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A Herpes Simplex Virus Type 2 Deleted for Glycoprotein D Enables Dendritic Cells to Activate CD4+ and CD8+ T Cells

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Herpes simplex virus type 2 (HSV-2) is highly prevalent in the human population producing significant morbidity, mainly because of the generation of genital ulcers and neonatal encephalitis. Additionally, HSV-2 infection significantly increases the susceptibility of the host to acquire HIV and promotes the shedding of the latter in the coinfected. Despite numerous efforts to create a vaccine against HSV-2, no licensed vaccines are currently available. A long-standing strategy, based on few viral glycoproteins combined with adjuvants, recently displayed poor results in a Phase III clinical study fueling exploration on the development of mutant HSV viruses that are attenuated in vivo and elicit protective adaptive immune components, such as antiviral antibodies and T cells. Importantly, such specialized antiviral immune components are likely induced and modulated by dendritic cells, professional antigen presenting cells that process viral antigens and present them to T cells. However, HSV interferes with several functions of DCs and ultimately induces their death. Here, we propose that for an attenuated mutant virus to confer protective immunity against HSV in vivo based on adaptive immune components, such virus should also be attenuated in dendritic cells to promote a robust and effective antiviral response.

We provide a background framework for this idea, considerations, as well as the means to assess this hypothesis. Addressing this hypothesis may provide valuable insights for the development of novel, safe, and effective vaccines against herpes simplex viruses.

Keywords: vaccine, dendritic cells, dendritic cell function, herpes simplex virus type 2, adaptive immunity, attenuation, T cell activation

INTRODUCTION

Herpes simplex virus type 2 (HSV-2) infects nearly 500 million people worldwide and is the main cause of genital ulcers in symptomatic individuals (1, 2). Importantly, infection may be transferred to neonates during birth, which may lead to life-threatening encephalitis (3). Although antivirals limit HSV-2 replication in the newborn, serious long-term neurologic sequelae may follow, despite treatment (4–6). HSV-2 is persistent in humans, establishing latency in neurons with periodic symptomatic or asymptomatic reactivations that shed infectious virus and significantly contribute to the spread of HSV-2 in the population (7, 8). Importantly, the risk of acquiring HIV is 3-fold higher among individuals that are HSV-2-seropositive (9, 10). In regions where HSV-2 infection is highly...
prevalent, it is estimated that nearly 50% of HIV infections may be attributed to previous HSV-2 infections (10, 11). Although oral antivirals limit the extent of the HSV reactivations, reduce virus shedding and shorten the duration of herpetic lesions, these drugs do not resolve persistent infection (12, 13). Thus, vaccines that prevent primary infection, block reactivation, and virus shedding are wanted to limit the spread of HSV-2 in the population and its multiple deleterious effects. Although important efforts have been undertaken for developing a vaccine against this virus and HSV-1, regrettably these attempts have failed so far.

**HSV-2 VACCINE APPROACHES**

Subunit vaccine candidates consisting of glycoprotein D from HSV-2 (gD-2), alone or in combination with other HSV envelope glycoproteins, as well as different adjuvants have predominated the HSV vaccine field for nearly 20 years (14–16). Such vaccine development efforts have mainly focused on gD-2 as the main viral target and are likely based on the fact that this glycoprotein is conserved among HSV-2 and HSV-1 isolates (17) and is essential for the entry of the virus into target cells both, immune and non-immune (18, 19). Additionally, HSV-2-infected individuals display high titers of anti-gD-2 antibodies indicating that this viral protein is highly immunogenic and highly visible to the immune system (20, 21). However, although antibodies directed against gD-2 after vaccination or natural exposure to the virus may display neutralizing activity in vitro (16, 22–25), their antiviral effects in vivo have seemingly been overestimated, as their presence in individuals not necessarily correlates with protective immunity (22, 26–28).

Because subunit vaccine candidates have failed so far at eliciting protective immunity against HSV-2 in clinical trials, other more traditional approaches, such as those based on attenuated mutant viruses have re-emerged as prophylactic alternatives for eliciting immunity against this virus (29). The notion that an attenuated HSV may achieve protective immunity against HSV-2 could be somewhat based on the fact that a weakened herpes virus, namely the varicella zoster virus Oka strain is currently used as a protective and therapeutic vaccine against varicella and shingles (30, 31). Nevertheless, its efficacy is modest, and it may be replaced in the short term by a subunit-based vaccine (32, 33). However, because attenuated HSV mutants have been relatively poorly explored as potential vaccines against HSV-2 this approach should be revisited.

At present, several attenuated viruses have been shown to be safe and confer protective immunity against HSV-2 in animal models. One example is an HSV mutant that has the nuclear localization sequence of the viral protein ICP0 deleted (0ΔNLS), which has been shown to be attenuated in vivo and induces protective antibodies targeted against numerous viral proteins (Table 1) (34–36). Another virus exhibiting very positive results in vivo and shown to confer protection against HSV infection,

| HSV mutant | Deletion or mutation | Outcome | Reference |
|------------|---------------------|---------|-----------|
| HSV-2 ΔgD-2 | Glycoprotein D (U6 gene) deleted from the genome. Complemented phenotypically with gD-1 (VD60 cells) | Protects against genital and skin challenges and blocks neuronal infection. Confers cross-protection against HSV-1. Antibody-mediated protection | (49, 50) |
| HSV-2 ΔTK | Viral thymidine kinase (TK, U23 gene) deleted | Protective, although non-optimal immunity in the mouse genital HSV infection model | (51, 52) |
| HSV-2 ICP10ΔPK | Protein kinase domain (PK) of the large subunit of HSV-2 ribonucleotide reductase (ICP10) deleted | Induction of a Th1 response and CD8+ cytotoxic T lymphocytes | (42) |
| HSV-2 ΔgH/FΔPK | Glycoprotein H (U22 gene) deleted from the genome. Complemented phenotypically with gH (Vero F cells) | Protection in the guinea pig model. Tested on individuals with symptomatic HSV-2. Neither virus shedding or recurrences rates were affected | (53, 54) |
| HSV-2 RAV 3995 | U55, U56, and RL1 genes deleted (deletion was done on both copies of the γ34.5 gene RL1) | Reduction in herpetic lesions and severity in the guinea pig model. Stimulates both, cell-mediated and humoral immune responses | (48) |
| HSV-2 d1S-29 | DNA replication helicase (U5 gene) and Infected Cell Protein 8 (ICP8, Major DNA-binding protein, U29 gene) deleted | Induces neutralizing antibodies and virus-specific CD8+ T cell responses in mice. Conferred protection in the guinea pig model | (55, 56) |
| HSV-2 AD 472 | U55-56, RL1 (gene for γ34.5), U43.5, and the U10-12 region deleted | Humoral and cellular immune response in mice and reduced frequency of herpetic reactivation in the guinea pig model | (47) |
| HSV-2 ΔgE | Envelope glycoprotein E (U8 gene) deleted | Reduced vaginal disease, viral titers, neuronal infection. However, protection was incomplete in the mouse infection model | (45) |
| HSV-2 gD+27 | Point mutations in the Nectin-1 binding domain of gD-2 (D215G, R222N, and F223I) | Impaired at infecting neurons. Provides protection in the mouse model, despite inducing modest titers of HSV-2-neutralizing antibodies in the serum | (44) |
| HSV-1 gC2 | Glycoprotein K (UL53 gene) and Envelope protein U20 (U20 gene) deleted | Induced protection through both, humoral and cellular responses in mice and conferred protection against genital challenges with HSV-1 and HSV-2 in rhesus macaques | (46) |
| HSV-2 0ΔNLS | Nuclear localization signal of the E3 ubiquitin-protein ligase ICP0 protein (RL2 gene) deleted | Induces protection in the mouse model through antibodies directed against numerous viral proteins. Elicits an antibody response against glycoprotein B and ICP viral proteins | (34, 35, 57) |
is an HSV-2 mutant virus designated d15-29, which has U15 and U129 deleted (Table 1) (37–39). Both attenuated viruses are being further studied in animal models and have transitioned into clinical trials (see Rational Vaccines Inc. and https://clinicaltrials.gov, respectively). Other attenuated HSV mutants that elicit protective immunity against infection with HSV in animal models are a mutant virus deleted at U129 which was designated ICP10ΔPK, because it has the protein kinase domain of the large subunit of HSV-2 ribonucleotide reductase (ICP10) deleted (40–43), an HSV-2 virus that has mutations in gD (44) which limit neuron infection, and an HSV mutant that has glycoprotein E (gE) deleted (45). Other mutant viruses tested as potential vaccines in animal models are HSV-1 VC2, which is a glycoprotein K (U153 gene) and envelope protein U20 (U20 gene)-deficient virus (46), AD 472 which has U455.5-56 (g3.54.5 gene), U435.5, and the U10-12 region deleted (47) and finally RAV 9395, a mutant virus with U55 and U56 genes deleted (48). Noteworthy, HSV-1 VC2 was recently tested in macaques with promising results (46).

Importantly, all these mutants have shown to be attenuated in animals (safe) and elicit either HSV-specific antibodies or HSV-specific T cells, or both, and confer protection against HSV-2 infection.

Because gD is essential for virus entry into target cells, deletion of this gene (ΔgD) should likely result in an attenuated virus that is impaired at infecting cells. Yet, if such virus is phenotypically complemented with gD protein on the surface of the virion, it would be capable of infecting cells, although its replication and progeny would likely be hampered. A virus with such characteristics (ΔgD/−/−pD1) was recently created and tested in animals and shown to be safe, highly immunogenic and confer protection against later challenges with high doses of clinical isolates of HSV-1 and HSV-2 in the skin and genital tissue (49, 50). These somewhat unexpected results may be partially explained by the fact that gD has been previously described to inhibit T cell proliferation and induce their death (58–61). Additionally, gD has been reported to decrease the cytotoxic activity of NK cells (62). Together, these findings suggest that gD may negatively modulate the induction of an effective antiviral response and thus, its deletion from the virion may promote more favorable immune responses.

Noteworthy, a mutant virus with glycoprotein H (U122 gene) deleted (ΔgH) conferred protection against primary HSV infection and reduced recurrent disease symptoms in an HSV infection animal model (53). However, once tested in a clinical setting, it failed to show a therapeutic response (Phase II clinical trial) (54). Another HSV mutant that displays incomplete protection in vivo after subcutaneous vaccination is a virus that is deleted for the viral thymidine kinase (TK). Indeed, although this mutant virus elicits anti-HSV antibodies and antiviral T cells when inoculated subcutaneously, these immune components only confer partial protection to genital infection in the mouse infection model (52).

Although most of the attenuated viruses described above elicit significant levels of protection in HSV animal infection models, which are mediated by adaptive immune components, such as anti-HSV antibodies and antiviral T cells, a specific correlate of protection against HSV has not been identified to date. Hence, it is currently unknown what would make a particular vaccine formulation better than another at conferring immunity and which one would have increased chances of being successful once applied to humans.

**DENDRITIC CELL INFECTION WITH HSV-2**

Dendritic cells are professional antigen presenting cells that play fundamental roles at establishing and regulating immune responses at the interface of innate and adaptive immunity (63). DCs are distributed within organs and tissues in the body and also located in the periphery, in tissues such as the skin and genital tract where they detect, capture, and process microbes and their antigens (64). Upon antigen capture, these cells migrate to the draining lymph nodes and display peptide fragments obtained from these microorganisms to antigen-specific T cells (63, 65). This DC–T cell interaction will educate T cells based on the integration of signals derived from membrane-bound and soluble molecules presented and secreted by DCs (66, 67). Attributes developed in T cells include the capacity to kill infected cells, secrete modulatory cytokines and regulate the functions of other immune cells, which will ultimately define the overall profile of the immune response elicited against an antigen (68). Therefore, many pathogens have evolved molecular mechanisms to hamper the function of DCs (69–71).

Noteworthy, HSV can interfere with DC function by blocking their maturation, migration to lymph nodes, promote the secretion of proinflammatory cytokines and inhibit normal autophagosome activity (Figure 1) (72–76). Furthermore, HSV-2 can limit DC presentation of viral antigens on MHC-I molecules by interfering with the transport of antigenic peptides from the cytoplasm to the endoplasmic reticulum and decrease the expression of T cell costimulatory molecules on the DC surface, thus impeding effective T cell activation (Figure 1) (74, 75, 77, 78). HSV can also block the activity of inducible nitric oxide synthase (iNOS) and NO production, by interacting with caveolin-1 (Cav-1) in DCs (Figure 1) (79). Additionally, and most importantly, HSV-2 elicits DC apoptosis early after infection, further limiting the chances of the host to establish an optimal antiviral T cell response (Figure 1) (17, 78, 80).

A recent report assessed the interaction of DCs with an attenuated HSV-1 mutant that induces protective immunity in vivo. Importantly, it was found that this virus was attenuated in DCs in vitro (i.e., innocuous, non-lethal) (81). Interestingly, the mutant virus expressed viral proteins in these cells despite limited genome replication, which was ultimately abrogated in these cells. Infection with this mutant virus was followed by DC maturation. Recently, we observed similar results with the ΔgD/−/−pD1 mutant virus (Retamal-Díaz et al., unpublished data). We found that this mutant virus was non-lethal to DCs and expressed non-structural proteins in these cells, despite the fact that viral genome replication was hampered. Furthermore, we found that although the ΔgH virus was attenuated in vivo, it was lethal to DCs in in vitro assays (Retamal-Díaz et al., unpublished data).

**STATEMENT OF HYPOTHESIS**

Based on the key role of DCs in establishing effective antiviral responses against pathogenic microbes and the negative effects
that HSV-2 exerts over these cells, we hypothesize that: **Attenuated viruses that confer protective immunity in vivo, namely those that rely on adaptive immune components, are attenuated in dendritic cells.** Similarly, the opposite may also be a valid hypothesis: **HSV-2 mutant viruses that are attenuated in DCs confer protective immunity in vivo.** We believe that such hypotheses have not been previously considered, at least not in an explicit manner. A recent study that assess the interaction of an attenuated HSV-1 mutant with DCs in vitro, which correlates with its protective properties in vivo is in line with this hypothesis (81), as well as data from our laboratory with the ΔgD−/−gD1, as described above. Thus, the hypothesis mentioned previously could be more specifically narrowed to: **A herpes simplex virus type 2 deleted for glycoprotein D that is safe in vivo and confers protective immunity, is attenuated in DCs and enables these cells to activate CD4+ and CD8+ T cells,** which could also apply to other HSV mutant viruses that confer protection in animal models (Table 1). **

**ASSUMPTION**

For this hypothesis to be consistent with previous reports, we consider that an important assumption should be taken into consideration. This assumption proposes that for the HSV-DC interaction to be considered as proestablishing protective immunity, the mutant virus should be able to express numerous of its genes within these cells, despite potential arrest of viral genome replication. This proposed condition arises from the observation that immunizations with UV-inactivated viruses are not protective, a finding which is true for either wild-type or mutant and attenuated viruses (50, 81). Data recently reported by others and data from our group support this notion (Retamal-Díaz et al., unpublished data) (81). Furthermore, this idea may be supported, at least partially by the fact that the expression of viral gene products from the virus genome will enable DCs to present a wider spectrum of viral antigens to T cells, extending beyond those present in the virion. Indeed, HSV encode at least 70 genes within 150,000 bp DNA, with half of the gene products present in the virion (82). Transcription and translation of viral genes within infected DCs may also translate into increased amounts of viral antigens being loaded by these cells onto MHC molecules for presentation to T cells. **

**SUGGESTED EXPERIMENTAL TESTS**

To assess the abovementioned hypotheses, we suggest a series of experiments with the HSV-2 ΔgD−/−gD1 mutant virus, as well
as other HSV mutants described in Table 1. All these mutant viruses have shown to confer at least some degree of protection to challenges with virulent HSV in vivo, in different HSV infection models. We propose that similar experiments also be performed with HSV mutants that have not shown satisfactory vaccination results in vivo. Interestingly, both human and murine DCs succumb to the negative effects of HSV-1 and HSV-2 and thus, either type of DC could be used in these assays, although experimenting with human DCs will likely be considered more insightful. The parental wild-type virus for each one of the mutant viruses assessed should be included in the experiments.

The experiments proposed below should allow assessing a wide range of key functions in DCs that are typically hampered after infections with wild-type virulent HSV.

**DC Viability**

DC viability has been previously shown to be severely compromised after inoculation with HSV-1 or HSV-2 (72, 80). This effect is observed at multiplicity of infections (MOIs) as low as 0.1. Hence, DCs may be inoculated with the different HSV viruses outlined in Table 1 and assessed for viability 24 and 48 h later with increasing MOIs by methods such as resazurin (e.g., AlamarBlue®) and viability dyes (e.g., Live/Dead®, Zombie®) that are fixable and can be safely assessed by flow cytometry. It is expected that viruses that have the potential to elicit robust protective immunity in vivo, will not significantly affect DC viability.

**DC Maturation**

DC maturation is the process by which these cells acquire a phenotype that promotes T cell activation. DC maturation usually relates to the expression of numerous activation markers on the surface of these cells, such as the expression of antigen-presenting molecules MHC-I and MHC-II, and T cell costimulatory molecules such as CD80, CD86, and CD83 (72, 83, 84). Additionally, DC maturation relates to the secretion of soluble molecules, namely cytokines such as IL-6, IL-10, IL-12, and TGF-β, which educate T cells and polarize them. Indeed, cytokine secretion by DCs not only evidences their maturation status, but also informs on the likelihood of the phenotype of T cells resulting from this interaction (67, 85). Other markers of DC maturation include increased antigen degradation, which may be assessed with exogenously added fluorescent-labeled proteins (e.g., labeled-ovalbumin) and the production of reactive oxygen species, which has been related to increased antigen-degradation capacity (86).

**DC-Mediated T Cell Activation**

Although anti-HSV T cells can be detected within HSV-infected humans (22, 87–89) and animals (49, 90–92) after natural exposure to the virus or infection with wild-type virus, numerous studies show that HSV hamper the capacity of DCs to activate these cells in vitro (72, 74, 80, 93). Furthermore, vaccination studies frequently show that T cell responses in animals vaccinated with protective formulations, such as attenuated HSV mutants, are enhanced as compared to those observed in animals challenged with wild-type virus alone. Hence, in vitro T cell activation by DCs may be considered as a readout of the DC–T cell activating capacity in vivo and an approach to quantify the magnitude of this activation (67, 94–98). Thus, to assess the potential of DCs pulsed with the mutant viruses to elicit protective T cell responses, virus-pulsed DC–T cell cocultures may be performed. For this, transgenic mouse antigen-specific T cells that either recognize viral antigens or exogenously added antigens can be used. Fortunately, there currently exist transgenic mice that harbor HSV-specific CD8+ T cells that recognize a HSV glycoprotein B (gB)-derived peptide on MHC-I (99) and HSV-specific CD4+ T cells that recognize a gD-derived peptide on MHC-II (100). Obviously, the latter could not be used for DCs inoculated with the ΔgD virus, as this virus does not encode gD. In this case, other transgenic mice that recognize foreign antigens on MHC-II, such as a peptide derived from the ovalbumin protein [e.g., OT-II (71)] may be used by pulsing DCs with the corresponding peptide at the time of coculture. Common readouts for T cell activation include the measurement of IL-2 secreted by T cells using ELISA, as well as the expression of CD69 and CD25 on the surface of these cells 24–48 h after coculture by flow cytometry (71).

**DC Migration In Vivo**

Recent studies indicate that HSV likely impairs DC function by interfering with their migration from the site of infection to the draining lymph nodes (101). Overall interference with this process, or the migration of specific DC subsets that are optimal for T cell activation in the lymph nodes seems to be due, at least in part, to the capacity of HSV to induce DC death (102). To assess whether protective HSV mutants recover this important DC function, or even enhance their migration, assays that consist on the local injection of virus and tracking dyes in the footpads or hind flank of limbs may be performed. These assays allow for the quantification of DC subsets that migrate from the periphery into the lymph nodes after infection (103). We expect HSV mutants that elicit protective immunity to promote the migration of DCs from the site of inoculation to the draining lymph nodes, either by increasing the amount of DCs that reach this secondary lymphoid organ or the migration of DC subtypes that are related to enhanced T cell activation (100, 101, 104).

**DC-Mediated T Cell Activation In Vivo**

A somewhat simple way to determine if an HSV mutant promotes enhanced DC function in vivo is to assess their capacity to activate T cells in this context, after interacting with mutant viruses. For this, DCs can be pulsed ex vivo with the viruses of interest and then inoculated into the animal to test for T cell activation (81). To exclude the possibility that T cell activation is occurring because inoculated virus-pulsed DCs that are moribund are being captured by non-injected DCs at the site of inoculation, the viability of DCs inoculated with the mutant virus needs to be verified before transferring these cells into the animals (78). If the mutant virus promotes DC function, these adoptively transferred DCs should promote T cell activation in vivo. T cells to be assessed in the animal can be those from the endogenous T cell repertoire, but also may be specific to HSV antigens, either using MHC-multimers or HSV-specific T cells, such as those outlined above to follow the small populations of T cells that are known to recognize HSV antigens (99, 100). Again, several T cell markers may be assessed which account for T cell activation, such as...
surface markers (e.g., CD69, CD25, CD71), intracellular cytokines (e.g., IL-2, interferon-gamma), and T cell proliferation, which may be assessed using a CFSE-dilution assay measured by flow cytometry (105). Furthermore, transferred cells may be followed thanks to endogenous surface markers, such as CD45.1/CD45.2 (52).

**DC-Mediated Anti-HSV Protective Immunity**

To assess the capacity of DCs inoculated with mutant HSV viruses to confer protective immunity against later exposure to HSV, we propose performing adoptive transfer experiments that consist on the injection of mutant virus-treated DCs into animals that will then be challenged with virulent HSV. Importantly, this type of assay devises numerous variables that require attention, such as the amount of DCs being transferred into the animals, the route of administration of these cells, and the number of transfers to be performed, among others. Furthermore, DCs from different sources may be used, such as *in vitro* differentiated DCs from the bone marrow (106, 107), DCs isolated from the spleens of naïve mice which then can be inoculated *ex vivo* with the mutant viruses (108, 109) or DCs purified from mice shortly after vaccination (81). Indeed, a recent report describes the use of the latter method for obtaining DCs inoculated with a mutant HSV-1 virus, particularly from the spleens 3 days after vaccination. These DCs were then transferred into naïve mice in three separate injections, which conferred protection (increased animal survival) against a challenge with a lethal dose of HSV-1 administered through the intranasal route (81). Importantly, we consider that for these assays in which virus-inoculated DCs are transferred into animals, an experimental group consisting of animals being transferred with DCs treated with wild-type virulent virus should also be included, as such treatment may confer some degree of protection to the animals because bystander DCs in the recipient animals could capture apoptotic HSV-infected inoculated DCs from the donor and present their antigens to immune cells (78). Noteworthy, the selection of a particular experimental method will depend, among others on the HSV infection model being evaluated (i.e., genital, skin, nasal or ocular infection, among others). Nevertheless, protective immunity after challenge can be measured, for instance by evaluating viral loads in neuronal and non-neuronal tissues (plaque forming units and viral loads by qPCR) and tissue pathology, among others (49, 50).

**CONCLUSION**

Although the deleterious effects of HSV over the functions of DCs were described nearly 15 years ago, with these interactions resulting in DC death, to date only few HSV mutants that could become future HSV vaccines have been tested directly on these cells (72, 80). We believe that because DCs are key determinants at mounting protective antiviral adaptive immune responses, namely anti-HSV T cells and B cells that secrete protective antiviral antibodies, assessing the interaction between HSV vaccine candidates and DCs could provide valuable insight for identifying correlates of protection for this pathogen. If this interaction proves to be important for acquiring protective immunity *in vivo*, assessing this outcome could help design and select for HSV mutants that elicit strong and protective anti-HSV immune responses. However, we cannot rule out the possibility that vaccine mutant viruses that induce apoptosis in DCs may be good vaccine candidates, as dying virus-infected DCs may be captured by bystander non-infected healthy DCs that present viral antigens effectively to T cells (78). Although the notion that pro-apoptotic mutant pathogens may promote protective immunity has been proposed before for other microbes (110–112), this scenario may be unlikely favorable in the context of HSV infection, as this outcome would somewhat resemble what already occurs upon natural infection of DCs with wild-type virus (72, 80). Thus, in such cases for apoptotic DCs derived from interactions with mutant viruses to be protective, they would need to differ in their immune-activating properties as compared to apoptotic DCs originating from infections with virulent virus. Such differences could relate to the secretion of cytokines released by dying cells, the expression of danger signals by apoptotic DCs and the repertoire of viral genes expressed in these cells, among others.

Taken together, we believe that the hypotheses proposed above are original and can be assessed. Furthermore, they could provide important insights on the mechanism of protection of certain HSV mutant viruses. Results from a recent study (81) and from our laboratory (Retamal-Díaz et al., unpublished data) suggest that the hypotheses proposed herein are likely plausible.

**AUTHOR CONTRIBUTIONS**

AR-D, AK, SB, and PG wrote and reviewed the manuscript.

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