. All the animals were monitored daily to observe their clinical symptoms. For weight observation group (n=10 per group), the mice were monitored daily after challenge to observe body weight and survival rate. For experimental sampling group (n=20 per group), the routine vaginal secretion, tissue and blood samples were collected 1 day, 3 days, 5 days, 7 days and 15 days after infection for subsequent testing and neutralization assays. Three mice were euthanized in each group at one time point. All remaining mice were euthanized at the end of the experiment.

## ELISPOT

An ELISPOT assay was performed with the Mouse IFN-γ/IL-4 ELISPOT Kit (Mabtech, Cincinnati, OH, USA) according to the manufacturer's protocol. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from mouse spleens by lymphocyte isolation (Dakewe, China) and plated in duplicate wells. Purified HSV2 ICP35 recombinant protein and the positive stimulus phytohemagglutinin (PHA) were added at a concentration of 5 μg/well to 96-well plates precoated with IFN-γ and IL-4. The plates were incubated at 37°C for 24 h in a carbon dioxide incubator. Then, the cells were removed, and the spots were developed. The colored spots were counted with an ELISPOT reader (CTL, Shaker Heights, OH, USA).

## Quantification of the Viral Load by q-RT-PCR

Total DNA was extracted from blood and tissue samples from the experimental mice with an Axygen® AxyPrep Body Fluid Viral DNA/RNA Miniprep Kit (Axygen®, USA). According to the protocol, the primers used for q-RT-PCR were designed to be specific for the gG sequences in the HSV2 genome (**Table 1**). q-RT-PCR was performed using the Takara Premix Ex Taq™ (Probe qPCR) Kit (TaKaRa, China).

## Electron Microscopy

HSV2 virus was purified by iodixanol density gradient centrifugation. Postfixation, en bloc contrasting, dehydration and embedding in epoxy resin were done following a standard protocol (Laue, 2010). Samples were stained with 1% phosphotungstic acid and observed by transmission electron microscopy (Hitachi, Kyoto, Japan).

## Histopathological Examinations

The organs of the experimental mice were fixed in 10% formalin, embedded in paraffin, sliced into 4-μm-thick sections and stained with hematoxylin and eosin (H&E). The morphology was assessed with an inverted microscope (Nikon).

## Statistical Analysis

The data are shown as the mean and standard deviation. GraphPad Prism software (San Diego, CA, USA) and the *t test* were used for the statistical analyses.

# RESULTS

## Recognition of Viral-Encoded Proteins by Convalescent Serum From HSV2-Infected Patients

Specific neutralizing antibodies, as certain indicators of antiviral immunity, reflect the recognition of key viral antigens, the main structural proteins that enable a virus to bind to receptors in the cell membrane, by the host immune system (Klasse, 2014). We performed protein 2-D electrophoresis and immunoblotting experiments with convalescent serum that was prepared by mixing 5 sera samples and showed a neutralizing antibody titer of 1:32 (**Supplemental Table 1**). Purified HSV2 was produced and harvested from infected Vero cells at different time points and was shown to contain various particles including those from different infectious stages (**Figure 1A**). This viral infectious sample was recognized by the convalescent serum, as indicated by the complicated antigenic map with multiple spots (**Figures 1B, C**). These spots were further identified using mass spectrometry, and various molecules of different sizes encoded by genes UL26.5 and UL26, including ICP35, VP22a and VP24, were recognized in the immunoblots (**Table 2**). In addition, other viral proteins, including some viral molecules that function during infection, such as TK, and structural components of the capsid, were recognized; however, only weak signals of glycoproteins that elicit neutralizing antibodies were observed in the blots (**Table 2**). Because ICP35 was thought to exist in immatured virus particles (Thomsen et al., 1995), we further purified the virus harvest using isodensity centrifugation to separate two different particles and performed immune-blotting with convalescent serum. The results showed that only trace levels of ICP35 were detected in matured particles compared to the other particles (**Figures 1D–F**), while the prokaryotic expressed ICP35 was recognized by convalescent serum in immune-blotting, either by immune serum of ICP35 (**Figure 1G**). To identify these data, we using convalescent serum and immune serum of expressed ICP35 traced the proteins encoded by UL26 gene during viral infection, the result of immune-fluorescent observation suggested that both sera were capable of recognizing the proteins encoded by UL26 gene including ICP35 during viral infection (**Figure 1H**). While the intact ICP35, VP22a and VP24 expressed in 293 cells were recognized by both sera (**Figure 1I**). These data comprehensively suggested that the viral proteins including ICP35 encoded by UL26 gene showed distinct immunogenicity during infection.

## ICP35 Elicits a Specific T Cell Response in HSV2-Infected Mice

Based on the ICP35 antigenic interaction with the antibodies in the convalescent serum, we used the prokaryotic expression method to produce a purified ICP35 protein and observed the relationship between its antigenicity and antiviral immunity in HSV2-infected mice. In ELISPOT assays to examine IFN-γ and IL-4 specificity, T cells isolated from the spleens of BALB/c mice infected with HSV2 were stimulated with the ICP35, and the proliferating T cell clones were counted. The results indicated that T cell stimulation with ICP35 protein clearly led to the proliferation of clones producing IFN-γ and clones producing IL-4. Similar results were obtained when T cells were stimulated with viral antigens (**Figures 2A, B**). This result suggested that the immune memory effect elicited by ICP35 played a role in host antiviral immunity after HSV2 infection.

## Study of the Immune Response Elicited by ICP35

To further immunologically characterize ICP35, we used BALB/c mice immunized with the prokaryotically expressed ICP35. The mice were administered three inoculations of 20 μg/dose into muscular tissue at intervals of 4 and 2-weeks (**Figure 3A**). The neutralizing antibodies were detected using a plaque assay at days 7 and 28 after the last immunization, and no positive results were observed at these two time points; however, the addition of serum to the assay restricted the sizes of the plaques, even if it did not decrease the virus titers (**Figure 3B**). ELISPOT was used to detect IFN-γ and IL-4 production at day 28 post immunization, and the results suggested a positive T cell response against this protein and against the purified virus harvested from infected Vero cells (**Figures 3C, D**). In a further study of the interaction between the serum and the viral antigen, viral particles harvested from infected Vero cells were incubated with the serum and were shown to contain viral genome but were noninfectious (**Figures 3E, F**). These results suggested that ICP35 is an effective viral antigenic protein but is not a neutralizing antigen.

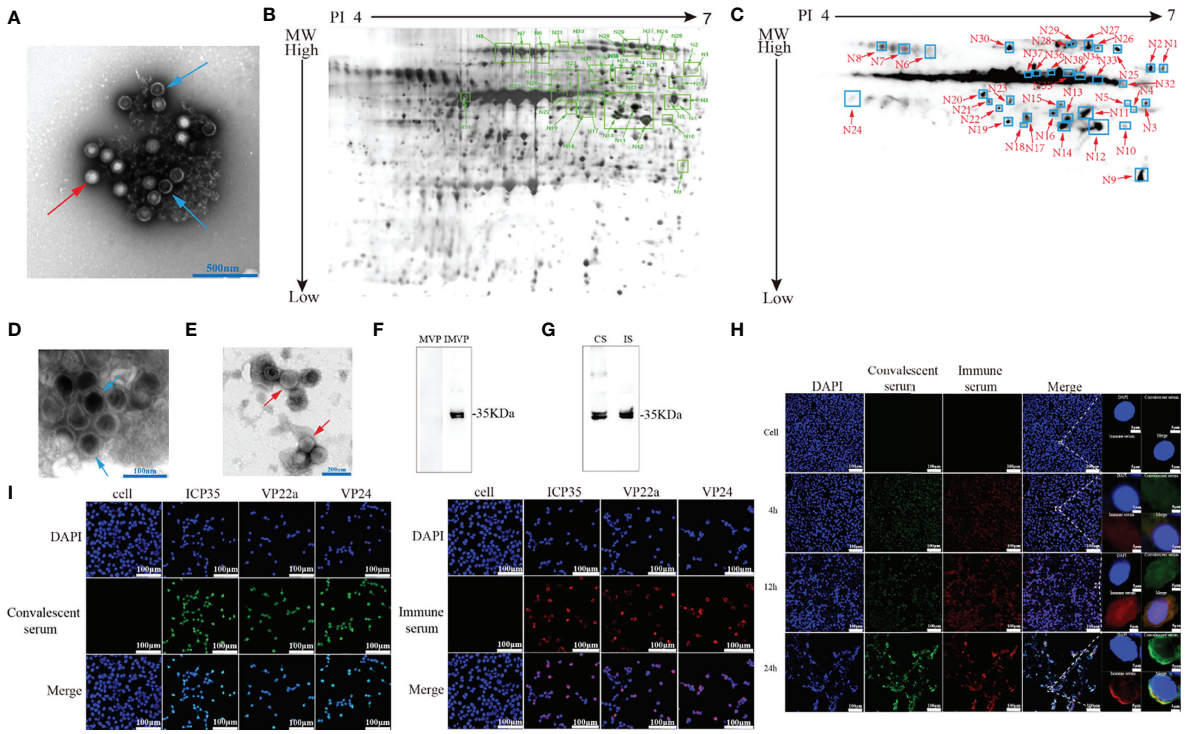


**FIGURE 1** | Viral-encoded proteins were recognized by the convalescent serum from HSV2-infected patients. **(A)** Electron microscopy of purified HSV2 virus; the blue arrow indicates immatured virus particles and the red arrow indicates matured virus particles. **(B)** 2-D electrophoresis of purified HSV2 virus. The glue was stained by silver dyeing. The number marked in the figure represents the protein neutralized by HSV2 patient convalescent serum according with **(C)**. The specific details are shown in the **Table 2**. MW refers to Molecular weight; PI refers to Isoelectric point. **(C)** Immunoblotting showed that the convalescent serum could identify viral proteins in the purified HSV2 virus. **(D)** Electron microscopy of purified HSV2 virus; the blue arrow indicates immatured virus particles. **(E)** Electron microscopy of purified HSV2 virus; the red arrow indicates matured virus particles. **(F)** The immune-blotting of matured and immatured virus particles with convalescent serum. The results showed that only trace levels of ICP35 were detected in matured particles compared to uncompleted particles. MVP refers to matured virus particles. IMVP refers to immatured virus particles. The convalescent serum was used as the primary antibody, and the secondary antibody was horseradish peroxidase labeled goat anti-human IgG (H+L) antibody. **(G)** The immune-blotting of the prokaryotic expressed ICP35 with convalescent serum and immune serum of ICP35. The convalescent serum and immune serum were used as the primary antibody, and the secondary antibody was horseradish peroxidase labeled goat anti-human IgG (H+L) antibody and goat anti-rabbit IgG (H+L) antibody. CS refers to convalescent serum. IS refers to immune serum of ICP35. **(H)** The results of the immunofluorescence detection with convalescent serum and immune serum of expressed ICP35 after HSV-2 virus infection. Vero cells were infected with HSV-2 at a MOI of 0.2, and at 4h, 12h, 24h postinfection, the samples were harvested. Blue represents nuclear staining (DAPI); green (Alexa Fluor® 488 Goat anti-human IgG) represents staining with the convalescent serum; red (Alexa Fluor® 647 Goat pAb to Rb IgG) represents staining with the immune serum. **(I)** The results of the immunofluorescence detection with convalescent serum and immune serum of ICP35 after transfecting eukaryotic vectors containing ICP35, VP22a and VP24 into 293 cells respectively. Blue represents nuclear staining (DAPI); red (Alexa Fluor® 647 Goat pAb to Rb IgG) represents staining with the immune serum; green (Alexa Fluor® 488 Goat anti-human IgG) represents staining with the convalescent serum.

## Immunization With ICP35 Attenuates the Clinical Symptoms in Mice Challenged With HSV2

To immunologically characterize HSV2, we infected BALB/c mice immunized with the expressed ICP35 protein with HSV2. The HSV2 HG52 strain was used to infect two groups of immunized mice *via* the vaginal route. The low-dose group received 1 x $10^4$ $CCID_{50}$/mouse while the high-dose group, which was used to assess the survival rate, received 2 x $10^4$ $CCID_{50}$/mouse, and similar groups were established as positive controls (**Figure 3A**). After infection, the mice were monitored daily to observe their symptoms. The mice in the immunized and control groups presented decreasing body weights. Beginning on day 7, this trend differed between the experimental and control groups (**Figure 4A**). By day 15, the experimental and control groups showed similar survival rates (**Figure 4B**). Interestingly, a difference was observed in the local tissues of the infected vaginas. In the immunized mice, these tissues presented weaker inflammatory reactions without exudation, but in the control mice, these tissues presented obvious inflammation with exudation (**Figure 4C**). The detection of viral load *via* vaginal swabs also supported the clinical features described above (**Figure 4D**). These results suggested that the immune response elicited by ICP35 contributed, to some extent, to antiviral immunity.

**TABLE 2 |** Mass spectrometric identification of 2-D protein gel electrophoresis spots.

| Spot number | Gene | Uniprot ID | Description |
|---|---|---|---|
| N1 | MCP | P89442 | Major capsid protein |
| N2 | MCP | P89442 | Major capsid protein |
| N3 | UL26.5 | G9I248 | Capsid scaffolding protein |
| N4 | UL42 | P89463 | DNA polymerase processivity factor |
| N5 | UL26.5 | G9I248 | Capsid scaffolding protein |
| N6 | TK | P89446 | Thymidine kinase |
| N7 | TK | P89446 | Thymidine kinase |
| N8 | TK | P89446 | Thymidine kinase |
| N9 | DBP | P89452 | Major DNA-binding protein |
| N10 | UL26.5 | G9I248 | Capsid scaffolding protein |
| N11 | UL26.5 | G9I248 | Capsid scaffolding protein |
| N12 | UL26.5 | G9I248 | Capsid scaffolding protein |
| N13 | UL26.5 | G9I248 | Capsid scaffolding protein |
| N14 | UL26.5 | G9I248 | Capsid scaffolding protein |
| N15 | UL26.5 | G9I248 | Capsid scaffolding protein |
| N16 | UL26.5 | G9I248 | Capsid scaffolding protein |
| N17 | UL26.5 | G9I248 | Capsid scaffolding protein |
| N18 | UL26.5 | G9I248 | Capsid scaffolding protein |
| N19 | UL26.5 | G9I248 | Capsid scaffolding protein |
| N20 | DBP | P89452 | Major DNA-binding protein |
| N21 | DBP | P89452 | Major DNA-binding protein |
| N22 | DBP | P89452 | Major DNA-binding protein |
| N23 | DBP | P89452 | Major DNA-binding protein |
| N24 | UL26.5 | G9I248 | Capsid scaffolding protein |
| N25 | DBP | P89452 | Major DNA-binding protein |
| N26 | DBP | P89452 | Major DNA-binding protein |
| N27 | DBP | P89452 | Major DNA-binding protein |
| N28 | DBP | P89452 | Major DNA-binding protein |
| N29 | UL26.5 | G9I248 | Capsid scaffolding protein |
| N30 | DBP | P89452 | Major DNA-binding protein |
| N31 | DBP | P89452 | Major DNA-binding protein |
| N32 | UL42 | P89463 | DNA polymerase processivity factor |
| N33 | UL42 | P89463 | DNA polymerase processivity factor |
| N34 | UL42 | P89463 | DNA polymerase processivity factor |
| N35 | DBP | P89452 | Major DNA-binding protein |
| N36 | DBP | P89452 | Major DNA-binding protein |
| N37 | DBP | P89452 | Major DNA-binding protein |
| N38 | gD | Q69467 | Envelope glycoprotein D |

## Viral Proliferation and the Resulting Pathological Damage in Various Tissues of ICP35 Protein-Immunized Mice

We sacrificed the immunized and control mice at 1, 3, 5, 7 and 15 days after low-dose viral challenge and detected the viral loads of various tissues. The results suggested that the viral loads in various tissues of the immunized mice, especially that in the vaginal tissue, were lower than those in the positive control mice, although these particles showed similar dynamic proliferative processes (**Figures 5A–J**). Histopathological observation suggested that viral challenge led to varying degrees of pathological inflammatory reactions in various tissues in the immunized and control mice (**Table 3**). Observation of the local vaginal tissue did not reveal invasive pathological lesions in the epithelial structure of the immunized mice, although there was infiltration of inflammatory cells; however, invasive tissue lesions associated with inflammatory reactions were found in the vaginal epithelial tissues of the control mice (**Figure 6**). These results suggested that the immunity induced by ICP35 did restrict viral pathogenesis to some extent.

# DISCUSSION

HSV2, a pathogen that spreads in populations *via* sexual contact and causes herpes progenitalis (Ahmed et al., 1982), has been studied due to its various strategies for interfering with the host immune system (Peng et al., 2009; Stefanidou et al., 2013; Stempel et al., 2019). Although our accumulated knowledge about these strategies allows us to understand some mechanisms by which the virus evades immune recognition and avoids immune attack, there are some unknown mechanisms that hinder the strategic study of HSV2 vaccines (Johnston et al., 2016). For instance, although specific neutralizing antibodies are elicited and are an important immunological indicator for the evaluation of individuals infected or immunized with HSV2 or vaccine antigens, these antibodies do not provide clinical protective effects during virus infection (Diebolder et al., 2014), suggesting a more complicated mechanism by which the virus interferes with the immune system. Based on this background, our work first investigated the recognition of virus-encoded protein antigens in infected cells by antibodies in the convalescent

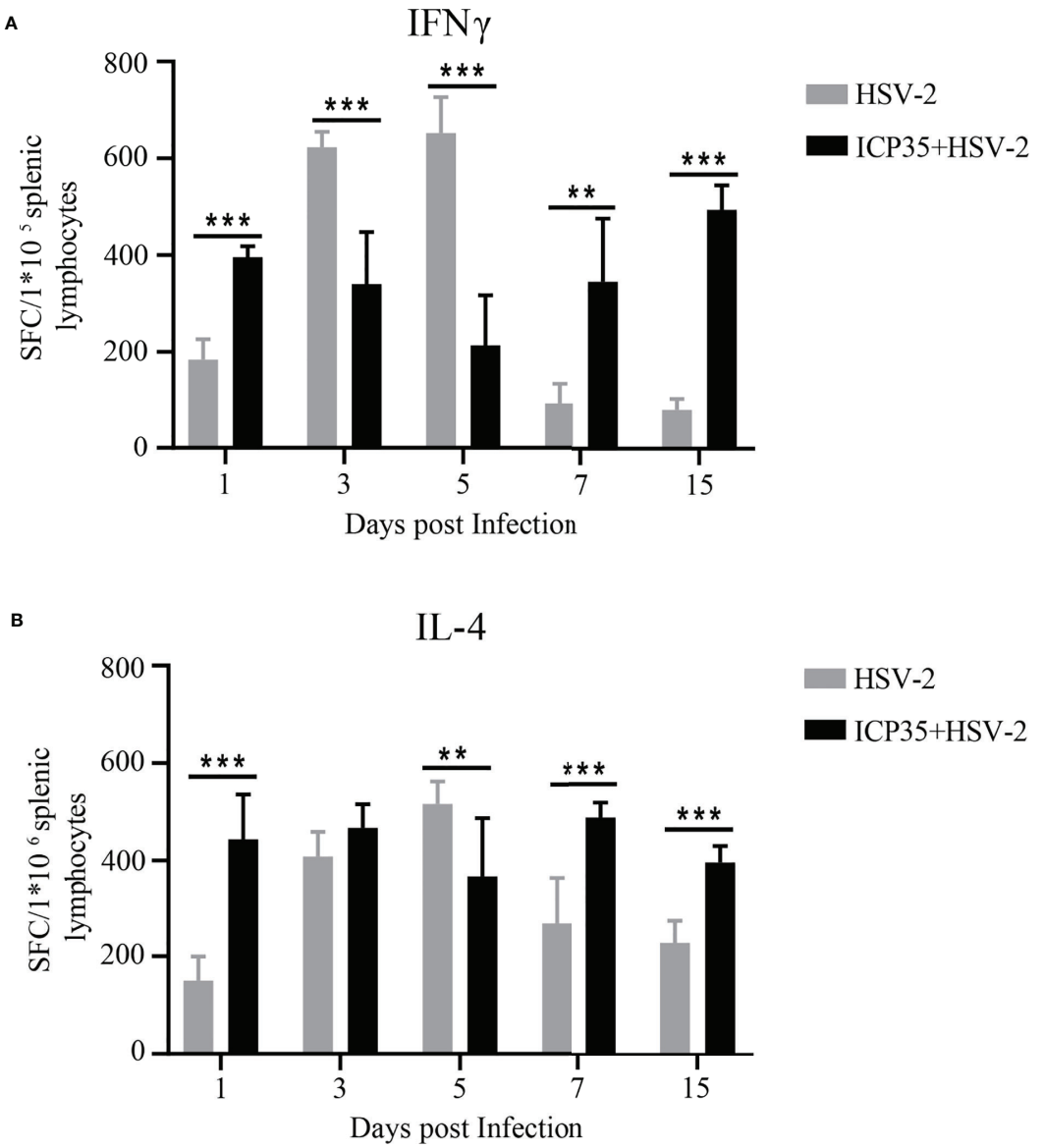


**FIGURE 2** | ICP35 elicited specific T cell response in mice. **(A)** The ELISPOT analysis of ICP35 elicited IFN-γ-secreting T cell responses within the splenic lymphocyte population in the immunized and control groups. Samples were run in duplicate. (n=3). **(B)** The ELISPOT analysis of ICP35 elicited IL-4- secreting T cell responses within the splenic lymphocyte population in the immunized and control groups. Samples were run in duplicate. (n=3). HSV-2, indicates the animal immunized with Al $(OH)_3$ and challenged with HSV-2 groups; ICP35+HSV-2, indicates the animal immunized with ICP35 and challenged with HSV-2 groups;. SFC, indicates the Spot-forming Cells. **P<0.01, ***P<0.001.

serum from HSV2-infected patients *via* 2-D electrophoresis and immunoblotting. We expected to primarily identify the remnant viral structural proteins with significant immunogenicity. Interestingly, we observed a clear trend that the antibodies in the convalescent serum recognized the viral capsid scaffold protein ICP35 and other similar proteins encoded by genes UL26.5 and UL26. ICP35 is thought to be a capsid structural protein that temporarily functions in capsid during viral assembly (Thomsen et al., 1995). Importantly, ICP35 actually is not expressed in mature virion (Thomsen et al., 1995), which means that it does not contribute to the antigenicity or immunogenicity of mature HSV2 virion. Our further work using the prokaryotic expression method and immunological studies characterized the antigenicity of this protein and showed that the elicited immunity was unable to control viral proliferation in mouse tissues *via* antibody and specific cytotoxic T lymphocyte (CTL) responses, although it did attenuate the clinical symptoms of local tissues to some

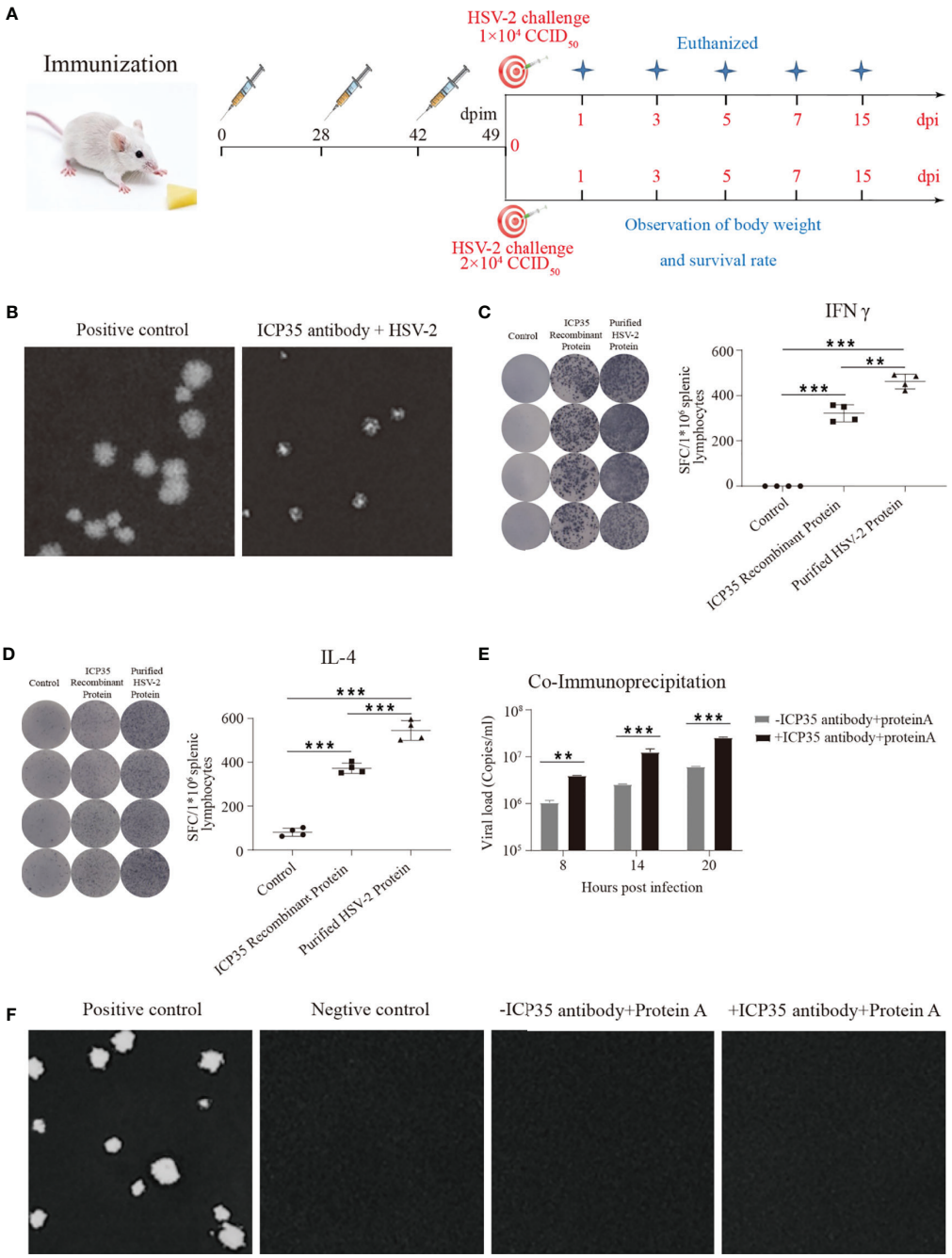


**FIGURE 3** | The integrated immune responses elicited by ICP35. **(A)** Schematic of ICP35 immunization and viral challenge protocols in the mice. **(B)** The addition of anti-ICP35 antibody restricted the sizes of the plaques. The positive control refers to HSV2 and normal serum samples; ICP35 antibody+HSV-2 refers to HSV2 and anti-ICP35 serum samples. **(C)** The ELISPOT analysis of ICP35 elicited IFN-γ-secreting T cell responses in ICP35 protein-immunized mice at day 28 after the last immunization (n=3). The spot diagram (left) and result (right) of IFN-γ were shown. Samples from the ICP35-immunized animals were stimulated with the ICP35 recombinant protein and purified HSV-2 protein. Samples were run in duplicate. **(D)** The ELISPOT analysis of ICP35 elicited IL-4-secreting T cell responses in ICP35 protein-immunized mice at day 28 after the last immunization (n=3). The spot diagram (left) and result (right) of IL-4 were shown. Samples from the ICP35-immunized animals were stimulated with the ICP35 recombinant protein and purified HSV-2 protein. Samples were run in duplicate. **(E)** Fluorescence quantitative PCR of protein A agarose gel particle sediment. -ICP35 antibody + protein A: the cell lysate supernatants infected HSV2 were incubated overnight without the anti-ICP35 antibody at 4°C; +ICP35 antibody + protein A: the cell lysate supernatants infected HSV2 were incubated overnight with the anti-ICP35 antibody at 4°C. **(F)** Plaque assay of protein A agarose gel particle sediment. The positive control refers to infection with HSV2 virus. The negative control refers to cells. -ICP35 antibody + protein A: the cell lysate supernatants infected HSV2 were incubated overnight without the anti-ICP35 antibody at 4°C; +ICP35 antibody + protein A: the cell lysate supernatants infected HSV2 were incubated overnight with the anti-ICP35 antibody at 4°C. **P<0.01, ***P<0.001.

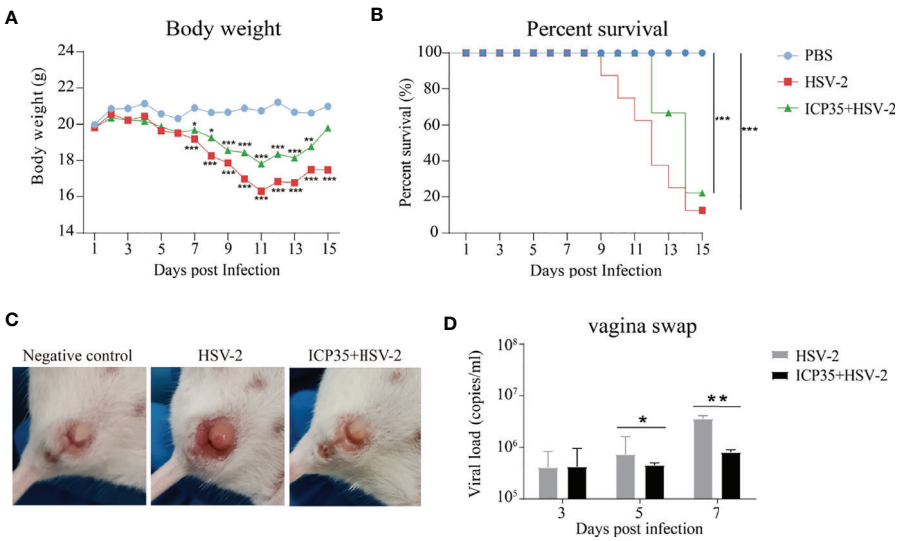


**FIGURE 4** | Immunization with ICP35 attenuated the clinical symptoms in mice challenged with HSV2. **(A)** The mice were monitored daily after challenge to observe their symptoms (n=10/group). Decreased body weight was observed in the immunized with ICP35 (ICP35+HSV-2), with Al $(OH)_3$ adjuvant groups (HSV-2) and PBS negative control groups (PBS), but beginning on day 7, this trend differed between the experimental and control groups. The data are the mean of ten independent animals. *P<0.05, **P<0.01, ***P<0.001 vs. PBS. **(B)** The survival rate of mice challenged with HSV2 was monitored daily (n=10/group). Death occurred in the Al $(OH)_3$ adjuvant groups (HSV-2) beginning on day 9, and death occurred in the ICP35 immunized group (ICP35+HSV-2) beginning on day 11. ***P<0.001. **(C)** The clinical symptoms of the mice challenged with HSV2 in the immunized with ICP35 (ICP35+HSV-2) and with Al $(OH)_3$ adjuvant groups (HSV-2). PBS group without challenge as negative control. **(D)** The detection of the viral load by vaginal swab in the immunized with ICP35 (ICP35+HSV-2) and with Al $(OH)_3$ adjuvant groups (HSV-2) after viral challenge. The data are the mean ± SD of three independent animals.

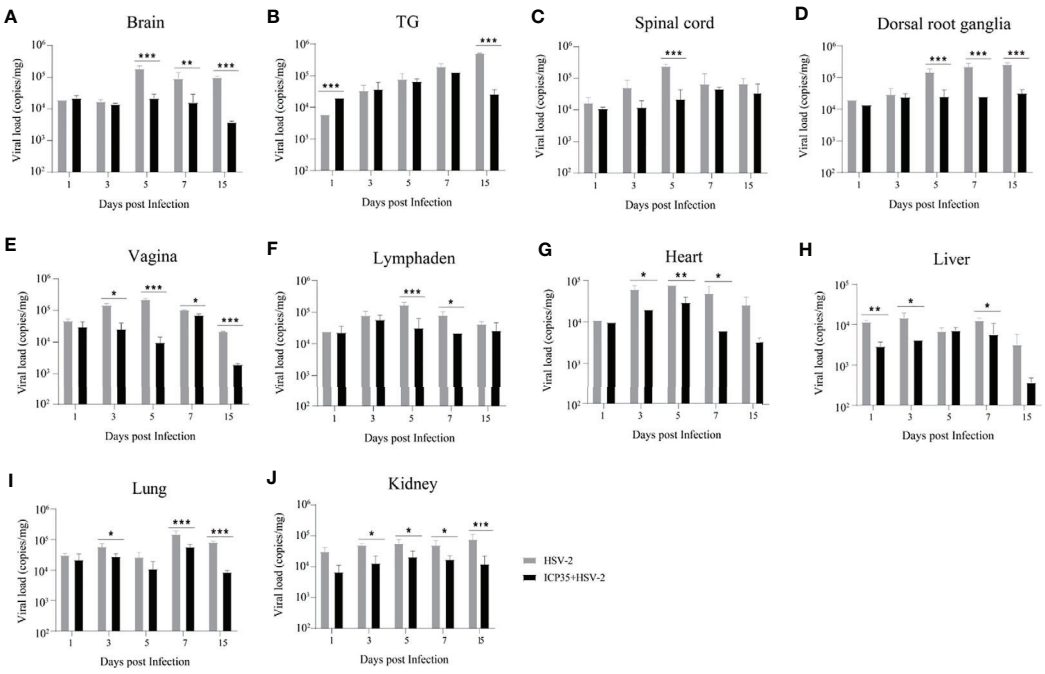


**FIGURE 5** | Viral proliferation in various tissues of the ICP35 protein-immunized or control mice. **(A–J)** The viral loads in various tissues of the immunized mice with ICP35 (ICP35+HSV-2), especially those in the vaginal tissues, were lower than those in various tissues of the mice with Al$(OH)_3$ adjuvant (HSV-2). **(A)** Brain, **(B)** TG, **(C)** Spinal cord, **(D)** Dorsal root ganglion, **(E)** Vagina, **(F)** Lymphaden, **(G)** Heart, **(H)** Liver, **(I)** Lung, **(J)** Kidney. The data shown in **(A–J)** are the mean ± SD of three independent animals. *P<0.05, **P<0.01, ***P<0.001.

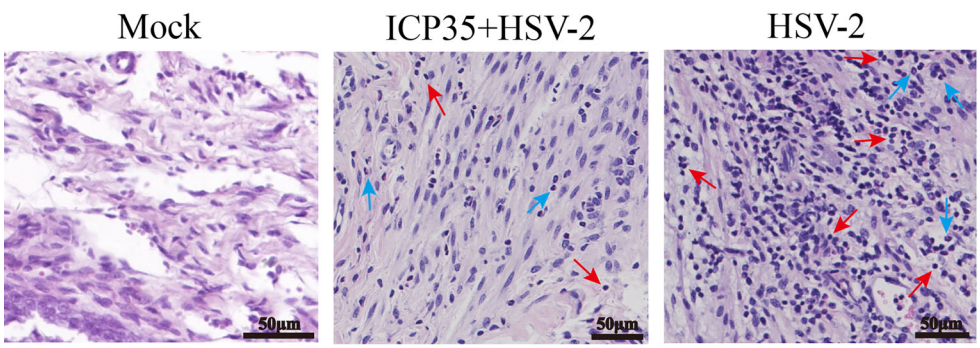


**FIGURE 6** | The resulting pathological damage in various tissues of the ICP35 protein-immunized or control mice. Histopathological observation of vaginal epithelial tissue from the PBS negative control (Mock), immunized with ICP35 (ICP35+HSV-2) and immunized with Al $(OH)_3$ adjuvant mice (HSV-2) after viral challenge. Inflammatory cell infiltration (red arrow: lymphocytes, blue arrow: neutrophils).

**TABLE 3** | Histopathological observation of pathological inflammatory reactions in various tissues in the immunized or control mice after viral challenge.

| Group | Tissues | Histopathological observation |
|---|---|---|
| HSV-2 | Brain | + |
| | TG | – |
| | Spinal cord | – |
| | Vagina | +++ |
| | Lymphaden | + |
| ICP35 protein+HSV-2 | Brain | + |
| | TG | – |
| | Spinal cord | – |
| | Vagina | ++ |
| | Lymphaden | + |

*-, Indicates no obvious abnormality; +, Indicates a small amount of inflammatory cell infiltration or bleeding; ++, Indicates medium inflammatory cell infiltration or bleeding, +++; Indicates lots of inflammatory cell infiltration or bleeding.*

extent. These data provided us with the technical possibility that the immunodominance of ICP35 might be used to design an integrated antigen with other viral glycoproteins, but further studies are needed to clarify the function of this protein in viral interference with host immunity.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Experimental Management Association of the IMB, CAMS. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Experimental Animal Management Association of the IMB, CAMS.

## AUTHOR CONTRIBUTIONS

QL, YZ, and WD conceived and designed the study. XL and JW performed the experiments and analyzed the data. TM, YG, YZ, LW, SF, XLX, GJ, PC, XXX, SD, JZ, DL, YL, LY, HZ, ML, HLZ, RG contributed reagents, materials and analysis tools. QL wrote and edited the manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.649722/full#supplementary-material

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ORIGINAL RESEARCH
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# Global CpG DNA Methylation Footprint in Kaposi's Sarcoma

*Guy Journo[1], Anuj Ahuja[1], David Dias-Polak[2], Yonatan Eran[1], Reuven Bergman[2] and Meir Shamay[1]**

*[1] Daniella Lee Casper Laboratory in Viral Oncology, Azrieli Faculty of Medicine, Bar-Ilan University, Safed, Israel, [2] Department of Dermatology, Rambam Health Care Campus, Haifa, Israel*

Kaposi's sarcoma-associated herpesvirus (KSHV), also familiar as human herpesvirus 8 (HHV-8), is one of the well-known human cancer-causing viruses. KSHV was originally discovered by its association with Kaposi's sarcoma (KS), a common AIDS-related neoplasia. Additionally, KSHV is associated with two B-lymphocyte disorders; primary effusion lymphoma (PEL) and Multicentric Castlemans Disease (MCD). DNA methylation is an epigenetic modification that is essential for a properly functioning human genome through its roles in chromatin structure maintenance, chromosome stability and transcription regulation. Genomic studies show that expressed promoters tend to be un-methylated whereas methylated promoters tend to be inactive. We have previously revealed the global methylation footprint in PEL cells and found that many cellular gene promoters become differentially methylated and hence differentially expressed in KSHV chronically infected PEL cell lines. Here we present the cellular CpG DNA methylation footprint in KS, the most common malignancy associated with KSHV. We performed MethylationEPIC BeadChip to compare the global methylation status in normal skin compared to KS biopsies, and revealed dramatic global methylation alterations occurring in KS. Many of these changes were attributed to hyper-methylation of promoters and enhancers that regulate genes associated with abnormal skin morphology, a well-known hallmark of KS development. We observed six-fold increase in hypo-methylated CpGs between early stage of KS (plaque) and the more progressed stage (nodule). These observations suggest that hyper-methylation takes place early in KS while hypo-methylation is a later process that is more significant in nodule. Our findings add another layer to the understanding of the relationship between epigenetic changes caused by KSHV infection and tumorigenesis.

**Keywords: Kaposi's sarcoma, CpG methylation, KS, KSHV, gene expression, promoter, enhancer**

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****Correspondence:***
*Meir Shamay*
*meir.shamay@biu.ac.il*

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## INTRODUCTION

Kaposi's sarcoma-associated herpesvirus (KSHV), also familiar as human herpesvirus 8 (HHV- 8), belongs to the γ-herpesvirus family and is one of the well-known human cancer-causing viruses (Chang et al., 1994; Purushothaman et al., 2016). KSHV was originally discovered by its association with Kaposi's sarcoma (KS) (Chang et al., 1994), a common AIDS-related neoplasia of endothelial/

mesenchymal origin (Boshoff et al., 1995; Li et al., 2018). Additionally, KSHV is associated with two B-lymphocyte disorders; primary effusion lymphoma (PEL) and Multicentric Castlemans Disease (MCD), which are characterized by proliferation of B-cells in body cavities and lymph nodes, respectively (Parravicini et al., 2000; Henke-Gendo and Schulz, 2004). Similar to other herpes viruses, KSHV infection can be characterized as lytic or latent. During lytic replication, virions are assembled and released from the cell. This process requires DNA synthesis together with expression of virion structural protein genes and results in death of the infected cell. Latent infection, however, is characterized by the persistence of the viral genome as a covalently closed circular episome DNA with very limited viral gene expression. In KS most of the cells are latently infected, but the few cells that turn on the lytic phase express lytic proteins with critical roles in the pathogenesis (Ganem, 2006; Zhang et al., 2015). KS is a low-grade vascular tumor that can involve the skin, mucosa, and viscera. There are four different epidemiologic-clinical forms of KS; Classic, Endemic, Immunosuppression-Associated and AIDS-Associated KS (Antman and Chang, 2000). In recent years there have been several advancements in our understanding of KS including promising targeted therapeutic agents, but despite these advances, KS still remains the most prevalent malignancy among patients with AIDS and continues to plague patients with drug-related or transplant-associated immunosuppression.

DNA methylation is an epigenetic modification that is essential for a properly functioning human genome through its roles in chromatin structure maintenance, chromosome stability and transcription regulation. Expressed promoters tend to be un-methylated whereas methylated promoters tend to be inactive (Antequera et al., 1990; Baylin, 2005). DNA methylation involves the transfer of a methyl group to cytosine in a CpG dinucleotide by DNA methyltransferases which create or maintain methylation patterns (Bestor, 2000). In mammalian cells, DNA methylation is added and maintained by a few DNA methyltransferases (DNMTs); DNMT1, DNMT3A and DNMT3B. DNMT1 acts as the "maintenance" methyltransferase, due to its preference for hemi-methylated DNA, which is abundant following DNA replication. DNMT3A and DNMT3B, often referred to as "*de-novo*" methyltransferases, are responsible for establishing patterns of DNA methylation. While enzymes that catalyze DNA methylation have been thoroughly studied, the enzymes and mechanisms of DNA de-methylation have remained elusive until recently. The TET (ten-eleven translocation) family proteins, has the ability to catalyze sequential oxidation reactions; converting 5-mC first to 5-hydroxymethylcytosine (5-hmC), then 5-formylcytosine, and finally 5-carboxylcytosine (5-caC) (Huang et al., 2014; Putiri et al., 2014). A following decarboxylation of 5-caC, by either a thymine-DNA glycosylase or other DNA repair enzyme, leads to removal of the methylated nucleotide and results in DNA de-methylation (Ito et al., 2010).

Few studies have shown the ability of KSHV to alter the methylation levels of specific cellular gene promoters. Transcription repression *via* CpG DNA hyper-methylation of p16INK4a (*CDKN2A*) (Platt et al., 2002), the TGF-beta type II receptor (*TbetaRII, TGFBR2*) (Di Bartolo et al., 2008), and PDZ-LIM domain-containing protein 2 (*PDLIM2*) (Sun et al., 2015) promoters have been detected in KSHV-infected primary effusion lymphoma (PEL) lines. Hyper-methylation of the H-cadherin (*CDH13*) promoter has been reported in LANA expressing endothelial cells and PEL (Shamay et al., 2006). In a previous study (Journo et al., 2018), we revealed the global CpG DNA methylation in KSHV-positive PEL cells and *de-novo* infected B cell line (BJAB219). Following KSHV infection, the virus imposes global hyper-methylation on the cellular genome while extensive global hypo-methylation seems to occur later on as cancer progresses to PEL. In a recent study (Naipauer et al., 2020), we have found global hypo-methylation and up regulation of tumor-driving genes during the process of KSHV dependent transformation in a mouse model for KSHV sarcomagenesis.

These previous studies in *de-novo* infected B-cells, KSHV-sarcomaregnesis mice model, and PEL suggested a wave of hyper-methylation following KSHV-infection, and hypo-methylation during the development of KSHV-associated transformation. In this study we were interested to reveal the methylome of the most frequent KSHV-associated malignancy, KS. To answer this question, we performed a global methylation analysis in KS and normal skin biopsies. Interestingly, we found that the percentage of hyper-methylation in KS is very similar to PEL, while hypo-methylation is very different. Furthermore, methylation and specifically hypo-methylation can differentiate plaque from nodule.

# MATERIALS AND METHODS

## Patient Samples

The Kaposi's sarcoma tissues and normal controls were reviewed and ethically approved by the institutional Helsinki committee at Rambam hospital (number 0391-15-RMB). Written informed consent was obtained by all participants. Punch biopsies of 4 mm were taken, and DNA was isolated using DNeasy Blood & Tissue Kit (QIAGEN) according to manufacturer procedure.

## DNA Isolation and Illumina MethylationEPIC BeadChip

Genomic DNA was isolated from cells using DNeasy Blood & Tissue Kit (QIAGEN). Next, gDNA samples were bisulfite converted (D5001 Zemo), and then hybridized to MethylationEPIC BeadChip (Illumina) according to manufacturer's protocol. The BeadChip array was performed in a single-base extension reaction, stained and imaged on an Illumina HiScan. The raw data was exported from GenomeStudio and normalized using ChAMP R pipeline (Morris et al., 2014) that has the ability to run a series of programs in which the output of one program is used as an input to the next one. The different programs decrease biases from known technical issues, such as adjustment for type I and type II probes, background correction and batch effects between chips. Statistical analysis was performed using limma program within ChAMP

pipeline and JMP-genomic software. The methylation analysis was performed at the Genomics Core Facility, BioRap Technologies (Rappaport Research Institute, Technion).

## DNA Methylation Data Analysis

Methylation rates of selected CpG sites were calculated (using GenomeStudio Methylation Module Software) as methylation β-value ranging from 0 (completely un-methylated) to 1 (completely methylated). Probes with a detection P-value of over 0.05 or blank β-value were excluded from further analyses. Differences in β-values (Δβ) between KS and control samples were determined as $\Delta\beta \geq 0.25$ and $\Delta\beta \leq -0.25$ and named as hyper and hypo-methylated, respectively (Kulis et al., 2015). To determine if these differentially methylated CpG are located in regulatory regions, genomic regions 100 bp up-stream and downstream of each CpG were analyzed in EnhancerAtlas (http://www.enhanceratlas.org/index.php) (Gao and Qian, 2020). To reduce the effect of redundant probes in the same regulatory region, probes residing within 200 bp were merged into a single interval using MergeBED (Galaxy bedtool, https://usegalaxy.org/) before the analysis on GREAT (http://great.stanford.edu/public/html/index.php) (McLean et al., 2010) to identify common functional processes and phenotypes. To generate the final lists of differentially methylated enhancers, two or more CpGs that were differentially methylated in the same enhancer region (up to 1000 bp apart) were merged using MergeBED. In the case of gene promoters, the final lists were based on the identified gene names. Gene-ontology analysis was performed on DAVID Bioinformatics Resources 6.8 (https://david.ncifcrf.gov/home.jsp) (Huang da et al., 2009).

## Data Access

Global methylation analysis obtained from the MethylationEPIC BeadChip (Illumina) analyses are available at: http://biodb.md.biu.ac.il/biu/shamay_lab_data.html.

# RESULTS

## Mapping the Human Methylome in Kaposi's Sarcoma

We have previously revealed the global methylation footprint in PEL cells and found that many cellular gene promoters become differentially methylated and hence differentially expressed in KSHV chronically infected PEL cell lines (Journo et al., 2018). PEL is originated from B cells and therefore might present a distinct methylation footprint from KS that is originated from either endothelial or mesenchymal cells (Boshoff et al., 1995; Li et al., 2018). We were interested to reveal the methylome of the most frequent KSHV-associated malignancy. For this analysis, we obtained six classic KS skin biopsies; three plaques (less progressed disease) and three nodules (more progressed disease), and two additional control skin biopsies. DNA isolated from skin biopsy was sheared, bisulfite treated and hybridized to the MethylationEPIC BeadChip. The EPIC array has the ability to cover over 850,000 CpG sites throughout the human genome, and every CpG site analyzed receives a methylation score known as beta value between 0 (un-methylated) and 1 (fully methylated) (Pidsley et al., 2016). The results of regression analysis of the raw beta values between the control samples detected correlation coefficient of $r>0.989$ (**Figure 1A**). The tight correlation between our two controls gave us confidence of the quality and reproducibility of our sample collection and methylation analysis. Regression analysis of the controls and plaques clearly shows methylation differences with hyper-methylation in plaque more pronounced (**Figure 1B**). Regression analysis of the controls and nodules shows more profound methylation differences with more extensive hyper-methylation and significant number of hypo-methylated probes (**Figure 1C**). We then applied all eight samples on a principal component analysis (PCA) of the global methylation beta values (**Table S1**). This analysis clearly differentiates KS from normal skin (**Figure 1D**). It seems that the methylation pattern reflects the progression of KS from plaque to nodule, since the nodule is distributed further away from the control, than the plaque.

## KS Disease Progression Can Be Determined by Global Hypo-Methylation Footprint

Looking deeper into the PCA (**Figure 1D**), we noticed one sample, plaque KS1, that represents an intermediate state between KS and normal skin, while the rest of the KS samples are distributed further away from the control skin. This might be explained due to the early stage (plaque) of sample KS1, a stage where infected cells are not always the major population within the lesion. We took this into consideration while further analyzing our data. We next decided to create a pie chart showing the percentage of hyper and hypo-methylated CpGs relative to control in two different stages of disease (Nodule and Plaque) (**Figure 2A**). This analysis revealed 2.71% (3.77% when sample KS1 was omitted) hyper-methylated and 0.29% (0.38% when sample KS1 was omitted) hypo-methylated sites in plaque, and 5.22% hyper-methylated and 1.8% hypo-methylated sites in nodule. While the hyper-methylation increased from 2.71% to 5.22% (1.9 fold), hypo-methylation increased from 0.29% to 1.8% (6.2 fold) during the process from plaque to nodule (**Figure 2B**). This observation suggests that hyper-methylation takes place early in KS while hypo-methylation is a later process that is more significant in nodule. When the probes were divided to those located within CpG-islands and non-island we have found significant preference for non-island over island in both hyper and hypo-methylated sites (**Figures 2C, D**). Interestingly, focusing solely on hypo-methylation of the non-CpG island sites, revealed a clear-cut difference between plaque and nodule (**Figure 2D**). Altogether, this supports the notion that hyper-methylation takes place in early stages of KS and accumulates as disease progresses, while hypo-methylation is a better indicator of disease progression, and this phenomenon is more substantial in non-CpG sites.

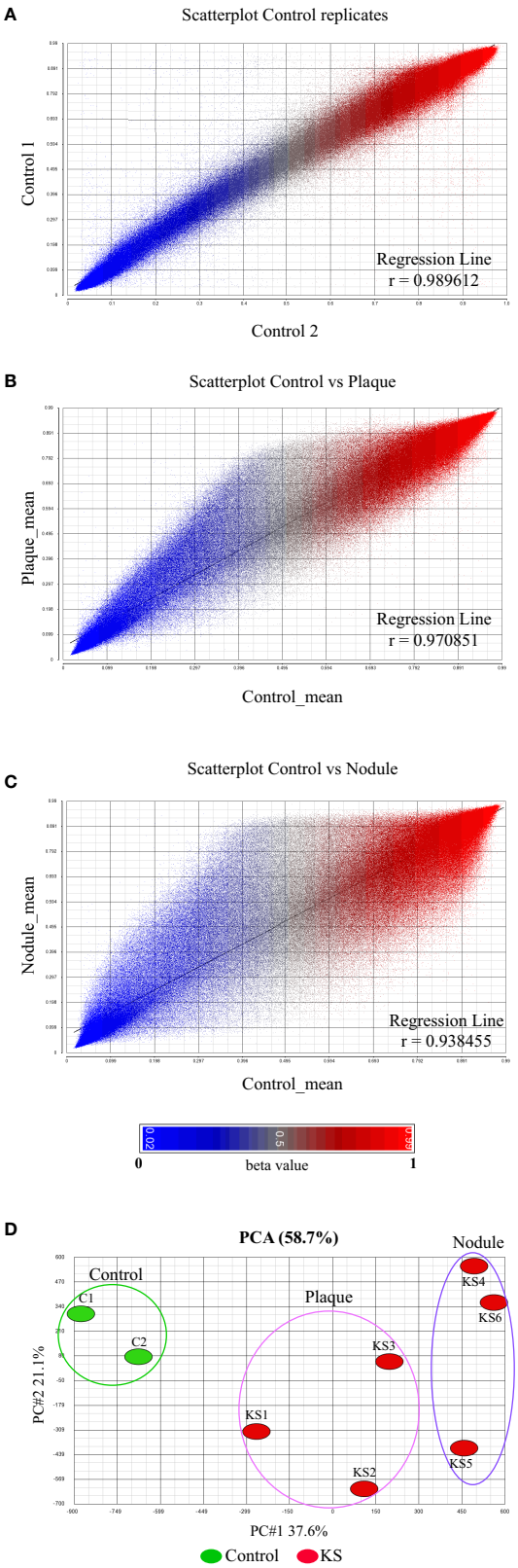



**FIGURE 1** | Continued

**FIGURE 1** | Global DNA methylation clearly distinguishes normal skin from KS. **(A–C)** The raw β value (presented between 0 and 1) of each CpG probe from the 850K BeadChip is represented by a single dot. Correlation coefficient analysis: **(A)** between biological replicates of uninfected skin controls showing close identity between replicates and **(B, C)** between uninfected skin control and KSHV infected KS samples at early tumor stage [Plaque **(B)**] and late tumor stage [Nodule **(C)**]. The raw β value (presented between 0 and 1) of each CpG probe from the 850K BeadChip is represented by a single dot. **(D)** Principal component analysis of the raw β values from the 850K BeadChip showing the variability between control and KS samples.

## Differentially Methylated Regulatory Elements in KS

The most differentially methylated probes between normal skin and KS are presented in a heatmap (**Figure 3A**), where the most changes are towards hyper-methylation in KS. At the top of the heatmap a small number of probes turn hypo-methylated, a phenomenon more profound in the nodule samples. To obtain deeper understanding of the methylation changes in KS, we identified differentially methylated CpGs in regulatory elements. Therefore, differentially methylated CpGs (a genomic region 100 bp up-stream and downstream of each CpG) were analyzed in EnhancerAtlas (http://www.enhanceratlas.org/index.php) (Gao and Qian, 2020). Out of the 19,242 hyper-methylated CpG in plaque, 3,838 (19.9%) CpG were located in 2,540 promoters and 3,577 (18.6%) were located in 3,063 enhancers. Out of the 580 hypo-methylated CpG in plaque, 6 (1%) were located in 6 promoters and 4 (0.7%) were in 4 enhancers. Analysis in nodule revealed that out of the 40,158 hyper methylated CpG, 9,477 (23.6%) were located in 5,154 promoters and 6,041 (15%) were located in 4,982 enhancers. Out of the 11,446 hypo-methylated CpG in nodule, 2,092 (18.3%) were located in 1,328 promoters and 485 (4.2%) were located in 432 enhancers (**Figures 3B, C** and **Table S2, S3**). In many cases more than one methylated probe was located in the same promoter/enhancer, this is the reason that the number of differentially methylated promoters/enhancers are smaller than the total number of probes within these elements. Several of the hyper-methylated promoters we identified in KS are among the few promoters that were previously reported and confirmed as hyper-methylated in KSHV-infected cells, such as the transforming growth factor–β type II receptor (TGFBR2/TβRII) (Di Bartolo et al., 2008), and EH Domain Containing 3 (EHD3) (Journo et al., 2018) in PEL, and Cadherin 13 (CDH13) (Shamay et al., 2006) in LANA expressing endothelial TIME cells. While around 20% of both hypo and hyper methylated CpGs in nodule are located in promoters, the enhancers are preferentially hyper-methylated (15%) relative to only 4% in hypo-methylation. This also revealed that more hypo-methylation changes are outside regulatory elements, and towards intergenic regions and gene body. This phenomenon is even more profound in plaque, where less than 2% of the hypo-methylation takes place in promoters and enhancers.

The 5,154 promoters and 4,982 enhancers which were hyper-methylated, and 1,328 promoters and 432 enhancers which were hypo-methylated in nodule were analyzed on GREAT (http://great.stanford.edu/public/html/index.php) (McLean et al., 2010) to identify common functional processes (**Table S4**) and

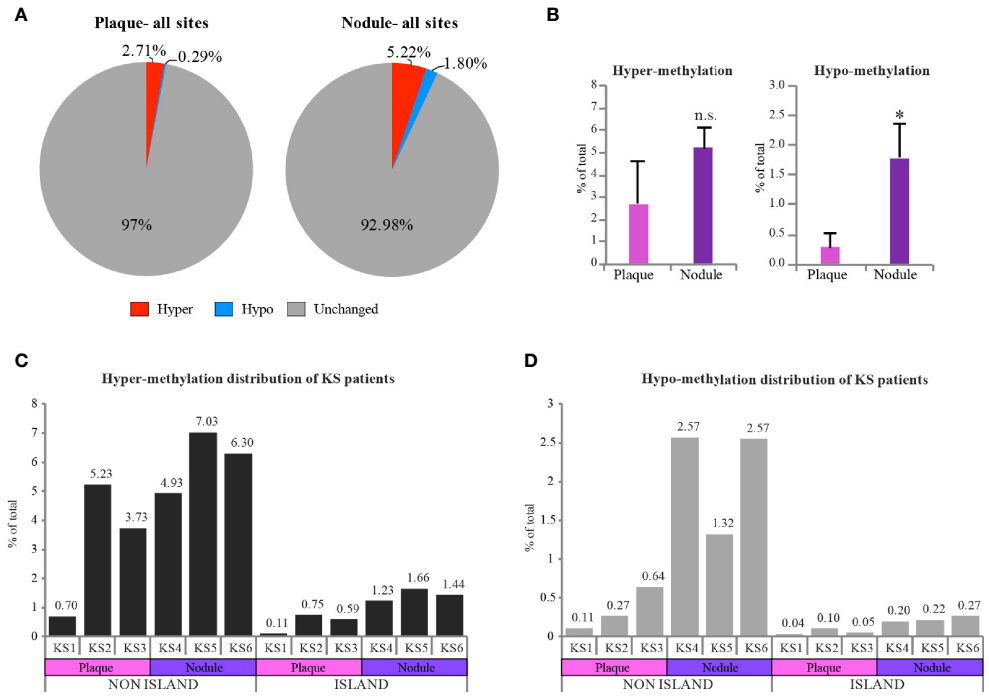



**FIGURE 2** | Hypo-methylation increases as KS progresses from plaque to nodule. **(A)** Pie charts represent the percentage of hyper and hypo-methylated CpGs relative to control skin in two different stages of disease (Nodule and Plaque). **(B)** Column charts represent hyper and hypo-methylation distribution of Plaque vs. Nodule compared to control (represented as mean of three samples +SD). **(C, D)** Column charts represent hyper and hypo-methylation distribution of KS samples compared to control in CpG island vs. non CpG island regions. Percentage of each individual sample is indicated above each column. Hyper and hypo-methylation threshold was set as $\Delta\beta \geq 0.25$ and $\Delta\beta \leq -0.25$ respectively. Two-tailed T-tests were performed. *$p < 0.05$; n.s., not significant.

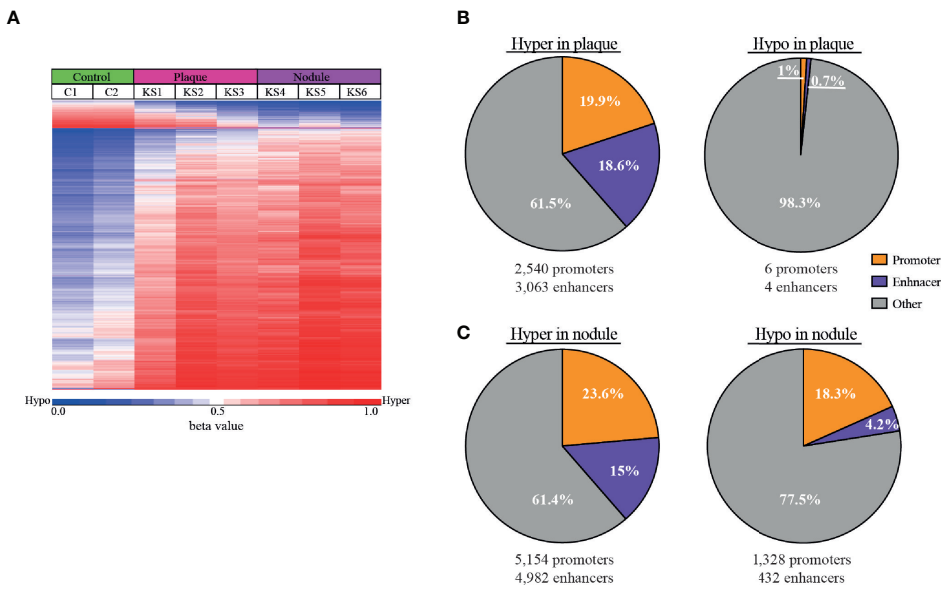



**FIGURE 3** | Hyper-methylation in KS is prevalent in enhancer regions. **(A)** Heat map of the most differentially methylated CpG sites (raw β values) comparing control and KS samples. Hyper (red) and hypo (blue) methylation threshold was set as $\Delta\beta \geq 0.25$ and $\Delta\beta \leq -0.25$ respectively with FDR ≤ 0.05. **(B, C)** Pie charts representing the percentage of hyper and hypo-methylated CpGs that are located within cellular promoters (orange) and enhancers (purple) for plaque **(B)** and nodule **(C)**. The number of cellular enhancers and promoters identified in our analysis are presented below.

phenotypes (**Table S5**). Biological processes in hyper-methylated promoters include cornification, establishment of skin barrier, hemiendosome assembly, and cell surface junction. Biological processes in hyper-methylated enhancers include regulation of mitochondrial membrane permeability, apoptotic signaling, keratinocytes differentiation, skin development, hair follicle development, and neuronal death. Hyper-methylated promoters include genes associated with phenotypes of abnormal keratinocyte differentiation, blistering and scaly skin. Hyper-methylated enhancers include genes associated with phenotypes of dermal atrophy, abnormal skin, hypotrichosis, nail dystrophy, epidermal acanthosis, abnormal epidermis stratum, thick epidermis, and abnormal wound healing. Altogether it seems that hyper-methylated genes control abnormal skin morphology, a characteristic phenotype of KS.

Biological processes in hypo-methylated promoters include regulation of immune response, leukocyte and lymphocyte activation, T-cell receptor, MHC I/II protein complex, and intermediate filaments. Biological processes in hypo-methylated enhancers include regulation of cell migration and motility, wound healing, angiogenesis, and epithelial to mesenchymal transition (EMT). Hypo-methylated promoters include genes associated with phenotypes of abnormality of lymphocytes, leukocytes, B-cell physiology, T-cell physiology, immunoglobulin levels, humoral immunity, neutrophil physiology, and lymph node physiology. Hypo-methylated enhancers include genes associated with phenotypes of abnormal blistering of the skin, angiogenesis, plasma cell number, lymph node morphology, second pharyngeal arch, small pharyngeal arch, and spine curvature. Altogether it seems that hypo-methylated genes control immune response phenotype, and genes that should be methylated in cells that are not pharyngeal or spine. Considering the important role of the EMT pathway (called EndMT in endothelial cells) in KS development, our study indicates that DNA methylation participate in the activation of this pathway by KSHV.

## Common CpG Methylation in PEL and KS

While the cell type of PEL is very different from KS, we were interested to see which of the methylation changes are common. Therefore, we compared the differentially methylated CpG between PEL (Journo et al., 2018) and KS and identified 199 hyper-methylated and 236 hypo-methylated promoters (**Tables S6**, **S7**). Gene-ontology analysis on DAVID Bioinformatics Resources 6.8 (https://david.ncifcrf.gov/home.jsp) (Huang da et al., 2009) for common hyper-methylated promoters identified alternative splicing, cell membrane, SH2 domain, tyrosine protein phosphatase, transcriptional repressor activity, transcriptional misregulation in cancer, and homeobox (**Table S8**). Several regulators of TGF-beta signaling were identified, such as TGFBR2, SMAD9, NEDD4L, and TGIF1. As mentioned above, TGFBR2 has been shown previously as hyper-methylated and repressed in KSHV-infected cells (Di Bartolo et al., 2008). In a previous study (Journo et al., 2018) we have found that several *DUSP* genes, such as *DUSP5*, *DUSP6*, and *DUSP10* were repressed and their promoter was hyper-methylated in PEL. Interestingly we found that *DUSP2, DUSP6, DUSP7, DUSP13, DUSP16*, and *DUSP28* were hyper-methylated in KS. Since DUSPs are dual specificity phosphatases that dephosphorylate MAPK, ERK, JNK and p38, their down-regulation in KSHV-infected cells may contribute to the activation of these kinases by KSHV. Gene-ontology analysis for common hypo-methylated gene promoters identified glycosylation, cell adhesion, plasma membrane, immunoglobulin, cellular defense response, and homeobox (**Table S8**). In addition, several GTP-binding superfamily and immuno-associated nucleotide (IAN) subfamily of nucleotide-binding proteins, GIMAP1, GIMAP4, GIMAP5, GIMAP7, and GIMAP8. The large number of proteins associated with cell adhesion, plasma membrane and immunoglobulin indicates that methylation changes within gene promoters have profound effect on the cell surface of KSHV-associated malignancies.

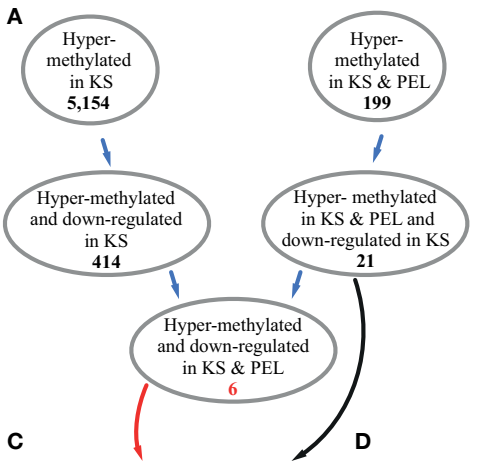
## CpG Methylation and Gene Expression in KS and PEL

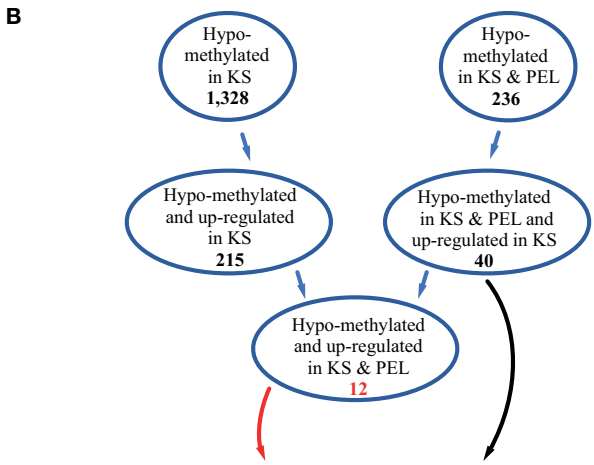
Next, we correlated the methylation changes we observed with gene expression. We intersected the differentially methylated gene promoters with a previous RNA-sequencing (RNA-seq) analysis performed on KS (Tso et al., 2018). Within the list of 5,154 hyper-methylated promoters, 414 were down-regulated in KS (**Figure 4A** and **Table S9**). On the more restricted list of 199 hyper-methylated promoters both in KS and PEL, 21 were down-regulated in KS (**Figure 4C**). Of these 21 genes, five are involved in metabolic pathways (ACSL1, ACSS2, ALDH2, ATP6V1C2, MGAT3) seven in acetylation (ACSL1, ACSS2, ALDH2, ELOVL5, BCAR3, NEDD4L, SYNGR1) and three are transcription factors (ZNF219, ZNF395, SOX8). We identified six genes that were hyper-methylated and repressed in both KS and PEL (**Figure 4C**).

Among the 1,328 hypo-methylated promoters, 215 were up-regulated in KS (**Figure 4B** and **Table S9**). On the more restricted list of 236 hypo-methylated promoters both in KS and PEL, 40 were up-regulated in KS, and 12 were up-regulated both in KS and PEL (**Figure 4D**). Of the 40 up-regulated in KS 21 are glycoproteins (ADAMTS5, CCR5, CD177, CD93, GPR1, NOX4, SLAMF8, CPVL, COL1A1, FAP, ITGA4, MGAT4A, MMP2, OLFML2B, OPCML, PRF1, ROBO4, SELPLG, SIGLEC12, SIRPB2, SIGIRR), six of them contain immunoglobulin domain (SLAMF8, OPCML, ROBO4, SIGLEC12, SIRPB2, SIGIRR) and three are metallopeptidases (ADAMTS5, FAP, MMP2). Here again, many of the hypo-methylated and up-regulated genes encode for cell membrane proteins.

# DISCUSSION

Here we present the cellular CpG DNA methylation in KS, the most common malignancy associated with KSHV. For this analysis we compared normal skin biopsy to KS samples of both plaque and nodule. The PCA analysis clearly differentiated normal skin from KS based on the cellular methylation data, indicating that indeed following KSHV infection and the development of KS there are significant changes in cellular DNA methylation. Moreover, these methylation changes can also differentiate less advanced disease (plaque) from more advanced disease (nodule). Comparison between normal skin and KS revealed that most changes are




C

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|---|---|
| ACSL1 | acyl-CoA synthetase long-chain family member 1 |
| ACSS2 | acyl-CoA synthetase short-chain family member 2 |
| ADGRG1 | Adhesion G Protein-Coupled Receptor G1(GPR56) |
| ALDH2 | aldehyde dehydrogenase 2 family (mitochondrial) |
| ATP6V1C2 | ATPase H+ transporting V1 subunit C2 |
| BCAR3 | breast cancer anti-estrogen resistance 3 |
| BCAS1 | breast carcinoma amplified sequence 1 |
| CRB3 | crumbs 3, cell polarity complex component |
| ELOVL5 | ELOVL fatty acid elongase 5 |
| FAM184A | family with sequence similarity 184 member A |
| GRB7 | growth factor receptor bound protein 7 |
| HSBP1L1 | heat shock factor binding protein 1 like 1 |
| KANK1 | KN motif and ankyrin repeat domains 1 |
| MGAT3 | mannosyl (beta-1,4-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase |
| NDRG4 | NDRG family member 4 |
| NEDD4L | developmentally down-regulated 4-like, E3 ubiquitin protein ligase |
| SLC29A2 | solute carrier family 29 member 2 |
| SOX8 | SRY-box 8 |
| SYNGR1 | synaptogyrin 1 |
| ZNF219 | zinc finger protein 219 |
| ZNF395 | zinc finger protein 395 |

D

| | | | |
|---|---|---|---|
| ADAMTS5 | ADAM metallopeptidase with thrombospondin type 1 motif 5 | IL32 | interleukin 32 |
| ASAP1 | ArfGAP with SH3 domain, ankyrin repeat and PH domain 1 | ITGA4 | integrin subunit alpha 4 |
| CCR5 | C-C motif chemokine receptor 5 (gene/pseudogene) | LCP2 | lymphocyte cytosolic protein 2 |
| CD177 | CD177 molecule | MGAT4A | mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase, isozyme A |
| CD93 | CD93 molecule | MMP2 | matrix metallopeptidase 2 |
| CMTM7 | CKLF like MARVEL transmembrane domain containing 7 | NOX4 | NADPH oxidase 4 |
| COL1A1 | collagen type I alpha 1 chain | OLFML2B | olfactomedin like 2B |
| CPVL | carboxypeptidase, vitellogenic like | OPCML | opioid binding protein/cell adhesion molecule like |
| ERG | ERG, ETS transcription factor | PRF1 | perforin 1 |
| ETS1 | ETS Proto-Oncogene 1 | PRR5L | proline rich 5 like |
| EXOC3L2 | exocyst complex component 3 like 2 | RARRES3 | retinoic acid receptor responder 3 |
| FAM107B | family with sequence similarity 107 member B | ROBO4 | roundabout guidance receptor 4 |
| FAP | fibroblast activation protein alpha | RTP4 | receptor transporter protein 4 |
| GIMAP4 | GTPase, IMAP family member 4 | SELPLG | selectin P ligand |
| GIMAP8 | GTPase, IMAP family member 8 | SIGIRR | single Ig and TIR domain containing |
| GMFG | glia maturation factor gamma | SIGLEC12 | sialic acid binding Ig like lectin 12 (gene/pseudogene) |
| GPR1 | G protein-coupled receptor 1 | SIRPB2 | signal regulatory protein beta 2 |
| GUCY1B3 | guanylate cyclase 1 soluble subunit beta | SLAMF8 | SLAM family member 8 |
| HMHB1 | histocompatibility minor HB-1 | VAMP5 | vesicle associated membrane protein 5 |
| IL16 | interleukin 16 | WIPF1 | WAS/WASL interacting protein family member 1 |

**FIGURE 4** | KS and PEL share common differentially methylated promoters. **(A)** The number of hyper-methylated promoters common in KS and PEL, and down-regulated are indicated. **(B)** The number of hypo-methylated promoters common in KS and PEL, and up-regulated are indicated. **(C)** Gene names and their descriptions for hyper-methylated promoters common in KS and PEL, and down-regulated in KS are indicated. Genes that are also down-regulated in PEL are indicated in red. **(D)** Gene names and their descriptions for hypo-methylated promoters common in KS and PEL, and up-regulated in KS are indicated. Genes that are also up-regulated in PEL are indicated in red.

towards hyper-methylation. Hyper-methylation starts earlier, while hypo-methylation increases as KS progress from plaque towards nodule. Accumulation of hypo-methylation changes have been observed also in a previous study that followed tumor development of endothelial cells by KSHV (Naipauer et al., 2020).

Global cellular CpG methylation analysis in PEL detected ~ 6% hyper-methylation (5.99% in BCBL1 and 6.24% in BC3) (Journo et al., 2018). The percentage of hyper-methylation detected in KS is very similar, with 5.22% in nodule. The observation that already following *de-novo* infection KSHV induced 4.4% hyper-methylation (Journo et al., 2018), supports the notion that KSHV imposes hyper-methylation on the cellular genome shortly after infection. We found that many of the differentially methylated promoters in KS were different from PEL, this was expected taking into account that the methylation pattern in B-cells is different from endothelial/mesenchymal cells. Even though, we identified 199 promoters that become hyper-methylated in both KS and PEL, suggesting these promoters are hyper-methylated regardless of the type of the infected tissue.

As opposed to hyper-methylation, hypo-methylation is very different between PEL and KS, while in PEL we observed up to 30% hypo-methylation (27.5% in BCBL1 and 30.16% in BC3), only 1.8% was detected in KS. Analysis in Epstein-Barr virus (EBV) infected cells revealed a similar phenomenon; in infected B-cells the majority of changes were towards hypo-methylation (22.75% hyper- and 77.25% hypo-methylated) (Zhang et al., 2018), while in epithelial (gastric cancer EBV positive vs negative) cells the majority of changes were towards hyper-methylation (83.2% hyper- and 16.8% hypo-methylated) (Zhao et al., 2013). In the case of EBV infected B-cells DNMT1 and

DNMT3B are downregulated, and DNMT3A is up-regulated (Leonard et al., 2011). While in EBV-positive epithelial cells high expression levels of DNMT1 and DNMT3B have been detected (Ksiaa et al., 2014). This change in DNMT expression along with changes in B-cell differentiation state might explain these changes. In KSHV infected PEL cells DNMT1 and DNMT3A were unchanged while DNMT3B was down-regulated (Journo et al., 2018). In KS vs normal skin the expression of DNMT1 and DNMT3B were unchanged while DNMT3A was up-regulated (Tso et al., 2018). The down-regulation of DNMTs in both EBV and KSHV infected B-cells might be one mechanism for the robust hypo-methylation observed in these cells.

The observation that 23.6% of the hyper-methylated CpGs were located in promoters and 15% were in enhancers, and 18.3% of the hypo-methylated CpGs were in promoters and 4.2% were in enhancers, suggests that these methylation changes might have an effect on cellular gene expression. Analysis of these promoters and enhancers on GREAT revealed that hyper methylated promoters and enhancers regulate genes associated with abnormal skin morphology a phenotype associated with KS development, while hypo-methylated promoters and enhancers regulate immune response genes and activation of B and T cells. We cannot exclude the possibility that some of these hypo-methylated promoter regions are the result of infiltrating immune cells into the lesion. Nevertheless, our study suggests that hypo-methylated enhancers regulate genes involved in EMT (in the case of endothelial cells called EndMT) an important process during the development of KS (Cheng et al., 2011; Gasperini et al., 2012), and might hint for the importance of methylation changes during KS development. To correlate methylation changes with gene expression, we intersected our methylation data with a published gene expression analysis in KS (Tso et al., 2018). We identified 414 hyper-methylated and repressed genes and 215 hypo-methylated and up-regulated genes in KS.

Most of the of the hyper- and hypo-methylated CpG are located at non-CpG island sites. This preference for non-CpG island sites was also observed in EBV infected cells (Zhang et al., 2018), but might results from the relatively small number of CpGs located in CpG-islands in the human genome and accordingly their relative representation in the MethylationEPIC BeadChip (Han et al., 2008; Pidsley et al., 2016). The differentially methylated promoters and enhancers identified here for KS, are only the first step in our understanding of CpG methylation in this tumor. Future studies should tackle the question why these specific promoters and enhancers become hyper-methylated in KSHV-infected cells.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: http://biodb.md.biu.ac.il/biu/shamay_lab_data.html.

## ETHICS STATEMENT

The studies involving human participants were ethically approved by the institutional Helsinki committee at Rambam hospital (number 0391-15-RMB). The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: GJ and MS. Performed the experiments: GJ, AA, and YE. Analyzed the data: GJ and MS. Contributed reagents/materials/analysis tools: DD-P and RB. Wrote the paper: GJ and MS. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.666143/full#supplementary-material

**Supplementary Table 1 |** Differentially methylated CpGs.

**Supplementary Table 2 |** Hyper methylated promoters and enhancers.

**Supplementary Table 3 |** Hypo methylated promoters and enhancers.

**Supplementary Table 4 |** Biological processes associated with differentially methylated regulatory elements.

**Supplementary Table 5 |** Phenotypes associated with differentially methylated regulatory elements.

**Supplementary Table 6 |** The common hyper-methylated gene promoters between KS and PEL.

**Supplementary Table 7 |** The common hypo-methylated gene promoters between KS and PEL.

**Supplementary Table 8 |** GO analysis.

**Supplementary Table 9 |** KS methylation and expression.

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