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Prospects of a novel vaccination strategy for human gamma-herpesviruses

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Abstract

Due to the oncogenic potential associated with persistent infection of human gamma-herpesviruses, including Epstein-Barr virus (EBV or HHV-4) and Kaposi's sarcoma-associated herpesvirus (KSHV or HHV-8), vaccine development has focused on subunit vaccines. However, the results using an animal model of mouse infection with a related rodent virus, murine gammaherpesvirus 68 (MHV-68, γ HV-68, or MuHV-4), have shown that the only effective vaccination strategy is based on live attenuated viruses, including viruses engineered to be incapable of establishing persistence. Vaccination with a virus lacking persistence would eliminate many potential complications. Progress in understanding persistent infections of EBV and KSHV, raises the possibility of engineering a live attenuated virus without persistence. Therefore, we should keep the option open for developing a live EBV or KSHV vaccine.

Keywords

Gamma-herpesviruses; vaccine; EBV; KSHV; MHV-68

Introduction

The human gamma-herpesviruses, Epstein-Barr virus (EBV or HHV-4) and Kaposi's sarcoma-associated herpesvirus (KSHV or HHV-8), are linked to several malignancies, especially in immuno-deficient patients, such as those with human immunodeficiency virus (HIV) infection or during organ transplantation. Tumorigenesis of gamma-herpesviruses is associated with the persistence of infection. Due to the oncogenic potential of EBV and KSHV, there is a strong need for a vaccine to prevent the establishment of persistent infections. Vaccines based on live attenuated viruses enable the presentation of a full repertoire of viral antigens in the context of active replication, and thus effectively elicit humoral and cell-mediated immune responses. A live virus vaccine for EBV or KSHV is generally considered to pose a great safety concern due to the tumorigenicity associated with viral persistence. As a result, most EBV vaccine development has been focused on subunit vaccines targeting the major viral envelope glycoprotein, gp350. In a recent clinical trial, a gp350-based vaccine demonstrated protective efficacy against symptomatic primary EBV infection but had limited effects on the overall infection rate [1]. As EBV and KSHV are

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large complex DNA viruses, superior in their ability to evade host immune responses, it is likely that subunit vaccines to individual viral proteins would not be sufficient to prevent infections of EBV or KSHV. Therefore, there is still a demand for a vaccine that can induce broad cellular and humoral immune responses against multiple viral antigens. However, EBV and KSHV are highly species-specific, and thus, the progress in the test and development of new vaccination strategies has been limited.

A goal of our laboratories is to exploit mouse infection with murine gammaherpesvirus 68 (MHV-68, also known as γ HV-68, or MuHV-4), a closely related virus to EBV and KSHV, as an experimental model to explore and test proof of principle vaccination strategies based on live-attenuated viruses. Although MHV-68 infection of mice may not recapitulate all aspects of human KSHV or EBV infection, MHV-68 shares remarkable similarities to EBV and KSHV in genomic sequence and biology. The knowledge obtained from this mouse infection model will be instrumental when considering a vaccination strategy for EBV and KSHV. For this purpose, our first focus was to genetically engineer a MHV-68 virus that was deficient in persistence, as viral persistence may cause the complications associated with the use of live viruses as vaccines. This persistence-deficient virus was generated and successfully used as a vaccine in mice to inhibit the establishment of persistent infection from the challenge wild-type virus [2]. The results provide an attractive vaccine strategy using genetically engineered live EBV or KSHV viruses that do not persist and therefore do not have the potential to cause tumors. Here, we will briefly review the molecular biology as well as immune control of gamma-herpesvirus persistent infections, and then discuss the prospects of live viral vaccines.

Herpesviruses

Herpesviruses are enveloped viruses that contain double-stranded DNA genomes larger than 100 kb with vast coding capacity. Their life cycles have two distinct phases, known as lytic replication and latency. During lytic replication, numerous viral genes are expressed in a highly-ordered manner, leading to the assembly and release of infectious particles, often resulting in the lysis of infected cells. Latency is the hallmark of herpesviruses and is characterized by limited gene expression without virion production, enabling the virus to escape or evade the host immune control while maintaining its genome and dwelling as a communicable agent in the infected cells. Interchanging between two phases of the life cycle has strategically allowed herpesviruses to efficiently establish life-long persistent infections in hosts.

Herpesviruses constitute an ancient virus family that has well-adapted to their hosts as demonstrated by their high human infection rates in most populations. The natural infection of herpesviruses has three stages: primary infection, latency and reactivation. Primary infection: Generally, herpesviruses are secreted into saliva and transmitted to a naïve host by the oral route [3, 4]. They are capable of replication within the oral epithelium. In immunocompetent hosts, primary infection is typically asymptomatic or causes a mild illness. However, primary infection of immunocompromised hosts is associated with diseases and can lead to fatal consequences. Latency: As primary infection resolves, herpesviruses establish latency in specific cell types where limited viral genes are expressed to avoid elimination by the host immune system. In a latent state, herpesviruses usually do not produce diseases. However, latency of some herpesviruses may result in benign or malignant tumors [5]. Reactivation: Periodically, herpesviruses reactivate from latency to enter a stage of active viral replication, producing and shedding infectious viruses. This reactivation and subsequent re-infection of naïve cells maintains a viral reservoir in the host for a lifetime. Factors triggering reactivation are poorly understood. Recurrent infection is

typically subclinical, but may induce severe and atypical diseases that differ from what is seen with primary infection, especially in hosts with a defective immune system.

The eight human herpesviruses identified so far are divided into three subgroups, alpha (α), beta (β) and gamma (γ) [6]. The α subgroup, including herpes simplex (HSV)-1, HSV-2, and varicella-zostere virus (VZV), establish latency in sensory nerve ganglia. Reactivation of HSV-1 is most commonly associated with cold sores, HSV-2 with genital lesions, and VZV with shingles. The β subgroup, human cytomegalovirus (HCMV), human herpesvirus (HHV)-6 and HHV-7, reside in monocytes, macrophages lymphocytes and the salivary gland during latency. HCMV primary infection is the major viral cause of congenital defects [7]. It is also a serious opportunistic pathogen in immunocompromised patients, causing symptomatic primary and recurrent diseases with risk of multi-organ failure as a result of disseminated infection. The γ subgroup, including KSHV and EBV, establish latency mainly in B-lymphocytes, but latent KSHV and EBV can also be found in endothelial and epithelial cells, respectively.

Human tumor-associated gamma-herpesviruses

EBV and KSHV are associated with several diseases, including benign and malignant tumors in a small portion of infected hosts. Although primary infection of EBV in young children is usually asymptomatic, infection during adolescence or adulthood is often associated with infectious mononucleosis (IM). Uncontrolled EBV reactivation in HIV/AIDS patients leads to oral hairy leukoplakia (OHL). Latent EBV infection is associated with Burkitt's lymphoma (BL) and nasopharyngeal carcinoma (NPC). BL, a B-cell lymphoma, is endemic to equatorial Africa where it is the most frequent type of childhood cancer. NPC, an epithelial tumor, has high incidence in certain regions of Southeast Asia and is one of the most common cancers among Chinese. Other types of malignancies, such as T-cell lymphomas, gastric carcinomas, and Hodgkin's disease, are also linked to EBV. KSHV was only identified in 1994 and is associated with Kaposi's sarcoma (KS), primary effusion lymphoma (PEL), and Multicentric Castleman's disease (MCD) [8–13], three tumors that frequently develop in AIDS patients. While PEL and MCD are of B-cell origin, KS is derived from endothelial cells. KSHV infection is uncommon (~3%) in western countries, however, it has high prevalence (ranging from 20 to 80%) in sub-Saharan Africa [14]. Also in Africa, a rare, but more aggressive and often fatal form of KS can occur in children. Now with the HIV-1 epidemic ongoing in the area, the overall incidence of KS is substantially increasing and KS has become one of the most common malignancies in adults [15–17].

Compared to general and healthy populations, virus-associated malignancies develop more frequently in the individuals whose immune system is compromised, such as by infection with HIV-1, or by using immunosuppressive drugs after receiving organ transplants. EBV-associated B-cell lymphomas and KSHV-associated KS are among the most common malignancies seen in AIDS patients. Post-transplant lymphoproliferative diseases (PTLD) are another type of life-threatening condition caused by EBV infections in transplant patients. Although there is a low incidence of diseases in the majority of infected people, for the high risk groups described here, a viral vaccine against EBV and KSHV is urgently needed.

Murine gammaherpesvirus-68 (MHV-68, γ HV-68 or MuHV-4)

Both EBV and KSHV are highly species-specific and do not infect small animals, such as mice and rats. Therefore, mouse infection of a closely related rodent virus, MHV-68/ γ HV-68, also known as MuHV-4, has provided a useful model to study virological and immunological aspects of gamma-herpesvirus infection in hosts. The MHV-68 genome has

been completely sequenced, and was found to share 80–90% sequence homology to KSHV and EBV [18–20]. MHV-68 infection in mice proceeds with three temporal stages [21]. Following intranasal inoculation of mice, MHV-68 first undergoes acute lytic replication in epithelial cells of the respiratory tract. Subsequently, the virus sets up latency in B-cells of the secondary lymphoid organs, peaking at two weeks after inoculation and accompanied by splenomegaly. MHV-68 then establishes a steady, low level persistent infection 4–6 weeks later. The massive lymphoproliferation at the peak of viral latency resembles several features of infectious mononucleosis that some humans experience with primary EBV infection [22, 23]. Furthermore, similar to EBV and KSHV, persistent infections of MHV-68 can lead to B-cell lymphoproliferative diseases in the mice with defective immune systems [24–26]. An additional distinct advantage of using MHV-68 as a model is the possibility of infecting various genetically modified mice and manipulating the immune system. Furthermore, unlike EBV and KSHV, MHV-68 readily undergoes lytic replication in a variety of cell lines, providing a model to study the lytic phase of gamma-herpesviruses.

Two distinct life-cycle phases of gamma-herpesviruses

Both phases of the viral cycle, latency and lytic replication, contribute to the pathogenesis of gamma-herpesviruses. In most virus-associated diseases, EBV and KSHV are found in their latent state while viral lytic replication is seen in OHL and MCD [27–29]. It is widely accepted that viral latency is directly linked to tumorigenesis. Several viral proteins expressed during latency are anti-apoptotic or growth-promoting and their primary function is believed to be supporting the survival and proliferation of latently infected cells in order to maintain viral persistence in hosts. However, this viral strategy also predisposes the cells to transformation. Lytic replication may have an important role in tumor development as well. While most tumor cells in KSHV-associated malignancies are latently infected, expression of viral lytic genes is detected in a small percentage of tumor cells [29–32]. This leads to the suggestion that lytic replication enhances the growth of latently infected cells in a paracrine fashion, especially by expressing the viral lytic proteins that are homologues of cellular cytokines and chemokines [31, 33–38]. In addition, lytic replication can contribute to tumorigenesis indirectly by producing infectious virions to re-infect naïve cells, thus replenishing the pool of latently infected cells, subsequently giving rise to transformed cells. The dynamic balance between the viral latent and lytic cycles is critical for persistent infection, transmission, and related pathogenesis.

Lytic replication of gamma-herpesviruses

Lytic replication of EBV and KSHV has largely been studied by reactivating the latent virus with chemical inducers (TPA and sodium butyrate) in cell lines that are derived from virus-infected B-cell lymphomas. In addition to reactivation in virus-infected cell lines, *de novo* infection of MHV-68 is commonly used to study viral lytic replication. These studies have established one important concept that the molecular master switch controlling lytic replication and latency is the virally encoded transcriptional activator expressed immediately upon reactivation. These viral proteins are ZEBRA and RTA of EBV, RTA of KSHV, and RTA of MHV-68. Although ZEBRA is unique to EBV, all three viral RTA proteins share significant sequence homology. Exogenous over-expression of these viral master switch proteins in their respective virus-infected cell lines is sufficient to disrupt viral latency and initiate the lytic replication cycle [39–44]. Therefore, the expression and function of these viral switch proteins control the viral life cycles, and are tightly and intricately regulated in both positive and negative ways.

Following the initiation of lytic replication, more than 80 viral genes are expressed in a cascade order. First immediate early (IE), then early (E), and finally late (L) viral genes are expressed, leading to the production of infectious viral particles and lysis of infected cells.

Unlike other viruses with smaller genome sizes, herpesviruses have multiple glycoproteins associated with the envelope of infectious viral particles, where complexes formed among glycoproteins mediate cell binding and entry. There are at least eight envelope glycoproteins for human gamma-herpesviruses [45, 46]. gp350 and gp220 of EBV, encoded by the same gene, are the most abundantly expressed envelope proteins and responsible for the attachment of EBV to B-cells [47, 48]. The antibodies against gp350/220 were shown to neutralize EBV infectivity [49]. Therefore, most EBV vaccine development has been focused on targeting gp350/gp220. However, as reviewed in-depth elsewhere [50], alternative envelope proteins may be used by EBV to interact with different target cells. In fact, although the anti-gp350 antibodies inhibit EBV infection of B-cells, these antibodies were found to enhance infections of epithelial cells [51, 52]. Two mechanisms for this enhancement have been proposed. One is that coating of EBV by IgA specific for gp350/gp220 promotes viral entry via the polymeric IgA-receptor on the surface of epithelial cells [51]. The other is that binding of gp350/gp220 by antibodies exposes other viral envelope proteins essential for infections of epithelial cells [52]. This cell type-specific requirement is also implicated in the binding of MHV-68 to B-cells and epithelial cells [53].

Latency of gamma-herpesviruses

During latency, herpesviruses express only a subset of genes and maintain their genome in a non-integrated, circular form (episome). Therefore, gamma-herpesviruses must have a mechanism to ensure the persistence of their genetic materials in proliferating cells, such as B-lymphocytes. This critical function is performed by EBNA1 of EBV, LANA (latency-associated nuclear antigen) of KSHV, and a LANA homologue of MHV-68. Despite of no amino acid sequence homology between EBNA1 and the two LANA homologues, all three exploit a similar molecular mechanism to distribute episomal viral genomes into both daughter cells following cell division. Both EBNA1 and LANA bind the terminal repeat region of their respective viral episome and tether the viral DNA to a cellular chromosome [54, 55]. As a result, the viral genome can be stably maintained in dividing cells without being integrated into a cellular chromosome. Studies based on EBNA1-deficient EBV [56] and LANA-deficient KSHV [57] mutant viruses have provided evidence that these two viral proteins are essential for gamma-herpesviruses to maintain their viral genomes in cells. Furthermore, LANA-deficient MHV-68 viruses were shown to be incapable of establishing latency in mice [58, 59]. Given the pivotal role of EBNA1 and LANA in the persistence of viral DNA, their expression is invariably detected during latency. EBNA1 and KSHV/LANA have also been implicated to play a role in tumorigenesis. Transgenic mice expressing EBNA1 or LANA developed B-cell lymphomas more frequently than age-matched controls [60, 61].

In addition to EBNA1, 11 other EBV genes can be detected during latency. They include the other five nuclear antigens (EBNA2-6), three membrane-associated proteins (LMP1, LMP2A, and LMP2B), and three RNA species (EBER1, EBER2, and BART). Particularly, LMP1 and LMP2A mimic constitutively active CD40 and BCR receptors, respectively, to promote the survival and growth of latently infected B-cells [62, 63]. Moreover, LMP1 alone is capable of transforming cells in culture [64]. Depending upon the types of associated diseases and infected cells, these 12 EBV latent genes are expressed in at least three different combinations, known as latency program I, II, and III. While the three RNA species and EBNA1 are expressed in all programs, LMP1 and LMP2 are found in both latency II and III, and EBNA2-6 are only in latency III. One prevailing hypothesis is that different latent gene expression programs allow EBV-infected naïve B-cells to differentiate into long-lived resting memory cells, where all EBV genes including EBNA1 are subsequently shut off, causing neither harmful effects to the host nor recognition by the host immune system [65]. This ideal co-existence between a virus and its host may explain why

more than 90% of the human population is infected by EBV but majority of infected individuals are healthy carriers without any disease expression.

In KSHV, a cluster of three viral genes, including LANA (ORF73), ORF72/vcyclin (a homologue of cellular cyclin D), and ORF71/vFLIP (a homologue of cellular FLICE inhibitory protein), are consistently expressed in latency-associated diseases and B-cell lines [66, 67]. ORF71/vFLIP activates NF- κ B signaling, which induces cellular transformation [68]. Although ORF72/vcyclin by itself does not have transforming activity, transgenic mice expressing ORF72/vcyclin can develop lymphomas if p53 is absent [69]. In addition to these three latent genes, there are the most abundant transcripts, T0.7, expressed during latency. The T0.7 transcripts encode three kaposin proteins [70–72], of which Kaposin A has oncogenic potential [73].

In addition to ORF73/LANA, the viral genes possibly expressed during latency of MHV-68 include ORF72/vcyclin, ORF74 (a viral GPCR), K3, and several MHV-68 specific ORFs, M2, M3 (a chemokine binding protein), M9, and M11 (a homologue of bcl-2) [74–78]. There are also seven non-coding tRNA-like molecules expressed during latency [79]. Because MHV-68 latent infection can be detected in macrophages, dendritic cells, and epithelial cells other than B-cells [80–82], it has been suggested that a distinct set of genes are expressed to establish latent infections in different cellular compartments [83]. The viral mutants with deficiency in K3, M2, M11/vbcl-2, or ORF73/LANA all showed reduced splenic latency [58, 84–89]. MHV-68 K3 is a homologue of a KSHV-specific gene, K3; and both K3 homologues down-regulate surface expression of MHC class I molecules [90]. M2, a gene specific to MHV-68, has several activities, including interacting with Vav proteins to regulate cellular signaling [91, 92], stimulating B-cell proliferation in an IL-10 dependent manner [93], and inhibiting the anti-viral activity of interferons [94, 95]. M11/vBcl-2, like its cellular counterpart, has both anti-apoptotic and anti-autophagic functions [89, 96–98].

Many of latency-associated genes of gamma-herpesviruses are able to transform cells when they are highly expressed and they may play a role in viral tumorigenesis. Nevertheless, one has to keep in mind that the viruses express these proteins to enhance the survival and growth of the latently infected cells as a part of the strategy to maintain persistent infections. Another interesting, possible function of the latent genes is to inhibit the initiation of viral lytic replication in order to sustain the latent phase. LMP2 prevents EBV reactivation induced by surface immunoglobulin cross-linking in latently infected B-cells [99]. LANA of KSHV represses the function of RTA, a viral protein that drives reactivation from latency to lytic replication [100], and consistently, a LANA-deficient virus has enhanced lytic gene expression [101]. Another latent gene, vFLIP, also blocks viral lytic replication [102].

Since the first virally encoded microRNAs (miRNAs) were found in 2004 [103], many more have been identified and currently there are 25 miRNA genes identified for EBV, 12 for KSHV, and 9 for MHV-68. The majority of viral miRNA genes are expressed during latency but their targets remain largely unknown. From the limited potential targets revealed so far, viral miRNAs appear to regulate the host immune responses, cellular proliferation and apoptosis, as well as the control of lytic replication and latency (for review see [104]). Interestingly, two KSHV miRNAs were found to reduce RTA expression [105, 106], indicating that negative regulation of the viral lytic switch protein is one of the strategies utilized by the virus to maintain latency.

Immune responses against gamma-herpesvirus infection

Most individuals infected by EBV and KSHV are able to utilize their immune systems to control the viral lifelong infection without manifesting any significant diseases. The critical role of T-cells in the control of gamma-herpesvirus infection is supported by that

malignancies caused by the outgrowth of virus-infected cells often emerge when T-cell function is compromised either by immunosuppressive drugs during organ transplantations or by chronic HIV-1 infections. In fact, it has been shown that adoptive transfer of EBV-specific T-cells can effectively treat PTLD where all nine EBV latent proteins are expressed, making PTLD an ideal target for immunotherapy [107, 108]. Understanding the immune responses in asymptomatic carriers and disease-associated patients is instrumental for the development of therapeutic immunological approaches to treat malignancies and ultimately viral vaccines to protect individuals from virus-associated diseases. The immune responses elicited by EBV infections are much more extensively studied than the responses elicited by KSHV, partly because KSHV is a newer virus and its prevalence (1–10%) is much lower than EBV (>90%) in most areas. In the following, we will briefly summarize the immune responses during gamma-herpesvirus infections.

CD8⁺ T-cell responses

CD8⁺ T-cells eliminate virus-infected cells through recognition of peptides derived from intracellular processing of viral proteins presented by MHC class I molecules on the surface. Studies have identified targets for CD8⁺ T-cell responses. During IM (primary symptomatic infection of EBV), strong responses are mounted towards immediate early and early but not late lytic proteins [109–112]. The responses to latent antigens are weaker and mostly towards EBNA3, 4 and 6, and to a lesser extent, towards LMP2 [111–113]. There are even fewer, though detectable, CD8⁺ T-cells specific for EBNA1, an antigen expressed in all latency programs. This low level of response is probably due to the intrinsic resistance of EBNA1 to proteasomal digestions, resulting in reduced antigen presentation by MHC class I molecules [114–116]. Nevertheless, it has also been shown that EBNA1-derived peptides can be produced via degradation of defective ribosomal products (DRiPs) generated from the translation process of EBNA1 [117, 118]. Consistent with this, EBNA1-specific CD8⁺ T-cell clones have been demonstrated to recognize virus-infected B-cells and release IFN- γ [119–121]. Some of such T-cell clones exhibit modest cytotoxicity to virus-infected cells while others inhibit the proliferation of infected cells without lysing them. These results indicate that the EBNA1-specific CD8⁺ T-cells can contribute to controlling EBV-latently infected cells *in vivo*, but to what extent remains to be determined. Healthy virus carriers, most likely contracting EBV years ago, have a similar response pattern to what is observed in IM patients, though the magnitude of responses is smaller. Their CD8⁺ T-cells are targeted towards the lytic antigens expressed in the early phase [111, 122, 123] and their responses to EBNA3, 4 and 6 are more frequently detected than their responses to other latent antigens [124–126].

For KSHV, the targets of CD8⁺ T-cells are less clear. Unlike what is seen in EBV-infected individuals, CD8⁺ T-cell responses against KSHV can be targeted to both early and late lytic proteins [127–129]. The latent antigen, LANA/ORF73, similar to EBNA1 of EBV, is able to evade the MHC class I antigen presentation pathway [130]. However, CD8⁺ T-cell responses to LANA/ORF73, though weak, have been identified [131]. Another latency-associated protein, K12/Kaposin, elicited a stronger response than LANA/ORF73 [128, 131, 132]. It has been shown that the individuals that developed KS appeared to have weaker, less diverse and less functional KSHV-specific CD8⁺ T-cell responses than those that have asymptomatic persistent infections [128, 129]. The results indicate a crucial role of CD8⁺ T cells in restricting the development of KSHV-associated diseases.

A systematic approach has been taken to examine CD8⁺ T-cell epitopes during MHV-68 infection [133, 134]. Both early and late lytic proteins are targets for the responses, similar to what was observed for KSHV infection in humans. Furthermore, it appears that the responses for individual epitopes can be roughly divided into two patterns by slow or fast

declines over time. The patterns do not correlate with whether the epitope is derived from early or late lytic protein. Instead, it has been postulated that the different patterns are due to whether the viral protein is presented only during the acute lytic phase or in both lytic and latent stages of infection. This is further supported by that the slow-declining responses decreased more rapidly in mice infected with a latency-deficient virus [133]. Importantly, there is no evidence for the loss of functionality of these T-cells, either the slow- or fast-declining virus-specific T-cells.

CD4⁺ T-cell responses

Traditionally, the recognition of CD4⁺ T-cells is via peptides derived from engulfed extracellular viral proteins, processed in lysosomes, and presented by MHC class II molecules on the cell surface. However, at least two alternative MHC class II presentation pathways have been recently described to process intracellular proteins, expanding the repertoire of peptides that can be displayed [135, 136]. CD4⁺ T-cells are important to support high affinity antibody production and maintain cytotoxic CD8⁺ T-cell responses. They can also perform direct effector activity on infected cells [137]. EBV-specific CD4⁺ T-cell clones have been shown to possess cytotoxic activity [138–141] and to inhibit proliferation of EBV-infected B-cells [142]. The difference among clones may be due to the levels of target peptides presented on the cell surface [139, 142]. The protein targets for CD4⁺ T-cells are less defined. Notably, unlike CD8⁺ T-cells, CD4⁺ T-cells frequently target EBNA1 [143, 144] and most healthy EBV carriers have EBNA1-specific CD4⁺ T-cells. These CD4⁺ T-cells are primarily Th1-polarized [145], secreting IFN- γ upon *in vitro* activation, and are able to kill the target cells that express EBNA-1, including BL-derived cells [140, 145]. The pivotal role of EBNA1-specific CD4⁺ T-cells in controlling the outgrowth of transformed infected cells is supported by the decrease of this response observed in children with BL in Africa [146] and in patients with EBV-associated lymphomas [147].

In addition to EBNA1, other CD4⁺ T-cell targets have been identified, including latent proteins as well as early and late lytic proteins [139, 142, 143, 148–150]. These EBV-specific CD4⁺ T-cell clones also secrete IFN- γ and lyse their respective target-expressing cells. The *in vivo* contribution of these effector CD4⁺ T-cells may depend upon the surface presentation level of the epitope that the T-cells recognize [139]. For KSHV, the CD4⁺ T-cell responses are largely uncharacterized. It seems that the responses are preferentially targeted to the early and late proteins [129] similar to what is found for CD8⁺ T-cells.

Virus-specific CD4⁺ T-cells are detected throughout persistent MHV-68 infection in mice [151]. They are essential for the host defense against MHV-68 since infected mice without CD4⁺ T-cells eventually lost control of viral infection and died within 4 months after infection [152]. Evidence supports an effector role of CD4⁺ T-cells in directly targeting MHV-68-infected cells in addition to the helper activity. In mice lacking B-cells and antibody production, lytic replication in the lung was controlled when CD8⁺ T-cells were depleted, but the mice succumbed to MHV-68 infection when both CD8⁺ T-cells and CD4⁺ T-cells were depleted [153]. Furthermore, this control from CD4⁺ T-cells was significantly reduced by the neutralization of IFN- γ . More recently, direct cytolytic activity of virus-specific CD4⁺ T-cells, though not strong, has been demonstrated as well [154]. It is possible that the effector functions of CD4⁺ T-cells can be mediated by two independent mechanisms, IFN- γ production and cytotoxicity. In fact, CD4⁺ T-cells from persistently infected mice do display two distinct effector populations, one producing IFN- γ and the other possessing killing activity [155]. Currently, two epitopes derived from viral late lytic proteins have been identified as targets for CD4⁺ T-cell responses [156]. Considering the

significance of effector CD4⁺ T-cells, it is important to make efforts to systematically identify the targeted epitopes.

Antibody responses

Although less emphasized, the antibody can play an important role in the control of viral infection, such as by neutralizing viral infectivity. The sera from healthy EBV carriers are capable of blocking viral infection of B-cells. This neutralizing activity of sera is mainly attributed to the antibodies against the two major viral envelope proteins, gp350 and gp220 [157]. Since EBV is primarily transmitted via an oral route, salivary gp350-specific antibody response was also examined and found to be present at low levels in a small percentage of healthy carriers. While the salivary IgA antibody specific for gp350 has a weak neutralization activity on B-cell infection [158], it can promote infection of epithelial cells [51, 52]. The antibody responses to other EBV proteins have also been detected, such as early lytic proteins, a viral capsid protein (VCA), as well as two latent proteins, EBNA1 and EBNA2. Their *in vivo* biological significance is unclear, but they have provided useful serological markers for diagnosis. Each status of infected individuals, healthy or sick with different EBV-associated diseases appears to have a distinct pattern of humoral responses [159]. Early studies found that EBV-specific IgA antibodies were elevated prior to NPC development [160], indicating that EBV reactivations occur at the mucosal surface before tumors emerge.

Neutralizing antibodies against KSHV are also elicited in KSHV-infected individuals. It was found that HIV-positive individuals who developed KS had lower KSHV neutralizing antibody responses but had higher total anti-KSHV IgG levels than the HIV-positive individuals without KS [161]. This indicates a critical role of neutralizing antibodies in preventing the emergence of KS. The underlying mechanisms remain to be determined. However, it is tempting to speculate that new infections of KSHV is important for KS development and perhaps in the context of the weaker cellular immunity caused by HIV-1 infection, neutralizing antibodies become a major defense against KSHV infections.

During MHV-68 intranasal infection, the neutralizing antibody and total virus-specific antibody slowly increase, reach the peak one month later, and remain strong without declines [162]. The numbers of IgG-antibody producing cells in the lymph nodes and spleen peak at 2–3 weeks after infection and decrease thereafter, but the numbers in bone marrow start to increase and stay high until later times [163]. No IgA-antibody producing cells are found in any of the above compartments. Although virus-specific antibody does not play a significant role during the acute infection phase, the contribution of humoral responses to the control of persistent MHV-68 infection has been demonstrated [81, 164–166].

Immune evasion genes

A long period of coexistence entails a delicate balance between virus persistent infections and host immune surveillance for mutual survival. Although both cellular and humoral responses are elicited, gamma-herpesviruses manage to escape the immune control and establish life-long infections. The central strategy, shared by all herpesviruses, is to exist in a latent state without alarming the host immune system. Moreover, the major latency-associated proteins of gamma-herpesviruses, such as EBNA1 of EBV and LANA of KSHV and MHV-68, have evolved to avoid antigen presentations by MHC class I molecules [114, 115, 130, 167]. It has been shown for MHV-68 that this evasion mechanism is essential for viral latency in the host [167]. The large size of the viral genomes has allowed herpesviruses to encode many immune evasion genes. For example, several viral proteins of gamma-herpesviruses are able to suppress the MHC class I antigen presentation, thereby protecting the virus from elimination by the host and inducing a suboptimal anti-viral immunity (for

review see [168–173]). Gamma-herpesviruses also can impair MHC class II presentation by using a viral host shutoff protein to reduce the level of MHC class II molecules [174, 175] and by using viral bcl-2 to inhibit Beclin1-mediated autophagy [176, 177], which is an important mechanism for cells to present intracellular proteins via MHC class II molecules [178]. Immune cells are often coordinated and regulated by cytokines and chemokines in response to infections. Gamma-herpesviruses encode homologues of cellular cytokines and chemokines, including vIL-10 of EBV [179] and viral C-C chemokine ligands (vCCLs, or MIPs) of KSHV [31, 37, 38], as well as chemokine binding protein, M3 of MHV-68 [180]. The function of these viral proteins is likely to modulate the host immune responses to favor viral infections [181–184]. Another remarkable observation is that gamma-herpesviruses appear to utilize multiple proteins to block the type I interferon production and responses, indicating a significant role of innate immunity in controlling viral infections. Recently, it was also found that seemingly dissimilar miRNAs encoded by several herpesviruses, including EBV and KSHV, target the same cellular protein, MICB, a stress-induced Natural Killer (NK) cell ligand, implying that NK cells play a role in the anti-viral host responses [185]. In Table 1, we list the viral immune evasion genes that are involved in antigen presentation and type I interferon responses. This knowledge will provide us with important guidance to improve the immunogenicity of viral vaccines.

Current developments in gamma-herpesvirus vaccines

Conventionally, vaccines are given to naïve individuals to prevent a disease. There are several highly effective preventive/prophylactic viral vaccines that drastically reduce once highly prevalent and devastating infections to close to elimination, such as polio, smallpox, and MMR vaccines. However, one prevailing notion is that there is no urgent need for gamma-herpesvirus vaccines since the majority of virus carriers do not develop diseases. We do not share this view because as pointed out previously, there are groups of individuals that are highly susceptible to life-threatening diseases associated with gamma-herpesviruses. These individuals include people with a high risk for HIV-1 infections, people living in BL and KS endemic areas, and people in Southern China. Post-exposure vaccination should be considered as a possibility for gamma-herpesviruses. Except for IM, most diseases develop many years after primary infection. Vaccines may be used to enhance natural immunity to mitigate the established persistent infections, thereby reducing the tumor risk. Post-exposure immunization could be particularly valuable for individuals with declining T-cell responses due to immunosenescence or immunosuppression, such as with HIV-1 infection. For example, the live varicella vaccine, the only licensed herpesvirus vaccine, has been shown to re-stimulate the pre-existing virus-specific T-cell responses elicited by natural infections [186] and represents a promising strategy to reduce herpes zoster and postherpetic neuralgia in older adults [187].

The viral envelope proteins that are the major targets of the neutralizing antibody responses are usually the prime candidates for vaccine development. For EBV, gp350/gp220 appears to be an ideal candidate for subunit vaccines. The antibodies against gp350/gp220 are able to neutralize EBV infectivity [49, 188] and are the major component of neutralizing activity of human sera [157]. Various vaccine formats based on gp350/gp220 were tested and shown to afford protection against EBV induced B-cell lymphomas in primates [189–191]. This success triggered great enthusiasm to proceed with clinical trials of the gp350-based vaccine in humans. One of the trials was conducted in infants immunized with vaccinia virus expressing gp350/220 [192]. The sera of all nine immunized infants contained gp350/220-specific antibodies as well as neutralizing activities. Six of the nine infants remained EBV seronegative while all of the 10 unvaccinated infants acquired EBV within 16 months after immunization. We do not know whether those six seronegative immunized individuals ever became infected with EBV after the conclusion of this study. The other trial was conducted

in healthy young adults receiving recombinant gp350 protein with adjuvants [1]. Within 18 months, 2 out of 86 immunized individuals and 8 out of 90 in the placebo control group developed IM, indicating a protective effect against EBV symptomatic infections. However, there was no significant effect on the EBV seroconversion rate. Whether the latent EBV loads of seroconverted individuals were different between vaccinated and unvaccinated groups was not reported. It is unclear why these two trials have different outcomes, but there were at least two major differences in the trial setups. First, the vaccine recipients were infants in the first trial and healthy young adults in the other. Second, gp350-based vaccine was delivered through a live viral vector in the first trial, which can induce both humoral and cellular immunity, while it was delivered in a purified protein format in the second trial, which preferentially elicits the antibody responses. In earlier vaccine tests using primates as a model, it was found that the protective effect of a gp350-based vaccine did not always correlate with the induction of neutralization activity [191]. Not until much later, gp350 was found to elicit cytotoxic CD4⁺ T-cell responses [149]. Therefore, it is possible that gp350/gp220-specific CD4⁺ T-cell responses contribute to the protection afforded by vaccines but such responses were induced to different levels in these two clinical trials.

The major goal of gamma-herpesvirus vaccines is to inhibit the establishment of latency, or even just reducing the steady level of latency will still be beneficial. It is unlikely that this goal can be achieved by gp350/gp220-based vaccines. Vaccines that target only latent antigens are another option but with limitations, as latency is considered to be immunologically “silent”. The major latent proteins are usually expressed at a low level and resistant to proteasome degradation for antigen presentation in order to minimize recognition by the immune system. Considering the complexity of potential viral antigens encoded by gamma-herpesviruses, it is likely that a collective immune response to multiple targets is needed to achieve protection. We may have to consider a vaccination approach, such as live attenuated viruses, to present a broad repertoire of epitopes in order to mobilize multiple arms of the immune system. However, development of such vaccine strategies has been limited because of the tumorigenicity of EBV and KSHV and because of the lack of a suitable and tractable animal model for EBV and KSHV.

Animal models for vaccine studies

Neither EBV nor KSHV infects small animals, but they can infect non-human primates. The cottontop tamarin infected with a high titer of EBV develops B-cell lymphoma, and these primates have been the most commonly used animal species for testing EBV vaccination strategies. KSHV infection of a New World primate species, common marmosets, was recently described [193]. In this model, B-cell hyperplasia was observed and a KS-like lesion was detected in one of the orally infected common marmosets. Undoubtedly, this primate model of KSHV infection will be invaluable for future vaccine studies. Other primate models, including the rhesus monkey models with infections of related monkey gamma-herpesviruses, such as EBV-related lymphocryptovirus (LCV) and KSHV-related Rhesus rhadinovirus (RRV), have been established as well [194, 195].

A more cost-effective and tractable *in vivo* model is based on mice. Previous accomplishments exploited immunodeficient mice transplanted with human hematopoietic tissues (thymus and liver) or stem cells to provide human target cells for KSHV and EBV infections [196–199]. The utility of this humanized model is further expanded when engraftment of human CD34⁺ hematopoietic stem cells permitted the reconstitution of most major components of the human immune system. Infection of these humanized mice with EBV elicited humoral and cellular immunity [198–201]. However, one has to keep in mind that these humanized models lack of non-immune human cells, such as epithelial cells, which potentially play an important role in EBV transmission at the mucosal site as well as

in persistent infections. The use of humanized mice for KSHV infections has not yet been reported.

Vaccine studies using MHV-68 infection of mice

We and others utilize mouse infection with MHV-68 as an *in vivo* model for gamma-herpesvirus infections, especially for exploring proof of principle vaccination strategies. Peptide and subunit vaccination based on lytic and latency-associated viral proteins that were identified as targets for T-cell and neutralization antibody responses reduced acute lung lytic replication and peak splenic latency of the challenge virus [202–207]. However, these vaccines did not affect the establishment of long-term latency. For example, the gp150/M7 of MHV-68 has some sequence homology to the EBV gp350 and binds to B-cells but not epithelial cells [208]. The efficacy of gp150-based vaccines was also tested [205]. Vaccination via a gp150 expressing vaccinia virus induced neutralizing antibody responses and almost abolished IM-like symptoms seen at the peak of latency in challenged mice. However, the challenge virus was still able to establish latency in the vaccinated mice with an unchanged long-term level. The result bears resemblance to the clinical trial of EBV gp350-based vaccines conducted in young adults. The lack of effects on long-term persistent infections from subunit vaccination strategies may not be unexpected, since the CD8⁺ T-cell responses elicited by MHV-68 infection are towards a variety of viral proteins and they may collectively contribute to the control of viral infections. Heat-inactivated MHV-68 virions were used for vaccination with the hope that this vaccine contains a variety of viral antigens and would be more effective. Disappointingly, the inactivated virions were also incapable of reducing long-term latency [209]. So far, the only vaccination strategy that can reduce the long-term latent load is based on live attenuated viruses designed to be deficient in latency [166, 210–213]. The rationale for this approach is that the vaccine viruses can undergo lytic replication and induce a broad spectrum of immune responses but would pose a minimal tumor risk to the host because of their severe defects in latency establishment.

Two principal strategies were exploited to achieve latency-deficiency. One strategy was to constitutively over-express RTA, thereby constantly driving the virus towards lytic replication [211, 214]. We previously constructed a recombinant MHV-68 virus with the insertion of an ectopic RTA expression cassette at the left end of the genome [211]. This virus was severely attenuated in splenic latency but had the capability of inducing protective immunity to prevent the challenge virus from establishing latency. Similar results were obtained by others using a recombinant virus that contains a CMV IE promoter in front of the RTA coding sequence [212]. One major drawback for this strategy is that the vaccine virus used by our group was found to re-gain the ability to establish latency by kicking out the inserted RTA cassette. The other strategy was to eliminate LANA/ORF73, thereby preventing the virus from establishing splenic latency [58, 85]. A potential concern for this strategy is that there may be latent reservoirs, such as memory B-cells, where cellular proliferation is rare and the latent viral genome would be maintained even in the absence of LANA/ORF73. Moreover, since gamma-herpesviruses encode several oncogenic proteins, if the viral DNA gets integrated into the cellular genome, which is an infrequent but detectable event, it can potentially transform cells. Taking all of these concerns into considerations, we combined both strategies and generated another vaccine virus by replacing the locus containing LANA/ORF73 and potentially oncogenic proteins, ORF72/v-cyclin and M11/vbcl-2 with the RTA expression cassette driven by a strong and constitutively active promoter [213]. This virus, named as AC-RTA, elicited virus-specific antibody responses and T-cell responses towards the viral lytic proteins to a similar extent as the wild-type virus. Notably, the T-cell response to a latent epitope mapped to M2 was drastically reduced to a background level, consistent with the lack of splenic latency of AC-RTA. Vaccination with the AC-RTA virus protected mice against latency establishment from the challenge

virus. One very important conclusion drawn from these studies using live attenuated viruses is that the protection against latency establishment can be afforded by the vaccine viruses that are deficient in latency. This would significantly improve the prospect of a live gamma-herpesvirus as a vaccine, because now the critical pathogenic component of the virus, latency, can be removed from the vaccine virus without losing the protective effects.

Having established the vaccination efficacy of the AC-RTA virus, there are two immediate questions to address. First, we will determine whether post-exposure vaccination of AC-RTA can modify the established infections. Persistent infections of herpesviruses are a dynamic balance between lytic replication and latency where both phases of the viral life cycle contribute to pathogenesis. As latent antigens are generally invisible to the host immune system, AC-RTA has a potential to boost the pre-existing immunity targeted to lytic proteins to help the host control viral persistent infections. Second, we will examine whether immunogenicity of the vaccine virus can be increased by removing the immune evasion genes. Some of the viral immune evasion genes, such as ORF36 and K3, are not required for viral replication in cell culture and thus their removal would not affect the production of the vaccine virus. In addition to previously known viral immune evasion genes, our laboratory recently identified multiple genes that inhibit the type I interferon (IFN) responses. Since type I interferons play an instrumental role in shaping the adaptive immune responses, we plan to remove the anti-IFN viral genes from the vaccine virus as well. Our preliminary results showed that the virus without these anti-IFN genes was severely attenuated in the cells with an intact innate response, but its replication could be rescued in the cells lacking of the ability to produce interferons. Therefore, these attenuating genetic changes are likely to reduce the acute lytic replication of the vaccine virus *in vivo*, providing the benefit of safety. However, the amount of antigens expressed usually determines the strength of the corresponding immunity. Therefore we need to determine whether a balance between immunogenicity and safety can be achieved with this new vaccination strategy.

Blueprints of live viral vaccines

Live vaccines are attenuated viruses that do not cause diseases. In the cases of gamma-herpesviruses, viral latent infection is directly associated with tumorigenesis and therefore, the live vaccine should not be able to establish latency. Without latency, the vaccine virus will not persist and will cause no tumors in the host. One would ask, 'Is it possible to generate a herpesvirus without latency?' Herpesviruses have evolved millions of years, and they have developed sophisticated mechanisms to establish life-long infections, making it seemingly unlikely to outwit these ancient viruses. Fortunately, all of these diligent studies on gamma-herpesviruses have taught us that the fundamental mechanism governing the viral life cycle, lytic replication or latency, lies with a viral transcription factor. We also learned that to maintain latency in proliferating cells, the virus must use a viral protein to ensure the distribution of the viral genomes. Combining these two pieces of knowledge, we now have a rationale design for a vaccine virus that would have only lytic replication but no latency. Even though the virus encodes several oncogenic proteins, the cells infected by the vaccine virus are designated to be lysed and little threat would be posed to the host. Nevertheless, the viral oncogenes can be removed from the live vaccine for safety concerns. If a live vaccine with neither a latent phase nor persistence can be generated, will it be capable of preventing the establishment of latency? Using the MHV-68 mouse infection model, we have demonstrated that protection can be achieved by a virus with only lytic replication [2]. Studies identifying the correlates of immune protection and characterizing the immune responses elicited by the vaccine virus are currently in progress. There is no doubt that these studies will have significant implications to the development of vaccines for EBV and KSHV.

Having presented strong arguments here, we would suggest that live attenuated EBV and KSHV viruses unable to establish latency are a viable option for vaccination. An efficient genetic engineering system for EBV and KSHV was developed and it is now possible to engineer the herpesviral genomic DNA and produce viruses with desired features. The results obtained from vaccination studies in the MHV-68 mouse infection model have provided us with the guidance for a rational design of latency-deficient EBV and KSHV viruses (Figure 1). A similar approach can be taken for KSHV to replace the locus spanning from K12 to ORF74, which includes three potential oncogenic proteins (Kaposin, vFLIP, vGPCR) and LANA/ORF73, with a RTA ectopic expression cassette. For EBV, EBNA1 will be deleted and a ZEBRA ectopic expression cassette will be inserted next to the terminal repeat to replace the region that contains oncogenic LMP1. In addition to removing the latency from vaccine viruses of EBV and KSHV, the immunogenicity may be improved by eliminating the viral immune evasion genes, especially those blocking MHC class I presentations.

If the live vaccine is used for post-exposure vaccination, it may be important for the live vaccine to present EBNA1 of EBV and LANA/ORF73 of KSHV that are expressed in all virus-associated tumors. Studies in EBV have indicated that the immune responses towards EBNA1 can be elicited and are important components to control the outgrowth of latently infected cells. EBNA1 and LANA/ORF73 have always been the focus of many studies. Therefore, we should be able to mine through the results of these studies and design a truncated protein that are unable to maintain the viral episome, transform cells or resist antigen presentation. Such a protein can be inserted as part of the live vaccine to increase the efficacy.

Concluding remarks

The lack of an amiable animal model for EBV and KSHV has inspired researchers using mouse infections with a related rodent gamma-herpesvirus, MHV-68, for studying many virological and immunological topics, especially for testing proof of principle vaccination strategies. In this review, we have described several aspects to consider for vaccine development in the perspective of similarities and differences among MHV-68, EBV and KSHV. Without a doubt, the knowledge we have gained from MHV-68 has provided significant implications to human gamma-herpesviruses. Most importantly, the only effective vaccination strategy tested so far to reduce the long-term latency of MHV-68 in mice is based on live viruses, including latency-deficient viruses. Therefore, we should reconsider the option of live attenuated viruses and make efforts to develop safe and effective live EBV and KSHV vaccines not only to induce protective immunity in naïve hosts, but also to enhance the immune control of virus carriers.

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Figure 1.

A rationale design of live vaccines for EBV and KSHV. A simplified representation for the genome organizations of MHV-68, KSHV, and EBV modified from Virgin *et al.* [19] is presented. The gray boxes indicate conserved gene blocks among three viruses. The latent genes are labeled green and the viral immediate early transcriptional factors are labeled red. The genes proposed to be deleted are indicated by a thin crossed line. The striped boxes at the end of viral genomes correspond to terminal repeats. In the EBV genome, there is the other striped box that represents the large internal repeat region which encodes latent genes, such as EBNA2 and EBNA5.

Table 1

Examples of immune evasion genes of EBV, KSHV, and MHV-68

T-cell responses	EBV		KSHV		MHV-68	
		ref		ref		ref
MHC Class I	BILF1	215	K3, K5	220,1	K3	90
	BGLF5/ORF37	174,5				
TAP transporter	BNLF2a	216			K3	232
MHC class II	BGLF5/ORF37	174,5				
Autophagy			vbcl-2/ORF16	177	vbcl-2/M11	176
Immunomodulators	vIL-10/BCRF-1	179	vCCL1-3	31,37,38	M3	222
Interferon responses						
Cellular IRF	BGLF4/ORF36	217	ORF36	222	ORF36	222
	BRLF1/RTA	218	RTA	223		
	LF2/ORF11	219	ORF45	224		
IFN signaling			vIRFs	225-30		
			ORF10	227	M2	94,5