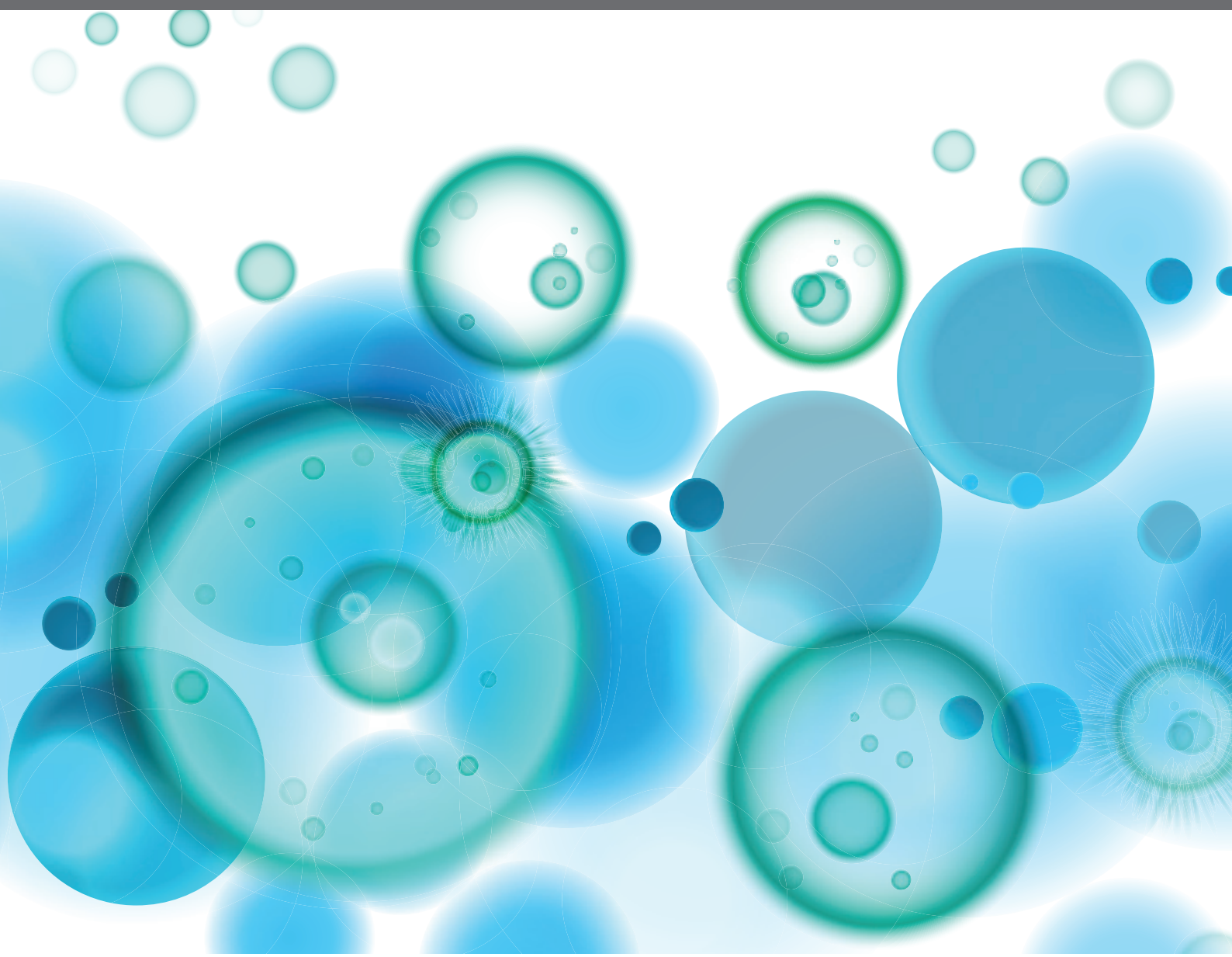
IMMUNITY AND IMMUNOPATHOGENESIS TO HERPESVIRUSES

EDITED BY: Susmit Suvas and Richard D. Dix
PUBLISHED IN: Frontiers in Immunology



frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88963-455-2
DOI 10.3389/978-2-88963-455-2

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

IMMUNITY AND IMMUNOPATHOGENESIS TO HERPESVIRUSES

Topic Editors:
Susmit Suvas, Wayne State University, United States
Richard D. Dix, Georgia State University, United States

Herpesviruses are a large group of double-stranded DNA viruses, which have evolved strategies to persist and disseminate widely throughout the human population. Unlike RNA viruses which have the ability to alter their antigenic expression profile to evade host immune responses, herpesviruses can establish life-long latency in the infected host. Herpesviruses are divided into alpha, beta and gamma herpesviruses sub-families. The human members of the alpha-herpesvirinae subfamily is comprised of herpes simplex virus-1 and 2 (HSV-1 and HSV-2) and of varicella-zoster virus (VZV). These viruses are considered neurotropic, as they can (i) infect nerve endings; (ii) traffic via neuronal axons and (iii) establish latency in neuronal nuclei. On the other hand, the members of the beta-herpesvirinae subfamily such as human cytomegalovirus (HCMV), human herpesviruses 6 and 7 (HHV-6 and HHV-7) are known to establish latent infections in immune cell types such as monocytes and T cells. Epstein-Barr Virus (EBV) is a member of gamma-herpesvirinae subfamily that establishes latency in B lymphocytes. Additionally, HHV-8 also known as Kaposi's Sarcoma-Associated Herpes virus (KSHV) is a γ-herpes virus which establishes latency in monocytes, dendritic cells, B lymphocytes and endothelial cells of the host.

Although members of the herpesviridae family share few properties, they differ significantly in terms of the expression of viral genes during the latent infection period, incidence of viral reactivation, the molecular mechanisms by which they evade the host immunity to establish latency, and the pathogenesis associated with viral reactivation. A better understanding of the virological and immunological events associated with herpesviruses infection should help in the development of prophylactic and therapeutic approaches to better manage these viral infections in patients worldwide.

Citation: Suvas, S., Dix, R. D., eds. (2020). Immunity and Immunopathogenesis to Herpesviruses. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88963-455-2

Table of Contents

frontiers in Immunology

REVIEW
published: 07 December 2018
doi: 10.3389/fimmu.2018.02895

Role of Herpes Simplex Virus Type 1 (HSV-1) Glycoprotein K (gK) Pathogenic CD8$^+$ T Cells in Exacerbation of Eye Disease

Ujjaldeep Jaggi[1], *Shaohui Wang*[1], *Kati Tormanen*[1], *Harry Matundan*[1], *Alexander V. Ljubimov*[2] *and Homayon Ghiasi*[1]*

[1] Department of Surgery, Center for Neurobiology and Vaccine Development, Cedars-Sinai Medical Center, Los Angeles, CA, United States, [2] Eye Program, Cedars-Sinai Medical Center, and David Geffen School of Medicine, Board of Governors Regenerative Medicine Institute, University of California, Los Angeles, Los Angeles, CA, United States

HSV-1-induced corneal scarring (CS), also broadly referred to as Herpes Stromal Keratitis (HSK), is the leading cause of infectious blindness in developed countries. It is well-established that HSK is in fact an immunopathological disease. The contribution of the potentially harmful T cell effectors that lead to CS remains an area of intense study. Although the HSV-1 gene(s) involved in eye disease is not yet known, we have demonstrated that gK, which is one of the 12 known HSV-1 glycoproteins, has a crucial role in CS. Immunization of HSV-1 infected mice with gK, but not with any other known HSV-1 glycoprotein, significantly exacerbates CS, and dermatitis. The gK-induced eye disease occurs independently of the strain of the virus or mouse. HSV-1 mutants that lack gK are unable to efficiently infect and establish latency in neurons. HSV-1 recombinant viruses expressing two additional copies of the gK (total of three gK genes) exacerbated CS as compared with wild type HSV-1 strain McKrae that contains one copy of gK. Furthermore, we have shown that an 8mer (ITAYGLVL) within the signal sequence of gK enhanced CS in ocularly infected BALB/c mice, C57BL/6 mice, and NZW rabbits. In HSV-infected "humanized" HLA-A*0201 transgenic mice, this gK 8mer induced strong IFN-γ-producing cytotoxic CD8$^+$ T cell responses. gK induced CS is dependent on gK binding to signal peptide peptidase (SPP). gK also binds to HSV-1 UL20, while UL20 binds GODZ (DHHC3) and these quadruple interactions are required for gK induced pathology. Thus, potential therapies might include blocking of gK-SPP, gK-UL20, UL20-GODZ interactions, or a combination of these strategies.

Keywords: ocular, eye disease, virus replication, corneal scarring, peptide, SPP, GODZ

OPEN ACCESS

Edited by:
Richard D. Dix, Georgia State University, United States

Reviewed by:
Lbachir Benmohamed, University of California, Irvine, United States
Clinton Jones, Oklahoma State University, United States

***Correspondence:**
Homayon Ghiasi
ghiasih@cshs.org

Specialty section:
This article was submitted to Viral Immunology, a section of the journal Frontiers in Immunology

Received: *25 October 2018*
Accepted: *26 November 2018*
Published: *07 December 2018*

Citation:
Jaggi U, Wang S, Tormanen K, Matundan H, Ljubimov AV and Ghiasi H (2018) Role of Herpes Simplex Virus Type 1 (HSV-1) Glycoprotein K (gK) Pathogenic CD8$^+$ T Cells in Exacerbation of Eye Disease. Front. Immunol. 9:2895. doi: 10.3389/fimmu.2018.02895

ROLE OF HSV-1 GLYCOPROTEINS IN PROTECTION AND DISEASE

HSV-1 encodes at least 85 genes (1) and 12 of these genes code for glycoproteins (1–6). These glycoproteins (gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, gM, and gN) are the major inducers and targets of humoral and cell-mediated immune responses following infection (4, 7–10). We have constructed recombinant baculoviruses expressing high levels of each of the 10 HSV-1 glycoprotein

genes (3–6, 11–20). Based on immunization studies in mice, we have classified these 10 baculovirus-expressed genes into four groups: (i) Immunization with gB, gC, gD, gE, or gI completely protects mice against lethal challenge (11–15); (ii) No significant protection was seen with gH, gJ, and gL (5, 6, 16–18); (iii) Immunization with gK leads to severe exacerbation of eye disease (3, 19, 20); and (iv) Immunization with gG also showed a tendency to be harmful (6, 16).

HERPES STROMAL KERATITIS (HSK)

HSV-1-induced CS, also broadly referred to as herpes stromal keratitis (HSK), can lead to blindness. HSV-1 is the leading cause of corneal blindness due to an infectious agent in developed countries (21–26). In the U.S., ∼30,000 people suffer recurrent ocular HSV episodes annually, requiring doctor visits, medication, and in severe cases, corneal transplants. It is estimated that 70–90% of American adults have antibodies to HSV-1 and/or HSV-2, and about 25% of these individuals have clinical symptoms upon routine clinical exam (21–26). HSV-1 is responsible for >90% of ocular HSV infections. The global incidence of Herpes Keratitis is roughly around 1.5 million including 40,000 new cases of severe visual impairment and blindness each year (27). A significant proportion (15–50%) of primary genital herpes is caused by HSV-1, and recent studies indicate that the proportion of first clinical episode genital herpes due to HSV-1 is increasing (28–30). Despite the frequent recurrence of ocular herpes, there are no vaccines available for HSV infections (31). In addition, no drug has been FDA approved for the prevention of ocular recurrences.

HERPES INFECTION AS AN IMMUNE-MEDIATED EVENT

Viral infections trigger the host immune response in a way that the immune system gets highly compromised (32). Chronic viral infections have evolved different mechanisms by which they escape the response of protective immune response presenting a serious challenge to the infected host (32). Many factors come into play, which are responsible for causing the spread of the disease and, if not properly managed, it can pose a serious threat to the host (33). Current therapies for treatment of ocular HSV-1 infection include the use of antiviral drugs and corticosteroids which can minimize the lesions but often lead to certain side effects (34); therefore, new measures need to be adopted. Studies on mouse models of ocular HSV-1 infection have unraveled many insights into the disease pathogenesis paving ways to future innovative therapies (35, 36). It is well-documented that HSV-1 pathology is a consequence of the immune response mounted by the host after virus infection and therefore, it is considered an immunopathological disease (37). During the course of HSV-1 infection, a series of events take place involving the replication of virus in the epithelial cells and formation of new blood vessels which accounts for the angiogenic response (33). The infectious virus is cleared from the eye by day 6–7 post infection, but secondary effects lead to the induction of strong cellular immune response with the appearance of immune cell infiltrates in the cornea resulting in damage to the eye (38, 39). Recent studies done in mice showed that HSV-1 infection also leads to corneal nerve damage/retraction, which results in loss of corneal sensitivity and blink reflexes and promotes HSK pathogenesis (40).

In this review, we will discuss the role of gK in HSV-1-induced CS and will propose new potential therapeutic approaches to reduce or control gK-induced CS.

gK AND ITS ROLE IN HERPES INFECTION

gK encoded by the UL53 gene is one of the HSV-1 glycoproteins and is expressed on the virions (1, 3, 41). gK is a highly hydrophobic 338-amino acid protein with a predicted molecular mass of 37 kDa (1). gK has a cleavable 30-amino-acid NH2-terminal signal sequence and is N-glycosylated on amino acids 48 and 58 (1, 42, 43). In HSV-1 infected cells, gK is expressed as a 39 kDa high-mannose precursor polypeptide, designated precursor gK (pgK), which is further glycosylated to produce a 41 kDa mature glycoprotein (41). When we expressed gK using a recombinant baculovirus, four gK-related baculovirus-expressed polypeptides of 29-, 35-, 38-, and 40-kDa were detected (3). The 35-, 38-, and 40-kDa species were susceptible to tunicamycin treatment revealing that they were N-glycosylated. The 35-kDa protein represented the cleaved and partially glycosylated peptide, whereas the 29-kDa protein represented the cleaved unglycosylated peptide. gK translated *in vitro* had a molecular mass of 36 kDa with four possible membrane-spanning regions (43, 44). Studies using insertion/deletion mutants have shown the importance of gK in virion morphogenesis and egress (45–47). Deletion of gK results in the formation of extremely rare microscopic plaques indicating that gK is required for virus replication, a concept that is supported by the observation that gK-deficient virus can only be propagated on complementing cells that express gK (45, 46).

gK shares 100% amino acid homology between different strains of HSV-1 (1, 48, 49). Similar to HSV-1 gK, HSV-2 is also 338 amino acids long but with ∼84% amino acid homology (1, 50, 51). In addition to HSV-1 and HSV-2, gK is also present in other members of alphaherpes viruses. The gK homologies between different alphaherpes viruses are shown in **Figure 1**. Protein sequence alignment is illustrated using clustal omega, in which we show that gK from Macacine Herpes Virus 1 (McHV-1), Bovine Herpes Virus 1 (BoHV-1) and Varicella zoster virus (HH3, VZV), share 66, 33 and 28% sequence homology with HSV-1 gK, respectively (**Figure 1**). Kousoulas' group reported that HSV-1 gK is a structural component of virion particles and demonstrated that gK is a Golgi complex-dependent glycosylated species (52). Previously, it was shown that HSV-1 UL20 is required to interact with gK for HSV-1 infection (53). Also, a similar study with Bovine herpes virus type 1 (BoHV-1), a member of the alphaherpes virus family, demonstrated that BoHV-1 gK and UL20 proteins function together in a manner similar to HSV-1 gK and UL20 in virus spread and infection. UL20 has a role in cell surface expression of gK but is not required

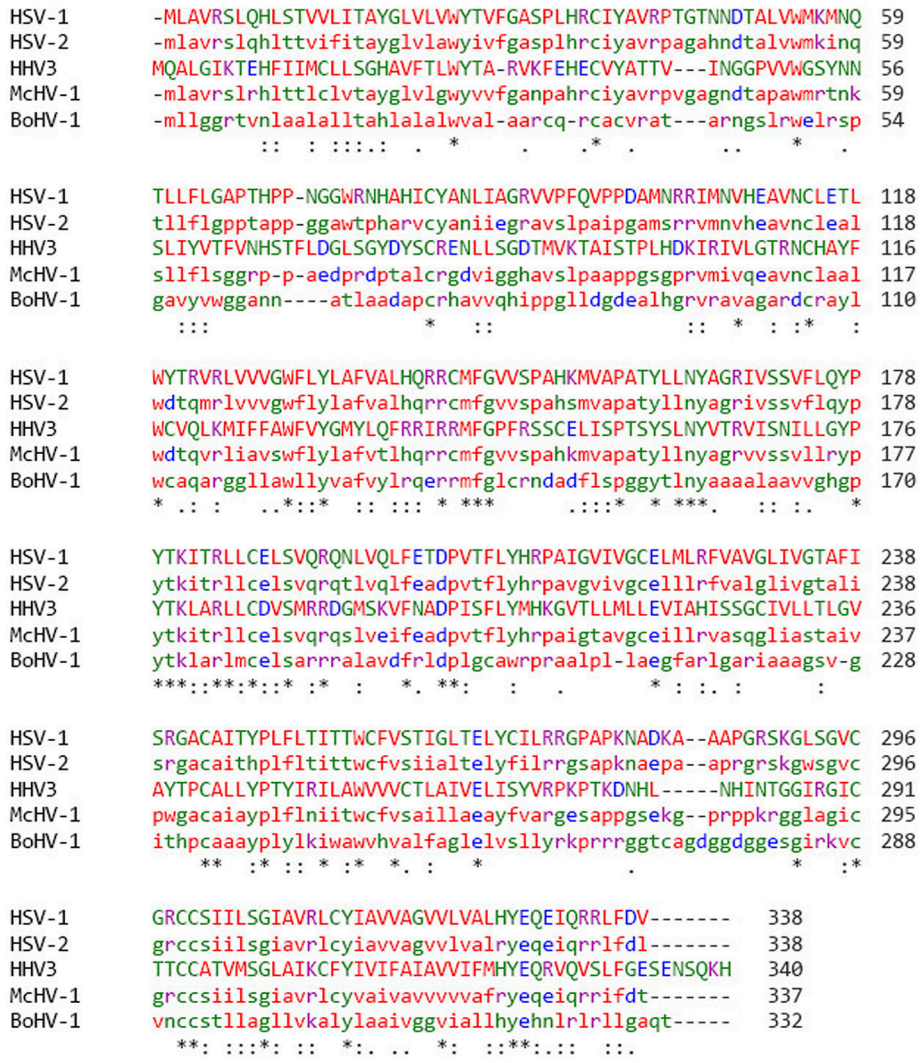


FIGURE 1 | gK protein sequence alignment in different strains of alphaherpes viruses. Protein sequence was aligned by clustal omega and percentage of amino acid homology was compared among different groups of Herpes viruses. HSV-1 has 85% homology with HSV-2, 66% homology with McHV-1, 34% homology with BoHV-1, and 28% homology with VZV. Stars (*) indicate that the amino acids sequences are the same.

for gK-mediated cell fusion (54). It has also been demonstrated that UL20 plays a critical role in virion envelopment, and virions lacking either gK or UL20 fail to form an envelope. A similar role has been assigned to HSV-1 UL37 protein in cytoplasmic virion envelopment, and it was shown that UL37 interacts with gK-UL20 protein complex in infected cells and facilitates in virion cytoplasmic envelope (55).

Recently, we reported that HSV-1 UL20 binds to and is palmitoylated by GODZ (also known as DHHC3), a Golgi apparatus-specific Asp-His-His-Cys (DHHC) zinc finger protein and an essential component of virus infectivity (56). Palmitoylation of UL20 is critical for gK cell surface localization. Thus, the use of GODZ dominant-negative mutant or GODZ shRNA can be a potential way of inhibiting the binding of UL20 to GODZ, which can affect gK localization and viral replication. We further showed the importance of GODZ in HSV-1 infection using knockout mice. GODZ$^{-/-}$ mice ocularly infected with HSV-1 had reduced ocular virus replication and reduced latency-reactivation as compared with wild type control mice. Our study also showed that the absence of GODZ resulted in blocking of palmitoylation of UL20 and affected the localization of gK along with the reduced expression levels of UL20, gK, and gB transcripts in the corneas of HSV-1 infected GODZ$^{-/-}$ mice (57).

Recently, it was shown that intramuscular injection with HSV-1 (F) mutant virus, which lacks the expression of gK conferred significant protection against either virulent HSV-1 strain McKrae or HSV-2 strain G intravaginal challenge in mice (58). To test if disruption of gK/UL20 interactions with gB would lead to reduced viral load, a recombinant virus (VC2) was

constructed with specific mutations in gK and its binding protein UL20. Intramuscular injection with VC2 indeed protected 100% of mice against virulent HSV-1 strain McKrae or HSV-2 strain G challenges by providing cross-reactive humoral and cellular immunity (59).

Additionally, gK binds with different affinity in different cell types (**Figure 2**) to signal peptide peptidase (SPP) also known as minor histocompatibility antigen H13 (60). To illustrate this binding, recombinant gKV5DI, gKV5DII, gKV5DIII, and gKV5DIV viruses were constructed expressing V5 epitope tags in frame within domains I, II, III, and IV of gK, respectively (52, 61). We infected rabbit skin (RS), HeLa and Vero cells with each virus and evaluated the co-localization of V5-gK and endogenous SPP. There was a strong co-localization in all the cell lines (RS, HeLa and Vero) when the V5 tag was expressed on cytoplasmic domains (II and III) compared to when it was expressed on extracellular domain (I and IV) (**Figure 2**). Binding of gK to SPP can be blocked by SPP inhibitors like aspirin, ibuprofen, L685, 458, $(Z\text{-}LL)_2$ ketone, and DAPT (62). These inhibitors significantly reduced viral replication in HSV-1 infected eye and reduced pathology. Thus, blocking the binding of SPP to gK can be one of the potential approaches toward treating HSV-1 induced CS (62).

gK AND VIRUS ENTRY

HSV-1 induced CS begins with the binding of viral glycoproteins to the host cell entry receptors. There are at least seven known receptors including herpes virus entry mediator (HVEM) as well as nectin-1, nectin-2, 3-O-sulfated heparan sulfate (3-OS-HS), paired immunoglobulin-like type 2 receptor (PILRα), non-muscle myosin heavy chain IIA (NMHC-IIA), and myelin-associated glycoprotein (MAG) (2, 63–72). For gK to potentiate its disease severity, the amino terminal of gK binds to the amino terminal of gB, which leads to the virus entry and disease progression (73). gB binds to Akt-1 during virus entry and it induces Akt phosphorylation and intracellular calcium release. A recent study done by Kousoulas' group showed that deletion of amino acids 31–68 within the amino terminus of gK inhibits gB binding to Akt-1 and thus blocks virus entry and its progression (74). Studies by the same group showed that both gK and PlLRα (paired immunoglobulin–like type 2 receptor α) bound gB in infected cells and that the association between gB-PILRα protein complex regulates membrane fusion of virus and the host cell which aids in virus penetration (75). Along with the role of amino terminus of gK in virus entry, a recent study described the role of two conserved N-linked glycosylation sites (N48 and N58) of gK in virus-induced cell fusion and replication (76). Mutation at N58 to alanine (N58A) resulted in extensive virus-induced cell fusion. The same group showed that mutation of cysteine residues within the amino terminus of gK, C37, and C114, led to significant reduction in virus production (76). In addition, gK plays a vital part in the recruitment of other viral glycoproteins into intracellular virus assembly. A recent study found that gM plays a major role in synergy with gK/UL20 in the incorporation of gD and gH/gL into mature virions (74).

ROLE OF gK-INDUCED CELLULAR RESPONSES

Adaptive immune responses play a major role in HSV-1 pathogenesis. The role of $CD8^+$ T cells in HSV-1 pathogenesis is currently unclear and needs deeper investigation. There are studies reporting that $CD8^+$ T cells play a protective role, whereas other studies show that $CD8^+$ T cells exacerbate the disease pathogenesis (77, 78). There is evidence supporting that gK is the only HSV-1 glycoprotein responsible for exacerbation of HSV-1 induced corneal scarring (CS). Research done by our team shows that a virus construct of HSV-gK^3 which is derived from the virulent HSV-1 strain McKrae mediates critical effects on HSV-1 pathogenicity in mice (79). Mice infected with HSV-gK^3 showed severe CS compared with control mice infected with wild type virus. HSV-gK^3 infected mice had elevated levels of virus replication and also had significantly higher number of $CD8^+$ T cells (79). Depletion of $CD8^+$ T cells and not $CD4^+$ T cells reduced CS in HSV-gK^3 infected mice to the level of wild

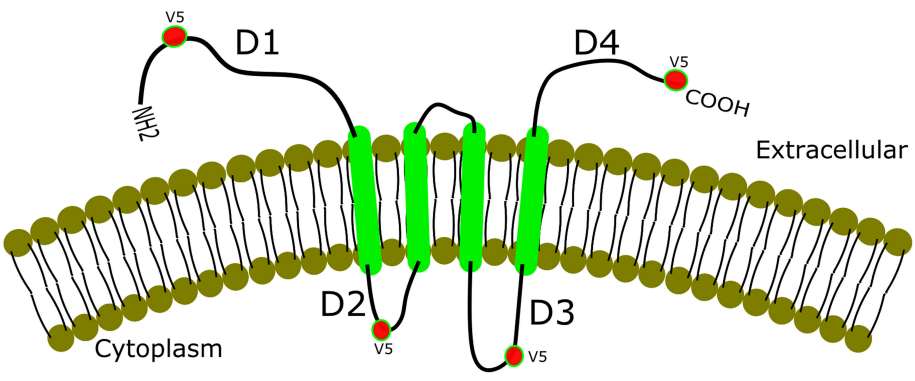


FIGURE 2 | Co-localization of gK and SPP. gK is a highly hydrophobic protein with four transmembrane domains. Epitope-tagging of four different domains of gK is shown with a strong co-localization of the two cytoplasmic domains (labeled D2 and D3 in the figure). Extracellular domains (D1 and D4), on the other hand, show weak or no co-localization with SPP in RS, HeLa, and Vero cell lines.

type infected mice. Overall, we have shown that exacerbation of eye disease in response to gK immunization or following ocular infection with recombinant viruses expressing additional copies of gK is associated with $CD8^+$T cell and not $CD4^+$T cell responses. Other studies have shown that $CD4^+$ T cells are involved in HSK (80–83). Thus, in the context of $CD8^+$-induced gK pathogenicity the role of $CD4^+$ T cells to disease or protection cannot be ruled out.

We previously looked into what region of gK participates in T cell proliferation and subsequently IFN-γ production (84). To this end, a panel of 33 overlapping peptides spanning all 338 amino acids of the gK polypeptide were produced. Splenocytes from mice were stimulated with each peptide individually both *in vivo* and *in vitro*. We found that out of 33 peptides, peptide 2 was involved in T cell proliferation and IFN-γ production *in vivo* and *in vitro* and accounted for 52% of CTL activity *in vivo*. The percentages of IFN-γ production by both $CD4^+$ T and $CD8^+$T cells *in vivo* and the CTL responses are illustrated in **Table 1**. *In vitro* results showed that $CD8^+$ T cells produced more IFN-γ compared to $CD4^+$ T cells. Our study confirmed that both $CD4^+$ and $CD8^+$T cells produced IFN-γ when stimulated with peptide 2, but IFN-γ production by $CD4^+$ T was $CD8^+$ T cell-dependent. In connection with our mapping studies (3, 79), we identified a highly conserved gK epitope (ITAYGLVL) within the peptide STVVLITAYGLVLVW, which served as an immunodominant gK T cell stimulatory region both *in vitro* and *in vivo* (84). This peptide is highly conserved between HSV-1 and HSV-2 strains. To investigate its role in HSV-1 infection, the octamer (8mer) was administered as an eye drop an hour before ocular infection. This resulted in a significant increase in viral replication leading to enhancement of CS, along with strong cytotoxic $CD8^+$ T cell responses and IFN-γ production (85). Mutations in the signal sequence of gK using recombinant viruses that expressed two additional copies of the mutated (MgK) or native (NgK) form of the gK blocked cell surface expression of gK in RS cells resulting in reduced reactivation and hence, less ocular disease when compared to RgK (revertant) virus. This study confirms the role of octamer within the signal sequence of gK in HSV-1 pathogenesis (86). Another study showed that the amino terminus of gK was essential for neuroinvasiveness and acute HSK using a recombinant HSV-1 (McKΔgK31-68), which was lacking the 38 amino acids from gK amino terminus. In McKΔgK31-68 mutant viral infection, there were no significant disease symptoms (87).

Hendricks's group looked at HSV-1-specific $CD8^+$ T cell repertoire in C57BL/6 mice that respond to 376 predicted HSV-1 $CD8^+$ T cell epitopes in C57BL/6 mice (88). Out of 376 HSV-1 $CD8^+$ T cell epitopes, only 19 ($gB_{498-505}$ and 18 subdominant epitopes) stimulated $CD8^+$T cells in spleen and TG of HSV-1 infected mice. The data in comparison to all these epitopes demonstrated that majority of the $CD8^+$T cells in spleen and TG of HSV-1 infected mice responded to $gB_{498-505}$ HSV-1 epitope and as expected the authors showed that gK peptide corresponding to aa 54–62 was recognized by $CD8^+$ effector T cells in TG and spleen of infected mice (88). So, collectively our study and Hendricks's group showed that $CD8^+$ T cells in C57BL/6 mice recognize various HSV-1 epitopes, especially gK but this is in contrast to human TG study in which it was indicated that the human TG is an immunocompetent environment for both $CD4^+$ and $CD8^+$ T cell recognition of diverse HSV-1 proteins expressed during latent infection (89). The infiltration of $CD4^+$ and $CD8^+$ T cells was measured in 15 TG of eight HSV-1 IgG seropositive donors by flow cytometry. It was found that there were equivalent numbers of $CD4^+$ and $CD8^+$ T cells, with a median ratio of $CD4^+$ and $CD8^+$ T cells of 0.99 (range 0.01–9.32). Also, peptide-specific $CD8^+$ T cell responses were detected in two TG which recognized four HLA-A*0101-restricted peptides: gL_{66-74}, $gK_{201-209}$ and two VP16 peptides, $VP16_{90-99}$, and $VP16_{479-488}$. It was concluded that human intra-TG HSV-1-specific $CD8^+$ T cell responses were directed to a relatively restricted number of viral proteins in

TABLE 1 | IFN-γ production and CTL activity from both $CD4^+$T and $CD8^+$T cells when stimulated with gK synthetic peptides[a].

Peptide	gK aa	$CD4^+IFN\gamma^+$	$CD8^+IFN\gamma^+$	CTL activity
1	MLAVRSLQHLSTVVL	2%	1%	9%
2	STVVLITAYGLVLVW	21%	8%	52%
3	LVLVWYTVFGASPLH	3%	2%	–
4	ASPLHRCIYAVRPTG	ND	ND	ND
5	VRPTGTNNDTALVWM	ND	ND	ND
6	ALVWMKMNQTLLFLG	ND	ND	ND
7	LLFLGAPTHPPNGGW	ND	ND	ND
8	PNGGWRNHAHICYAN	ND	ND	ND
9	ICYANLIAGRVVPFQ	ND	ND	ND
10	VVPFQVPPDAMNRRI	ND	ND	ND
11	MNRRIMNVHEAVNCL	ND	ND	ND
12	AVNCLETLWYTRVRL	ND	ND	ND
13	TRVRLVVVGWFLYLA	ND	ND	ND
14	FLYLAFVALHQRRCM	ND	ND	ND
15	QRRCMFGVVSPAHKM	ND	ND	ND
16	PAHKMVAPATYLLNY	ND	ND	ND
17	YLLNYAGRIVSSVFL	ND	ND	ND
18	SSVFLQYPYTKITRL	ND	ND	ND
19	KITRLLCELSVQRQN	ND	ND	ND
20	VQRQNLVQLFETDPV	ND	ND	ND
21	ETDPVTFLYHRPAIG	ND	ND	ND
22	RPAIGVIVGCELMLR	ND	ND	ND
23	ELMLRFVAVGLIVGT	ND	ND	ND
24	LIVGTAFISRGACAI	ND	ND	ND
25	GACAITYPLFLTITT	ND	ND	ND
26	LTITTWCFVSTIGLT	ND	ND	ND
27	TIGLTELYCILRRGP	ND	ND	ND
28	LRRGPAPKNADKAAA	ND	ND	ND
29	DKAAAPGRSKGLSGV	ND	ND	ND
30	GLSGVCGRCCSIILS	ND	ND	ND
31	SIILSGIAVRLCYIA	ND	ND	ND
32	LCYIAVVAGVVLVAL	ND	ND	ND
33	VLVALHYEQEIQRRL	ND	ND	ND

[a] *Splenocytes from naive BALB/c mice were prepared and tested for in vivo cytolytic activity as was reported (84). Cells were pulsed with respective peptides for 18 h and cytolytic activity was measured by FACS analysis. ND, Not detected.*

each person (89). $CD8^+$ T cell depletion in gK immunized mice resulted in reduced severity of gK-induced CS in mice infected with wild type HSV-1 strain McKrae (90). The underlying mechanism of $CD8^+$ T cell pathology in HSV-1 infected gK-immunized mice was confirmed by the presence of $CD8^+CD25^+$ regulatory T cells in cornea of gK immunized mice (78). Thus, similar to our results, the published studies confirmed our finding that gK induces $CD8^+$ T cell responses and this response is contributing to enhancement of eye disease. This is probably the reason why depletion of $CD8^+$ T cell but not $CD4^+$ T cells reduced gK exacerbation of eye disease (79).

Previous studies revealed that gK sera caused antibody-dependent enhancement (ADE) of HSV-1 infection, which may explain the higher viral load in the corneas of gK-vaccinated mice (91). ADE differs from the usual process of virus entry where virus enters the host cells by binding of the viral glycoproteins to the cellular receptors. In ADE, IgG binds to a virus allowing the virus-antibody complex to attach to the host cells containing Fc receptors. A comparative study between HSK sera and non-HSK sera indicated that about 75% of found neutralizing antibodies were associated with gB, gC, gD, gE, and gI. It was shown by ELISA that sera from HSK group had significantly higher anti-gD and anti-gK antibodies than sera from non-HSK group. Similarly, when mice were immunized with gD+gK, levels of neutralizing antibody titers in immunized mice were reduced by ∼30% in comparison to mice immunized with gD alone. This is in agreement with data showing that mice immunized with gD showed T_H1 response whereas mice immunized with gK exhibited a $T_H1 + T_H2$ response. $T_H1 + T_H2$ response in gK-immunized mice enhances the eye pathology (79).

ROLE OF gK IN HSV-1 CHRONIC INFECTION

One of the hallmarks of HSV-1 infection is the ability of the virus to establish latency in sensory neurons of an infected host (92–96). In neurons, expression of more than 80 genes of HSV-1 that occurs during lytic infection is drastically modified. The latency-associated transcript (LAT) is the only gene product consistently detected in abundance during latency in infected mice, rabbits, and humans (92–94, 97, 98). In mice, spontaneous reactivation occurs at extremely low levels and infectious virus is rarely detected. When mouse TGs are removed at autopsy and explant co-cultivated in tissue culture with indicator cells, latent virus reactivates and can be observed by the detection

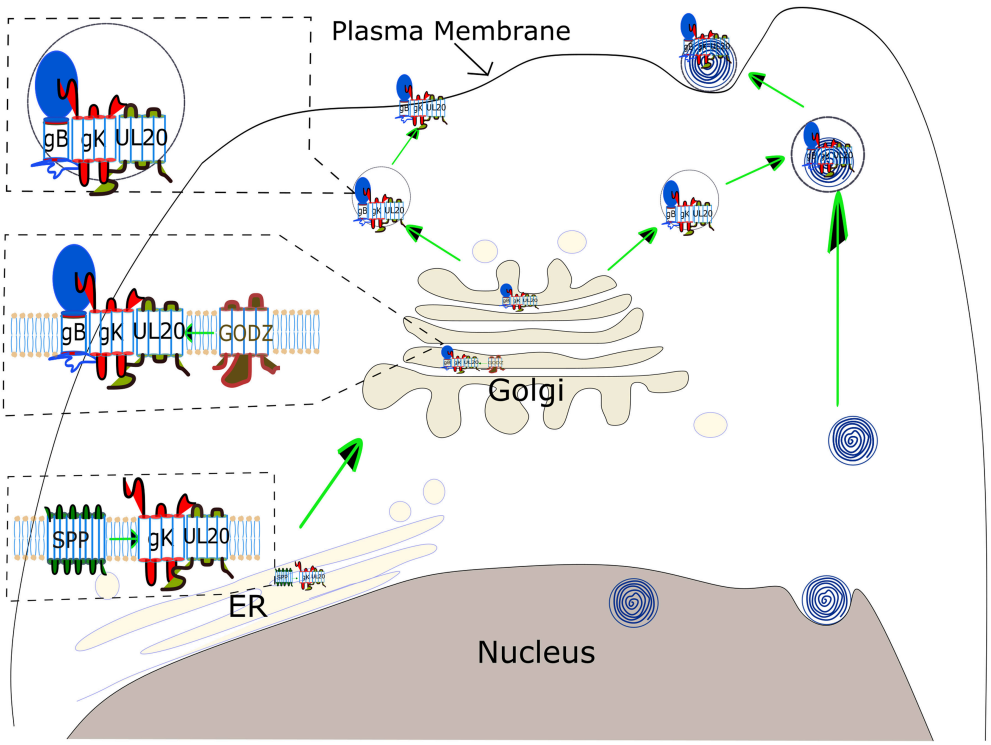


FIGURE 3 | Schematic view of gK transportation and its role in virus egress. gK binds to SPP in the ER, which is necessary for virus replication, although the precise binding domain between these two proteins has not been identified yet. gK has a signal sequence in its N-terminus, however, it is not clear if this gK signal peptide is cleaved by SPP. gK is then transported to the Golgi via a UL20-dependent pathway. UL20 is palmitoylated by the host *cis*-Golgi protein GODZ, and this post-translational modification by GODZ is necessary for transport of the gK, and UL20 complex to the plasma membrane and virus infectivity. The gK and UL20 complex is also required for gB transportation to the cell surface. The complex of three proteins, gB, gK, and UL20, is either assembled into virus capsid emerging from the nucleus in a vesicle derived from TGN (**Upper right**) or transported directly to the plasma membrane (**Upper left**).

of cytopathic effects (CPE) on the indicator cell monolayer. Reactivation from latency is not immediate, and typically CPE is not detected during the first 2–3 days of explant co-cultivation. In contrast, when cell-free lysates of latently infected TG are plated on indicator cells, CPE is not seen (20, 99). This indicates that there was no infectious virus present in the TGs and confirms that reactivation from latency by co-cultivation requires explant of intact neurons (100, 101). We previously reported that vaccination of BALB/c mice with the baculovirus-expressed gK or passive transfer of anti-gK purified IgG to naïve BALB/c mice causes severe exacerbation of HSV-1 induced CS following ocular challenge (3, 19). In addition, a productive chronic infection, rather than a latent infection, is found in most TGs (20). Similar to gK immunization or anti-gK IgG transfer, ocular challenge of naive $A_{\beta}^{-/-}$ but not $\beta_2 m^{-/-}$ mice with HSV-1 did not result in chronic infections. Surprisingly, however, when $A_{\beta}^{O/O}$ mice were vaccinated even with media alone or adjuvant alone prior to ocular challenge, a chronic, rather than a latent, infection was seen (102). When SCID mice which lack both T and B cells, are challenged ocularly with HSV-1, the surviving mice have a chronic, rather than a latent infection in their TG, with significant amounts of infectious virus (103). Thus, gK enhancement of eye disease may be associated with suppression of a certain protective arm of immune response, while enhancing the harmful arm.

From the studies done above, we can make an observation that both gK and LAT plays an important role in pathogenesis of CS. Where LAT is directly involved in reactivation of the virus which leads to pathogenicity, gK follows an indirect approach toward pathology by binding to SPP, which is known to cause virus infectivity and activation of $CD8^{+}$T cells which in turn produce high amounts of IFN-γ and cytotoxic effects. We have also studied that deletion of gK in neural cell cultures leads to inhibition of virus to undergo transport in anterograde or retrograde directions, in short inhibiting the reactivation of virus. gK is known to cause severe immunopathology including cornea scaring, its effect on nerve damage can be detrimental to the host. A recent report shows that deletion of gK can significantly attenuate nerve damage caused by HSV-1 infection (104).

POSSIBLE USE OF gK FOR CONTROL OF HSV-1 INDUCED CS

Many steps have been evaluated in resolving the lesions caused after HSV-1 infection such as administering anti-viral drugs and using corticosteroids, which provide limited control of viral replication and are also known to cause side effects (105). Drugs like trifluridine and ganciclovir are being extensively used for patients with HSV-1 infection along with topical acyclovir to control active viral replication (106). Therefore, we need effective measures to control virus reactivation. It would be more clinically beneficial if new means are developed to prevent the initiation of pathogenesis. As discussed above, HSV-1 gK binds to SPP and UL20, while UL20 binds to GODZ (56, 60). Therefore, blocking the binding of gK to SPP, gK to UL20, or UL20 to GODZ or their combinations could be used to block HSV infectivity and pathogenesis. For example, previously we have shown that blocking the binding of gK to SPP by using SPP inhibitors can reduce CS in infected mice.

CONCLUSIONS

The journey of combating HSV-1 induced CS has started long ago, although many areas of the path of virus pathogenesis still remain unexplored (107). Seroprevalence studies have illustrated that the majority of individuals in the United States are infected with HSV-1 (108). This review focused on the role of HSV gK in the progression of disease severity. Published studies have clearly demonstrated the participation of gK in the exacerbation of CS and the immune response to gK in this process as a major pathogenic mechanism. A model of gK activity is illustrated in **Figure 3**. gK interacts with SPP in the endoplasmic reticulum (ER), and this interaction may be necessary for transport of gK from the ER to the Golgi. In the Golgi, gK interacts with gB and UL20. Palmitoylation of UL20 by GODZ either facilitates transport of the gB- gK- UL20 complex to the plasma membrane or viral packaging (**Figure 3**). Research is in progress to inhibit the function of gK in causing HSV-1 induced CS but further studies are required. Clearly, an exciting approach would be inhibiting the binding of gK to SPP, gK to UL20, and UL20 interactions with GODZ supports the goal of controlling HSK pathogenesis.

AUTHOR CONTRIBUTIONS

UJ, KT, and HG writing and editing. SW, HM, and AL editing. SW and KT designing figures.

FUNDING

This study was supported by Public Health Service NIH grant R01 EY013615.

REFERENCES

1. McGeoch DJ, Dalrymple MA, Davison AJ, Dolan A, Frame MC, McNab D, et al. The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. *J Gen Virol.* (1988) 69:1531–74. doi: 10.1099/0022-1317-69-7-1531
2. Spear PG, Eisenberg RJ, Cohen GH. Three classes of cell surface receptors for alphaherpesvirus entry. *Virology* (2000) 275:1–8. doi: 10.1006/viro.2000.0529
3. Ghiasi H, Slanina S, Nesburn AB, Wechsler SL. Characterization of baculovirus-expressed herpes simplex virus type 1 glycoprotein. *J Virol.* (1994) 68:2347–54.
4. Ghiasi H, Kaiwar R, Nesburn AB, Slanina S, Wechsler SL. Expression of seven herpes simplex virus type 1 glycoproteins (gB, gC, gD, gE, gG, gH, and gI): comparative protection against lethal challenge in mice. *J Virol.* (1994) 68:2118–26.
5. Ghiasi H, Kaiwar R, Slanina S, Nesburn AB, Wechsler SL. Expression and characterization of baculovirus expressed herpes simplex virus type 1 glycoprotein. *Arch Virol.* (1994) 138:199–212. doi: 10.1007/BF01379126
6. Ghiasi H, Bahri S, Nesburn AB, Wechsler SL. Protection against herpes simplex virus-induced eye disease after vaccination with seven individually expressed herpes simplex virus 1 glycoproteins. *Invest Ophthalmol Vis Sci.* (1995) 36:1352–60.

7. Burke RL. Development of a herpes simplex virus subunit glycoprotein vaccine for prophylactic and therapeutic use. *Rev Infect Dis.* (1991) 13:S906–11. doi: 10.1093/clind/13.Supplement_11.S906
8. Burke RL. Contemporary approaches to vaccination against herpes simplex virus. *Curr Top Microbiol Immunol.* (1992) 179:137–58. doi: 10.1007/978-3-642-77247-4_9
9. El Kasmi, Lippe R. Herpes simplex virus 1 gN partners with gM to modulate the viral fusion machinery. *J Virol.* (2015) 89:2313–23. doi: 10.1128/JVI.03041-14
10. Striebinger H, Zhang J, Ott M, Funk C, Radtke K, Duron J, et al. Subcellular trafficking and functional importance of herpes simplex virus type 1 glycoprotein M domains. *J Gen Virol.* (2015) 96:3313–25. doi: 10.1099/jgv.0.000262
11. Ghiasi H, Nesburn AB, Kaiwar R, Wechsler SL. Immunoselection of recombinant baculoviruses expressing high levels of biologically active herpes simplex virus type 1 glycoprotein D. *Arch Virol.* (1991) 121:163–78. doi: 10.1007/BF01316752
12. Ghiasi H, Kaiwar R, Nesburn AB, Slanina S, Wechsler SL. Baculovirus-expressed glycoprotein E (gE) of herpes simplex virus type- 1 (HSV-1) protects mice against lethal intraperitoneal and lethal ocular HSV-1 challenge. *Virology* (1992) 188:469–76. doi: 10.1016/0042-6822(92)90500-O
13. Ghiasi H, Kaiwar R, Nesburn AB, Wechsler SL. Baculovirus expressed herpes simplex virus type 1 glycoprotein C protects mice from lethal HSV-1 infection. *Antiviral Res.* (1992) 18:291–302. doi: 10.1016/0166-3542(92)90062-A
14. Ghiasi H, Kaiwar R, Nesburn AB, Wechsler SL. Expression of herpes simplex virus type 1 glycoprotein I in baculovirus:preliminary biochemical characterization and protection studies. *J Virol.* (1992) 66:2505–9.
15. Ghiasi H, Kaiwar R, Nesburn AB, Wechsler SL. Expression of herpes simplex virus type 1 glycoprotein B in insect cells. Initial analysis of its biochemical and immunological properties. *Virus Res.* (1992) 22:25–39. doi: 10.1016/0168-1702(92)90087-P
16. Ghiasi H, Kaiwar R, Nesburn AB, Wechsler SL. Baculovirus-expressed glycoprotein G of herpes simplex virus type 1 partially protects vaccinated mice against lethal HSV-1 challenge. *Virology* (1992) 190:233–9. doi: 10.1016/0042-6822(92)91209-D
17. Ghiasi H, Kaiwar R, Nesburn AB, Wechsler SL. Baculovirus-expressed glycoprotein H of herpes simplex virus type 1 (HSV-1) induces neutralizing antibody and delayed type hypersensitivity responses, but does not protect immunized mice against lethal HSV-1 challenge. *J Gen Virol.* (1992) 73:719–22. doi: 10.1099/0022-1317-73-3-719
18. Ghiasi H, Nesburn AB, Cai S, Wechsler SL. The US5 open reading frame of herpes simplex virus type 1 does encode a glycoprotein (gJ). *Intervirology* (1998) 41:91–7. doi: 10.1159/000024919
19. Ghiasi H, Cai S, Slanina S, Nesburn AB, Wechsler SL. Nonneutralizing antibody against the glycoprotein K of herpes simplex virus type-1 exacerbates herpes simplex virus type-1-induced corneal scarring in various virus-mouse strain combinations. *Invest Ophthalmol Vis Sci.* (1997) 38:1213–21.
20. Ghiasi H, Cai S, Nesburn AB, Wechsler SL. Vaccination with herpes simplex virus type 1 glycoprotein K impairs clearance of virus from the trigeminal ganglia resulting in chronic infection. *Virology* (1996) 224:330–3. doi: 10.1006/viro.1996.0537
21. Dawson CR. Ocular herpes simplex virus infections. *Clin Dermatol.* (1984) 2:56–66. doi: 10.1016/0738-081X(84)90066-X
22. Barron BA, Gee L, Hauck WW, Kurinij N, Dawson CR, Jones DB, et al. Herpetic eye disease study. a controlled trial of oral acyclovir for herpes simplex stromal keratitis. *Ophthalmology* (1994) 101:1871–82. doi: 10.1016/S0161-6420(13)31155-5
23. Wilhelmus KR, Dawson CR, Barron BA, Bacchetti P, Gee L, Jones DB, et al. Risk factors for herpes simplex virus epithelial keratitis recurring during treatment of stromal keratitis or iridocyclitis. Herpetic eye disease study group. *Br J Ophthalmol.* (1996) 80:969–72. doi: 10.1136/bjo.80.11.969
24. Liesegang TJ. Classification of herpes simplex virus keratitis and anterior uveitis. *Cornea* (1999) 18:127–43. doi: 10.1097/00003226-199903000-00001
25. Liesegang TJ. Herpes simplex virus epidemiology and ocular importance. *Cornea* (2001) 20:1–13. doi: 10.1097/00003226-200101000-00001
26. Hill TJ. Ocular pathogenicity of herpes simplex virus. *Curr Eye Res.* (1987) 6:1–7. doi: 10.3109/02713688709020060
27. Farooq AV, Shukla D. Herpes simplex epithelial and stromal keratitis: an epidemiologic update. *Survey Ophthalmol.* (2012) 57:448–62. doi: 10.1016/j.survophthal.2012.01.005
28. Roberts CM, Pfister JR, Spear SJ. Increasing proportion of herpes simplex virus type 1 as a cause of genital herpes infection in college students. *Sex Transm Dis.* (2003) 30:797–800. doi: 10.1097/01.OLQ.0000092387.58746.C7
29. Auslander BA, Biro FM, Rosenthal SL. Genital herpes in adolescents. *Semin Pediatr Infect Dis.* (2005) 16:24–30. doi: 10.1053/j.spid.2004.09.008
30. Singh AE, Romanowski B, Wong T, Gourishankar S, Myziuk L, Fenton J, et al. Herpes simplex virus seroprevalence and risk factors in 2 Canadian sexually transmitted disease clinics. *Sex Transm Dis.* (2005) 32:95–100. doi: 10.1097/01.olq.0000151415.78210.85
31. Koelle DM, Ghiasi H. Prospects for developing an effective vaccine against ocular herpes simplex virus infection. *Curr Eye Res.* (2005) 30:929–42. doi: 10.1080/02713680500313153
32. Zuniga EI, Macal M, Lewis GM, Harker JA. Innate and adaptive immune regulation during chronic viral infections. *Annual Rev Virol.* (2015) 2:573–97. doi: 10.1146/annurev-virology-100114-055226
33. Biswas PS, Rouse BT. Early events in HSV keratitis–setting the stage for a blinding disease. *Microbes Infect.* (2005) 7:799–810. doi: 10.1016/j.micinf.2005.03.003
34. Knickelbein JE, Hendricks RL, Charukamnoetkanok P. Management of herpes simplex virus stromal keratitis: an evidence-based review. *Survey Ophthalmol.* (2009) 54:226–34. doi: 10.1016/j.survophthal.2008.12.004
35. Mancini M, Vidal SM. Insights into the pathogenesis of herpes simplex encephalitis from mouse models. *Mamm Genome* (2018) 29:425–45. doi: 10.1007/s00335-018-9772-5
36. Brun P, Scarpa M, Marchiori C, Conti J, Kotsafti A, Porzionato A, et al. Herpes simplex virus type 1 engages toll like receptor 2 to recruit macrophages during infection of enteric neurons. *Front Microbiol.* (2018) 9:2148. doi: 10.3389/fmicb.2018.02148
37. Metcalf JF, Kaufman HE. Herpetic stromal keratitis-evidence for cell-mediated immunopathogenesis. *Am J Ophthalmol.* (1976) 82:827–34. doi: 10.1016/0002-9394(76)90057-X
38. Daheshia M, Kanangat S, Rouse BT. Production of key molecules by ocular neutrophils early after herpetic infection of the cornea. *Exp Eye Res.* (1998) 67:619–24. doi: 10.1006/exer.1998.0565
39. Thomas J, Gangappa S, Kanangat S, Rouse BT. On the essential involvement of neutrophils in the immunopathologic disease: herpetic stromal keratitis. *J Immunol.* (1997) 158:1383–91.
40. Yun H, Rowe AM, Lathrop KL, Harvey SA, Hendricks RL. Reversible nerve damage and corneal pathology in murine herpes simplex stromal keratitis. *J Virol.* (2014) 88:7870–80. doi: 10.1128/JVI.01146-14
41. Hutchinson L, Goldsmith K, Snoddy D, Ghosh H, Graham FL, Johnson DC. Identification and characterization of a novel herpes simplex virus glycoprotein, gK, involved in cell fusion. *J Virol.* (1992) 66:5603–9.
42. Debroy C, Pederson N, Person S. Nucleotide sequence of a herpes simplex virus type 1 gene that causes cell fusion. *Virology* (1985) 145:36–48. doi: 10.1016/0042-6822(85)90199-0
43. Ramaswamy R, Holland TC. *In vitro* characterization of the HSV-1 UL53 gene product. *Virology* (1992) 186:579–87. doi: 10.1016/0042-6822(92)90024-J
44. Mo C, Holland TC. Determination of the transmembrane topology of herpes simplex virus type 1 glycoprotein K. *J Biol Chem.* (1997) 272:33305–11. doi: 10.1074/jbc.272.52.33305
45. Foster TP, Kousoulas KG. Genetic analysis of the role of herpes simplex virus type 1 glycoprotein K in infectious virus production and egress. *J Virol.* (1999) 73:8457–68.
46. Hutchinson L, Johnson DC. Herpes simplex virus glycoprotein K promotes egress of virus particles. *J Virol.* (1995) 69:5401–13.
47. Hutchinson L, Roop-Beauchamp C, Johnson DC. Herpes simplex virus glycoprotein K is known to influence fusion of infected cells, yet is not on the cell surface. *J Virol.* (1995) 69:4556–63.
48. Watson G, Xu W, Reed A, Babra B, Putman T, Wick E, et al. Sequence and comparative analysis of the genome of HSV-1 strain McKrae. *Virology* (2012) 433:528–37. doi: 10.1016/j.virol.2012.08.043

49. Bowen CD, Renner DW, Shreve JT, Tafuri Y, Payne KM, Dix RD, et al. Viral forensic genomics reveals the relatedness of classic herpes simplex virus strains KOS, KOS63, and KOS79. *Virology* (2016) 492:179–86. doi: 10.1016/j.virol.2016.02.013
50. McGeoch DJ, Cunningham C, McIntyre G, Dolan A. Comparative sequence analysis of the long repeat regions and adjoining parts of the long unique regions in the genomes of herpes simplex viruses types 1 and 2. *J Gen Virol.* (1991) 72:3057–75. doi: 10.1099/0022-1317-72-12-3057
51. Dolan, Jamieson FE, Cunningham C, Barnett BC, McGeoch DJ. The genome sequence of herpes simplex virus type 2. *J Virol.* (1998) 72:2010–21.
52. Foster TP, Rybachuk GV, Kousoulas K. Glycoprotein K specified by herpes simplex virus type 1 is expressed on virions as a Golgi complex-dependent glycosylated species and functions in virion entry. *J Virol.* (2001) 75:12431–8. doi: 10.1128/JVI.75.24.12431-12438.2001
53. Foster TP, Chouljenko VN, Kousoulas KG. Functional and physical interactions of the herpes simplex virus type 1 UL20 membrane protein with glycoprotein. *J Virol.* (2008) 82:6310–23. doi: 10.1128/JVI.00147-08
54. Foster TP, Alvarez X, Kousoulas KG. Plasma membrane topology of syncytial domains of herpes simplex virus type 1 glycoprotein K (gK): the UL20 protein enables cell surface localization of gK but not gK-mediated cell-to-cell fusion. *J Virol.* (2003) 77:499–510. doi: 10.1128/JVI.77.1.499-510.2003
55. Jambunathan N, Chouljenko D, Desai P, Charles AS, Subramanian R, Chouljenko VN, et al. Herpes simplex virus 1 protein UL37 interacts with viral glycoprotein gK and membrane protein UL20 and functions in cytoplasmic virion envelopment. *J Virol.* (2014) 88:5927–35. doi: 10.1128/JVI.00278-14
56. Wang S, Mott KR, Wawrowsky K, Kousoulas KG, Luscher B, Ghiasi H. Binding of HSV-1 UL20 to GODZ affects its palmitoylation and is essential for infectivity and proper targeting and localization of UL20 and gK. *J Virol.* (2017) 91:e00945–17. doi: 10.1128/JVI.00945-17
57. Wang S, Mott KR, Cilluffo M, Kilpatrick CL, Murakami S, Ljubimov AV, et al. The absence of DHHC3 affects primary and latent herpes simplex virus 1 infection. *J Virol.* (2018) 92:e01599–17. doi: 10.1128/JVI.01599-17
58. Iyer AV, Pahar B, Chouljenko VN, Walker JD, Stanfield B, Kousoulas KG. Single dose of glycoprotein K (gK)-deleted HSV-1 live-attenuated virus protects mice against lethal vaginal challenge with HSV-1 and HSV-2 and induces lasting T cell memory immune responses. *Virol J.* (2013) 10:317. doi: 10.1186/1743-422X-10-317
59. Stanfield BA, Stahl J, Chouljenko VN, Subramanian R, Charles AS, Saied AA, et al. A single intramuscular vaccination of mice with the HSV-1 VC2 virus with mutations in the glycoprotein K and the membrane protein UL20 confers full protection against lethal intravaginal challenge with virulent HSV-1 and HSV-2 strains. *PLoS ONE* (2014) 9:e109890. doi: 10.1371/journal.pone.0109890
60. Allen SJ, Mott KR, Matsuura Y, Moriishi K, Kousoulas KG, Ghiasi H. Binding of HSV-1 glycoprotein K (gK) to signal peptide peptidase (SPP) is required for virus infectivity. *PLoS ONE* (2014) 9:e85360. doi: 10.1371/journal.pone.0085360
61. Jayachandra S, Baghian A, Kousoulas KG. Herpes simplex virus type 1 glycoprotein K is not essential for infectious virus production in actively replicating cells but is required for efficient envelopment and translocation of infectious virions from the cytoplasm to the extracellular space. *J Virol.* (1997) 71:5012–24.
62. Allen SJ, Mott KR, Ghiasi H. Inhibitors of signal peptide peptidase (SPP) affect HSV-1 infectivity *in vitro* and *in vivo*. *Exp Eye Res.* (2014) 123:8–15. doi: 10.1016/j.exer.2014.04.004
63. Satoh T, Arii J, Suenaga T, Wang J, Kogure A, Uehori J, et al. PILRalpha is a herpes simplex virus-1 entry coreceptor that associates with glycoprotein. *Cell* (2008) 132:935–44. doi: 10.1016/j.cell.2008.01.043
64. Arii J, Goto H, Suenaga T, Oyama M, Kozuka-Hata H, Imai T, et al. Non-muscle myosin IIA is a functional entry receptor for herpes simplex virus-1. *Nature* (2010) 467:859–62. doi: 10.1038/nature09420
65. Suenaga T, Satoh T, Somboonthum P, Kawaguchi Y, Mori Y, Arase H. Myelin-associated glycoprotein mediates membrane fusion and entry of neurotropic herpesviruses. *Proc Natl Acad Sci USA.* (2010) 107:866–71. doi: 10.1073/pnas.0913351107
66. Yoon M, Zago A, Shukla D, Spear PG. Mutations in the N termini of herpes simplex virus type 1 and 2 gDs alter functional interactions with the entry/fusion receptors HVEM, nectin-2, and 3-O-sulfated heparan sulfate but not with nectin-1. *J Virol.* (2003) 77:9221–31. doi: 10.1128/JVI.77.17.9221-9231.2003
67. Montgomery RI, Warner MS, Lum BJ, Spear PG. Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. *Cell* (1996) 87:427–36. doi: 10.1016/S0092-8674(00)81363-X
68. Taylor JM, Lin E, Susmarski N, Yoon M, Zago A, Ware CF, et al. Alternative entry receptors for herpes simplex virus and their roles in disease. *Cell Host Microbe* (2007) 2:19–28. doi: 10.1016/j.chom.2007.06.005
69. Shukla D, Liu J, Blaiklock P, Shworak NW, Bai X, Esko JD, et al. A novel role for 3-O-sulfated heparan sulfate in herpes simplex virus 1 entry. *Cell* (1999) 99:13–22. doi: 10.1016/S0092-8674(00)80058-6
70. O'Donnell CD, Kovacs M, Akhtar J, Valyi-Nagy T, Shukla D. Expanding the role of 3-O sulfated heparan sulfate in herpes simplex virus type-1 entry. *Virology* (2010) 397:389–98. doi: 10.1016/j.virol.2009.11.011
71. Karaba AH, Kopp SJ, Longnecker R. Herpesvirus entry mediator and nectin-1 mediate herpes simplex virus 1 infection of the murine cornea. *J Virol.* (2011) 85:10041–7. doi: 10.1128/JVI.05445-11
72. Agelidis AM, Shukla D. Cell entry mechanisms of HSV: what we have learned in recent years. *Future Virol.* (2015) 10:1145–54. doi: 10.2217/fvl.15.85
73. Musarrat F, Jambunathan N, Rider PJF, Chouljenko VN, Kousoulas KG. The amino terminus of herpes simplex virus 1 glycoprotein K (gK) is required for gB binding to Akt, release of intracellular calcium, and fusion of the viral envelope with plasma membranes. *J Virol.* (2018) 92:e01842–17. doi: 10.1128/JVI.01842-17
74. Lau SY, Crump CM. HSV-1 gM and the gK/pUL20 complex are important for the localization of gD and gH/L to viral assembly sites. *Viruses* (2015) 7:915–38. doi: 10.3390/v7030915
75. Chowdhury S, Chouljenko VN, Naderi M, Kousoulas KG. The amino terminus of herpes simplex virus 1 glycoprotein K is required for virion entry via the paired immunoglobulin-like type-2 receptor alpha. *J Virol.* (2013) 87:3305–13. doi: 10.1128/JVI.02982-12
76. Rider PJF, Naderi M, Bergeron S, Chouljenko VN, Brylinski M, Kousoulas KG. Cysteines and N-glycosylation sites conserved among all alphaherpesviruses regulate membrane fusion in herpes simplex virus 1 infection. *J Virol.* (2017) 91:e00873–17. doi: 10.1128JVI.00873-17
77. Stuart PM, Summers B, Morris JE, Morrison LA, Leib DA. CD8(+) T cells control corneal disease following ocular infection with herpes simplex virus type 1. *J Gen Virol.* (2004) 85:2055–63. doi: 10.1099/vir.0.80049-0
78. Allen SJ, Mott KR, Ljubimov AV, Ghiasi H. Exacerbation of corneal scarring in HSV-1 gK-immunized mice correlates with elevation of CD8+CD25+ T cells in corneas of ocularly infected mice. *Virology* (2010) 399:11–22. doi: 10.1016/j.virol.2009.12.011
79. Mott KR, Perng GC, Osorio Y, Kousoulas KG, Ghiasi H. A Recombinant herpes simplex virus type 1 expressing two additional copies of gK Is more pathogenic than wild-type virus in two different strains of mice. *J Virol.* (2007) 81:12962–72. doi: 10.1128/JVI.01442-07
80. Iijima N, Iwasaki A. Access of protective antiviral antibody to neuronal tissues requires CD4 T-cell help. *Nature* (2016) 533:552–6. doi: 10.1038/nature17979
81. Newell CK, Sendele D, Rouse BT. Effects of CD4+ and CD8+ T-lymphocyte depletion on the induction and expression of herpes simplex stromal keratitis. *Reg Immunol.* (1989) 2:366–9.
82. Niemialtowski MG, Godfrey VL, Rouse BT. Quantitative studies on CD4+ and CD8+ cytotoxic T lymphocyte responses against herpes simplex virus type 1 in normal and beta 2-m deficient mice. *Immunobiology* (1994) 190:183–94. doi: 10.1016/S0171-2985(11)80268-8
83. Niemialtowski MG, Rouse BT. Phenotypic and functional studies on ocular T cells during herpetic infections of the eye. *J Immunol.* (1992) 148:1864–70.
84. Osorio Y, Mott KR, Jabbar AM, Moreno A, Foster TP, Kousoulas KG, et al. Epitope mapping of HSV-1 glycoprotein K (gK) reveals a T cell epitope located within the signal domain of gK. *Virus Res.* (2007) 128:71–80. doi: 10.1016/j.virusres.2007.04.007
85. Mott KR, Chentoufi AA, Carpenter D, Benmohamed L, Wechsler SL, Ghiasi H. The role of a glycoprotein K (gK) CD8+ T-cell epitope of herpes simplex virus on virus replication and pathogenicity. *Invest Ophthalmol Vis Sci.* (2009) 50:2903–12. doi: 10.1167/iovs.08-2957

86. Matundan HH, Mott KR, Akhtar AA, Breunig JJ, Ghiasi H. Mutations within the pathogenic region of herpes simplex virus 1 gK signal sequences alter cell surface expression and neurovirulence. *J Virol.* (2015) 89:2530–42. doi: 10.1128/JVI.03506-14
87. Saied AA, Chouljenko VN, Subramanian R, Kousoulas KG. A replication competent HSV-1(McKrae) with a mutation in the amino-terminus of glycoprotein K (gK) is unable to infect mouse trigeminal ganglia after cornea infection. *Curr Eye Res.* (2014) 39:596–603. doi: 10.3109/02713683.2013.855238
88. St Leger AJ, Peters B, Sidney J, Sette A, Hendricks RL. Defining the herpes simplex virus-specific CD8+ T cell repertoire in C57BL/6 mice. *J Immunol.* (2011) 186:3927–33. doi: 10.4049/jimmunol.1003735
89. van Velzen M, Jing L, Osterhaus AD, Sette A, Koelle DM, Verjans GM. Local CD4 and CD8 T-cell reactivity to HSV-1 antigens documents broad viral protein expression and immune competence in latently infected human trigeminal ganglia. *PLoS Pathog.* (2013) 9:e1003547. doi: 10.1371/journal.ppat.1003547
90. Osorio Y, Cai S, Hofman FM, Brown DJ, Ghiasi H. Involvement of CD8+ T cells in exacerbation of corneal scarring in mice. *Curr Eye Res.* (2004) 29:145–51. doi: 10.1080/02713680490504632
91. Ghiasi H, Perng GC, Nesburn AB, Wechsler SL. Antibody-dependent enhancement of HSV-1 infection by anti-gK sera. *Virus Res.* (2000) 68:137–44. doi: 10.1016/S0168-1702(00)00165-9
92. Stevens JG. Human herpesviruses: a consideration of the latent state. *Microbiol Rev.* (1989) 53:318–32.
93. Wechsler SL, Nesburn AB, Watson R, Slanina S, Ghiasi H. Fine mapping of the major latency-related RNA of herpes simplex virus type 1 in humans. *J Gen Virol.* (1988) 69:3101–6. doi: 10.1099/0022-1317-69-12-3101
94. Wechsler SL, Nesburn AB, Watson R, Slanina SM, Ghiasi H. Fine mapping of the latency-related gene of herpes simplex virus type 1: alternative splicing produces distinct latency-related RNAs containing open reading frames. *J Virol.* (1988) 62:4051–8.
95. Fraser NW, Valyi-Nagy T. Viral, neuronal and immune factors which may influence herpes simplex virus (HSV) latency and reactivation. *Microb Pathog.* (1993) 15:83–91. doi: 10.1006/mpat.1993.1059
96. Phelan D, Barrozo ER, Bloom DC. HSV1 latent transcription and non-coding RNA:A critical retrospective. *J Neuroimmunol.* (2017) 308:65–101. doi: 10.1016/j.jneuroim.2017.03.002
97. Dressler GR, Rock DL, Fraser NW. Latent herpes simplex virus type 1 DNA is not extensively methylated *in vivo*. *J Gen Virol.* (1987) 68:1761–5. doi: 10.1099/0022-1317-68-6-1761
98. Rock DL, Nesburn AB, Ghiasi H, Ong J, Lewis TL, Lokensgard JR, et al. Detection of latency-related viral RNAs in trigeminal ganglia of rabbits latently infected with herpes simplex virus type 1. *J Virol.* (1987) 61:3820–6.
99. Ghiasi H, Nesburn AB, Wechsler SL. Vaccination with a cocktail of seven recombinantly expressed HSV-1 glycoproteins protects against ocular HSV-1 challenge more efficiently than vaccination with any individual glycoprotein. *Vaccine* (1996) 14:107–12. doi: 10.1016/0264-410X(95)00169-2
100. Cook ML, Bastone VB, Stevens JG. Evidence that neurons harbor latent herpes simplex virus. *Infect Immun.* (1974) 9:946–51.
101. Gordon YJ, Rock DL. Co-cultivation versus blot hybridization for the detection of trigeminal ganglionic latency following corneal inoculation with HSV-1 strains of varying TK expression and pathogenicity. *Curr Eye Res.* (1984) 3:1097–100. doi: 10.3109/02713688409000807
102. Ghiasi H, Cai S, Nesburn AB, Wechsler SL. MHC-II but not MHC-I responses are required for vaccine-induced protection against ocular challenge with HSV-1. *Curr Eye Res.* (1997) 16:1152–8. doi: 10.1076/ceyr.16.11.1152.5104
103. Valyi-Nagy T, Deshmane SL, Raengsakulrach B, Nicosia M, Gesser RM, Wysocka M, et al. Herpes simplex virus type 1 mutant strain in1814 establishes a unique, slowly progressing infection in SCID mice. *J Virol.* (1992) 66:7336–45.
104. He J, Cosby R, Hill JM, Bazan HE. Changes in corneal innervation after HSV-1 latency established with different reactivation phenotypes. *Curr Eye Res.* (2017) 42:181–6. doi: 10.3109/02713683.2016.1167919
105. Klysik K, Pietraszek A, Karewicz A, Nowakowska M. Acyclovir in the treatment of herpes viruses - a review. *Curr Med Chem.* (2018) 25. doi: 10.2174/0929867325666180309105519
106. Gordon YJ, Cheng KP, Araullo-Cruz T, Romanowski E, Johnson BJ, Blough HA. Efficacy of glycoprotein inhibitors alone and in combination with trifluridine in the treatment of murine herpetic keratitis. *Curr Eye Res.* (1986) 5:93–9. doi: 10.3109/02713688609015097
107. Hazlett LD, Hendricks RL. Reviews for immune privilege in the year 2010: immune privilege and infection. *Ocul Immunol Inflamm.* (2010) 18:237–43. doi: 10.3109/09273948.2010.501946
108. Bradley H, Markowitz LE, Gibson T, McQuillan GM. Seroprevalence of herpes simplex virus types 1 and 2–United States, 1999-2010. *J Infect Dis.* (2014) 209:325–33. doi: 10.1093/infdis/jit458

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 17 December 2018
doi: 10.3389/fimmu.2018.02922

Blockade of LAG-3 Immune Checkpoint Combined With Therapeutic Vaccination Restore the Function of Tissue-Resident Anti-viral CD8$^+$ T Cells and Protect Against Recurrent Ocular Herpes Simplex Infection and Disease

Soumyabrata Roy[1], *Pierre-Grégoire Coulon*[1], *Ruchi Srivastava*[1], *Hawa Vahed*[1], *Grace J. Kim*[1], *Sager S. Walia*[1], *Taikun Yamada*[1], *Mona A. Fouladi*[1], *Vincent T. Ly*[1] *and Lbachir BenMohamed*[1,2,3]*

[1] *Laboratory of Cellular and Molecular Immunology, School of Medicine, Gavin Herbert Eye Institute, University of California, Irvine, Irvine, CA, United States,* [2] *Department of Molecular Biology and Biochemistry, University of California, Irvine, Irvine, CA, United States,* [3] *Institute for Immunology, School of Medicine, University of California, Irvine, Irvine, CA, United States*

OPEN ACCESS

Edited by:
Richard D. Dix, Georgia State University, United States

Reviewed by:
Homayon Ghiasi, Cedars-Sinai Medical Center, United States
Deepak Shukla, University of Illinois at Chicago, United States

***Correspondence:**
Lbachir BenMohamed
Lbenmoha@uci.edu

Specialty section:
This article was submitted to Viral Immunology, a section of the journal Frontiers in Immunology

Received: *06 October 2018*
Accepted: *28 November 2018*
Published: *17 December 2018*

Citation:
Roy S, Coulon P-G, Srivastava R, Vahed H, Kim GJ, Walia SS, Yamada T, Fouladi MA, Ly VT and BenMohamed L (2018) Blockade of LAG-3 Immune Checkpoint Combined With Therapeutic Vaccination Restore the Function of Tissue-Resident Anti-viral CD8$^+$ T Cells and Protect Against Recurrent Ocular Herpes Simplex Infection and Disease. Front. Immunol. 9:2922. doi: 10.3389/fimmu.2018.02922

Recurrent viral diseases often occur after the viruses evade the hosts' immune system, by inducing exhaustion of antiviral T cells. In the present study, we found that functionally exhausted herpes simplex virus type 1 (HSV-1) -specific CD8$^+$ T cells, with elevated expression of lymphocyte activation gene-3 (LAG-3), an immune checkpoint receptor that promotes T cell exhaustion, were frequent in symptomatic (SYMP) patients with a history of numerous episodes of recurrent corneal herpetic disease. Similarly, following UV-B induced virus reactivation from latency the symptomatic wild-type (WT) B6 mice that developed increase virus shedding and severe recurrent corneal herpetic disease had more exhausted HSV-specific LAG-3$^+$CD8$^+$ T cells in both trigeminal ganglia (TG) and cornea. Moreover, a therapeutic blockade of LAG-3 immune checkpoint with antagonist antibodies combined with a therapeutic immunization with $gB_{498-505}$ peptide immunodominant epitope of latently infected B6 mice significantly restored the quality and quantity of functional HSV-1 $gB_{498-505}$ specific CD8$^+$ T cells in both TG and cornea and protected against UV-B induced recurrent corneal herpes infection and disease. In contrast to dysfunctional HSV-specific CD8$^+$ T cells from WT B6 mice, more functional HSV-specific CD8$^+$ T cells were detected in LAG-3$^{-/-}$ deficient mice and were associated with less UV-B induced recurrent corneal herpetic disease. Thus, the LAG-3 pathway plays a fundamental role in ocular herpes T cell immunopathology and provides an important immune checkpoint target that can synergizes with T cell-based therapeutic vaccines against symptomatic recurrent ocular herpes.

Keywords: herpes simplex type 1, CD8$^+$ T cells, LAG-3, immune check point, recurrent, therapeutic, animal model, humans

INTRODUCTION

A staggering 3.72 billion individuals worldwide are infected with herpes simplex virus type 1 (HSV-1), a prevalent human viral pathogen (1–3). Herpes infection and reactivation cause complications which range from mild, such as cold sores and genital lesion, to grave, such as permanent brain damage from encephalitis in adults and neonates, and blinding recurrent corneal herpetic disease (4). After a primary acute infection of the cornea, HSV-1 travels up sensory neurons to the trigeminal ganglia (TG) where it establishes lifelong latency in its host (5–9). Potentially blinding keratitis occurring from recurrent corneal herpetic disease results from the reactivation of latent virus from neurons of the TG, anterograde transportation to nerve termini, and re-infection of the cornea (8, 9).

Controlling the establishment of HSV-1 latency and preventing reactivation from TG involves dynamic crosstalk between the virus and CD8$^+$ T cells within the latently infected TG microenvironment (5, 6, 8–10). However, the molecular mechanisms by which such interactions occur remain to be fully elucidated. HSV-specific CD8$^+$ T cells are selectively activated and retained in the tissues of latently infected TG (6, 8, 9). On one hand, HSV-specific CD8$^+$ T cells can significantly reduce reactivation in TG explant from latently infected mice (5, 9), apparently by interfering with virus replication and spread following the initial molecular events of reactivation (5, 8, 9). On the other hand, HSV-1 can manage to reactivate in the face of an often-sizable pool of virus-specific CD8$^+$ T cells in the TG, apparently by interfering with the quality and quantity of CD8$^+$ T cells that reside in the TG (6, 9, 11). Thus, the virus appears to keep CD8$^+$ T cells "in check" using among several mechanisms, functional impairment of T cells (i.e., exhaustion), which is usually the result of prolonged exposure of T cell to high levels of viral antigens, as occurs during productive chronic infections (12, 13). Many viruses, including HSV-1, appear to reactivate from latency and sustain their productive infection by inducing functional exhaustion of antiviral CD8$^+$ T cells (10, 12, 14–17).

Total or partial loss of T cell function occurs following repetitive HSV-1 latent/reactivation cycles, sporadic events that occur in latently infected trigeminal ganglia (TG) (10, 18, 19). T cell dysfunction requires two signals: (1) T cell receptors (TCR) engaged by MHC presenting an HSV epitope (16); and (2) T cell co-inhibitory receptors engaged by their ligands expressed on infected cells (e.g., infected sensory neurons of TG) (10, 20). When T cell dysfunction develops under conditions of repetitive exposure to viral antigens it is called exhaustion [reviewed in (21)]. This is usually linked with the expression of a long list of T cell co-inhibitory receptors including: programmed death-1 (PD-1), T cell immunoglobulin mucin-(TIM)-3, lymphocyte activation gene-3 (LAG-3, also known as CD223), T cell immunoreceptor with Ig and ITIM domains (TIGIT), P-selectin glycoprotein ligand-1 (PSGL-1), 2B4 (also known as CD244), glucocorticoid-induced TNFR-related protein (GITR, also known as TNFRSF18), CD160, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4, also known as CD152), B- and T-lymphocyte attenuator (BTLA also known as CD272), and V-domain immunoglobulin suppressor of T cell activation (VISTA) [reviewed in (16, 22)]. In humans, sporadic molecular reactivations of latent HSV-1 from sensory neurons of the TG is accompanied by chronic CD8$^+$ T cell infiltrates (23–27). The cellular and molecular immune mechanisms that control the HSV-1 latency-reactivation cycle remain to be fully elucidated. Nevertheless, at least a portion of these virus reactivations in the TG appears to be controlled by CD8$^+$ T cell-mediated mechanisms (8, 10, 28). Many of these ganglia-resident CD8$^+$ T cells express PD-1 (25). However, there is not much information: (*i*) on the exhaustion states of HSV-specific CD8$^+$ T cells that reside in the TG of HSV-1 seropositive individuals; nor (*i*) on the phenotypic and functional exhaustion characteristics of HSV-specific CD8$^+$ T cells from symptomatic (SYMP) individuals (who develop frequent, recurrent herpetic disease) and asymptomatic (ASYMP) individuals (who never experience any recurrent herpetic disease despite being infected). Based on these collective observations, we hypothesized that: (*i*) HSV-1 latently infected TG, with spontaneous or UV-B induced sporadic virus reactivation, would harbor CD8$^+$ T cells that express at least some of the T cell co-inhibitory receptors above and exhibit functional exhaustion; and (*ii*) therapeutic blockade of the highly expressed T cell inhibitory receptors, to restore the function of TG-resident anti-viral CD8$^+$ T cells combined with a therapeutic vaccination to further boost the number and the function of HSV-specific CD8$^+$ T cells that reside in TG would markedly improve clinical outcomes and protect against recurrent corneal herpes infection and disease.

In the present study, we tested the above hypotheses by: (*i*) Comparing phenotypic and functional exhaustion of peripheral blood-derived HSV-specific CD8$^+$ T cells from SYMP and ASYMP individuals; and (*ii*) Studying phenotypic and functional exhaustion of cornea and TG-derived HSV-specific CD8$^+$ T cells using our established mouse model of recurrent ocular herpes. In this model, UV-B irradiation of the cornea of latently infected B6 mice induces HSV-1 reactivation from latently infected TG, as measured by shedding of reactivated virus in tears, in turn leading to recurrent herpetic corneal disease (29, 30). We found that: (*i*) Both PD-1 and LAG-3 co-inhibitory receptors were expressed at significantly higher levels on HSV-specific CD8$^+$ T cells from SYMP individuals, with severe recurrent corneal disease, compared to ASYMP individuals with no disease; (*ii*) Higher prevalence of HSV-specific LAG-3$^+$CD8$^+$ T cells and PD-1$^+$CD8$^+$ T cells were present in SYMP individuals compared to ASYMP individuals; (*iii*) In the B6 mouse model of recurrent ocular herpes, following UV-B induced reactivation, most effector CD8$^+$ T cells from the cornea and TG expressed higher levels of LAG-3 and PD-1; (*iv*) This phenotype correlated with functional exhaustion of HSV-specific CD8$^+$ T cells and with increased virus reactivation, as measured by shedding of reactivated virus in tears, and severe recurrent cornea herpetic disease; and (*v*) Blockade of LAG-3 pathway combined with therapeutic immunization of latently infected B6-albino mice reversed the exhaustion of HSV-specific CD8$^+$ T cells, in both TG and cornea, associated with protection against UV-B induced recurrent corneal herpes infection and disease.

TABLE 1 | Cohorts of HLA-A*02:01 positive, HSV seropositive symptomatic and asymptomatic individuals enrolled in the study.

Subject-level characteristic	All subjects ($n = 39$)
Gender [no. (%)]:	
Female	15 (51%)
Male	14 (49%)
Race [no. (%)]	
Caucasian	19 (66%)
Non-Caucasian	10 (34%)
Age [median (range) years]	39 (21–67 years)
HSV status [no. (%)]	
HSV-1 seropositive	29 (100%)
HSV-2 seropositive	0 (0%)
HSV-1 and -2 seropositive	0 (0%)
HSV seronegative	10 (100%)
HLA [no. (%)]	
HLA-A*02:01 positive	24 (83%)
HLA-A*02:01 negative	5 (17%)
Herpes disease status [no. (%)]	
Asymptomatic (ASYMP)	19 (66%)
Symptomatic (SYMP)	10 (34%)

Definition of symptomatic and asymptomatic individuals are detailed in Materials and Methods.

Overall, our findings suggest that: (*i*) Besides PD-1, the LAG-3 pathway plays a fundamental role in controlling herpes T cell immunity; (*ii*) Blockade of the LAG-3 pathway provides an important immune checkpoint that can synergize with T cell-based therapeutic herpes vaccines to protect against recurrent ocular herpes.

MATERIALS AND METHODS

Human Study Population

All clinical investigations in this study were conducted according to the Declaration of Helsinki. All subjects were enrolled at the University of California, Irvine under approved Institutional Review Board-approved protocols (IRB#2003-3111 and IRB#2009-6963). Written informed consent was received from all participants prior to inclusion in the study.

During the last 15 years (i.e., January 2003 to July 2018), we have screened 875 individuals for HSV-1 and HSV-2 seropositivity. Patients were segregated into SYMP and ASYMP individuals based on the inclusion criteria as previously described (2, 32–33). Among the large cohort of SYMP and ASYMP individuals, 16 HLA-A*02:01 positive patients (8 ASYMP and 8 SYMP) were enrolled in this study (**Table 1**). SYMP and ASYMP groups were matched for age, gender, serological status, and race. The HLA-A2 status was confirmed by PBMC staining with 2 μl of anti-HLA-A2 mAb (clone BB7.2; BD Pharmingen Inc., San Diego, CA), at 4°C for 30 min. The cells were washed and analyzed by flow cytometry using a LSRII (Becton Dickinson, Franklin Lakes, NJ). The acquired data were analyzed with FlowJo software (BD Biosciences, San Jose, CA).

Human Peripheral Blood Mononuclear Cells (PBMC) Isolation

Individuals (negative for HIV, HBV, and with or without any HSV infection history) were recruited at the UC Irvine Institute for Clinical and Translational Science (ICTS). Between 40 and 100 mL of blood was drawn into Vacutainer® Tubes (Becton Dickinson). The serum was isolated and stored at −80°C for the detection of anti-HSV-1 and HSV-2 antibodies, as we have previously described (31). PBMCs were isolated by gradient centrifugation using leukocyte separation medium (Life Sciences, Tewksbury, MA). The cells were then washed in PBS and re-suspended in complete culture medium consisting of RPMI1640, 10% FBS (Bio-Products, Woodland, CA) supplemented with 1x penicillin/streptomycin/L-glutamine, 1x sodium pyruvate, 1x non-essential amino acids, and 50 μM of 2-mercaptoethanol (Life Technologies, Rockville, MD). Freshly isolated PBMCs were also cryo-preserved in 90% FCS and 10% DMSO in liquid nitrogen for future testing.

Human T Cells Flow Cytometry Assays

The following anti-human antibodies were used for the flow cytometry assays: CD3 A700 (clone SK7; BioLegend, San Diego, CA), CD8 PE-Cy7 (clone SK1; BioLegend) PD-1 FITC (clone EH12.2H7; BioLegend), LAG-3 PerCPCy5.5 (clone 11C3C65; BioLegend), For the surface stain, mAbs against cell markers were added to a total of 1×10^6 cells in 1X PBS containing 1% FBS and 0.1% sodium azide (FACS buffer) for 45 min at 4°C. After washing twice with FACS buffer, cells were fixed in PBS containing 2% paraformaldehyde (Sigma-Aldrich, St. Louis, MO). For each sample, 100,000 total events were acquired on the BD LSRII. Ab capture beads (BD Biosciences) were used as individual compensation tubes for each fluorophore in the experiment. To define positive and negative populations, we used fluorescence minus controls for each fluorophore. Furthermore, we optimized gating by examining known negative cell populations for background expression levels similar to that used in our previous work (7). Briefly, we gated single cells, dump cells, viable cells (Aqua Blue), lymphocytes, CD3$^+$ cells, and CD8$^+$ cells before finally gating human epitope-specific CD8$^+$ T cells using HSV-specific tetramers (**Figure S1**). Data analysis was performed using FlowJo software (BD Biosciences, San Jose, CA). Statistical analyses were done using GraphPad Prism version 5 (La Jolla, CA).

Tetramer/VP11/12 Peptide Staining

Fresh PBMCs were analyzed for the frequency of CD8$^+$ T cells recognizing the VP11/12 peptide/tetramer complexes, as we previously described (32–35). The cells were incubated with VP11/12 peptide/tetramer complex for 30–45 min at 37°C. The cell preparations were then washed with FACS buffer and stained with FITC-conjugated anti-human CD8 mAb (BD Pharmingen). The cells were then washed and fixed with 1% paraformaldehyde in PBS and subsequently acquired on a BD LSRII. Data were analyzed using FlowJo version 9.5.6 (Tree Star).

Mice

Female B6(Cg)-*Tyr*$^{c-2J}$/J, or B6-albino mice and LAG-3-deficient mice (LAG-3$^{-/-}$ mice) (6 to 8 weeks old; on the C57BL/6 background) and female C57BL/6 (B6) wild-type (WT) mice (6 to 8 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, ME). Animal studies were performed conforming to the *Guide for the Care and Use of Laboratory Animals* (28). Experiments were conducted with the approval of the Institutional Care and Use Committee of University of California Irvine (Irvine, CA).

Virus Production and the Ocular Challenge of Mice With HSV-1

HSV-1 (strain McKrae) was grown and tittered on rabbit skin (RS) cells as described previously (20–22). All types of mice were ocularly infected with either with 2×10^5 PFU (acute phase studies) or 1×10^6 PFU (reactivation studies) of strain McKrae via eye drops. Following ocular infection, mice were monitored for ocular herpes virus infection and disease.

Immunization With Immunodominant gB$_{498-505}$ Peptide SSIEFARL

Age-matched female mice of each type were assorted in various groups ($n = 10$/group). As per the experimental plan, groups of mice were immunized subcutaneously (s.c.) with the immunodominant gB$_{498-505}$ peptide SSIEFARL delivered with the promiscuous CD4+ T helper (Th) epitope PADRE and CpG1826 adjuvant on day 18 post-infection (PI) followed by a booster dose on day 25 PI. All immunizations were carried out with 100 uM of each peptide.

UV-B Induced Reactivation of HSV-1 From Latency in Mice

Thirty-five days post-infection, when latency was fully established, reactivation of latent HSV-1 infection was induced following UV-B irradiation in all groups of mice (30). TM20 Chromato-Vu transilluminator (UVP, San Gabriel, CA), which emits UV-B at a peak wavelength of 302 nm was used for the purpose. Anesthetized [Intraperitoneal (IP) injection of ketamine/xylazine mouse cocktail 0.1 mL/20 g mouse containing 87.5 mg/kg ketamine and 12.5 mg/kg xylazine] mice were placed on the transilluminator, and each mouse was positioned on a piece of cardboard containing a hole the same size as the mouse's eye. This allowed just the eyes to be irradiated by the UV-B source. Each eye was irradiated with 250 mJ/cm^2 of UV-B light (60-s exposure on the transilluminator).

PD-1 and LAG-3 Blockade in Mice

Anti-PD-1 mAb (RMPI-14) and anti-LAG-3 mAb (C9B7W) were purchased from BioXcell (West Lebanon, NH). For acute phase studies, WT B6 mice were ocularly infected with 2×10^5 PFU of strain McKrae and treated on day 3, 5, and 7 with IP injection of 200 μg of anti-PD-1 mAb or anti-LAG-3 mAb during the acute phase. For reactivation studies, in some designated groups, UV-B irradiation was performed on day 35 and subsequently treated on day 37, 39, and 41 with IP injection of 200 μg of anti-LAG-3 mAb.

Monitoring of Ocular Herpes Infection and Disease in Mice

Virus shedding during the acute phase and that induced by UV-B irradiation was quantified in eye swabs collected every day during the acute phase and post-UV-B irradiation (up to day 8). Eyes were swabbed using moist type 1 calcium alginate swabs and frozen at −80°C until titrated on RS cell monolayers, as described previously (30–34). Animals were examined for signs of recurrent corneal herpetic disease by slit lamp camera (Kowa American Corporation, Torrance CA 90502), for 30 days post UV-B radiation; this was performed by investigators who were blinded to the treatment regimen of the mice and scored according to a standard 0–4 scale (0 = no disease; 1 = 25%; 2 = 50%; 3 = 75%; 4 = 100%) as previously described (30, 31). Total disease score of each day in each group of mice till 30-days post-UV-B exposure was noted. Cumulative graphs of eye disease were generated by dividing the total score of each day per group of mice by total number of eyes in each group and adding the value to that obtained in the succeeding day and continuing till day 30 post-UV-B. Similarly, cumulative graphs of the number of eyes showing recurrent keratitis were done by dividing the total number of eyes showing disease per group of mice (irrespective of disease severity) by the total number of eyes in each group and adding the value to that obtained in the following day and continuing till 30-days post-UV-B. Average of the total score of each group for each of the 30 days post UV-B was calculated by dividing the total score of each day by the total number of eyes in each group.

Isolation of Lymphocytes

Mice from all groups were euthanized, and the spleen, cornea, and TG were individually harvested. Cornea and TG tissues were digested in complete medium containing 2.5-mg/ml collagenase type IV (Sigma Chemical Co., St. Louis, MO). Digestion was accomplished by incubation at 37°C with shaking for 30 min. After digestion, tissues and cells were filtered through a sterile gauze mesh and washed with RPMI 1640 medium. Spleen homogenates were prepared by pressing the tissue through a sterile mesh screen into 10 ml of PBS under aseptic conditions. Single-cell suspensions thus prepared from spleen, TG and cornea were analyzed using flow cytometry (**Figure S1**).

Mice Flow Cytometry Analysis

The following anti-mouse antibodies were used: CD3 FITC (clone17A2; Biolegend), CD8 PerCP (clone 53-6.7; BD), CD107a FITC (clone 1D4B; BD), CD107b FITC (clone Ha1/29; BD), IFN-γ-PE (clone XMG1.2; BioLegend), and Ki-67 PE/Cy7 (clone 16A8; BioLegend). Both surface and intracellular staining were performed similarly to the human study as described above.

Tetramer/gB$_{498-505}$ Staining

Cells harvested from spleen, TG and cornea were analyzed for the frequency of CD8^{+}T cells recognizing gB$_{498-505}$ peptide tetramer complex similarly to the human study as aforementioned.

Statistical Analysis

Data for each assay were compared by ANOVA and Student's *t*-test using GraphPad Prism version 5 (La Jolla, CA). Differences between the groups were identified by ANOVA and multiple comparison procedures, as we previously described (33, 34). Data are expressed as the mean ± SD. Results were considered statistically significant at P value of ≤ 0.05.

RESULTS

HSV-Specific CD8$^+$ T Cells, With Elevated Expression of PD-1 and LAG-3, Are Frequent in Symptomatic Patients With Recurrent Herpetic Disease

The characteristics of the symptomatic (SYMP) and asymptomatic (ASYMP) study population used in this present study, with respect to gender, age, HLA-A*02:01 frequency distribution, HSV-1/HSV-2 seropositivity and status of ocular and genital herpetic diseases are presented in **Table 1** and detailed in the *Materials* and *Methods section*. Since HSV-1 is the main cause of ocular herpes, only individuals who are HSV-1 seropositive and HSV-2 seronegative were enrolled in the present study. HSV-1 seropositive individuals were divided into two groups: (*i*) ten HLA-A*02:01 positive, HSV-1-infected ASYMP individuals who have never had any clinically detectable herpes disease; and (*ii*) ten HLA-A*02:01 positive HSV-1-infected SYMP individuals with a history of numerous episodes of well-documented recurrent clinical herpes diseases, such as herpetic lid lesions, herpetic conjunctivitis, dendritic or geographic keratitis, stromal keratitis, and iritis consistent with rHSK, with one or more episodes per year for the past 5 years. Only SYMP patients who were not on Acyclovir or other anti-viral or anti-inflammatory drug treatments at the time of blood sample collections were enrolled. One patient had over two severe recurrent episodes during the last 10 years that necessitated multiple corneal transplantations.

We first sought to determine whether there is any differential frequency of HSV-specific CD8$^+$ T cells expressing exhaustion markers PD-1 and LAG-3 between SYMP and ASYMP individuals. Blood-derived HSV-1 VP11/12$_{66-74}$ epitope specific CD8$^+$ T cells from SYMP and ASYMP ($n = 8$, each) individuals were analyzed by flow cytometry for the expression of LAG-3 and PD-1. A tetramer specific to the immunodominant VP11/12$_{66-74}$ epitope was used to decipher the expression of LAG-3 and PD-1 uniquely on HSV-specific T cell (instead of bulk CD8$^+$ T cells). As shown in **Figure 1A**, there were no observed differences in the frequency of VP11/12$_{66-74}$ epitope-specific CD8$^+$T cells between ASYMP (1.1%) and SYMP (1.5%) individuals. However, VP11/12$_{66-74}$ epitope-specific LAG-3$^+$CD8$^+$T cells and PD-1$^+$CD8$^+$T cells appeared to be more frequent in SYMP compared to ASYMP individuals (**Figures 1B,C**). Moreover, as shown in **Figure 1D**, elevated expression levels of LAG-3 and PD-1 were detected in VP11/12$_{66-74}$ epitope-specific CD8$^+$T cells of SYMP patients compared to ASYMP healthy individuals, as depicted by a significant difference in mean fluorescent intensity (MFI) of LAG-3 and PD-1 expression.

Altogether these results indicate that both PD-1 and LAG-3 markers of exhaustion are highly expressed in HSV-specific CD8$^+$ T cells from SYMP patients that are clinically diagnosed with the repetitive recurrent ocular herpetic disease. This data is in agreement with the functional impairment of VP11/12$_{66-74}$-specific CD8$^+$ T cells we have previously reported on in SYMP individuals (2). Since LAG-3 and PD-1 markers are strong determinants of functional exhaustion, this denotes that exhaustion of antigen-specific CD8$^+$ T cells in SYMP individuals may be a potential cause of the suboptimal immunity, often associated with symptomatic shedding.

Because of ethical and practical complexities in obtaining cornea- and trigeminal ganglia- (TG) derived CD8$^+$ T cells in humans, we were limited to using blood-derived CD8$^+$ T cells in humans. However, the phenotype and function of human blood-derived CD8$^+$ T cells may not reflect tissue-resident CD8$^+$ T cells. For these reasons, the remainder of this study utilized our established mouse model of acute and UV-B induced recurrent ocular herpes to determine the phenotypic and functional exhaustion of TG- and cornea-resident CD8$^+$ T cells and their association with acute and recurrent ocular herpes. Since our results above on human blood-derived CD8$^+$ T cells suggests high frequencies of HSV specific CD8$^+$ T cells expressing LAG-3 and PD-1 in SYMP individuals; next, we determined the kinetics of LAG-3 and PD-1 expression in cornea and TG following HSV-1 infection in mice.

Increased Frequency and Number of HSV Specific LAG-3$^+$CD8$^+$ T Cells in the Cornea and TG of Ocular Herpes Infected Mice

A group of 40 mice were infected with 2×10^5 pfu of HSV-1 strain McKrae. Mice ($n = 10$) were sacrificed during acute and latent phases at five different time points (i.e., days 3, 8, 14, 23, and 41). Cornea and TG were harvested, and the frequencies of HSV specific (gB$_{498-505}$) CD8$^+$ T cells expressing LAG-3 and PD-1 exhaustion markers were analyzed by FACS. The frequencies of HSV specific (gB$_{498-505}$) CD8$^+$ T cells expressing LAG-3 appeared to increase starting on day 3 during acute infection in both cornea (33.1%) and TG (12.1%) (**Figures 2A–E**). The highest frequencies of HSV specific (gB$_{498-505}$) CD8$^+$ T cells expressing LAG-3 were detected on day 23 during latency in both cornea (53.2%) and TG (27.2%), and those seem to persist until day 41 of latency (**Figures 2B,C**). Similarly, the frequencies of HSV specific (gB$_{498-505}$) CD8$^+$ T cells expressing PD-1 increased in both the cornea and TG starting on day 3 during acute infection in both cornea (18.8%) and TG (13.3%) (**Figures 2D,E**). Further heightened expression of PD-1 was observed late in acute phase on day 14 in both cornea (41.2%) and TG (34.6%) and gradually diminished by day 41 during latency (**Figures 2D,E**). Intriguingly, high frequencies of LAG-3$^+$CD8$^+$ T cells, but not of PD-1$^+$CD8$^+$ T cells, were found in the cornea. In contrast, similar frequencies of LAG-3$^+$CD8$^+$ T cells and PD-1$^+$CD8$^+$ T cells were detected in the TG.

Altogether, these findings suggest that similar to HSV-1 infected SYMP humans: (*i*) HSV-specific CD8$^+$ T cells in

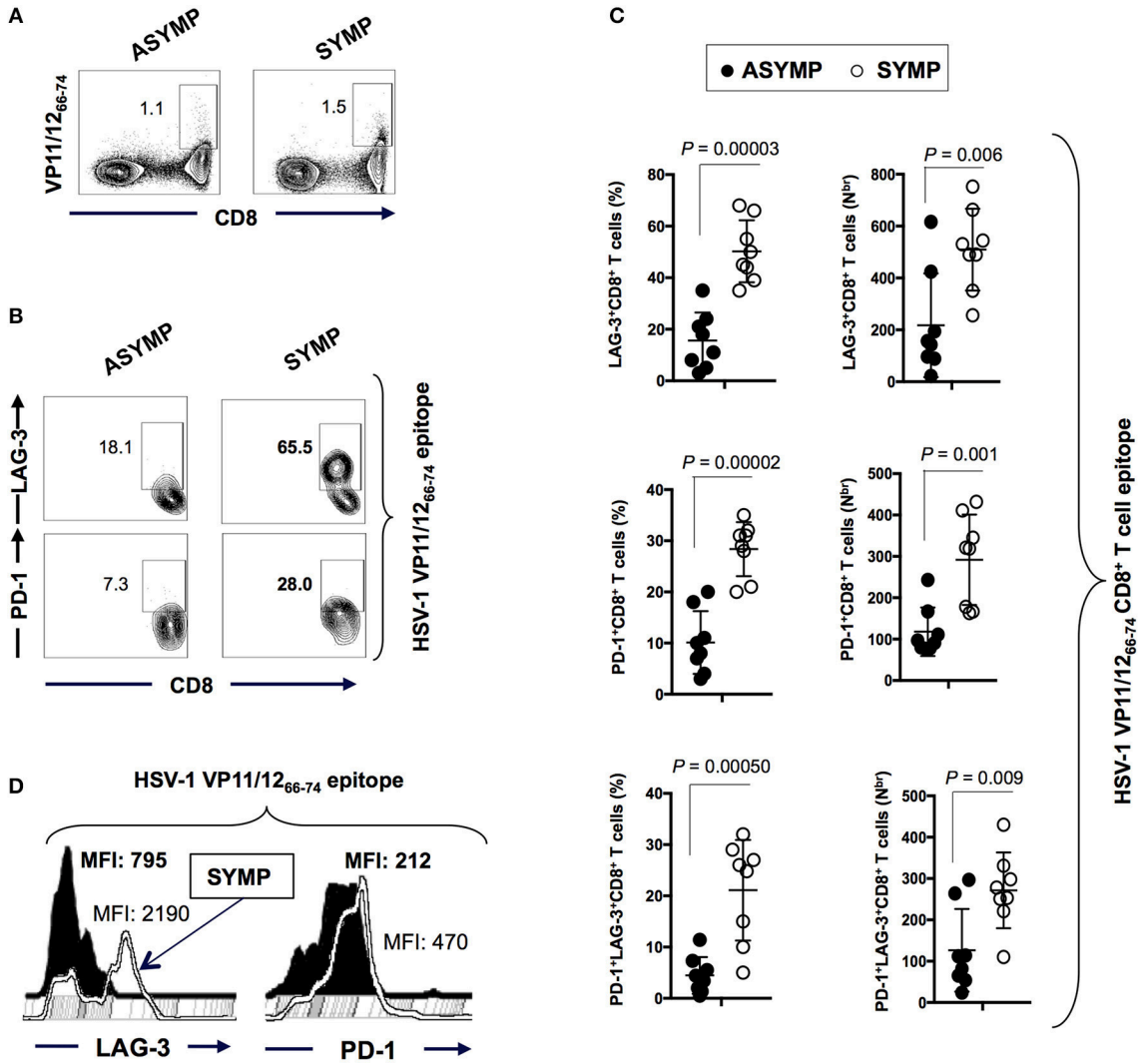


FIGURE 1 | Frequency of HSV-1 VP11/12$_{66-74}$ epitope-specific LAG-3^{+}CD8^{+}T cells and PD-1^{+}CD8^{+}T cells in ASYMP vs. SYMP individuals. **(A)** Representative FACS plot of the frequencies of HSV-1 VP11/12$_{66-74}$ tetramer-specific CD8^{+} T cells in ASYMP vs. SYMP individuals. **(B)** Representative FACS plot of the frequencies of LAG-3^{+}CD8^{+}T cells and PD-1^{+}CD8^{+}T cells in ASYMP and SYMP individuals. **(C)** Average percentages (*left panels*) and the absolute number (*right panels*) of HSV-1 VP11/12$_{66-74}$ tetramer-specific PD-1^{+} CD8^{+} T cells, LAG-3^{+} CD8^{+} T cells and PD-1^{+}LAG-3^{+}CD8^{+} T cells in ASYMP and SYMP individuals. **(D)** level of expression of LAG-3 and PD-1 receptors onCD8^{+} T cells from ASYMP vs. SYMP individuals, depicted as Mean fluorescent intensity (MFI). Results are representative of two independent experiments in each individual. The indicated *P*-values, calculated using the unpaired *t*-test, show statistical significance between SYMP and ASYMP individuals.

infected cornea and TG of mice show elevated expression of LAG-3 and PD-1 exhaustion markers; and (*ii*) a conspicuous involvement of the LAG-3 and PD-1 pathways in mediating CD8^{+} T cell exhaustion during the latent phase of symptomatic herpes infection.

Blockade of LAG-3 and PD-1 During Acute Phase Controls Herpes Infection and Disease and Strengthens the Anti-viral Immune Response

We next studied the effect of blocking LAG-3 and PD-1 using antagonist mAbs, on viral infection, disease, and anti-viral CD8^{+} T cell response during the acute phase of HSV-1 infection. A group of 40 WT B6 mice were infected with 2×10^5 pfu of HSV-1 strain McKrae. Mice were intraperitoneally (i.p.) injected with 200 μg of anti-LAG-3 mAb ($n = 10$) and 200 μg of anti PD-1 mAb ($n = 10$) at 3 different time points [i.e., days 3, 5, and 7 post-infection (PI)].

Following the blockade of both LAG-3 and PD-1, a significant decrease in viral replication and disease was evidenced ($P < 0.05$, **Figures 3A,B**, **4A,B**). This was associated with a significant decrease in the severity of primary ocular disease, as shown in average of 10 mice (**Figures 3B, 4B,** *right panels*) and in representative eye disease pictures (**Figures 3B, 4B,** *left panels*). Further the number and function of HSV-1 gB$_{495-505}$ specific

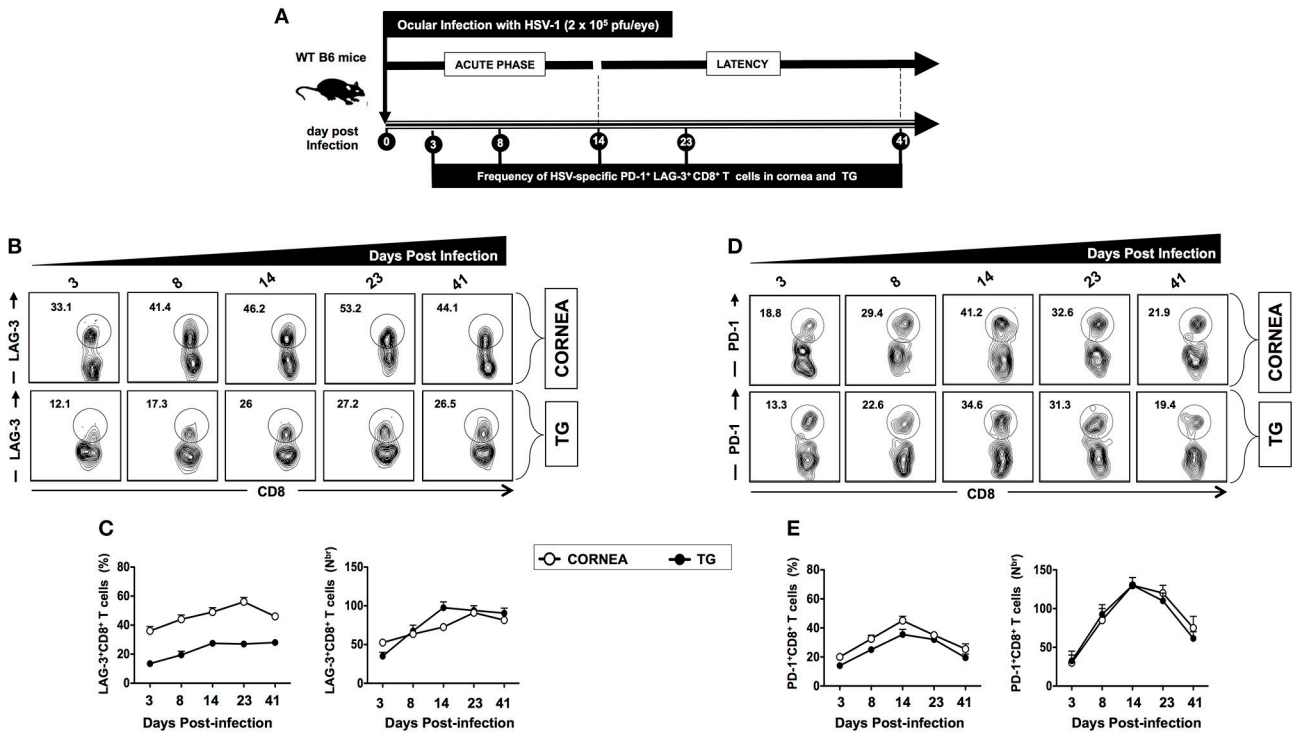


FIGURE 2 | Kinetics of LAG-3 and PD-1 expression on HSV-specific CD8+ T cells following ocular herpes virus infection. **(A)** Schematic representation of the timeline for HSV-1 infection and assays on phenotypic exhaustion of HSV-specific CD8$^+$ T cells in WT B6 mice (6–8 weeks old, $n = 40$), following ocular infection on day 0 with 2×10^5 pfu of HSV-1 strain McKrae, as described in *Materials* and *Methods*. A group of 10 mice were sacrificed on days 3, 8, 14 (acute phase), 23, and 41 (latent phase) post-infection (PI). Subsequently, cornea and TG were harvested, and the frequencies of the HSV specific ($gB_{498-505}$) CD8$^+$ T cells expressing LAG-3 and PD-1 exhaustion markers were determined by FACS. **(B)** Representative FACS plot and **(C)** average percentages and absolute numbers of HSV-specific CD8$^+$ T cells expressing LAG-3 are detected in cornea and TG at different time points. **(D)** Representative FACS plot, and **(E)** average percentages and absolute numbers of HSV-specific CD8$^+$ T cells expressing PD-1 detected in cornea and TG at different time points. The results are representative of two independent experiments.

CD8$^+$ T cells detected from αLAG-3 (**Figures 3C–J**) and αPD-1(**Figures 4C–J**) mAb treated mice revealed an increase in both the frequency and number of $gB_{498-505}$ specific CD8$^+$ T cells, IFN-γ^+CD8$^+$ T cells, CD107$^+$CD8$^+$ T cells, and Ki-67$^+$CD8$^+$ T cells, as compared to isotype and mock treated control groups. Representative FACS plots showed heightened frequencies of $gB_{498-505}$ specific CD8$^+$ T cells (αLAG-3: 10% vs. Isotype: 8%, Mock: 6.9%; αPD-1: 12% vs. Isotype: 8%, Mock: 6.9%), IFN-γ^+CD8$^+$ T cells (αLAG-3: 15.6% vs. Isotype: 6%, Mock: 5.2%; αPD-1: 10.1% vs. Isotype: 6%, Mock: 5.2%), CD107$^+$CD8$^+$ T cells (αLAG-3: 13% vs. Isotype: 5.8%, Mock: 4.7%; αPD-1: 9.6% vs. Isotype: 6%, Mock: 5.2%) and Ki-67$^+$CD8$^+$ T cells (αLAG-3: 10.8% vs. Isotype: 6.8%, Mock: 5.7%; αPD-1: 11.6% vs. Isotype: 6%, Mock: 5.2%) in αLAG-3 mAb (**Figures 3C–J**) and αPD-1 mAb (**Figures 4C–J**) treated groups in comparison to controls. The corresponding average percentage and average absolute number of $gB_{498-505}$ tetramer specific CD8$^+$ T cells; IFN-γ^+ CD8$^+$ T cells; CD107$^+$CD8$^+$ T cells and Ki-67$^+$CD8$^+$ T cells are also shown for αLAG-3 (**Figures 3C–J**) and αPD-1 (**Figures 4C–J**) treated groups in comparison to controls. No systemic or local side effect was detected following the blockade of both LAG-3 and PD-1 pathways.

Altogether, these results suggest that blockade of the LAG-3 and PD-1 pathways of exhaustion can be a promising strategy to combat ocular herpes. As PD-1 blockade is already widely reported to combat persistent pathogens including HSV and is the most thoroughly investigated immune checkpoint pathways, our results essentially reinforce the earlier studies, and henceforth we focused on the lesser investigated LAG-3 pathway to combat HSV reactivation.

Combination of a Therapeutic LAG-3 Blockade and Therapeutic Immunization Restored the Function of HSV-Specific CD8$^+$ T Cells in Cornea and TG Associated With a Reduction in Recurrent Ocular Herpes

Subsequently, we determined whether LAG-3 blockade after HSV-1 reactivation from latently infected mice would reduce viral shedding and disease and restore antiviral immune response. We used our novel UV-B model of virus reactivation and preferred albino B6(Cg)-*Tyr*$^{c-2J}$/J mice over WT B6 mice, as they are known to be more susceptible to reactivation and recurrent corneal disease. Four groups of B6(Cg)-*Tyr*$^{c-2J}$/J mice ($n = 10$/group) were latently infected with 1×10^6 pfu of McKrae as described in the *Materials* and *Methods* and were

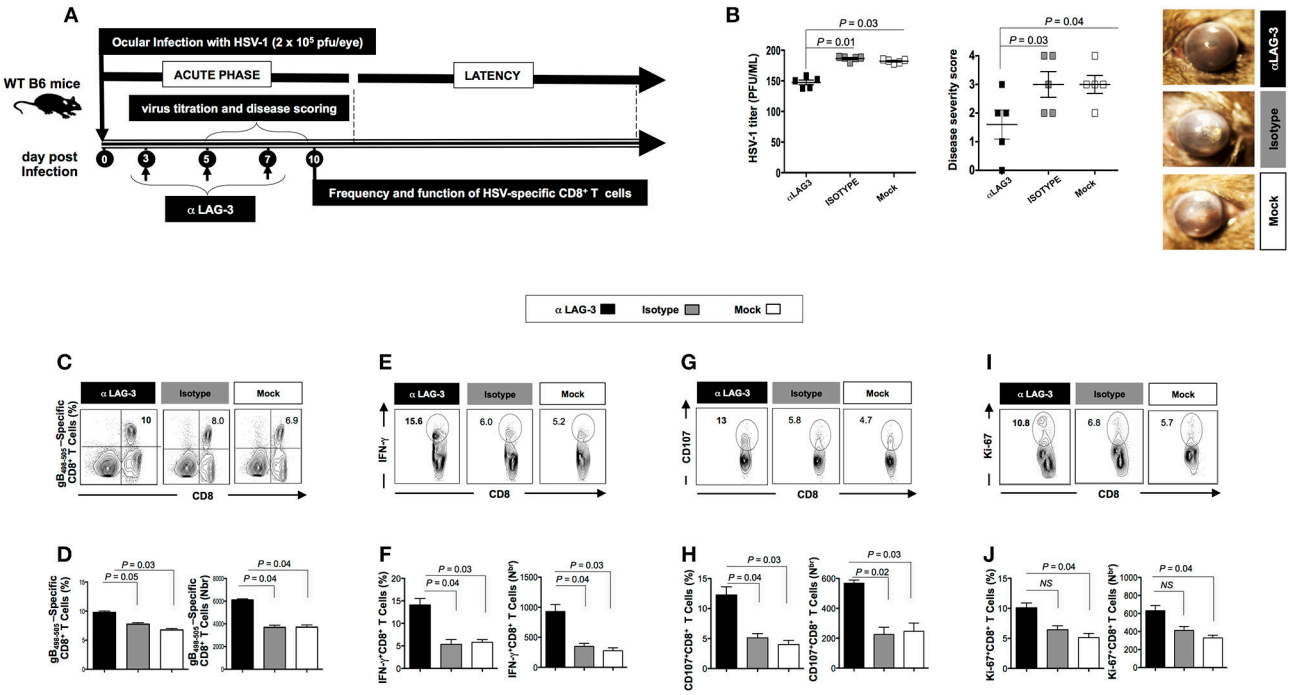


FIGURE 3 | Effects of the LAG-3 blockade on herpes infection, disease and antiviral CD8+ T cell response during the acute phase of ocular herpes infection. **(A)** Schematic representation of HSV-1 ocular infection, blockade LAG-3 mAb treatment, virological and immunological analyses in 30 WT B6 mice (6–8 weeks old) following ocular infection on day 0 with 2 × 10^5 pfu of HSV-1 (strain McKrae) as detailed in the *Materials* and *Methods* section. **(B)** Quantification of infectious virus particles in the eye swabs by standard plaque assay on RS cells and acute eye disease scoring on a scale of 1–4. Mean viral load (pfu/ml), mean disease score and representative eye disease pictures after the terminal day of the blockade (day 8) are shown. Representative FACS plots, average percentage and average absolute number of **(C,D)** HSV- specific ($gB_{498-505}$) CD8^{+} T cells **(E,F)** HSV-specific IFNy^{+}CD8^{+} T cells **(G,H)** HSV-specific CD107^{+}CD8^{+} T cells **(I,J)** HSV-specific Ki-67^{+}CD8^{+} T cells in the spleen of mice from each group. The results are representative of two independent experiments. The indicated *P*-values, calculated using the unpaired *t*-test, show statistical significance between mAb treated and control groups.

then segregated as follows: (1) a group of αLAG-3 + $gB_{498-505}$ mice was therapeutically immunized during latency on days 18 and 25 PI with $gB_{498-505}$ CD8^{+} T cell epitope mixed with the CD4^{+} T helper epitope PADRE and then treated three times with αLAG-3 on days 3, 5, and 7 post UV-B exposure (i.e., days 37, 39, and 41 PI, respectively); (2) a group of αLAG-3 alone, in which mice were non-immunized but therapeutically treated with αLAG-3 as in 1; (3) a group of $gB_{498-505}$ alone, in which mice were only immunized with $gB_{498-505}$ CD8^{+} T cell epitope mixed with the CD4^{+} T helper epitope PADRE; and (4) a group of Mock controls, in which the mice were neither immunized nor treated with αLAG-3. All the three forms of therapeutic interventions: αLAG-3 + $gB_{498-505}$/PADRE, αLAG-3 alone, $gB_{498-505}$/PADRE alone significantly reduced the manifestation of UV-B induced recurrent disease compared to mock, but the combination therapy of αLAG-3 + $gB_{498-505}$/PADRE showed the most significant effect (**Figures 5A–D**). This is clear from both the cumulative reactivation score (**Figure 5B**) and the cumulative number of eyes with recurrent disease (**Figure 5C**). The average score of each day per group detected till day 30 post UV-B exposure also revealed significant difference between the combination therapy of αLAG-3 + $gB_{498-505}$ vaccine vs. αLAG-3 alone ($P = 0.05$), αLAG-3+$gB_{498-505}$ vaccine vs. $gB_{498-505}$ vaccine alone ($P = 0.04$), and αLAG-3+$gB_{498-505}$ vaccine vs. mock ($P = 0.03$) (**Figure 5D**). Meanwhile, the average degree of viral shedding following the final αLAG-3 treatment (day 8 post UV-B) showed significant difference between combination therapy of αLAG-3 + $gB_{498-505}$ vs. αLAG-3 alone ($P = 0.04$), αLAG-3+$gB_{498-505}$ vs. $gB_{498-505}$ alone ($P = 0.03$), and αLAG-3 + $gB_{498-505}$ vs. mock ($P = 0.02$) (**Figure 5E**). Representative eye pictures showed significant differences in disease severity amongst all the groups (**Figure 5F**).

At the end of monitoring recurrent disease on day 30 post UV-B, we sacrificed the mice of all groups, harvested mononuclear cells (MNC's) from cornea and TG, as described in *Materials* and *Methods* and determined the number and function of HSV-specific CD8^{+} T cells. Representative FACS plots showed increased frequencies of $gB_{498-505}$ specific CD8^{+} T cells (Cornea: αLAG-3 + $gB_{498-505}$ vaccine: 25.4% vs. αLAG-3 alone: 20.3%, $gB_{498-505}$ vaccine alone: 18.5%, Mock: 12.8%; TG: αLAG-3+$gB_{498-505}$ vaccine: 28.9% vs. αLAG-3 alone: 18.1%, $gB_{498-505}$ vaccine alone: 21.3%, Mock: 15%), IFN-γ^{+}CD8^{+} T cells (Cornea: αLAG-3 + $gB_{498-505}$ vaccine: 23% vs. αLAG-3 alone: 18.2%, $gB_{498-505}$ vaccine alone: 16%, Mock: 9%; TG: αLAG-3 + $gB_{498-505}$ vaccine: 40.1% vs. αLAG-3 alone: 34.5%, $gB_{498-505}$ vaccine alone: 29.1%, Mock: 20.2%), CD107^{+}CD8^{+}

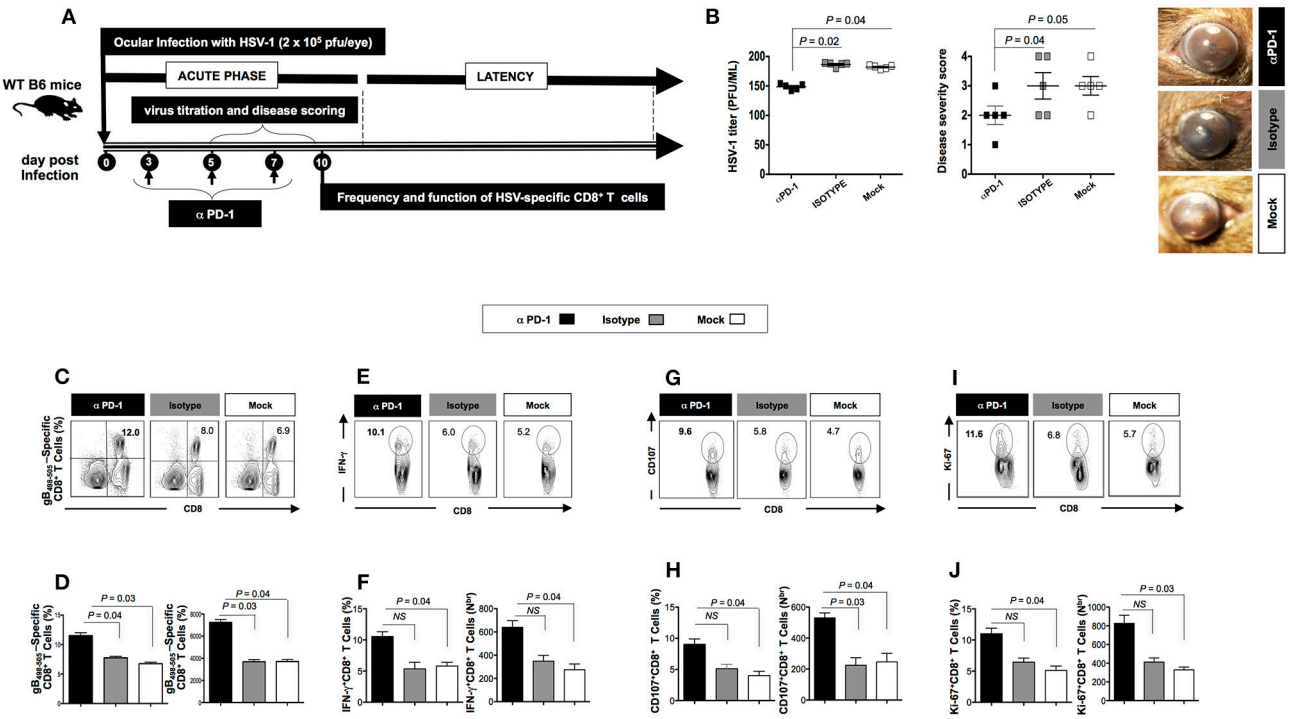


FIGURE 4 | Effect of the PD-1 blockade on herpes infection, disease and antiviral immune response during the acute phase of ocular herpes virus. **(A)** Schematic representation of HSV-1 ocular infection, blockade PD-1 mAb treatment, virological and immunological analyses in 30 WT B6 mice (6–8 weeks old) following ocular infection on day 0 with 2 × 10^5 pfu of HSV-1 (strain McKrae) as detailed in the *Materials* and *Methods* section **(B)** Quantification of infectious virus particles in the eye swabs by standard plaque assay on RS cells and acute eye disease scored on a scale of 1–4. Mean viral load (pfu/ml), mean disease score and representative eye disease pictures after the terminal day of the blockade (day 8) are shown. Representative FACS plots, average percentage and average absolute number of **(C,D)** HSV- specific ($gB_{498-505}$) CD8$^+$ T cells **(E,F)** HSV- specific IFNγ$^+$CD8$^+$ T cells **(G,H)** HSV-specific CD107$^+$CD8$^+$ T cells **(I,J)** HSV-specific Ki-67$^+$CD8$^+$ T cells in the spleen of mice from each group. The results are representative of 2 independent experiments. The indicated *P*-values, calculated using the unpaired *t*-test, show statistical significance between mAb treated and control groups.

T cells (Cornea: αLAG-3 + $gB_{498-505}$ vaccine: 24% vs. αLAG-3 alone: 19%, $gB_{498-505}$ vaccine alone: 16.1%, Mock: 8.1%; TG: αLAG-3 + $gB_{498-505}$ vaccine: 34.2% vs. αLAG-3 alone: 28.2%, $gB_{498-505}$ vaccine alone: 24.2%, Mock: 13.1%), and Ki-67$^+$CD8$^+$ T cells (Cornea: αLAG-3 + $gB_{498-505}$ vaccine: 42% vs. αLAG-3 alone: 34.3%, $gB_{498-505}$ vaccine alone: 28%, Mock: 19%; TG: αLAG-3 + $gB_{498-505}$ vaccine: 18% vs. αLAG-3 alone: 13%, $gB_{498-505}$ vaccine alone: 10.2%, Mock: 5%) in combination therapy of αLAG-3 + $gB_{498-505}$ vaccine group when compared with the other groups (**Figures 6A,C**). The corresponding average percentage and average absolute number of $gB_{498-505}$ tetramer specific CD8$^+$ T cells; IFN-γ$^+$ CD8$^+$ T cells; CD107$^+$CD8$^+$ T cells and Ki-67$^+$CD8$^+$ T cells are also shown for all the groups (αLAG-3+$gB_{498-505}$ vaccine, αLAG-3 alone, $gB_{498-505}$ vaccine alone, and mock) in cornea (**Figure 6B**) and TG (**Figure 6D**).

Taken together, these results suggest that the combined effect of the blockade and immunization (1) significantly combats the manifestation of disease severity during reactivation of HSV-1 from latency and (2) significantly restores HSV specific immunity in the resident tissues during latency that underscores the observed protection from recurrent ocular herpes.

Therapeutic Immunization Improves HSV-Specific CD8$^+$ T Cell Response in the Cornea and TG and Protects Against Recurrent Ocular Herpes in LAG-3$^{-/-}$ Mice

Two groups of mice (WT B6 and LAG-3$^{-/-}$ deficient mice (n = 10/group) were latently infected with 1 × 10^6 pfu of McKrae. One group of WT B6 and one of LAG-3$^{-/-}$ mice were immunized with the immunodominant $gB_{498-505}$ peptide mixed with the promiscuous CD4$^+$Th epitope PADRE and CpG_{1826} adjuvant as detailed in the *Materials* and *Methods* section (**Figure 7A**). The groups were then divided as follows: (1) LAG-3$^{-/-}$ mice + $gB_{498-505}$ vaccine; (**2**) LAG-3$^{-/-}$ mice + Mock vaccine; (3) WT mice + $gB_{498-505}$ vaccine; and (4) WT mice + Mock vaccine.

As shown in **Figures 7B–D**, the groups LAG-3$^{-/-}$ + $gB_{498-505}$ vaccine, LAG-3$^{-/-}$ + Mock vaccine, WT + $gB_{498-505}$ vaccine showed significant reduction of recurrent disease compared to WT + Mock vaccine. Moreover, the group of mice that received the combination therapy of the LAG-3$^{-/-}$ + $gB_{498-505}$ vaccine showed the most significant effect on recurrent corneal herpetic disease. This is evident from both the cumulative reactivation score (**Figure 7B**) and the cumulative number of eyes with recurrent disease (**Figure 7C**). As shown in **Figure 7D**

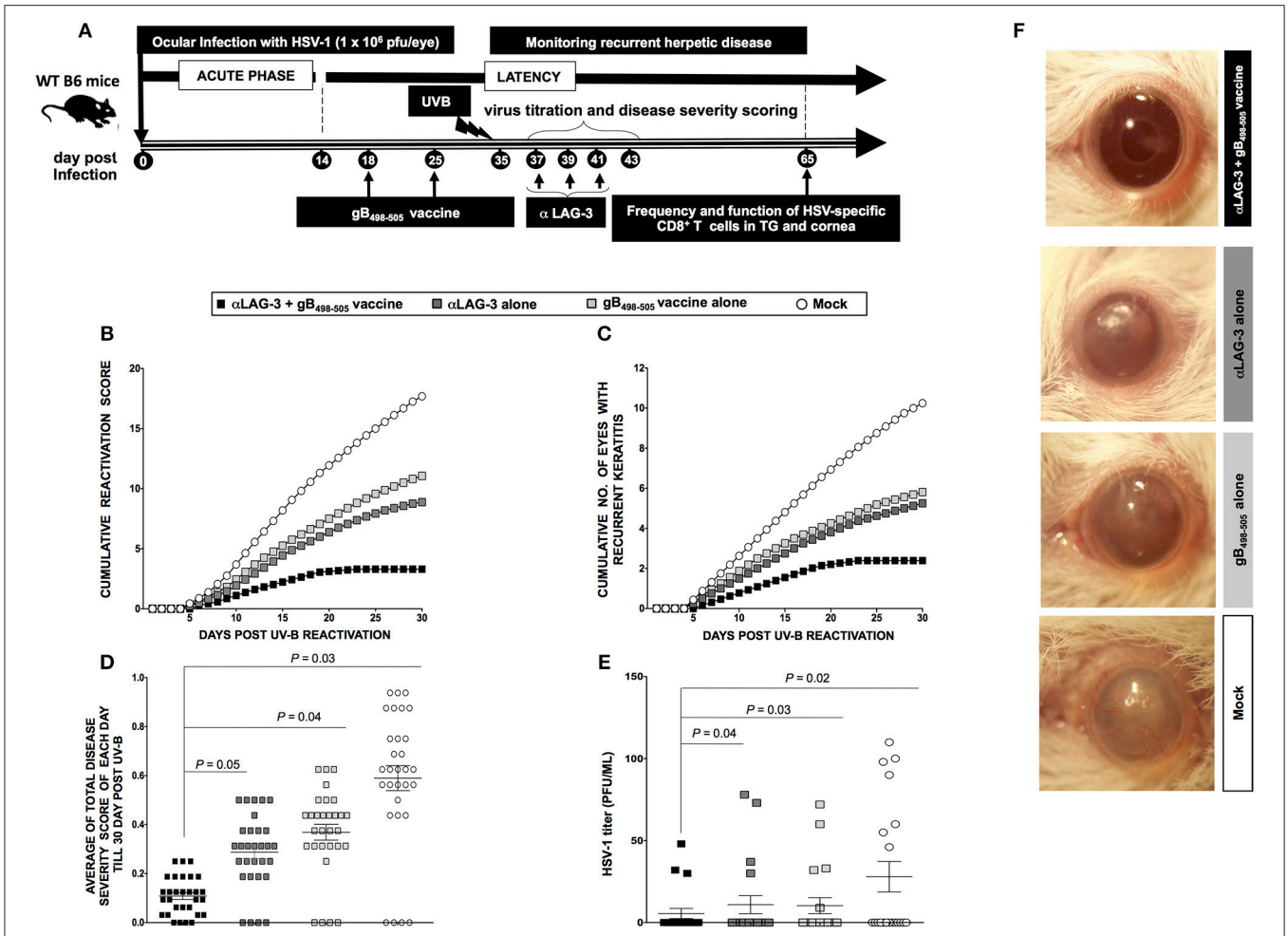


FIGURE 5 | Blockade of LAG-3 following UV-B induced HSV-1 reactivation reduces recurrent ocular herpes. **(A)** Schematic representation of UV-B induced reactivation, blockade by mAb treatment, virological and immunological analyses, and recurrent eye disease scoring in 40 B6(Cg)-*Tyr*$^{c-2J}$/J mice (6–8 weeks old) categorized into α-LAG-3 + $gB_{498-505}$, α-LAG-3 alone, $gB_{498-505}$ alone, and mock groups on the basis of different immunization strategies following ocular infection on day 0 with 1 × 10^6 pfu of HSV-1 (strain McKrae). **(B)** Cumulative reactivation score till 30-day post UV-B. **(C)** The cumulative number of eyes showing recurrent disease till 30-day post UV-B. **(D)** Average of total eye disease score of each day till 30-day post UV-B. **(E)** Average viral shedding evaluation by plaque assay after the last day of LAG-3mAb treatment (day 8 post UV-B). **(F)** Representative eye pictures of recurrent disease. Results are representative of two independent experiments. The indicated *P*-values, calculated using the unpaired *t*-test, show statistical significance between α-LAG-3 + $gB_{498-505}$ and other groups.

the average of total score of each day per group, detected up to 30-day post UV-B induced reactivation, showed a significant difference between; LAG-$3^{-/-}$ + $gB_{498-505}$ vaccine vs. LAG-$3^{-/-}$ + Mock ($P = 0.04$), LAG-$3^{-/-}$ + $gB_{498-505}$ vaccine vs. WT + $gB_{498-505}$ vaccine ($P = 0.03$), LAG-$3^{-/-}$ + $gB_{498-505}$ vaccine vs. WT + Mock ($P = 0.02$).

Significance differences in virus shedding were found between LAG-$3^{-/-}$ + $gB_{498-505}$ vaccine vs. LAG-$3^{-/-}$ + Mock ($P = 0.03$), LAG-$3^{-/-}$ + $gB_{498-505}$ vaccine vs. WT + $gB_{498-505}$ vaccine ($P = 0.03$), LAG-$3^{-/-}$ + $gB_{498-505}$ vaccine vs. WT + Mock ($P = 0.02$) (**Figure 7E**). Recurrent disease was also significantly reduced in LAG-$3^{-/-}$ + $gB_{498-505}$ vs. all the other groups (**Figure 7F**).

On day 30 post UV-B exposure (i.e., at the end of monitoring recurrent disease) the mice of all groups were sacrificed, mononuclear cells (MNC's) from cornea and TG were harvested and antiviral CD8^{+} T cell responses were compared between groups. From the representative FACS plots heightened frequencies for $gB_{498-505}$ specific CD8^{+} T cells (Cornea: LAG-$3^{-/-}$ + $gB_{498-505}$ vaccine: 44% vs. LAG-$3^{-/-}$ + Mock: 35.9%, WT + $gB_{498-505}$ vaccine: 35%, WT + Mock: 23%; TG: LAG-$3^{-/-}$ + $gB_{498-505}$ vaccine: 35% vs. LAG-$3^{-/-}$ + Mock: 29%, WT + $gB_{498-505}$ vaccine: 25%, WT + Mock: 17%), IFN-γ^{+}CD8^{+} T cells (Cornea: LAG-$3^{-/-}$ + $gB_{498-505}$ vaccine: 43% vs. LAG-$3^{-/-}$ + Mock: 35%, WT + $gB_{498-505}$ vaccine: 28%, WT + Mock: 20%; TG: LAG-$3^{-/-}$ + $gB_{498-505}$ vaccine: 27.6% vs. LAG-$3^{-/-}$ + Mock: 22.2%, WT + $gB_{498-505}$ vaccine: 18%, WT + Mock: 13%), CD107^{+}CD8^{+} T cells (Cornea: LAG-$3^{-/-}$ + $gB_{498-505}$ vaccine: 68.4% vs. LAG-$3^{-/-}$ + Mock: 60.2%, WT + $gB_{498-505}$ vaccine: 55.2%, WT + Mock: 42%; TG: LAG-$3^{-/-}$ +

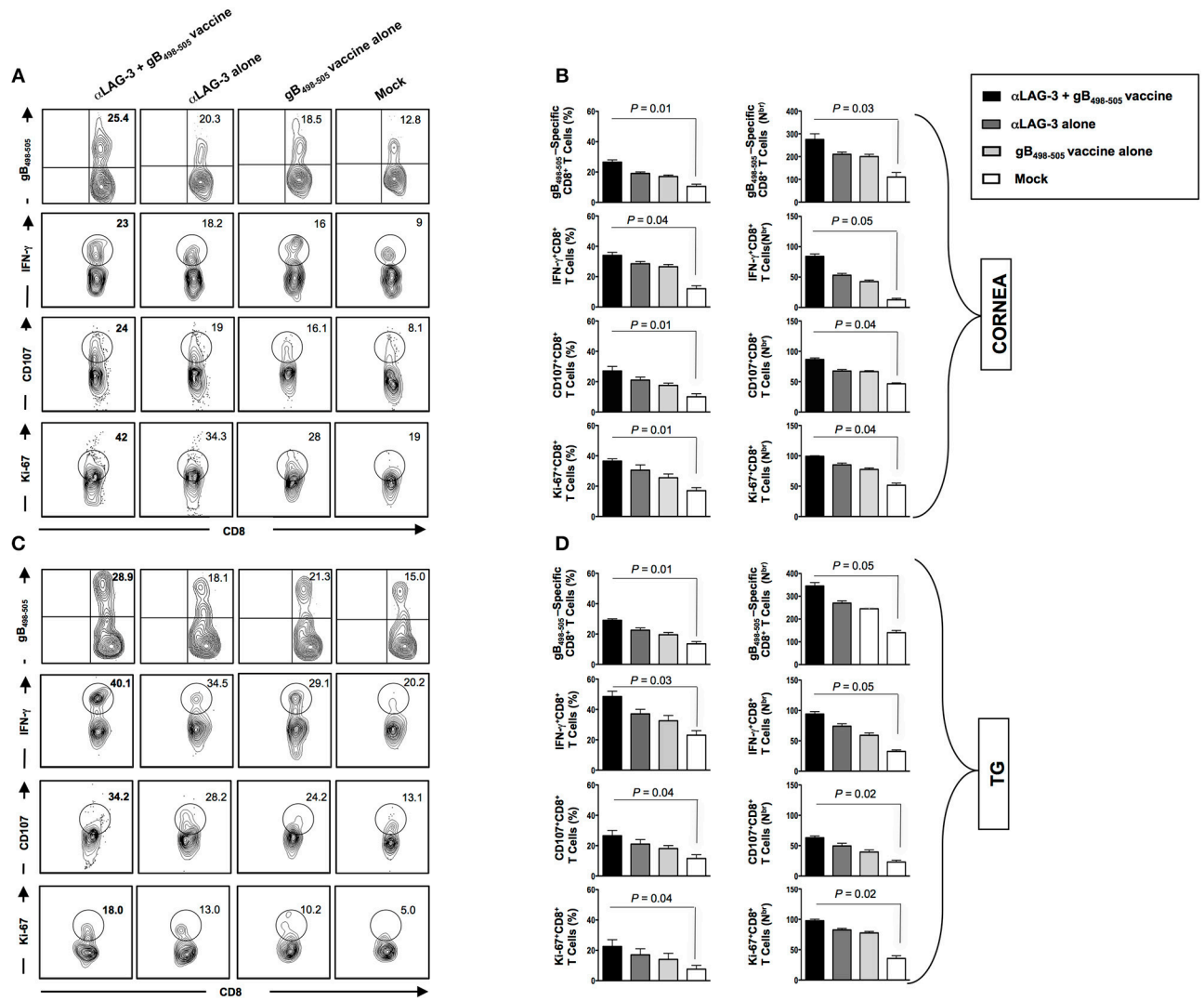


FIGURE 6 | Reduction in recurrent disease by blockade of LAG-3 is associated with better anti-viral immune response in cornea and TG. Representative FACS plots, average percentage and average absolute number of HSV- specific ($gB_{498-505}$) CD8^{+} T cells; HSV- specific IFNy^{+}CD8^{+} T; HSV-specific CD107^{+}CD8^{+} T cells; HSV-specific Ki-67^{+}CD8^{+} T cells against four immunization strategies namely α-LAG-3 +$gB_{498-505}$ vaccine, α-LAG-3 alone, $gB_{498-505}$ vaccine alone, and mock in **A,B** cornea and **(C,D)** TG are shown. Results are representative of 2 independent experiments. The indicated *P*-values are calculated using the unpaired *t*-test, show statistical significance between α-LAG-3 + $gB_{498-505}$ vaccine and mock groups.

$gB_{498-505}$ vaccine: 56.2% vs. LAG-3$^{-/-}$ + Mock: 47.3%, WT + $gB_{498-505}$ vaccine: 41.2%, WT + Mock: 32%), and Ki-67^{+}CD8^{+} T cells (Cornea: LAG-3$^{-/-}$ + $gB_{498-505}$ vaccine: 38% vs. LAG-3$^{-/-}$ + Mock: 32.2%, WT + $gB_{498-505}$ vaccine: 25.6%, WT + Mock: 19%; TG: LAG-3$^{-/-}$ + $gB_{498-505}$ vaccine: 28% vs. LAG-3$^{-/-}$ + Mock: 21.3%, WT + $gB_{498-505}$ vaccine: 16.2%, WT + Mock: 11%) were observed in combination therapy of LAG-3$^{-/-}$ + $gB_{498-505}$ vaccine group when compared with the other groups (**Figures 8A,C**). The corresponding average percentage and average absolute number of $gB_{498-505}$ tetramer specific CD8^{+} T cells; IFN-γ^{+} CD8^{+} T cells; CD107^{+}CD8^{+} T cells and Ki-67^{+}CD8^{+} T cells are also shown for all the groups (LAG-3$^{-/-}$ mice + $gB_{498-505}$, LAG-3$^{-/-}$ mice + Mock, WT mice + $gB_{498-505}$, WT mice + Mock) in cornea (**Figure 8B**) and TG (**Figure 8D**). Taken together, these results essentially substantiate our earlier observations on the blockade of LAG-3.

DISCUSSION

An immune-surveillance role for the trigeminal ganglia-resident HSV-specific CD8^{+} T cells that decrease virus reactivations has been established (9, 10, 28, 36–38). However, the mechanisms of CD8^{+} T cell dynamics in recurrent ocular herpetic disease remain to be fully elucidated. Key knowledge gaps that remain include: (*i*) How CD8^{+} T cells protect from reactivation, virus shedding, and recurrent disease; and (*ii*) The immune evasion

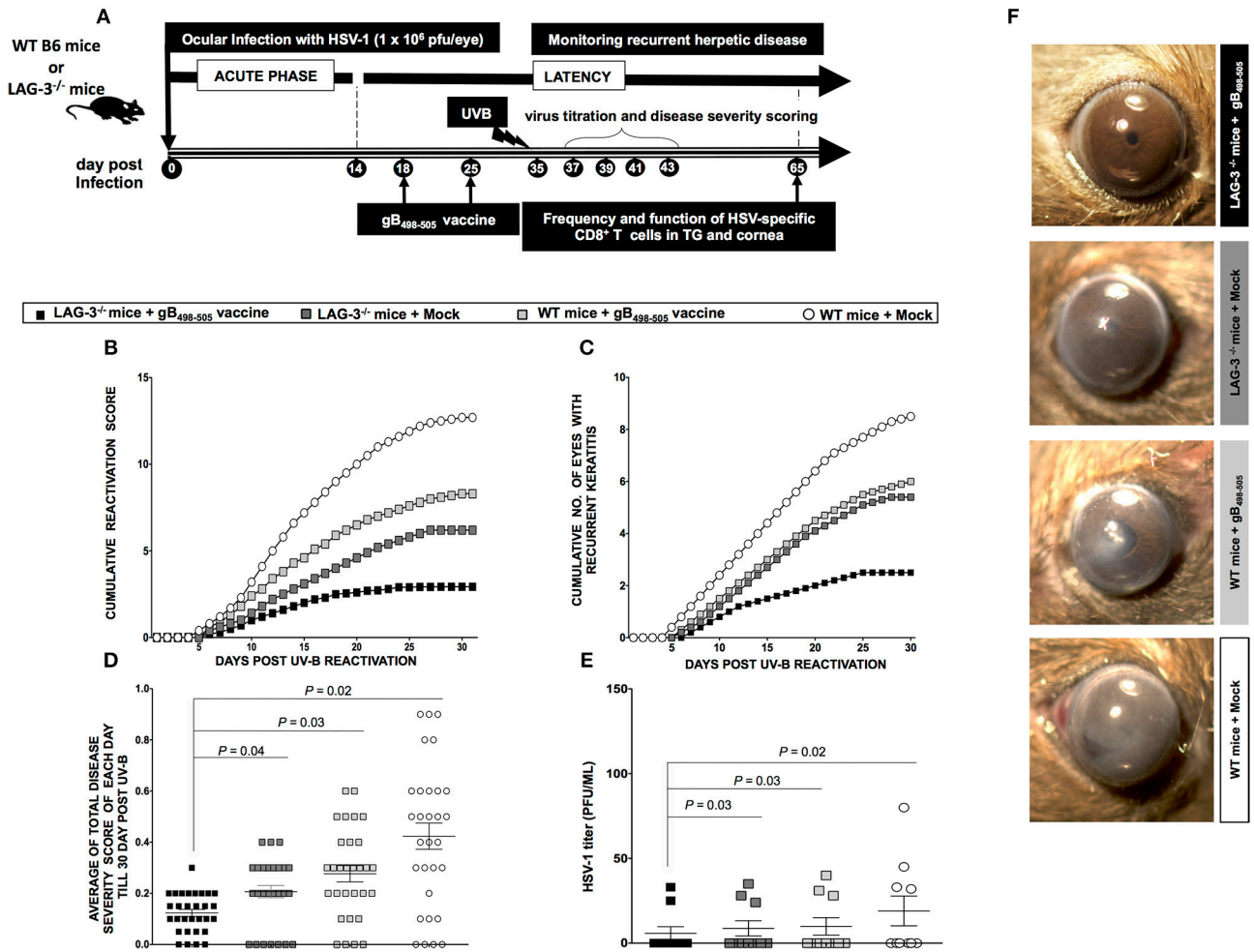


FIGURE 7 | LAG-3$^{-/-}$ mice show less recurrent ocular herpes compared to WT B6 mice following UV-B induced HSV-1 reactivation. **(A)** Schematic representation of UV-B induced reactivation, virological and immunological analyses, following HSV-1 ocular infection of 20 WT B6 and 20 LAG-3$^{-/-}$ mice (6–8 weeks old) on day 0 with 1 × 10^{6} pfu of HSV-1 (strain McKrae). In one group; WTs B6 mice (n = 10) and LAG-3$^{-/-}$ mice (n = 10) are immunized; while in the other group, WTs B6 (n = 10) and LAG-3$^{-/-}$ (n = 10) mice remain non-immunized. **(B)** The cumulative reactivation score till day 30 post UV-B. **(C)** The cumulative number of eyes showing recurrent disease till day 30 post UV-B. **(D)** Average of total eye disease score of each day till day 30 post UV-B. **(E)** Average viral shedding measured by plaque assay at day 8 post UV-B. **(F)** Representative eye pictures of recurrent disease. Results are representative of 2 independent experiments. The indicated P-values, calculated using the unpaired t-test, show statistical significance between LAG-3$^{-/-}$ + gB$_{498-505}$ and other groups.

strategies evolved by the virus as a counter-defense against the host's CD8+ T cells. How does the virus evade CD8^{+} T cell immune surveillance to allow efficient reactivation from latency? This report shows that: (*i*) Humans with recurrent clinical ocular herpes upregulate expression of the inhibitory receptors PD-1 and LAG-3 on their HSV-specific CD8^{+} T cells; (*ii*) Compared to ASYMP individuals, SYMP individuals have a significant number of PD-1^{+}CD8^{+} T cells and LAG-3^{+}CD8^{+} T cells; (*iii*) HSV-1 specific CD8^{+} T cells from SYMP patients, but not from healthy ASYMP individuals, were functionally exhausted; (*iv*) In mice latently infected with HSV-1(strain McKrae), there is an increase in cornea- and TG-resident LAG-3^{+}CD8^{+} and PD-1^{+}CD8^{+} T cells. LAG-3^{+}CD8^{+} T and PD-1^{+}CD8^{+} T cells increased in both TG and cornea as early as 3 days of acute infection, and on day 14-post-infection (just after the acute infection has cleared), there were significantly more LAG-3^{+}CD8^{+} T cells and PD-1^{+}CD8^{+} T cells in both the cornea and TG. However, on days 23 to 41, during latent infection, the number of both LAG-3^{+}CD8^{+} T cells and PD-1^{+}CD8^{+} T cells started to decline. To our knowledge, this is the first report showing: (*i*) LAG-3-related functional exhaustion of HSV-1 specific CD8^{+} T cells in both TG and cornea of latently infected mice; (*ii*) A significant increase in functionally exhausted LAG-3^{+}CD8^{+} T cells in SYMP patients as well as in B6 mice that developed increased virus shedding and severe recurrent corneal herpetic disease following UV-B induced reactivation.

This study also confirms our previous finding showing that HSV-1 infection in B6 mice results in accumulation of virus-specific exhaustion CD8^{+}T cells, expressing PD-1, in latently-infected trigeminal ganglia (TG) (10, 15, 28). More importantly,

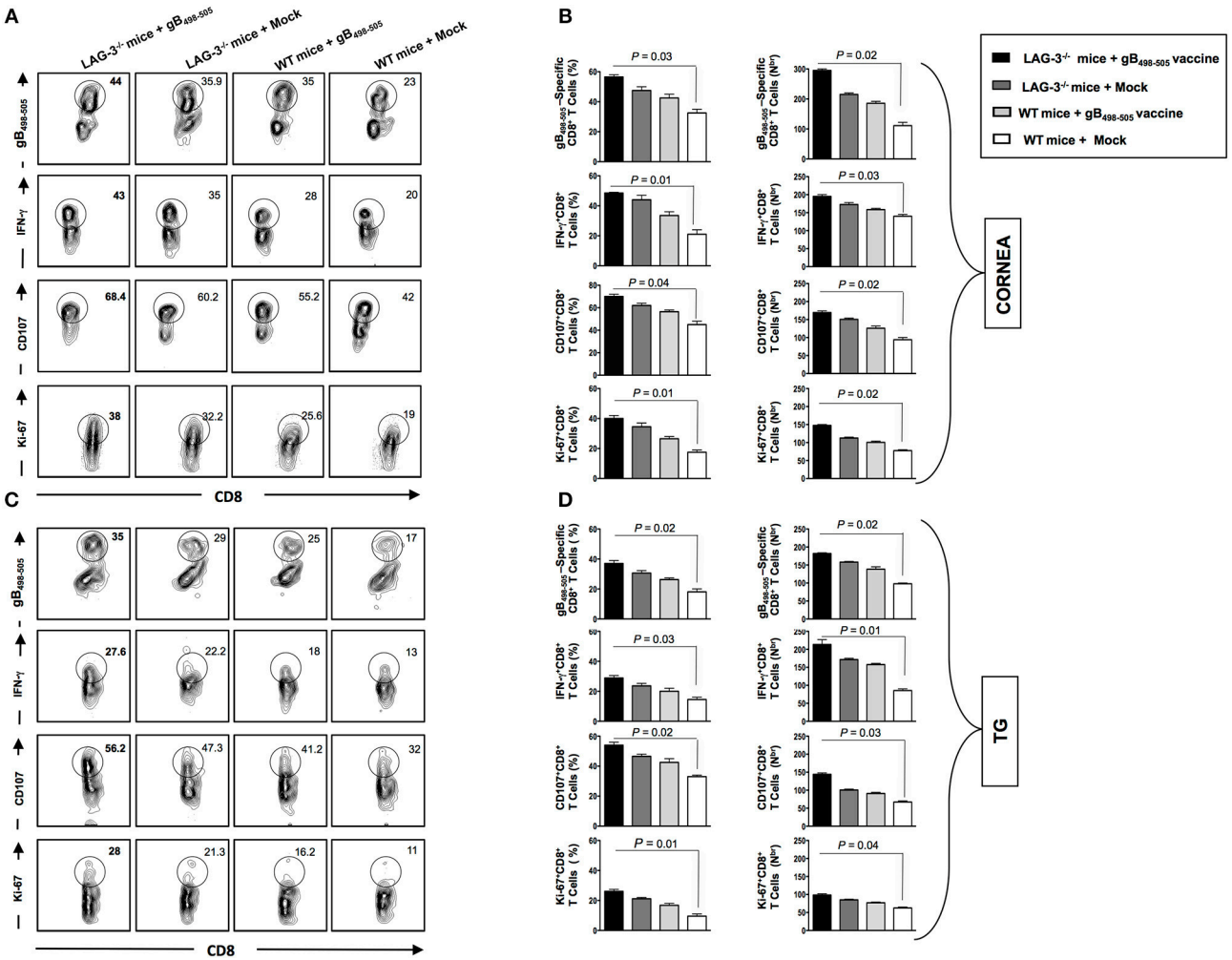


FIGURE 8 | Reduction in recurrent disease in LAG-3$^{-/-}$ mice is associated with better anti-viral immune response in cornea and TG. The mice are monitored till day 30 post UV-B and subsequently sacrificed to process cornea, and TG for immunological analysis as mentioned in the *Materials and Methods*. Representative FACS plots, average percentage and average absolute number of HSV- specific ($gB_{498-505}$) CD8^{+} T cells; HSV- specific IFNγ^{+}CD8^{+} T; HSV-specific CD107^{+}CD8^{+} T cells; HSV-specific Ki-67^{+}CD8^{+} T cells detected in **A,B** cornea and **(C,D)** TG are shown. The results are representative of 2 independent experiments. The indicated *P*-values, calculated using an unpaired *t*-test, show statistical significance between LAG-3$^{-/-}$ + $gB_{498-505}$ vaccine and mock groups.

we show that combination of a therapeutic LAG-3 blockade and therapeutic immunization restored the function of HSV-specific CD8^{+} T cells in cornea and TG associated with a reduction in recurrent ocular herpes, following UV-B induced reactivation in latently infected B6 mice. Thus, in addition to previously described immune evasion mechanisms (5, 8, 15, 28, 29, 29, 39–44), our data reveal a novel mechanism by which HSV-1 evades the protective host immune responses through dampening and dysregulating LAG-3^{+}CD8^{+} T cell function. Moreover, the LAG-3 pathway plays a fundamental role in ocular herpes T cell immunity, thus providing an important immune checkpoint target that can be combined with T cell-based therapeutic vaccines to improve protection against recurrent ocular herpes.

A protective CD8^{+} T cell response to viral infection depends upon T cell receptor (TCR) stimulation along with costimulatory signals (45). Dysregulation in positive and negative co-stimulatory signals affects the magnitude of CD8^{+} T cell response (45–47). The LAG-3 receptor is a negative T cell co-stimulatory molecule that is highly expressed on dysfunctional virus specific CD8^{+} T cells and interacts with MHC-II (48). In this report, we found that LAG-3 is highly expressed on HSV-specific CD8^{+} T cells from symptomatic (SYMP) individuals, in which HSV-1 reactivation often causes painful recurrent corneal disease (49–52). In contrast, LAG-3 was comparatively low on HSV-specific CD8^{+} T cells from asymptomatic (ASYMP) individuals in which virus reactivation never causes recurrent disease (31, 34, 52, 53). Our results in humans suggest that the magnitude of the HSV-specific CD8^{+} T cell immunity, after ocular HSV-1 reactivation, is subject to control by the LAG-3 pathway.

The high level of LAG-3 expression were detected on HSV-1 VP11/12$_{66-74}$ epitope-specific human CD8^{+} T cells as well

on CD8$^+$ T cells specific to four additional HSV-1 epitopes from VP13/14, gB, UL43, and UL44 proteins (Coulon et al., Manuscript in preparation). However, no upregulation was detected on bulk CD8$^+$ T cells, suggesting that the observed T cell exhaustion is restricted to herpes-specific LAG3$^+$CD8$^+$ T cells. The ultimate underlying molecular mechanism by which LAG-3 pathway led to CD8$^+$ T cell exhaustion is not defined by the present study. However, as illustrated in **Figure S2**, the findings are consistent with a potential role of LAG-3 pathway in exhaustion of HSV-specific CD8$^+$ T cells and that mAbs blocking of such pathways reverse such dysfunction associated with protection from recurrent ocular herpes. MHC class II is the main ligand of LAG-3 receptor (54). MHC-II appears to bind with higher affinity to LAG-3 compared to CD4 molecule a, competition that is expected to destabilized the TCR/CD4/MHC-II interaction (55, 56). Doing so, the LAG-3 pathway negatively regulates the function and homeostasis of CD4$^+$ T cells (57, 58). Moreover, LAG-3 pathway also regulates the function and homeostasis of CD8$^+$ T cells during chronic viral infection (48). The mechanisms by which LAG-3 pathway regulated CD8$^+$ T cells function/dysfunction remain to be fully elucidated. It is also unclear whether LAG-3 competition with MHC class II makes HSV-specific CD4$^+$ T cells more exhausted than HSV-specific CD8$^+$ T cells, mainly because of lack of CD4$^+$ T cell specific tetramers that would help quantify the function HSV-specific CD4$^+$ T cells. Nevertheless, we found that regulation of CD8$^+$ T cell exhaustion by LAG-3 and PD-1 inhibitory pathways was non-redundant, as blockade of the T cell inhibitory receptors LAG-3 and PD-1 simultaneously and synergistically improved CD8$^+$ T cell responses and diminished HSV-1 load and recurrent disease in HLA Tg mice (Roy et al., under review). Thus, antiviral CD8$^+$ T cell responses during herpes viral infection appears to be regulated by complex patterns of co-expressed inhibitory receptors.

During HSV-1 neuronal latency in the TG of mice and humans, a small number of latently infected neurons are surrounded by CD8$^+$ T cells (9, 59–61). Since CD8$^+$ T cells are presumably attracted to these neurons by viral antigens, it is assumed that the neurons surrounded by CD8$^+$ T cells are those in which the virus has initiated the early stages of reactivation from latency. In mice, experimental reactivation of HSV-1 from latency is typically accomplished by explanting TG into tissue culture media for up to 14 days and testing for the appearance of infectious (i.e., reactivated) virus (29, 29, 30). In this TG explant induced reactivation model, depleting CD8$^+$ T cells with specific mAb leads to the detection of more reactivated virus (9, 62). Conversely, addition of exogenous CD8$^+$ T cells reduces detection of reactivated virus (6, 9, 11). Thus, with wild type HSV-1, CD8$^+$ T cells in the TG are apparently able to reduce the detection of infectious reactivated virus. Following T cell activation *in vivo*, many co-stimulatory and inhibitory receptors are upregulated on T cells. It is possible that inhibitory interactions between the LAG-3 receptor on T cells and its respective ligand on APCs, such infected DCs and MΦ, at the time of priming are attenuating the effector T cell response. In fact, we found that HSV-1 infection of mice lacking LAG-3 expression on hematopoietic cells (i.e., LAG-3$^{-/-}$ deficient mice) generated more HSV-specific IFN-γ-producing cytotoxic CD107$^+$CD8$^+$ T cells compared to wild type (WT) infected mice. Moreover, we demonstrated that blocking the LAG-3 *in vivo* following administration of anti-LAG-3-specific mAbs at the time of T cell priming significantly enhanced the number of HSV-1 gB$_{498--505}$-specific CD8$^+$ T cells in resident tissues. We also demonstrated that blocking the LAG-3 pathway at the time of priming increases the frequency of IFN-γ-secreting HSV-1 gB$_{498--505}$-specific CD8$^+$ T cells and improves their cytotoxic potential. It is possible that the inhibitory receptor LAG-3 might be involved in regulating the effector function of HSV specific CD8$^+$ T cells.

The recent success of therapies targeting immune checkpoints to treat many cancers has driven a reappearance of interest in therapies targeting immune checkpoints against chronic infections and diseases (63–65). While still in its early stages, basic and clinical data suggest that blockade of CTLA-4 and PD-1 can be beneficial in the treatment of chronic HIV, HBV, and HCV infection, as well as other chronic diseases. Furthermore, novel inhibitory receptors such as TIM-3, LAG-3, and TIGIT are the potential next wave of checkpoints that can be manipulated for the treatment of chronic infections (64). However, caution should be taken when blocking immune checkpoint pathways that help keep the body's immune responses in check. Releasing the "brakes" on the immune system over-activate effector CD8$^+$ T cells that might cause tissue damage. Both PD-1 and LAG-3 play important roles during the normal immune response to prevent autoimmunity. Nevertheless, in the present study we found that mAbs therapies blocking LAG-3 immune checkpoint safely and efficiently led to significant reductions of recurrent corneal herpetic disease following UV-B induced reactivation in B6 mice latently infected with HSV-1. The improved clinical outcome of LAG-3 blockade in mice with established UV-B induced recurrent herpes was directly associated with a multifaceted enhancement of both the numbers of function antiviral CD8$^+$ T cells. Moreover, this report shows for the first time that a combination of a therapeutic blockade of LAG-3 immune checkpoint and a therapeutic vaccination leads to the generation of functional HSV-specific CD8$^+$ T cells in latently infected TG and cornea associated with an even more reduction in virus reactivation and recurrent disease in latently infected mice, following UV-B induced reactivation. No systemic or local side effects were observed following PD-1 and LAG-3 blockade in HSV-1 infected mice pointing to the safety of this treatment. The precise mechanisms by which LAG-3 blockade results in robust numerical and functional enhancement of effector CD8$^+$ T cells remain to be discovered. Our data also demonstrate that the "exhausted" phenotype (i.e., PD-1$^+$CD8$^+$ T cells and LAG-3$^+$CD8$^+$ T cells) was predominantly established prior to terminal cell differentiation, at the stage of memory-like T cells. It is likely that compartmentalization of inhibitory receptor expression predicts distinct cellular responses to inhibitory receptor blockade. For example, LAG-3 blockade may preferentially act on the terminally differentiated CD8$^+$ T cells. On the other hand, PD-1 blockade will act on both the memory-like CD8$^+$ T cells and on the terminally differentiated CD8$^+$ T cells. Regardless of the mechanisms, since HSV-1

specific CD8$^+$ T cells in SYMP individuals appeared to be functionally exhausted with a significant number of PD-1$^+$CD8$^+$ and LAG-3$^+$CD8$^+$ T cells, blockade of the LAG-3 and PD-1 signaling transduction pathways provide new therapeutic options for herpes infected symptomatic patients.

The immune checkpoints are often divided into a first and a second-generation (66-70). The classic examples of the first-generation checkpoints are PD-1/PD-L1 and CTLA4 and of the second-generation checkpoints are LAG-3, TIGIT, VISTA, and TIM-3. While blocking of the first-generation molecules is widely employed, a recent shift in focus toward targeting the second-generation molecules is noteworthy, as resistance to first generation therapies are amply reported and a combination of the two show synergistic and non-redundant effects. Some recent reports identify LAG-3, other than the widely studies PD-1, as a powerful inhibitory receptor whose blockade improves T cell immunity and limits diseases (45, 64, 66–68). Blackburn et al. (48) showed that T cell co-expressing multiple inhibitory receptors correlated with a more severe exhaustion and a greater disease load and blockade of LAG-3 and PD-1 show a synergistic effect. LAG-3 is shown to regulate homeostatic proliferation of CD8$^+$ T cells and potentiate the suppressor function of regulatory T cells. The KIEELE motif in the cytoplasmic region of LAG-3 has been shown to play a decisive role in its inhibitory effect, although the detailed mechanism is still poorly understood (69, 70).

In this report, we found that the percentage and absolute number of HSV specific IFN-γ^+CD8$^+$T cells, CD107$^+$CD8$^+$T cells and Ki-67$^+$CD8$^+$T cells were all significantly decreased in TG and cornea of latently infected mice following UV-B induced reactivation compared to latently infected mice with no induced reactivation (*data not shown*). This indicated phenotypic and functional exhaustion of both cytokine expression and cytotoxic activity of these CD8$^+$ T cells. To our knowledge, this is the first report to show significantly more phenotypically and functionally exhausted HSV-specific CD8$^+$ T cells in both the TG and cornea of latently infected mice following UV-B induced reactivation. Thus, our original hypotheses that increasing the number of exhausted CD8+ T cells in the TG and cornea led to HSV-1 escape from the control of CD8$^+$ T cells was correct.

The increased CD8$^+$ T cell exhaustion in TG and cornea of mice infected with HSV-1 could be due to increased viral antigens in these tissues. However, during latency in mouse TGs, less than 1 neuron/TG had detectable viral Ag by immunostaining (8, 71, 72). In addition, if spontaneous reactivation occurs in the mice TG, it is minimal (73). Thus, even though CD8$^+$ T cells are much more sensitive to Ag than the antibodies used for immunostaining, the very low un-sustained Ag level that appears to be the situation in the mice TGs is unlikely to result in exhaustion of CD8$^+$ T cells. Thus, it seems unlikely that the CD8$^+$ T cell exhaustion detected was due to the viral Ag load, unless additional factors contributed to immune stimulation. For example, cornea- and TG-resident HSV-specific CD8$^+$ T cells could have a higher functional avidity (ability to respond to low epitope density) than their counterparts in the periphery (18). Alternatively, CD8$^+$ T cell exhaustion may suggest that there is a lot more viral Ag present in the TG and cornea of mice latently infected with HSV-1 than has been previously thought. The HSV-gB$_{498-505}$-epitope and B6 mice were chosen to detect HSV-1 specific CD8$^+$ T cells in this study because in this mouse strain the majority (over 60%) of CD8$^+$ T cells are directed to this single immunodominant epitope (74–76). The phenotypic and functional exhaustion of CD8$^+$ T cells specific to other mouse HSV-1 or human CD8$^+$ T cell epitopes in humans still remains to be determined. Using our HLA transgenic mouse model (6, 77), we are currently in the process of assessing the exhaustion of CD8$^+$ T cells specific to a set of immunodominant and sub-dominant human CD8$^+$ T cell HSV-1 epitopes that we have recently identified as being recognized by these animals.

Both SYMP and ASYMP individuals shed the virus in tears as a result of sporadic reactivation, but only the SYMP individuals manifest lifelong recurrences of herpetic disease, usually multiple times a year and often require continuous antiviral therapy (i.e., Acyclovir and derivatives). In this study, we applied blockade during a brief span following the UV-B induced reactivation and as our results suggest, an appropriate strategy to limit recurrent keratitis in SYMP humans, would be to monitor the SYMP patients for reactivation episodes and apply the blockade therapy during those brief phases of recurrences. Prior therapeutic immunization is expected to reinforce the effect of the blockade. Ideally, several rounds of treatment are expected to boost the generation of polyfunctional CD8$^+$ T cells in the TG and cornea, improving their versatility. As impaired T cells responses are among the potential causes of symptomatic shedding (3, 78, 79), generation of sturdier polyfunctional T cells in the TG and cornea is expected to restrain or even nullify future harmful reactivation episodes. However, translational hurdles of the study are the safety and timing of the therapy. Another hurdle would be the delivery of mAbs in appropriate amounts to the immunologically recalcitrant sites, TG and cornea. Since our observations indirectly presuppose a significant delivery of mAbs to TG and cornea, it is likely that the timing of delivery is a crucial. In addition, inflammation associated with reactivation episodes increases tissue permittivity. Thus, with optimum dosage through right route of administration at an appropriate time of recurrences will likely make for best delivery of blocking mAbs to the targeted tissues. Such optimal delivery would interfere with virus reactivation from TG and stop or at least reduce recurrent corneal herpetic disease.

In summary, the present study demonstrates, for the first time, that the cornea and TG from HSV-1 infected mice, with UV-B-induced recurrent corneal disease, present more exhausted HSV-specific PD-1$^+$CD8$^+$ T cells and LAG-3$^+$CD8$^+$ T cells. Since functional HSV-specific CD8$^+$ T cells appear to be important in decreasing reactivation from latency (9), the higher number of functional HSV-specific CD8$^+$ T cells was detected following treatment with mAbs that block the LAG-3 pathway associated reduced reactivation and less severe recurrent disease as compared to mock-treated mice. This is also the first study to report that combination of the LAG-3 immune checkpoint blockade together with a therapeutic vaccination leads to generation of functional HSV-specific CD8$^+$ T cells in latently infected TG and cornea associated with even more reduction in virus reactivation and recurrent disease. Blockade of the LAG-3 pathway in combination with vaccination may

have great therapeutic promise and open up the possibilities of designing novel combination therapies. This includes therapeutic vaccination and the blockade of T cell exhaustion in confronting HSV-1 reactivation and cure of potentially blinding recurrent ocular herpes.

ETHICS STATEMENT

The manuscript, which has not been submitted elsewhere, does contain both human studies and animal studies, which conform to the Guides for IRB and IACUC published by the US National Institute of Health.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: SR, P-GC, RS, LB. Performed the experiments: SR, P-GC, RS, HV, GK, SW, TY, MF, VL. Analyzed the data: SR, P-GC, RS, LB. Contributed reagents, materials, analysis tools: SR, P-GC, RS, LB. Wrote the paper: SR, P-GC, RS, LB.

FUNDING

This work is supported by Public Health Service Research R01 Grants EY026103, EY019896 and EY024618 from National Eye Institute (NEI) and R21 Grant AI110902 from National Institutes of allergy and Infectious Diseases (NIAID), by The Discovery Center for Eye Research (DCER) and by a Research to Prevent Blindness (RPB) grant.

ACKNOWLEDGMENTS

This work is dedicated to the memory of late Professor Steven L. Wechsler Steve (1948–2016), whose numerous pioneering works on herpes infection and immunity laid the foundation to this line of research. We thank the NIH Tetramer Facility (Emory University, Atlanta, GA) for providing the Tetramers used in this study; Diane Capobianco (RN) from UC Irvine's Institute for Clinical and Translational Science (ICTS) for helping with blood drawing from HSV-1 seropositive symptomatic and asymptomatic individuals and Dr. Prakash, Swayam for help in editing this manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02922/full#supplementary-material

REFERENCES

1. Samandary S, Kridane-Miledi H, Sandoval JS, Choudhury Z, Langa-Vives F, Spencer D, et al. Associations of HLA-A, HLA-B and HLA-C alleles frequency with prevalence of herpes simplex virus infections and diseases across global populations: implication for the development of an universal CD8+ T-cell epitope-based vaccine. *Hum Immunol.* (2014) 75:715–29. doi: 10.1016/j.humimm.2014.04.016
2. Srivastava R, Khan AA, Spencer D, Vahed H, Lopes PP, Thai NT, et al. HLA-A02:01-restricted epitopes identified from the herpes simplex virus tegument protein VP11/12 preferentially recall polyfunctional effector memory CD8+ T cells from seropositive asymptomatic individuals and protect humanized HLA-A*02:01 transgenic mice against ocular herpes. *J Immunol.* (2015) 194:2232–48. doi: 10.4049/jimmunol.1402606
3. Srivastava R, Hernandez-Ruiz M, Khan AA, Fouladi MA, Kim GJ, Ly VT, et al. CXCL17 chemokine-dependent mobilization of CXCR8(+)CD8(+) effector memory and tissue-resident memory T cells in the vaginal mucosa is associated with protection against genital herpes. *J Immunol.* (2018). 200:2915–26. doi: 10.4049/jimmunol.1701474
4. Agelidis AM, Shukla D. Cell entry mechanisms of HSV: what we have learned in recent years. *Fut Virol.* (2015) 10:1145–54. doi: 10.2217/fvl.15.85
5. Mott KR, Bresee CJ, Allen SJ, BenMohamed L, Wechsler SL, Ghiasi, H. Level of herpes simplex virus type 1 latency correlates with severity of corneal scarring and exhaustion of CD8+ T cells in trigeminal ganglia of latently infected mice. *J Virol.* (2009) 83:2246–54. doi: 10.1128/JVI.02234-08
6. Chentoufi AA, Dasgupta G, Christensen ND, Hu J, Choudhury ZS, Azeem A, et al. A novel HLA (HLA-A*0201) transgenic rabbit model for preclinical evaluation of human CD8+ T cell epitope-based vaccines against ocular herpes. *J Immunol.* (2010) 184:2561–71. doi: 10.4049/jimmunol.0902322
7. Chentoufi AA, Zhang X, Lamberth K, Dasgupta G, Bettahi I, Nguyen A, et al. HLA-A*0201-restricted CD8+ cytotoxic T lymphocyte epitopes identified from herpes simplex virus glycoprotein D. *J Immunol.* (2008) 180:426–37. doi: 10.4049/jimmunol.180.1.426
8. Allen SJ, Hamrah P, Gate DM, Mott KR, Mantopoulos D, Zheng L, et al. The role of LAT in increased CD8+ T cell exhaustion in trigeminal ganglia of mice latently infected with herpes simplex virus type 1. *J Virol.* (2011) 85:4184–97. doi: 10.1128/JVI.02290-10
9. Knickelbein JE, Khanna KM, Yee MB, Baty CJ, Kinchington PR, Hendricks RL. Noncytotoxic lytic granule-mediated CD8+ T cell inhibition of HSV-1 reactivation from neuronal latency. *Science* (2008) 322:268–71. doi: 10.1126/science.1164164
10. Chentoufi AA, Dervillez X, Dasgupta G, Nguyen C, Kabbara KW, Jiang X, et al. The herpes simplex virus type 1 latency-associated transcript inhibits phenotypic and functional maturation of dendritic cells. *Viral Immunol.* (2012) 25:204–15. doi: 10.1089/vim.2011.0091
11. Hoshino Y, Pesnicak L, Cohen JI, Straus SE. Rates of reactivation of latent herpes simplex virus from mouse trigeminal ganglia *ex vivo* correlate directly with viral load and inversely with number of infiltrating CD8+ T cells. *J Virol.* (2007) 81:8157–64. doi: 10.1128/JVI.00474-07
12. Mueller SN, Ahmed R. High antigen levels are the cause of T cell exhaustion during chronic viral infection. *Proc Natl Acad Sci USA.* (2009) 106:8623–8. doi: 10.1073/pnas.0809818106
13. Wherry EJ, Ha SJ, Kaech SM, Haining WN, Sarkar S, Kalia V, et al. Molecular signature of CD8+ T cell exhaustion during chronic viral infection. *Immunity* (2007) 27:670–84. doi: 10.1016/j.immuni.2007.09.006
14. Khan AA, Srivastava R, Chentoufi AA, Geertsema R, Thai NT, Dasgupta G, et al. Therapeutic immunization with a mixture of herpes simplex virus 1 glycoprotein D-derived "asymptomatic" human CD8+ T-cell epitopes decreases spontaneous ocular shedding in latently infected HLA transgenic rabbits: association with low frequency of local PD-1+ TIM-3+ CD8+ exhausted T cells. *J Virol.* (2015) 89:6619–32. doi: 10.1128/JVI.00788-15
15. Srivastava R, Dervillez X, Khan AA, Chentoufi AA, Chilukuri S, Shukr N, et al. The herpes simplex virus latency-associated transcript gene is associated with a broader repertoire of virus-specific exhausted CD8+ T cells retained within the trigeminal ganglia of latently infected HLA transgenic rabbits. *J Virol.* (2016) 90:3913–28. doi: 10.1128/JVI.02450-15

16. Day CL, Kaufmann DE, Kiepiela P, Brown JA, Moodley ES, Reddy S, et al. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* (2006) 443:350–4. doi: 10.1038/nature 05115
17. Barber DL, Wherry EJ, Masopust D, Zhu B, Allison JP, Sharpe AH, et al. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* (2006) 439:682–7. doi: 10.1038/nature04444
18. Frank GM, Lepisto AJ, Freeman ML, Sheridan BS, Cherpes TL, Hendricks RL. Early CD4(+) T cell help prevents partial CD8(+) T cell exhaustion and promotes maintenance of Herpes Simplex Virus 1 latency. *J Immunol.* (2010) 184:277–86. doi: 10.4049/jimmunol.0902373
19. Allen SJ, Mott KR, Zandian M, Ghiasi H. Immunization with different viral antigens alters the pattern of T cell exhaustion and latency in herpes simplex virus type 1-infected mice. *J Virol.* (2010) 84:12315–24. doi: 10.1128/JVI.01600-10
20. Teigler JE, Zelinskyy G, Eller MA, Bolton DL, Marovich M, Gordon AD, et al. Differential inhibitory receptor expression on T cells delineates functional capacities in chronic viral infection. *J Virol.* (2017) 91:e1263–1217. doi: 10.1128/JVI.01263-17
21. Wherry EJ. T cell exhaustion. *Nat Immunol.* (2011) 12:492–9. doi: 10.1038/ni.2035
22. Anderson AC, Joller N, Kuchroo VK. Lag-3, Tim-3, and TIGIT: co-inhibitory receptors with specialized functions in immune regulation. *Immunity* (2016) 44:989–1004. doi: 10.1016/j.immuni.2016.05.001
23. Held K, Junker A, Dornmair K, Meinl E, Sinicina I, Brandt T, et al. Expression of herpes simplex virus 1-encoded microRNAs in human trigeminal ganglia and their relation to local T-cell infiltrates. *J Virol.* (2011) 85:9680–5. doi: 10.1128/JVI.00874-11
24. Held K, Derfuss T. Control of HSV-1 latency in human trigeminal ganglia-current overview. *J Neurovirol.* (2011) 17:518–27. doi: 10.1007/s13365-011-0063-0
25. van Velzen M, Laman JD, Kleinjan A, Poot A, Osterhaus AD, Verjans GM. Neuron-interacting satellite glial cells in human trigeminal ganglia have an APC phenotype. *J Immunol.* (2009) 183:2456–61. doi: 10.4049/jimmunol.0900890
26. Derfuss T, Arbusow V, Strupp M, Brandt T, Theil D. The presence of lytic HSV-1 transcripts and clonally expanded T cells with a memory effector phenotype in human sensory ganglia. *Ann N Y Acad Sci.* (2009) 1164:300–4. doi: 10.1111/j.1749-6632.2009.03871.x
27. Theil D, Derfuss T, Paripovic I, Herberger S, Meinl E, Schueler O, et al. Latent herpesvirus infection in human trigeminal ganglia causes chronic immune response. *Am J Pathol.* (2003) 163:2179–84. doi: 10.1016/S0002-9440(10)63575-4
28. Chentoufi AA, Kritzer E, Tran MV, Dasgupta G, Lim CH, Yu DC, et al. The herpes simplex virus 1 latency-associated transcript promotes functional exhaustion of virus-specific CD8+ T cells in latently infected trigeminal ganglia: a novel immune evasion mechanism. *J Virol.* (2011) 85:9127–38. doi: 10.1128/JVI.00587-11
29. BenMohamed L, Osorio N, Srivastava R, Khan AA, Simpson JL, Wechsler SL. Decreased reactivation of a herpes simplex virus type 1 (HSV-1) latency-associated transcript (LAT) mutant using the *in vivo* mouse UV-B model of induced reactivation. *J Neurovirol.* (2015) 21:508–17. doi: 10.1007/s13365-015-0348-9
30. BenMohamed L, Osorio N, Khan AA, Srivastava R, Huang L, Krochma JJ, et al. Prior corneal scarification and injection of immune serum are not required before ocular HSV-1 infection for UV-B induced virus reactivation and recurrent herpetic corneal disease in latently infected mice. *J Neurovirol.* (2015) 41:747–56. doi: 10.3109/02713683.2015.1061024
31. Chentoufi AA, Binder NR, Berka N, Durand G, Nguyen A, Bettahi I, et al. Asymptomatic human CD4+ cytotoxic T-cell epitopes identified from herpes simplex virus glycoprotein B. *J Virol.* (2008) 82:11792–802. doi: 10.1128/JVI.00692-08
32. Long D, Skoberne M, Gierahn TM, Larson S, Price JA, Clemens V, et al. Identification of novel virus-specific antigens by CD4(+) and CD8(+) T cells from asymptomatic HSV-2 seropositive and seronegative donors. *Virology* (2014) 464–465:296–311. doi: 10.1016/j.virol.2014.07.018
33. Hosken N, McGowan P, Meier A, Koelle DM, Sleath P, Wagener F, et al. Diversity of the CD8+ T-cell response to herpes simplex virus type 2 proteins among persons with genital herpes. *J Virol.* (2006) 80:5509–15. doi: 10.1128/JVI.02659-05
34. Dasgupta G, Chentoufi AA, Kalantari M, Falatoonzadeh P, Chun S, Lim CH, et al. Immunodominant "asymptomatic" herpes simplex virus 1 and 2 protein antigens identified by probing whole-ORFome microarrays with serum antibodies from seropositive asymptomatic versus symptomatic individuals. *J Virol.* (2012) 86:4358–69. doi: 10.1128/JVI.07107-11
35. Kalantari-Dehaghi M, Chun S, Chentoufi AA, Pablo J, Liang L, Dasgupta G, et al. Discovery of potential diagnostic and vaccine antigens in herpes simplex virus 1 and 2 by proteome-wide antibody profiling. *J Virol.* (2012) 86:4328–39. doi: 10.1128/JVI.05194-11
36. St Leger AJ, Hendricks RL. CD8(+) T cells patrol HSV-1-infected trigeminal ganglia and prevent viral reactivation. *J Neurovirol.* (2011) 17:528–34. doi: 10.1007/s13365-011-0062-1
37. St Leger AJ, Jeon S, Hendricks RL. Broadening the repertoire of functional herpes simplex virus type 1-specific CD8+ T cells reduces viral reactivation from latency in sensory ganglia. *J Immunol.* (2013) 191:2258–65. doi: 10.4049/jimmunol.1300585
38. Jeon S, St Leger AJ, Cherpes TL, Sheridan BS, Hendricks RL. PD-L1/B7-H1 regulates the survival but not the function of CD8+ T cells in herpes simplex virus type 1 latently infected trigeminal ganglia. *J Immunol.* (2013) 190:6277–86. doi: 10.4049/jimmunol.1300582
39. Allen SJ, Mott KR, Chentoufi AA, BenMohamed L, Wechsler SL, Ballantyne CM, et al. CD11c controls herpes simplex virus 1 responses to limit virus replication during primary infection. *J Virol.* (2011) 85:9945–55. doi: 10.1128/JVI.05208-11
40. Mott KR, Chentoufi AA, Carpenter D, BenMohamed L, Wechsler SL, Ghiasi H. The role of a glycoprotein K (gK) CD8+ T-cell epitope of herpes simplex virus on virus replication and pathogenicity. *Invest Ophthalmol Vis Sci.* (2009) 50:2903–12. doi: 10.1167/iovs.08-2957
41. Perng GC, Maguen B, Jin L, Mott KR, Kurylo J, BenMohamed L, et al. A novel herpes simplex virus type 1 transcript (AL-RNA) antisense to the 5′ end of the latency-associated transcript produces a protein in infected rabbits. *J Virol.* (2002) 76:8003–10. doi: 10.1128/JVI.76.16.8003-8010.2002
42. Smiley JR. Herpes simplex virus virion host shutoff protein: immune evasion mediated by a viral RNase? *J Virol.* (2004) 78:1063–8. doi: 10.1128/JVI.78.3.1063-1068.2004
43. Carr DJ, Harle P, Gebhardt BM. The immune response to ocular herpes simplex virus type 1 infection. *Exp Biol Med.* (2001) 226:353–66. doi: 10.1177/153537020122600501
44. Paladino P, Mossman KL. Mechanisms employed by herpes simplex virus 1 to inhibit the interferon response. *J Interferon Cytokine Res.* (2009) 29:599–607. doi: 10.1089/jir.2009.0074
45. Chihara N, Madi A, Kondo T, Zhang H, Acharya N, Singer M, et al. Induction and transcriptional regulation of the co-inhibitory gene module in T cells. *Nature* (2018) 558:454–9. doi: 10.1038/s41586-018-0206-z
46. Stojanovic A, Correia MP, Cerwenka A. The NKG2D/NKG2DL axis in the crosstalk between lymphoid and myeloid cells in health and disease. *Front Immunol.* (2018) 9:827. doi: 10.3389/fimmu.2018.00827
47. Vingerhoets J, Dohlsten M, Penne G, Colebunders R, Sansom D, Bosmans E, et al. Superantigen activation of CD4+ and CD8+T cells from HIV-infected subjects: role of costimulatory molecules and antigen-presenting cells (APC). *Clin Exp Immunol.* (1998) 111:12–9. doi: 10.1046/j.1365-2249.1998.00465.x
48. Blackburn SD, Shin H, Haining WN, Zou T, Workman CJ, Polley A, et al. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nat Immunol.* (2009) 10:29–37. doi: 10.1038/ni.1679
49. Schiffer JT, Abu-Raddad L, Mark KE, Zhu J, Selke S, Koelle DM, et al. Mucosal host immune response predicts the severity and duration of herpes simplex virus-2 genital tract shedding episodes. *Proc Natl Acad Sci USA.* (2010) 107:18973–8. doi: 10.1073/pnas.1006614107
50. Dervillez X, Qureshi H, Chentoufi AA, Khan AA, Kritzer K, Yu DC, et al. "Asymptomatic" HLA-A*02:01-restricted epitopes from herpes simplex virus glycoprotein B preferentially recall polyfunctional CD8+ T cells from seropositive asymptomatic individuals and protect HLA transgenic mice against ocular herpes. *J Immunol.* (2013) 191:5124–38. doi: 10.4049/jimmunol.1301415

51. Dervillez X, Gottimukkala C, Kabbara KW, Nguyen C, Badakhshan T, Kim SM, et al. Future of an "asymptomatic" T-cell epitope-based therapeutic herpes simplex vaccine. *Fut Virol.* (2012) 7:371–8. doi: 10.2217/fvl.12.22
52. Dasgupta G, Nesburn AB, Wechsler SL, BenMohamed L. Developing an asymptomatic mucosal herpes vaccine: the present and the future. *Fut Microbiol.* (2010) 5:1–4. doi: 10.2217/fmb.09.101
53. Chentoufi AA, BenMohamed L. Future viral vectors for the delivery of asymptomatic herpes epitope-based immunotherapeutic vaccines. *Fut Virol.* (2010) 5:525–8. doi: 10.2217/fvl.10.44
54. Huard B, Mastrangeli R, Prigent P, Bruniquel D, Donini S, El-Tayar N, et al. Characterization of the major histocompatibility complex class II binding site on LAG-3 protein. *Proc Natl Acad Sci USA.* (1997) 94:5744–9. doi: 10.1073/pnas.94.11.5744
55. Huard B, Prigent P, Pages F, Bruniquel D, Triebel F. T cell major histocompatibility complex class II molecules down-regulate CD4+ T cell clone responses following LAG-3 binding. *Eur J Immunol.* (1996) 26:1180–6. doi: 10.1002/eji.1830260533
56. Huard B, Prigent P, Tournier M, Bruniquel D, Triebel F. CD4/major histocompatibility complex class II interaction analyzed with CD4- and lymphocyte activation gene-3 (LAG-3)-Ig fusion proteins. *Eur J Immunol.* (1995) 25:2718–21. doi: 10.1002/eji.1830250949
57. Huard B, Tournier M, Hercend T, Triebel F, Faure F. Lymphocyte-activation gene 3/major histocompatibility complex class II interaction modulates the antigenic response of CD4+ T lymphocytes. *Eur J Immunol.* (1994) 24:3216–21. doi: 10.1002/eji.1830241246
58. Huard B, Gaulard P, Faure F, Hercend T, Triebel F. Cellular expression and tissue distribution of the human LAG-3-encoded protein, an MHC class II ligand. *Immunogenetics* (1994) 39:213–7. doi: 10.1007/BF002 41263
59. Verjans GM, Hintzen RQ, van Dun JM, Poot A, Milikan JC, Laman JD, et al. Selective retention of herpes simplex virus-specific T cells in latently infected human trigeminal ganglia. *Proc Natl Acad Sci USA.* (2007) 104:3496–501. doi: 10.1073/pnas.0610847104
60. Theil D, Arbusow V, Derfuss T, Strupp M, Pfeiffer M, Mascolo A, et al. Prevalence of HSV-1 LAT in human trigeminal, geniculate, and vestibular ganglia and its implication for cranial nerve syndromes. *Brain Pathol.* (2001) 11:408–13. doi: 10.1111/j.1750-3639.2001.tb00408.x
61. Derfuss T, Segerer S, Herberger S, Sinicina I, Hufner K, Ebelt K, et al. Presence of HSV-1 immediate early genes and clonally expanded T-cells with a memory effector phenotype in human trigeminal ganglia. *Brain Pathol.* (2007) 17:389–98. doi: 10.1111/j.1750-3639.2007.00088.x
62. Miyajima K, Knickelbein MB, Nakajima A. Stern-gerlach study of multidecker lanthanide-cyclooctatetraene sandwich clusters. *J Phys Chem A* (2008) 112:366–75. doi: 10.1021/jp0766196
63. Kim N, Kim HS. Targeting checkpoint receptors and molecules for therapeutic modulation of natural killer cells. *Front Immunol.* (2018) 9:041. doi: 10.3389/fimmu.2018.02041
64. De Sousa Linhares, Leitner J, Grabmeier-Pfistershammer K, Steinberger P. Not all immune checkpoints are created equal. *Front Immunol.* (2018) 9:1909. doi: 10.3389/fimmu.2018.01909
65. Vilgelm AE, Johnson DB, Richmond A. Combinatorial approach to cancer immunotherapy: strength in numbers. *J Leukoc Biol.* (2016) 100:275–90. doi: 10.1189/jlb.5RI0116-013RR
66. Lichtenegger FS, Rothe M, Schnorfeil FM, Deiser K, Krupka C, Augsberger C, et al. Targeting LAG-3 and PD-1 to enhance T cell activation by antigen-presenting cells. *Front Immunol.* (2018) 9:385. doi: 10.3389/fimmu.2018.00385
67. Foy SP, Sennino B, dela Cruz T, Cote JJ, Gordon EJ, Kemp F, et al. Poxvirus-based active immunotherapy with PD-1 and LAG-3 dual immune checkpoint inhibition overcomes compensatory immune regulation, yielding complete tumor regression in mice. *PLoS ONE* (2016) 11:e0150084. doi: 10.1371/journal.pone.0150084
68. Durham NM, Nirschl CJ, Jackson CM, Elias J, Kochel CM, Anders RA, et al. Lymphocyte activation gene 3 (LAG-3) modulates the ability of CD4 T-cells to be suppressed *in vivo*. *PLoS ONE* (2014) 9:e109080. doi: 10.1371/journal.pone.0109080
69. Workman CJ, Vignali DA. The CD4-related molecule, LAG-3 (CD223), regulates the expansion of activated T cells. *Eur J Immunol.* (2003) 33:970–9. doi: 10.1002/eji.200323382
70. Workman CJ, Dugger KJ, Vignali DA. Cutting edge: molecular analysis of the negative regulatory function of lymphocyte activation gene-3. *J Immunol.* (2002) 169:5392–5. doi: 10.4049/jimmunol.169.10.5392
71. Ellison AR, Yang L, Voytek C, Margolis TP. Establishment of latent herpes simplex virus type 1 infection in resistant, sensitive, and immunodeficient mouse strains. *Virology* (2000) 268:17–28. doi: 10.1006/viro.1999.0158
72. Feldman LT, Ellison AR, Voytek CC, Yang L, Krause P, Margolis TP. Spontaneous molecular reactivation of herpes simplex virus type 1 latency in mice. *Proc Natl Acad Sci USA.* (2002) 99:978–83. doi: 10.1073/pnas.022301899
73. Gebhardt BM, Halford WP. Evidence that spontaneous reactivation of herpes virus does not occur in mice. *Virol J.* (2005) 2:67–73. doi: 10.1186/1743-422X-2-67
74. Blaney JE Jr, Nobusawa E, Brehm MA, Bonneau RH, Mylin LM, Fu TM, et al. Immunization with a single major histocompatibility complex class I-restricted cytotoxic T-lymphocyte recognition epitope of herpes simplex virus type 2 confers protective immunity. *J Virol.* (1998) 72:9567–74.
75. Bonneau RH, Salvucci LA, Johnson DC, Tevethia SS. Epitope specificity of H-2Kb-restricted, HSV-1-, and HSV-2-cross-reactive cytotoxic T lymphocyte clones. *Virology* (1993) 195:62–70. doi: 10.1006/viro.1993.1346
76. Cose SC, Kelly JM, Carbone FR. Characterization of diverse primary herpes simplex virus type 1 gB-specific cytotoxic T-cell response showing a preferential V beta bias. *J Virol.* (1995) 69:5849–52.
77. Dasgupta G, Chentoufi AA, Nesburn AB, Wechsler SL, BenMohamed L. New concepts in herpes simplex virus vaccine development: notes from the battlefield. *Expert Rev Vaccines* (2009) 8:1023–35. doi: 10.1586/erv.09.60
78. Srivastava R, Khan AA, Garg S, Syed SA, Furness JN, Vahed H, et al. Human asymptomatic epitopes identified from the herpes simplex virus tegument protein VP13/14 (UL47) preferentially recall polyfunctional effector memory CD44high CD62Llow CD8+ TEM cells and protect humanized HLA-A*02:01 transgenic mice against ocular herpesvirus infection. *J Virol.* (2017) 91:e01793-16. doi: 10.1128/JVI.01793-16
79. Srivastava R, Khan AA, Chilukuri S, Syed SA, Tran TT, Furness J, et al. CXCL10/CXCR3-dependent mobilization of herpes simplex virus-specific CD8+ TEM and CD8+ TRM cells within infected tissues allows efficient protection against recurrent herpesvirus infection and disease. *J Virol.* (2017) 91:e00278-17. doi: 10.1128/JVI.00278-17

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 19 December 2018
doi: 10.3389/fimmu.2018.02932

Herpes Simplex Virus Type 2 Infection-Induced Expression of CXCR3 Ligands Promotes CD4$^+$ T Cell Migration and Is Regulated by the Viral Immediate-Early Protein ICP4

Mudan Zhang[1], Xu Deng[2,3], Xinmeng Guan[2,3], Lanlan Geng[4], Ming Fu[2,3], Binman Zhang[2,3], Rui Chen[2], Huimin Hu[2,3], Kai Hu[2], Di Zhang[2,3], Mei Li[2,3], Yalan Liu[2], Sitang Gong[4] and Qinxue Hu[2,5]**

[1] The Joint Center of Translational Precision Medicine, Guangzhou Institute of Pediatrics, Guangzhou Women and Children's Medical Center, Wuhan Institute of Virology, Chinese Academy of Science, Wuhan, China, [2] State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China, [3] University of Chinese Academy of Sciences, Beijing, China, [4] Department of Gastroenterology, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, Guangzhou, China, [5] Institute for Infection and Immunity, St George's University of London, London, United Kingdom

OPEN ACCESS

Edited by:
Richard D. Dix,
Georgia State University,
United States

Reviewed by:
Deepak Shukla,
University of Illinois at Chicago,
United States
Lbachir Benmohamed,
University of California, Irvine,
United States

***Correspondence:**
Qinxue Hu
qhu@wh.iov.cn
Sitang Gong
sitangg@126.com

Specialty section:
This article was submitted to Viral Immunology, a section of the journal Frontiers in Immunology

Received: 16 July 2018
Accepted: 29 November 2018
Published: 19 December 2018

Citation:
Zhang M, Deng X, Guan X, Geng L, Fu M, Zhang B, Chen R, Hu H, Hu K, Zhang D, Li M, Liu Y, Gong S and Hu Q (2018) Herpes Simplex Virus Type 2 Infection-Induced Expression of CXCR3 Ligands Promotes CD4$^+$ T Cell Migration and Is Regulated by the Viral Immediate-Early Protein ICP4. Front. Immunol. 9:2932. doi: 10.3389/fimmu.2018.02932

HSV-2 infection-induced CXCR3 ligands are important for the recruitment of virus-specific CD8$^+$ T cells, but their impact on CD4$^+$ T cell trafficking remains to be further determined. Given that recruitment of CD4$^+$ T cells to infection areas may be one of the mechanisms that account for HSV-2 infection-mediated enhancement of HIV-1 sexual transmission, here we investigated the functionality of HSV-2 infection-induced CXCR3 ligands CXCL9, CXCL10, and CXCL11 *in vivo* and *in vitro*, and determined the viral components responsive for such induction and the underlying mechanisms. We first found that the expression of CXCR3 ligands CXCL9, CXCL10, and CXCL11 was increased in mice following vaginal challenge with HSV-2, while CXCL9 played a predominant role in the recruitment of CD4$^+$ T cells to the vaginal foci of infected mice. HSV-2 infection also induced the production of CXCL9, CXCL10, and CXCL11 in human cervical epithelial cells. Of note, although HSV-2 induced the expression of all the three CXCR3 ligands, the induced CXCL9 appeared to play a predominant role in promoting CD4$^+$ T cell migration, reflecting that the concentrations of CXCL10 and CXCL11 required for CD4$^+$ T cell migration are higher than that of CXCL9. We further revealed that, ICP4, an immediate-early protein of HSV-2, is crucial in promoting CXCR3 ligand expression through the activation of p38 MAPK pathway. Mechanistically, ICP4 binds to corresponding promoters of CXCR3 ligands via interacting with the TATA binding protein (TBP), resulting in the transcriptional activation of the corresponding promoters. Taken together, our study highlights HSV-2 ICP4 as a vital viral protein in promoting

CXCR3 ligand expression and CXCL9 as the key induced chemokine in mediating $CD4^+$ T cell migration. Findings in this study have shed light on HSV-2 induced leukocyte recruitment which may be important for understanding HSV-2 infection-enhanced HIV-1 sexual transmission and the development of intervention strategies.

Keywords: HSV-2, CXCR3 ligands, $CD4^+$ T cells, recruitment, ICP4

INTRODUCTION

Herpes simplex virus type 2 (HSV-2), a large enveloped dsDNA virus, affects ∼500 million people worldwide and acquires an annual rate of close to 25 million (1), resulting in up to 40% human adults living with HSV-2 latency (2, 3). HSV-2 infections are known to be restricted to mucosal and keratinized epithelia and neuronal ganglia, and cause genital herpes (4) with sexual transmission being the main route (5). Human immunodeficiency virus type 1 (HIV-1) causes destruction of the immune system, leading to acquired immune deficiency syndrome (AIDS) (6). In 2016, there were 1.8 million new HIV-1 infections globally, adding up to a total of 36.7 million people living with HIV-1 (7). The majority of HIV-1 infections are acquired by genital mucosal exposure, with sexual transmission as the leading mode of HIV-1 infection worldwide (8).

Due to the high positive-incidence of HSV-2 and common routes of transmission with HIV-1, mucosal HIV-1/HSV-2 co-infections attract more and more attention. Epidemiological studies show that HSV-2 infection results in an ∼3-fold increased risk of HIV-1 acquisition (9, 10), but the underlying mechanisms remain to be determined. One of the mechanisms that HSV-2 infection increases the probability of HIV-1 acquisition is the generation of lesions at HSV-2-infection sites, which provides a chance for HIV-1 to contact the target cells in the epidermis and dermis (11). Moreover, the number of $CD4^+$ T cells at the infection sites is increased following HSV-2 infection, which may further facilitate HIV-1 to infect these target cells (12–14). However, it is still not fully elucidated concerning the mechanism of $CD4^+$ T cell migration induced by HSV-2.

Chemokine CXCL9 is a member of the CXC family and plays an important role in the chemotaxis of $CXCR3^+$ immune cells. CXCR3 is a chemokine receptor that is rapidly induced on activated naive cells and sustains highly expression on Th1-type $CD4^+$ T cells and effector $CD8^+$ T cells (15). CXCR3 could be activated by three interferon-inducible ligands CXCL9 (MIG), CXCL10 (IP-10), and CXCL11 (I-TAC). Although CXCR3 could also be activated by CXCL4 and CXCL4L1, these two chemokines are released by platelets and have been implicated in atherogenesis and acute coronary syndrome (16). It is known that the upregulation of CXCR3 ligands is positively associated with a variety of tumors, inflammatory diseases, and infectious diseases such as AIDS (17). Although the expression of CXCL9 and CXCL10 has been shown to be increased in the cervical tissues of mice infected by HSV-2, the study on mice focused on the roles of recruited $CD8^+$ T cells in control of HSV-2 infection (18–20). Our previous study demonstrated that CXCL9 levels in cervical mucus from HSV-2–positive women were significantly increased and that CXCL9 induced by HSV-2 infection in cervical epithelial cells can enhance the migration of $CD4^+$ T cells (14). Although HSV-2-induced expression of CXCL10 and CXCL11 was previously reported (19, 21), the significance of HSV-2-induced CXCR3 ligands *in vivo* and the molecular mechanisms underlying the HSV-2-induced expression of CXCR3 ligands, in particular CXCL10 and CXCL11, have yet to be addressed. Furthermore, HSV-2 component(s) responsible for the induction and the underlying mechanism remain to be fully investigated.

In the current study, we found that expression of mouse CXCR3 ligands was increased following vaginal challenge with HSV-2 in mice. In addition, HSV-2-induced CXCL9 played a crucial role in promoting $CD4^+$ T cell migration to the vaginal foci of infected mice. In human cervical epithelial cells, HSV-2 infection induced the production of CXCL10 and CXCL11 in addition to CXCL9. Although CXCL10 and CXCL11 were induced following HSV-2 infection, the migration of $CD4^+$ T cells was mainly dependent on HSV-2 infection-induced CXCL9, reflecting that the concentrations of CXCL10 and CXCL11 required for $CD4^+$ T cell migration are higher than that of CXCL9. Moreover, HSV-2 immediate-early protein ICP4 (also known as RS1) appeared to be the vital viral component to induce the production of CXCR3 ligands. We further explored the molecular mechanisms underlying ICP4–induced CXCR3 ligand expression, revealing that ICP4 binds to corresponding promoters of CXCR3 ligands to activate their transcription by interaction with TBP. Our study together has shed light on the molecular mechanisms underlying HSV-2-induced $CD4^+$ T cell accumulation in mucosal infection sites, which may be crucial for understanding HSV-2 infection-enhanced HIV-1 sexual transmission and the development of intervention strategies.

Abbreviations: HSV-2, herpes simplex virus type 2; HIV-1, human immunodeficiency virus type 1; AIDS, acquired immune deficiency syndrome; PBMC, peripheral blood monocyte cell; C/EBP-β, CCAAT/enhancer-binding protein-β; MIG, monokine induced by gamma-interferon; IP-10, interferon-induced protein-10; I-TAC, interferon-inducible T-cell alpha chemoattractant; TFIIB, transcription factor II B; TBP, TATA binding protein; TFIID, transcription factor II D; Ultraviolet, UV.

MATERIALS AND METHODS

Viruses, Cell Lines, Antibodies, and Inhibitors

HSV-2 (G strain) was obtained from LGC standards and propagated in African green monkey kidney cells (Vero). Virus stocks were aliquoted and stored at −80°C before

used for infection. Ultraviolet (UV)-inactivated HSV-2 was obtained by exposure to ultraviolet irradiation for 15 min. HSV-2 titration was determined by plaque assay on confluent Vero monolayers (53). ME180, PM1, and Vero cells were obtained from American Tissue Culture Collection. Human cervical epithelial cell line ME180 and Vero cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Life Technologies, 11965, Australia) supplemented with 10% FBS, 100 units/mL penicillin and 100 units/mL streptomycin at 37°C in a 5% CO2 incubator. Human T cell line PM1 cells were cultured in RPMI-1640 medium (HyClone, SH30809.01B, USA) supplemented with 10% FBS, 100 units/mL penicillin and 100 units/mL streptomycin at 37°C in a 5% CO2 incubator. Abs against p38, phospho-p38, and β-actin, respectively, were purchased from Santa Cruz Biotechnology (sc-7149, sc-101759 and sc-81178, USA). Ab against phospho-C/EBP-β was purchased from Cell Signaling Technology (3084S, USA). Inhibitors specifically against ERK (PD98059), JNK (SP600125), and p38 (SB203580), respectively, were purchased from Merck Millipore (19-143, 420119, and 559389, USA). Abs against HA and Flag tag were purchased from Sigma-Aldrich (H6908 and F1804, USA). Ab against Proliferating Cell Nuclear Antigen (PCNA) and TATA binding protein (TBP) were from Proteintech (10205-2-AP and 22006-1-AP, Wuhan, China). Rabbit normal IgG and Cy3-conjugated goat anti-mouse IgG were purchased from BOSTER (BA1031 and BA1045, Wuhan, China). Abs against mouse CD4, CXCL9, CXCL10, and CXCL11 were purchased from R&D Systems (MAB554, AF-492-NA, AF-466-NA, and AF-572, USA). Abs against ICP4, ICP27, gB, and HSV-2 were from Abcam (ab96431, ab53480, ab6506, and ab21112, England). Ab against gD was from Santa Cruz Biotechnology (sc-69802, USA).

Plasmid Construction

HSV-2 genome was extracted from the cells infected with HSV-2 for 48 h using QIAamp DNA Blood Mini Kit (Qiagen, 51104, Germany). The expression plasmids of US1, RS1, US12, UL54, and RL2, and the reporter of CXCL9 were described previously (14, 22). The open reading frames (ORFs) were amplified by PCR with the primers shown in **Table S1**. The reporters of CXCL10 and CXCL11 were amplified with forward primers (CXCL10 Luc-F and CXCL11 Luc-F) and reverse primers (CXCL10 Luc-R and CXCL11 Luc-R), respectively. The sequences of primers were showed in **Table S1**. An N-terminal HA or Flag tag was introduced into ICP4 by the forward primer. N-terminal Flag tag was introduced into UL20, UL46, UL47, UL48, UL56, UL49A, US4, US7, or RL1 by the forward primer. The promoter reporters were cloned into pGL3-basic. Unless otherwise described, other PCR products were cloned into pcDNA3.1(+) (Invitrogen) and the constructed expression plasmids were named UL20, RS1-HA (ICP4-HA), RS1-Flag (ICP4-Flag), UL46, UL47, UL48, UL56, UL49A, US4, US7, RL1, UL20-Flag, UL46-Flag, UL47-Flag, UL48-Flag, UL56-Flag, UL49A-Flag, US4-Flag, US7-Flag, and RL1-Flag, respectively. The constructs were verified by DNA sequencing (Sunny Biotechnology, Shanghai, China).

HSV-2 Challenge and Sampling

Animal experiments were approved by the Institutional Animal Care and Use Committee and performed in accordance with the guidelines of the Hubei Laboratory Animal Science Association. In brief, female BALB/c mice (6–8 wk old) were purchased from Beijing HFK Biotechnology (Beijing, China) and maintained in specific pathogen–free conditions with food and water supplied. Seven days prior to challenge, each mouse was injected with 2 mg progesterone in intraperitoneal, subcutaneous, and intramuscular sites to ensure that each mouse rapidly entered the estrous cycle (23). After the estrous cycle, the mouse vaginal mucosal epithelia became thinner and were more susceptible to HSV-2. One day prior to challenge, the neutralizing Abs against CXCL9 (2 μg, R&D Systems, MAB554, USA), CXCL10 (2 μg, R&D Systems, MAB554, USA), and CXCL11 (2 μg, R&D Systems, MAB554, USA) were delivered to the vagina of mice, respectively, or in combination. Mice were anesthetized with pentobarbital sodium and challenged intravaginally with 10 μL/mouse HSV-2 at a concentration of 6×10^7 PFU/mL. Mice challenged with medium alone were set as background controls. The signs of mouse vagina were observed at days 3, 5, and 7 post HSV-2 challenge. Vaginal ulcers arose at day 7 in infected mice but not in the control group. Seven days after challenge, vaginal lavage fluids were collected using a vaginal Transferpettor by washing the vagina three times with sterile PBS plus protease inhibitors (Roche, 11697498001, Germany) in a total volume of 100 μL/mouse. Collected samples were centrifuged (15,000 × g, 10 min at 4°C), and supernatants were aliquoted and stored at −80°C until use. Thereafter, mice were sacrificed by neck dislocation. The cervical-vaginal tissues (Y type, two fallopian tube in the upper and vagina in the lower) were excised according to the characteristics of mouse physiological structure and collected under sterile conditions. The tissues were fixed in 4% formaldehyde followed by immunohistochemistry analysis. The collection of vaginal lavages or tissues was performed by the same people.

CBA for Human CXCL9, CXCL10 and CXCL11, and Mouse CXCL9 and CXCL10

ME180 cells in 6-well plates were transfected with empty vector or plasmid expressing ICP4 for 24 h. In some cases, ME180 cells were infected or mock-infected with HSV-2 for 24 h. Cell supernatants were collected and centrifuged to remove cell debris. Cytometric Bead Assay (CBA) was carried out to quantify secreted human CXCL9, CXCL10, and CXCL11 using the BD Cytometric Bead Array Human Soluble Protein Flexset Kit according to the manufacturer's instructions. Briefly, 50 μL diluted standards or undiluted samples were added into labeled tubes followed by the addition of 50 μL mixed beads. At 1 h post-incubation, 50 μL PE conjugated detection antibody was added into all tubes followed by incubation for 2 h at room temperature. All tubes were then washed with 1 mL washing buffer and centrifuged at 1,200 rpm for 5 min. The supernatants were removed and the beads were resuspended with 300 μL washing buffer. The concentration of mouse CXCL9 and CXCL10 in the vaginal lavage fluids was detected using the LEGENDplex™

Cytometric Bead Array mouse proinflammation Chemokine Mix and Match Subpanel according to the manufacturer's instructions. Briefly, 25 μL assay buffer was added into all tubes, followed by the addition of 25 μL diluted standard or 25 μL undiluted sample to each labeled tube. Thereafter, 25 μL mixed beads and 25 μL detection antibodies were added into all tubes followed by incubation for 2 h at room temperature with shaking. All the tubes were then incubated for 30 min at room temperature after the addition of 25 μL SA-PE solution. Beads were spun down (1,100 rpm, 5 min at room temperature) and washed with 1× washing buffer. The beads were resuspended with 200 μL of 1× washing buffer. All the samples were read on the BD LSRFortessa™ Flow Cytometer.

ELISA for Mouse CXCL11

The concentration of CXCL11 in the vaginal lavage fluids of mice was detected using Mouse CXCL11 ELISA Kit (BOSTER, EK0738, China). The standard of CXCL11 was provided in the Kit. Fifty microliter of undiluted fluids were tested for mouse CXCL11 detection according to the manufacturer's instructions.

Immunohistochemistry

Immunohistochemistry analysis of mouse cervical-vaginal tissues was conducted as described previously (24, 25). Briefly, the specimens obtained from challenged mice were fixed in 4% formaldehyde for 24 h at room temperature, embedded in paraffin, and cut into 3-mm sections. For detection of $CD4^+$ T cells in cervical-vaginal samples, slides were first dewaxed in xylene and rehydrated in a descendant ethanol scale. Ag retrieval was subsequently performed using Antigen Retrieval Reagent Basic Kit (R&D Systems, CTS013, USA) for 30 min in a water bath according to the manufacturer's instructions, and endogenous peroxidase was blocked by 3% H_2O_2 for 10 min at room temperature. Immunohistochemistry staining was performed using Cell & Tissue Staining Kit (R&D Systems, CTS017, USA) according to the manufacturer's instructions. $CD4^+$ T cells were detected by rabbit anti-mouse CD4 Ab. HSV-2 infection was detected by goat anti-HSV-2 polyclonal Ab. The colorimetric reaction was developed by adding 3, 3′-diaminobenzidine (DAB) at room temperature. For immunofluorescence detection of ICP4 and CXCR3 ligands or HSV-2 in cervical-vaginal samples, slides were first treated as the above instruction. ICP4 was detected with rabbit anti HSV-2 ICP4 Ab. Mouse CXCL9, CXCL10, and CXCL11 were detected by goat anti-mouse CXCL9, CXCL10, and CXCL11 Abs, respectively. HSV-2 was detected by goat anti-HSV-2 polyclonal Ab. FITC-conjugated goat anti-mouse, Cy3 conjugated donkey anti-goat and anti-rabbit (Beyotime, A0568, A0502, and A0516, China) secondary Abs were used in subsequent detection. The images were acquired using the Hungary 3DHISTECH apparatus (Pannoramic MIDI).

Dual Luciferase Report (DLR) Assay

ME180 cells were seeded in 24-well plates overnight and co-transfected with empty vector or plasmid encoding ICP4, Renilla luciferase plasmid phRL-TK and reporter plasmid CXCL9-Luc, CXCL10-Luc or CXCL11-Luc. Transfections were carried out using X-tremeGENE™ HP DNA Transfection Reagent (Roche, 6366236001, Germany) according to the manufacturer's instructions. At 24 h post-transfection, cells were harvested and lysed. The lysates were used for measuring firefly and Renilla luciferase activities using the Dual-Luciferase Reporter Assay System (Promega, E1980, USA) according to the manufacturer's instructions. For some experiments, ME180 cells were co-transfected with reporter plasmid CXCL9-Luc, CXCL10-Luc or CXCL11-Luc, and phRL-TK, followed by infection with HSV-2 or ultraviolet-inactivated HSV-2 at an MOI of 1. At 24 h post-infection, the enzymatic activities of Firefly and Renilla luciferase were measured. Values for the samples were normalized using Renilla luciferase values and expressed as fold increase of the value induced in cells transfected with empty vector or mock-infected with DMEM.

RNA Isolation and Quantitative PCR

Cells were collected and total RNA was extracted using RNA isolation kit (MN, 740955, Germany) according to the manufacturer's instructions. The cDNA was synthesized by Moloney murine leukemia virus transcriptase (Promega, M170B, USA). The newly synthesized cDNA was used as the template for amplifying the genes of CXCR3 ligands and GAPDH. The primer pairs for CXCL9, CXCL10, and CXCL11 were named CXCL9-F/CXCL9-R, CXCL10-F/CXCL10-R, and CXCL11-F/CXCL11-R (**Table S1**). GAPDH was used as an internal control and amplified with primers GAPDH-F and GAPDH-R (**Table S1**). Relative real-time quantitative PCR was performed on an ABI StepOne apparatus using a SYBR Green Real-Time PCR Master Mix (Toyobo, QPK-201, Japan) according to the following conditions: 95°C for 1 min, followed by 40 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 45 s. The expression difference was calculated on the basis of $2^{-\Delta\Delta Ct}$ values.

Western Blot

Western blot analysis was performed as described previously (22). Briefly, cytoplasmic and nuclear proteins were isolated using the Nucleus and Cytoplasm Protein Extraction Kit (Beyotime, P0028, China). In some cases, cells were lysed with lysis buffer (Life technologies, 87788, USA). Cell extracts were subjected to 10 or 15% SDS-PAGE and transferred onto PVDF membranes (Millipore 0.45 μm or 0.22 μm) followed by blocking with 5% non-fat milk in Tris-buffered saline-Tween (TBST, 50mM Tris-HCl pH 7.5, 200mM NaCl, 0.1% (v/v) Tween-20) at room temperature for 2 h. The membrane was clipped according to the molecular weight of the protein, and then probed with an appropriate primary antibody at room temperature for 2 h. After three washes with TBST, the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (BOSTER, BA1054, China), goat anti-mouse IgG (BOSTER, BA1051, China) or donkey anti-goat IgG (Beyotime, A0181, China) at room temperature for 1 h. Protein bands were visualized by exposure to FluorChem HD2 Imaging System (Alpha Innotech) after the addition of chemiluminescent substrate (Beyotime, P0018, China). Protein molecular weight

markers were purchased from Thermo Fisher (26616, USA) and YEASEN (20352, China).

Isolation and Culture of PBMCs and $CD4^+$ T Cells

All protocols involving human subjects were reviewed and approved by the local Research Ethics Committee of Wuhan Institute of Virology, Chinese Academy of Sciences. Informed written consents from the human subjects were obtained in this study. Both sexes were used and the donors were free of HSV-1 and HSV-2. PBMCs were isolated from healthy donors by using a Ficoll-Hypaque density gradient. $CD4^+$ T cells were separated from PBMCs using $CD4^+$ Cell Negative Isolation Kit according to the manufacturer's protocol (Miltenyi Biotec, 130-096-533, Germany). PBMCs and $CD4^+$ T cells were activated by stimulating with 1 μg/mL PHA (Sigma-Aldrich, L4144, USA) and 20 U/mL IL-2 (PeproTech, 200-02, USA). PBMCs and $CD4^+$ T cells cultured in complete RPMI 1640 containing 20 U/mL IL-2 were used as controls for flow cytometry. PBMCs and $CD4^+$ T cells were harvested at day 7 and used in subsequent assays.

Chemotaxis Assay

Chemotaxis assay was performed using 24-well Transwell plates (Costar, 3415, USA). One milliliter supernatants from ME180 cells which were mock-infected or infected with HSV-2, or mock-transfected or transfected with ICP4 expressing plasmid were added to the lower chamber. To examine the roles of CXCR3 and CXCR3 ligands in mediating cell migration, supernatants or cells were incubated with anti-CXCL9 (10 μg/mL, R&D Systems, MAB392, USA), -CXCL10 (2 μg/mL, R&D Systems, MAB266, USA), -CXCL11 (2 μg/mL, R&D Systems, MAB672, USA) or -CXCR3 (1 μg/mL, R&D Systems, MAB160, USA) neutralizing Abs, respectively, for 1 h, according to the manufacturer's instructions. Activated PBMCs and $CD4^+$ T cells (5×10^5) in 100 μL RPMI-1640 medium were added to the upper chamber. The chambers were incubated for 2 h at 37°C in a 5% CO2 incubator. Cell migrated to the lower chambers were collected and counted using an automatic cell counter (Bio-Rad).

Flow Cytometry

PBMCs and $CD4^+$ T cells were collected and resuspended with 3% FBS on ice for 10 min. Hundred microliter cell suspension (1×10^6) was prepared for one test. Two microliter BV421 conjugated mouse anti-human CD25 (BD biosciences, 562443, USA), BB515 conjugated mouse anti-human CD4 (BD biosciences, 564419, USA) and APC conjugated mouse anti-human CD69 (BD biosciences, 560967, USA) Abs or PE conjugated mouse anti-human CXCR3 Ab (BD biosciences, 560928, USA) were added into the corresponding samples, followed by incubation on ice for 15 min. Background staining was assessed by isotype-matched control Abs, including BV421 conjugated mouse IgG1 (BD biosciences, 562438, USA), BB515 conjugated mouse IgG1 (BD biosciences, 564416, USA), APC conjugated mouse IgG1 (BD biosciences, 555751, USA), and PE conjugated mouse IgG1 (BD biosciences, 555749, USA). Cells were washed with 1× PBS for three times. Three hundred microliter cell suspension was filtrated through a 200-mesh membrane and performed on BD LSRFortessa™ Flow Cytometer. Data were analyzed using BD FACSDiva software (BD Biosciences).

Immunofluorescence Assay

ME180 cells were seeded in 35-mm dishes with glass bottom and transfected with HA-tagged plasmid expressing ICP4. At 24 h post-transfection, cells were fixed with 4% formaldehyde and permeabilized with 0.2% Triton X-100. After three washes with 1× PBS, cells were blocked in PBS containing 5% BSA at 4°C overnight. Thereafter, cells were incubated with mouse anti-HA Ab at a dilution of 1:100 at 37°C for 1 h. Following three washes with 1× PBS, cells were then incubated with Cy3-conjugated goat anti-mouse IgG (Beyotime, A0521, China) at a dilution of 1:50 for 1 h at 37°C. Cells were subsequently washed and incubated with DAPI for 10 min at 37°C. After washes, cells were incubated with anti-fluorescence quenching reagent (Beyotime, P0126, China) and observed under a fluorescence microscope (Olympus IX51).

Chromatin Immunoprecipitation (ChIP)

ME180 cells in 6-well plates were transfected with HA-tagged plasmid expressing ICP4 or empty vector. At 24 h post-transfection, ChIP assay was performed as described previously (22) according to the manufacturer's instructions (Millipore, 17-409, Germany). The purified DNA was used as a template for PCR detection of the promoter sequences of CXCR3 ligands with primer pairs CXCL9 pro-F/CXCL9 pro-R, CXCL10 pro-F/CXCL10 pro-R, and CXCL11 pro-F/CXCL11 pro-R, respectively (**Table S1**).

Co-immunoprecipitation (Co-IP) Assay

ME180 cells in 6-well plates were transfected with HA-tagged ICP4 expression plasmid or empty vector. At 24 h post-transfection, cells were harvested and lysed on ice for 10 min in 200 μL of lysis buffer (50 Mm Tris (PH 8.0), 150 mM NaCl, 1% NP40) containing protease inhibitor cocktail (Roche, 11697498001, Germany). To eliminate nonspecific binding of other proteins, the samples were pretreated with dynabeads Protein G (Invitrogen, 10003D, USA) for 2 h at room temperature followed by separation prior to Co-IP assay. Meanwhile, 2 μg rabbit anti-HA Ab or control rabbit Ab was diluted in 200 μL PBS with 1% Tween-20 (PBST) and added to fresh dynabeads protein G. After incubation with rotation for overnight at 4°C, dynabeads-Ab complexes were washed once with 200 μL PBST before mixed with the pretreated samples, followed by overnight incubation with rotation at 4°C to allow the formation of dynabeads-Ab-Ag complexes. The complexes were washed three times with PBST and target antigens were eluted by boiling and subjected to western blot analysis.

Statistical Analysis

All experiments were repeated at least three times and the data are presented as mean ± S.D. with each condition performed in triplicate or in duplicate unless otherwise specified. Data analyses were performed with GraphPad Prism 5 software (GraphPad). Comparison between two groups was analyzed by two tailed

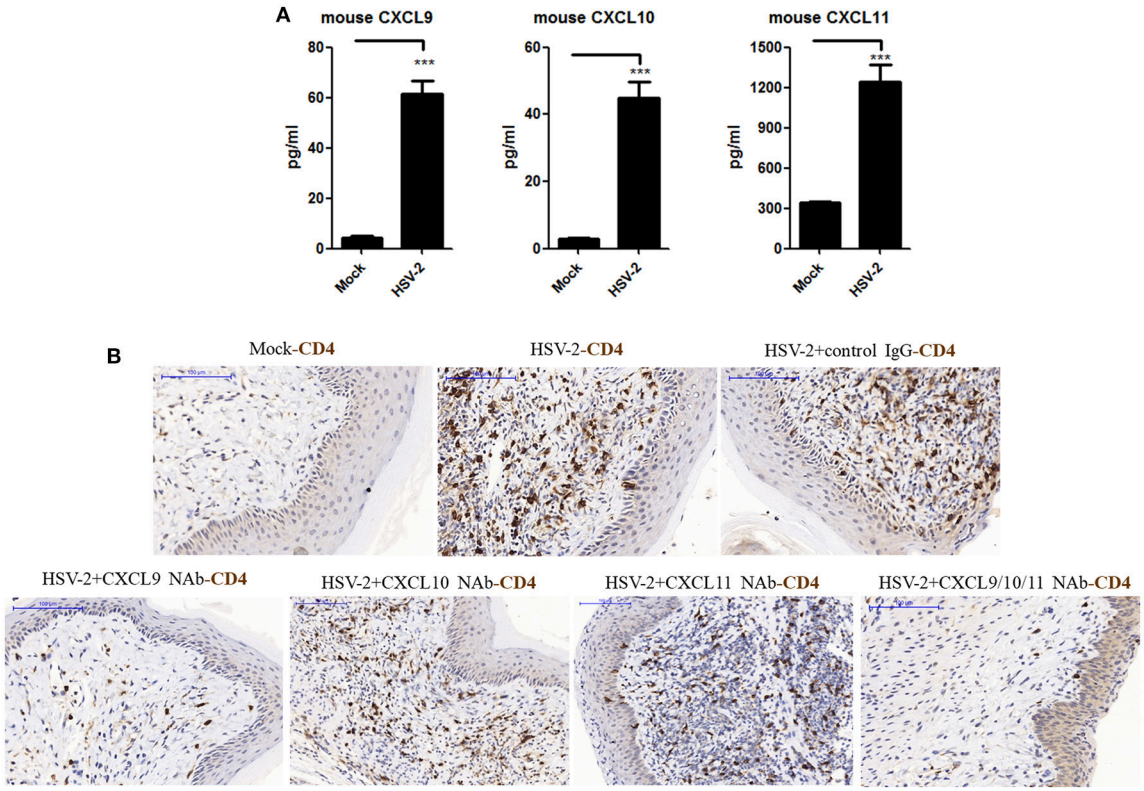


FIGURE 1 | Contribution of HSV-2 infection-induced CXCR3 ligands to $CD4^+$ T cell infiltration into mouse vagina. Seven days prior to HSV-2 challenge, BALB/c mice were injected with progesterone in multiple sites. One day prior to HSV-2 challenge, CXCL9, CXCL10, and CXCL11 neutralizing antibodies were delivered to the vagina of mice, alone or in combination, while isotype matched control IgG was used as the control. Mice were then anesthetized with pentobarbital sodium and challenged intravaginally with 10 μL/ mouse HSV-2 at a concentration of 6×10^7 PFU/ml or mock- challenged. Vaginal lavage fluids and cervical-vaginal tissues were collected at day 7 after challenge. **(A)** HSV-2 infection induces the production of mouse CXCR3 ligands. The protein levels of CXCL9 and CXCL10 ligands in vaginal lavage fluids were measured by CBA, and the protein level of CXCL11 was detected by ELISA. **(B)** CXCL9 mediates the migration of $CD4^+$ T cells to the vaginal foci of infected mice. $CD4^+$ T cells in infection foci were detected using anti-CD4 Ab by IHC. The scale bar indicates 100 μm. Data shown are mean ± S.D. ($n = 5$ mice/group) of three independent experiments **(A)**. $^{***}p < 0.001$. One representative out of three independent experiments is shown **(B)**.

unpaired Student's *t*-test, whereas comparisons among more than two groups were analyzed by one-way ANOVA with the Turkey's test. $P < 0.05$ was considered statistically significant.

RESULTS

Contribution of HSV-2 Infection-induced CXCR3 Ligands to $CD4^+$ T Cell Infiltration Into Mouse Vagina

The expression of CXCL9 and CXCL10 has been shown to be increased in the cervical tissues of mice infected by HSV-2 in previous studies (20), which mainly focused on the recruitment of activated $CD8^+$ T cells and its contribution to the control of HSV-2 infection. However, the impact of HSV-2 infection on $CD4^+$ T cell migration in mice remains to be further addressed. To assess this, mice were challenged with HSV-2 vaginally, and vaginal lavage fluids and cervical-vaginal tissues of the mice were collected for subsequent assessment. We confirmed a productive infection of HSV-2 in mouse vagina by immunohistochemistry and immunofluorescence-histochemistry assays (**Supplementary Material Figures 1A,B**), while Cytometric Bead Array (CBA) showed that the production of mouse chemokines CXCL9 and CXCL10 was significantly increased (**Figure 1A**). ELISA also indicated the enhancement of CXCL11 in mice challenged with HSV-2 (**Figure 1A**). Meanwhile, immunohistochemistry (IHC) assays showed that the number of $CD4^+$ T cells was significantly increased in the vaginal foci of infected mice (**Figure 1B**). To identify which chemokine plays a vital role in $CD4^+$ T cell recruitment, mice were vaginally treated with the neutralizing Ab against CXCL9, CXCL10, or/and CXCL11 before HSV-2 challenge. The number of $CD4^+$ T cells was dramatically decreased after the administration of CXCL9 neutralizing antibody to the vagina of mice (**Figure 1B**). Although the migration of $CD4^+$ T cells was almost completely abolished after administration of a combination of neutralizing Abs against CXCL9, CXCL10, and CXCL11 into the vaginal tissue, $CD4^+$ T cells were still significantly recruited to the infection foci after the administration of CXCL10 or CXCL11 neutralizing

antibody (**Figure 1B**). These data together indicate that HSV-2 vaginal infection of mice increases the expression of CXCR3 ligands CXCL9, CXCL10 and CXCL11, and the migration of $CD4^+$ T cells to the vaginal foci is mainly mediated by CXCL9.

HSV-2 Infection Induces the Production of CXCR3 Ligands in Human Cervical Epithelial Cells

Although HSV-2-induced expression of CXCL10 and CXCL11 was previously reported (19, 21), it remains to be addressed as to how HSV-2 induces the expression CXCR3 ligands in human mucosal epithelial cells. Epithelial cells are the primary HSV-2 target cells during sexual transmission. Having demonstrating the correlation of CXCR3 ligands with $CD4^+$ T cell migration in mice, we next addressed the underlying mechanism in cellular models. Our previous study showed that HSV-2 infection of human epithelial cells induces CXCL9 expression (14). To investigate the association between HSV-2 infection and the induction of CXCR3 ligands, human cervical epithelial cell line ME180 was used for assessing CXCR3 ligand expression at promoter, mRNA and protein levels. Our results indicated that HSV-2 infection significantly activated not only the promoter of CXCL9 but also the promoters of CXCL10 and CXCL11 (**Figure 2A**). We next assessed whether HSV-2 productive infection is necessary for the transcriptional activation of CXCR3 ligands. The results showed that UV-inactivated HSV-2 did not significantly induce the transcriptional activation of CXCL9 and CXCL10. Although CXCL11 appeared to be induced by UV-inactivated HSV-2, the level of induction was low (**Figure 2A**). In agreement, several HSV-2 proteins were undetectable following UV inactivation (**Supplementary Material Figure 2**). These results together indicated that HSV-2 productive infection is essential for the induced production of CXCR3 ligands. To further confirm the effect of HSV-2 on the induction of CXCL10 and CXCL11, we next investigated the mRNA and protein levels of CXCL10 and CXCL11 following HSV-2 infection. Relative real-time PCR assay and CBA showed that HSV-2 infection significantly promoted the production of CXCL9, CXCL10, and CXCL11 at both mRNA (**Figure 2B**) and protein levels (**Figure 2C**).

HSV-2 Infection-induced CXCL9 Plays a Predominant Role in Mediating $CD4^+$ T Cell Migration

It is known that CXCR3 is highly expressed on $CD4^+$ T cells and CXCL9, CXCL10 or CXCL11 could activate $CXCR3^+$ T cells (15). We previously demonstrated the functionality of HSV-2-induced CXCL9 in chemotacting $CD4^+$ T cells (14). To assess the functionality of HSV-2–induced CXCL10 and CXCL11 in human cells, chemotaxis assay was performed using activated human peripheral blood mononuclear cells (PBMCs) and $CD4^+$ T cells. The percentage of $CD4^+$ cells was 95.6 and 33.4% in $CD4^+$ T cells and PBMCs, respectively (**Supplementary Material Figure 3**). We also confirmed that $CD4^+$ T cells and PBMCs were activated by PHA prior to the onset of chemotaxis assay (**Supplementary Material Figure 4**). ME180 cells were infected with HSV-2 for 24 h, and the chemotactic activity of supernatants was determined. In response to supernatants from HSV-2–infected cells, the migratory activity of PBMCs (**Figure 3B**) and $CD4^+$ T cells (**Figure 3C**) was significantly increased. Cell migration was almost abolished upon the addition of anti-CXCL9 neutralizing Ab to supernatants from HSV-2–infected cells (**Figures 3B,C**), whereas a control Ab, anti-CXCL10 or CXCL11 neutralizing Ab did not have such effect, which is accordance with a previous study (26), indicating the critical role of CXCL9 in inducing $CD4^+$ T cell migration. To further confirm the observation, a neutralizing Ab against CXCR3 was mixed well with cells for 1 h and then added into the upper chamber in chemotaxis assay, showing that the migration of $CD4^+$ T cells was significantly reduced (**Figure 3D**). We also demonstrated that HSV-2 infection did not regulate the expression of CXCR3 by flow cytometry assay (**Supplementary Material Figure 5A**).The concentrations of CXCL9, CXCL10, and CXCL11 in the supernatants of HSV-2-infected ME180 cells used for chemotaxis assays were detected by CBA (**Figure 3A**), showing that CXCL10 and CXCL11 were produced at levels not less than that of CXCL9. We therefore conducted the chemotaxis assay using recombinant CXCL9, CXCL10, or CXCL11 at the similar concentration as that induced by HSV-2 infection. The results indicated that recombinant CXCL9 induced the migration of $CD4^+$ T cells at a low concentration of 48 pg/ml, whereas CXCL10 and CXCL11 had no significant impact on $CD4^+$ T cell migration at the concentrations of 55 pg/mL and 175 pg/mL, respectively (**Figure 3E**). Nevertheless, CXCL10 or CXCL11, at a much higher concentration than that induced by HSV-2 infection, did promote the migration of $CD4^+$ T cells (**Figures 3F,G**), indicating that the concentrations of CXCL10 and CXCL11 required for $CD4^+$ T cell migration are higher than that of CXCL9. Taken together, our results together indicated that HSV-2 infection-induced CXCL9 likely plays a predominant role in mediating $CD4^+$ T cell migration.

HSV-2 ICP4 Promotes the Production of Human CXCR3 Ligands

UV-inactivation attenuated the ability of HSV-2 to induce CXCR3 ligand production, indicating that productive HSV-2 infection is necessary for the induced production of CXCR3 ligands. Given the complexity of HSV-2 genome which contains over 70 genes (27, 28), we next investigated which viral components are responsible for the induction of CXCR3 ligands during HSV-2 infection. ME180 cells were co-transfected with individual HSV-2 protein expression vector and the promoter reporter of CXCL9, CXCL10, or CXCL11. Dual luciferase reporter assay (DLR) indicated that HSV-2 immediate-early protein ICP4 significantly activated the promoters of CXCR3 ligands (**Figure 4A**). The expression of all tested viral proteins was assessed by Western Blot (**Figure 4B**). To further confirm the role of ICP4 on transcriptional activation, ME180 cells were transfected with ICP4 expressing plasmid, and cell supernatants were collected for CBA while mRNAs were extracted for reverse transcription PCR. Relative real-time quantitative PCR indicated

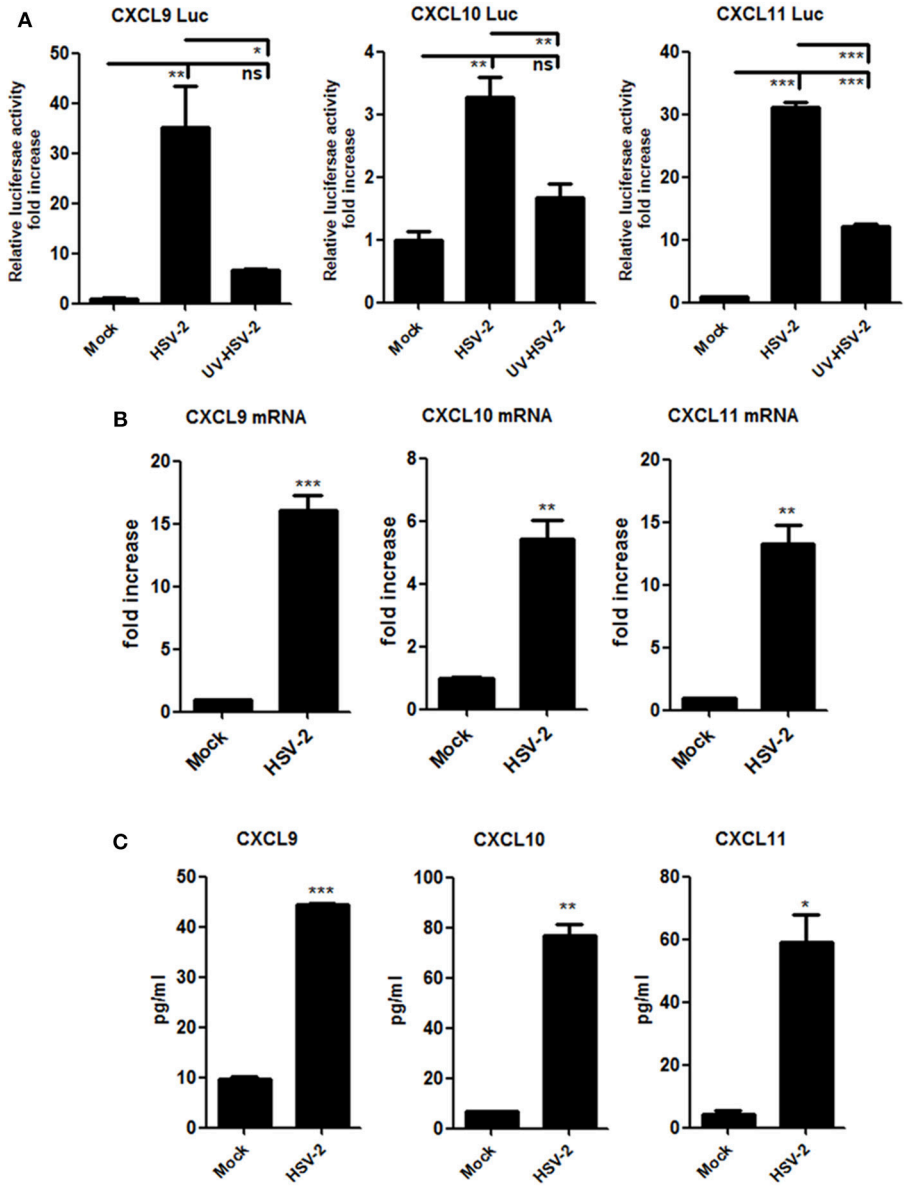


FIGURE 2 | HSV-2 infection induces the production of CXCR3 ligands in human cervical epithelial cells. **(A)** HSV-2 infection activates the promoters of human CXCR3 ligands. ME180 cells in 24-well plates were co-transfected with 150 ng CXCL9-Luc, CXCL10-Luc or CXCL11-Luc, and 15 ng internal control plasmid phRL-TK. At 4 h post-transfection, cells were infected with HSV-2 or ultraviolet-inactivated HSV-2 (UV-HSV-2) at an MOI of 1 for 24 h. DLR assay was performed. Values for the samples were normalized using Renilla luciferase values and expressed as fold increase of the value induced in mock-infected samples. **(B)** HSV-2 infection induces the mRNA production of CXCR3 ligands. ME180 cells in 6-well plates were infected with HSV-2 at an MOI of 1 for 24 h. Cells were harvested and total RNA was extracted. The expression of CXCR3 ligands and GAPDH was evaluated by relative real-time quantitative PCR. The Ct values of GAPDH among all groups were equable and not overloaded. mRNA copies of CXCR3 ligands were normalized using GAPDH and expressed as fold increase of the value for the mock-infected control. **(C)** HSV-2 infection induces the production of CXCR3 ligands. As depicted in **(B)**, cell supernatants were collected, and the protein level of CXCR3 ligands was measured by CBA. Data shown are mean ± S.D. of three independent experiments (A, B, and C). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

that HSV-2 ICP4 enhanced the expression of CXCR3 ligands at mRNA level (**Figure 4C**). CBA showed that HSV-2 ICP4 enhanced the production of CXCR3 ligands at protein level (**Figure 4D**). Subsequent chemotaxis assay was performed to assess the role of ICP4-induced CXCR3 ligands in promoting the migration of activated PBMCs or $CD4^+$ T cells. ME180 cells were transfected with ICP4 expressing plasmid, and the chemotactic activity of supernatants was determined. In response to supernatants from ICP4–transfected cells, the migratory activity of PBMCs (**Figure 4E**) and $CD4^+$ T cells (**Figure 4F**) was significantly increased. Cell migration was significantly reduced upon the addition of anti-CXCL9 (**Figures 4E,F**) neutralizing Ab to supernatants from ICP4 expressing plasmid–transfected cells or anti-CXCR3 neutralizing Ab (**Figure 4G**) to cell

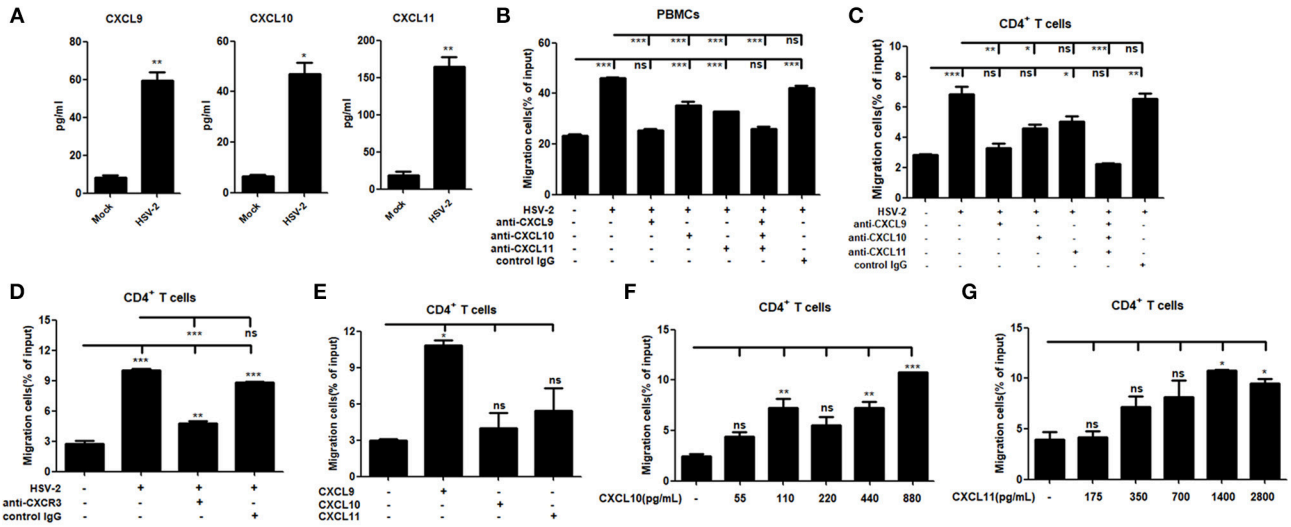


FIGURE 3 | HSV-2 infection-induced CXCL9 plays a predominant role in mediating $CD4^+$ T cell migration. **(A)** The concentrations of CXCR3 ligands in the supernatants of ME180 cells infected with HSV-2 or mock-infected with DMEM were detected by CBA. **(B,C)** CXCL9 induced by HSV-2 recruits the migration of PBMCs **(B)** and $CD4^+$ T cells **(C)**. ME180 cells in 6-well plates were infected with HSV-2 at an MOI of 1 for 24 h. Cell supernatants were collected and added to the lower chamber of transwell plates in the absence or presence of anti-CXCL9, –CXCL10, or/and –CXCL11 neutralizing Ab or control Ab for 1 h. **(D)** Neutralization of CXCR3 reduces the migration of $CD4^+$ T cells induced by HSV-2 infection. ME180 cells in 6-well plates were infected with HSV-2 at an MOI of 1 for 24 h. Cell supernatants were collected and added to the lower chamber of transwell plates. The activated $CD4^+$ T cells were incubated with RPMI 1,640 medium containing anti-CXCR3 neutralizing Ab for 1 h and placed in the upper chamber. **(E)** Recombinant CXCL9 significantly induces the migration of $CD4^+$ T cells. DMEM containing recombinant CXCL9, CXCL10, or CXCL11 (48 pg/mL, 55 pg/mL and 175 pg/mL, respectively; the lowest concentration induced by HSV-2 infection) was added to the lower chamber of transwell plates. **(F,G)** Recombinant CXCL10 or CXCL11 mediates the migration of $CD4^+$ T cells in a dose-dependent manner. DMEM containing recombinant CXCL10 or CXCL11 was added to the lower chamber of transwell plates. CXCL10 or CXCL11 was started from 55 pg/mL and 175 pg/mL, respectively, at a concentration gradient of two times. The activated $CD4^+$ T cells were placed in the upper chamber. After 2 h incubation, cells migrated to lower chambers were collected and counted using an automatic cell counter. Cells migration was expressed as percentage of input. Input cells in the upper chamber were 5×10^5. Data shown are mean ± S.D. of three independent experiments **(A–G).** ns, not significant, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

suspension. In addition, the expression of CXCR3 in ICP4 expressing cells was also detected by flow cytometry assay, showing that ICP4 did not induce the expression of CXCR3 (**Supplementary Material Figure 5B**). These results indicated that the immediate-early protein ICP4 of HSV-2 promotes the production of human CXCR3 ligands, of which CXCL9 plays a predominant role in mediating $CD4^+$ T cell migration.

HSV-2 ICP4 Regulates the Expression of CXCR3 Ligands Via the p38 MAPK Signaling Pathway

It is known that HSV-2 could activate MAPK pathway to regulate the expression of downstream genes (29). Our previous study demonstrated that HSV-2-mediated up-regulation of CXCL9 involves the p38 MAPK signaling pathway (14). To investigate whether MAPK pathway is involved in HSV-2-mediated transcriptional activation of CXCL10 and CXCL11 or ICP4-mediated transcriptional activation of CXCL9, CXCL10, and CXCL11, ME180 cells were pretreated with or without PD98059 (ERK inhibitor), SP600125 (JNK inhibitor) or SB203580 (p38 inhibitor), and then transfected with CXCR3 ligand reporter plasmid followed by infection with HSV-2 or co-transfected with CXCR3 ligand reporter plasmid and ICP4 expression plasmid. DLR assay showed that pretreatment of cells with SB203580, but not with PD98059 or SP600125, significantly decreased HSV-2–mediated activation of CXCL9 (**Figure 5A**), CXCL10 (**Figure 5B**), and CXCL11 (**Figure 5C**) promoters. In accordance, ICP4–mediated activation of CXCL9 (**Figure 5D**), CXCL10 (**Figure 5E**), and CXCL11 (**Figure 5F**) promoters was also decreased after pretreatment with SB203580. In our previous study, we found that HSV-2 infection could induce the phosphorylation of p38 and CCAAT/enhancer-binding protein-β (C/EBP-β). We then determined the impact of ICP4 on the activation of p38 MAPK pathway. ME180 cells were transfected with ICP4 expressing plasmid, and then the total or phosphorylation level of p38 and the phosphorylation level of C/EBP-β were examined by western blot assay. The results showed that, like HSV-2, ICP4 increased the phosphorylation level of p38 (**Figure 5G**). Taken together, these results suggest that HSV-2 ICP4–induced CXCR3 ligand expression in human cervical epithelial cells is mediated through the activation of p38 MAPK pathway.

HSV-2 ICP4 Binds to the Promoters of CXCR3 Ligands by Interaction With TBP

Although HSV-2 ICP4 induces the expression of CXCR3 ligands via the p38 MAPK signaling pathway, it does not affect the

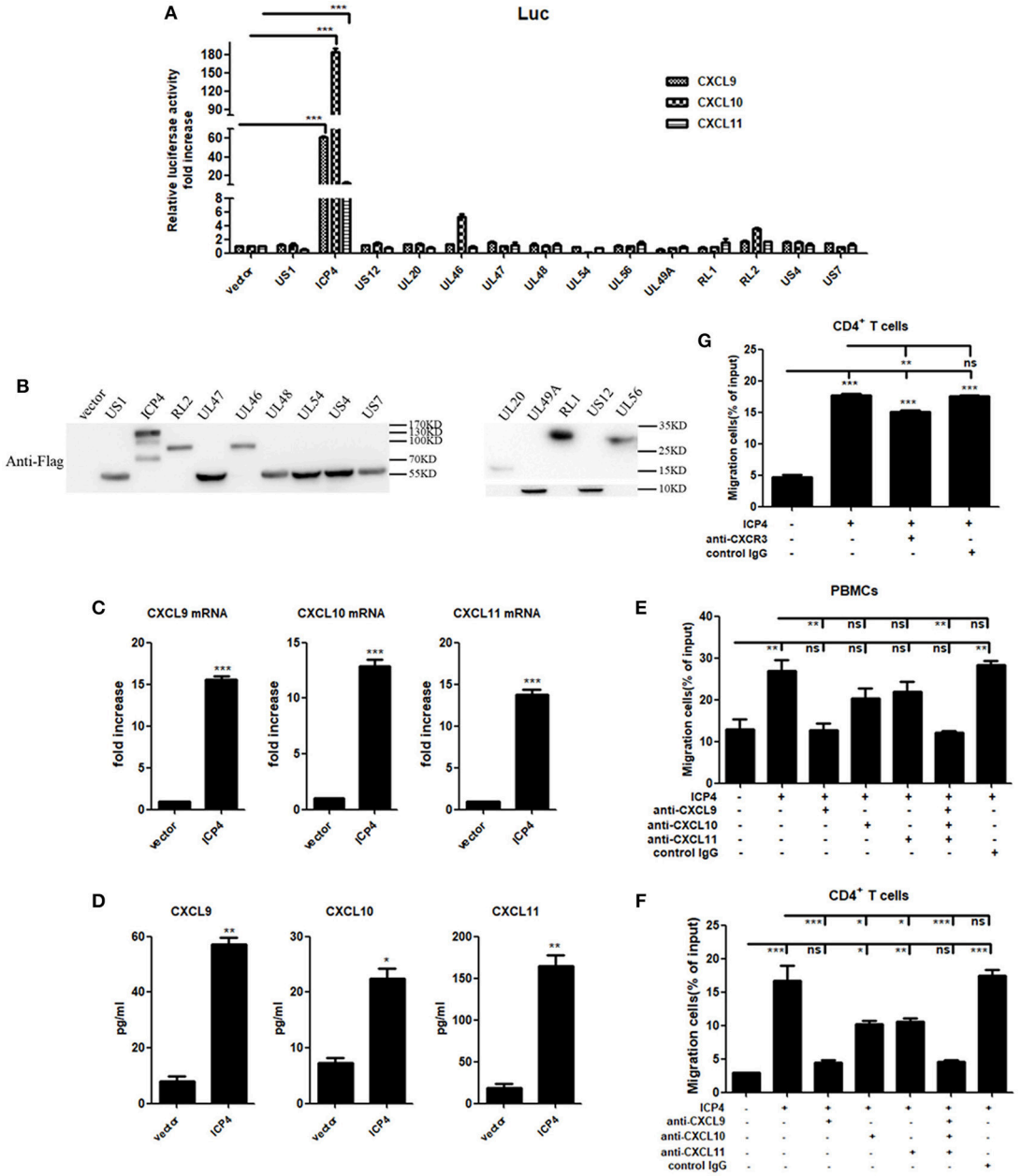


FIGURE 4 | HSV-2 ICP4 promotes the production of human CXCR3 ligands. **(A)** ICP4 induces the activation of CXCR3 ligand promoters. ME180 cells in 24-well plates were transfected with 300 ng expression plasmid of HSV-2 gene or empty vector together with 150 ng CXCR3 ligand reporter and 15 ng phRL-TK. At 24 h post-transfection, DLR assay was performed. Values for the samples were normalized using Renilla luciferase values and expressed as fold increase of the value induced in cells transfected with empty vector. **(B)** The expression of HSV-2 genes was detected using anti-Flag Ab by Western Blot. ME180 cells were transfected with 3 μg HSV-2 gene expression plasmid for 24 h. The proteins were collected and detected using mouse anti-Flag Ab. **(C)** ICP4 induces the mRNA production of CXCR3 ligands. ME180 cells in 6-well plates were transfected with 3 μg ICP4 expression plasmid for 24 h. Cells were harvested and total RNA was extracted. The expression of CXCR3 ligands and GAPDH gene was evaluated by relative real-time quantitative PCR. The Ct values of GAPDH among all groups were equable and not overloaded. mRNA copies of CXCR3 ligands were normalized using GAPDH and expressed as fold increase of the value for the empty vector-transfected control. **(D)** ICP4 induces the production of CXCR3 ligands. As depicted in **(C)**, cell supernatants were collected, and the protein levels of CXCR3 ligands were measured by CBA. **(E,F)** CXCL9 induced by ICP4 recruits the migration of PBMCs **(E)** and $CD4^+$ T cells **(F)**. ME180 cells in 6-well plates were transfected with 3 μg ICP4 expression plasmid for 24 h. Cell supernatants were collected and added to the lower chamber of transwell plates in the absence or presence of anti-CXCL9, –CXCL10, or/and –CXCL11 neutralizing Ab or control Ab for 1h. **(G)** Neutralization of CXCR3 reduces the migration of $CD4^+$ T cells induced by ICP4. ME180 cells in 6-well plates were infected with HSV-2 at an MOI of 1 for 24 h. Cell supernatants were collected and added to the lower chamber of transwell plates. The activated $CD4^+$ T cells were incubated with RPMI 1,640 medium containing anti-CXCR3 neutralizing Ab for 1 h and placed in the upper chamber. As depicted in **Figure 3**, cells migrated to lower chambers were counted. Cells migration was expressed as percentage of input. One representative out of three independent experiments is shown **(B)**. Data shown are mean ± S.D. of three independent experiments **(A,C–G)**. ns, not significant, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

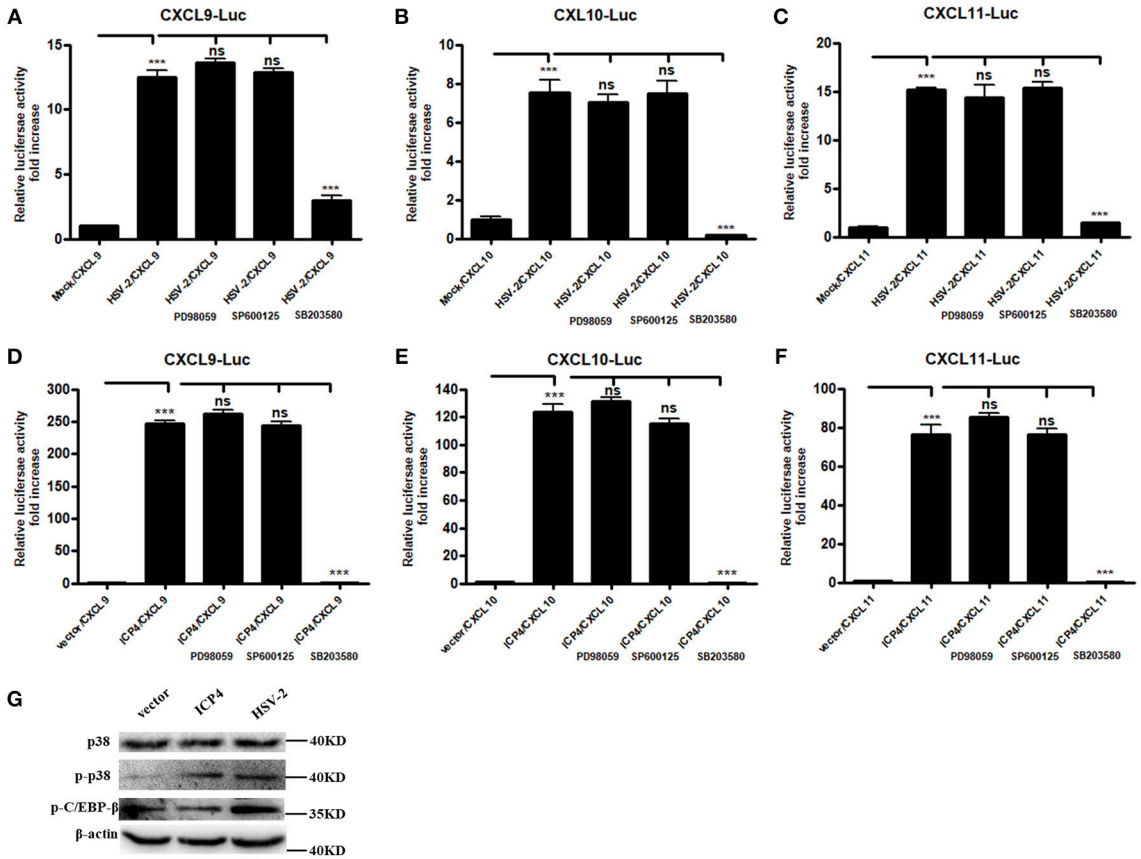


FIGURE 5 | HSV-2 ICP4 regulates the expression of CXCR3 ligands via the p38 MAPK signaling pathway. **(A–C)** HSV-2 regulates the expression of CXCL9 **(A)**, CXCL10 **(B)**, and CXCL11 **(C)** via p38/MAPK signaling pathway. ME180 cells in 24-well plates were co-transfected with 150 ng CXCR3 ligand reporter and 15 ng phRL-TK. At 4 h post-transfection, cells were infected with HSV-2 at an MOI of 1 and supplemented with inhibitor PD98059, SP600125, or SB203580. DLR assay was performed at 24 h post-transfection. Values for the samples were normalized using Renilla luciferase values and expressed as fold increase of the value induced in mock-infected samples. **(D–F)** HSV-2 ICP4 regulates the expression of CXCL9 **(D)**, CXCL10 **(E),** and CXCL11 **(F)** via p38/MAPK signaling pathway. ME180 cells in 24-well plates were co-transfected with 300 ng empty vector or ICP4 expression plasmid together with 150 ng CXCR3 ligand reporter and 15 ng phRL-TK. At 4 h post-transfection, cells were cultured in complete DMEM supplemented with inhibitor PD98059, SP600125, or SB203580. DLR assay was performed at 24 h post-transfection. Values for the samples were normalized using Renilla luciferase values and expressed as fold increase of the value induced in cells transfected with empty vector. **(G)** ICP4 activates p38 MAPK signaling pathway. ME180 cells were transfected with 3 μg ICP4 expression plasmid. The protein level of p38, phospho-p38 (p-p38) or phospho-C/EBP-β (p-C/EBP-β) was detected by Western Blot. Data shown are mean ± S.D. of three independent experiments **(A–F)**. ns, not significant, $^{***}p < 0.001$. One representative out of three independent experiments is shown **(G)**.

phosphorylation level of C/EBP-β (**Figure 5G**), suggesting a novel mechanism involved in ICP4-mediated production of CXCR3 ligands. Previous studies have demonstrated that ICP4 is essential for virus growth (30) and functions as a transcriptional activator in some cases (31–34). It is probable that ICP4 binds to the promoters of CXCR3 ligands in the nucleus which results in their transcriptional activation. ICP4 must be located in the nucleus to act as a transcriptional factor. To test this hypothesis, we first analyzed the nucleotide sequence of ICP4, revealing several nuclear localization sequences (NLSs) (**Figure 6A**). ME180 cells were transfected with HA-tagged ICP4, and examined by indirect immunofluorescence (IF) and western blot assay, showing that ICP4 was indeed located in the nucleus (**Figures 6B,C**). In agreement, ICP4 was also located in the nucleus in the context of HSV-2 infection (**Supplementary Material Figure 6**). Meanwhile, at 24 h post-transfection with HA-tagged ICP4, ME180 cells were collected for chromatin immunoprecipitation (ChIP) assay. The ChIP assay indicated that ICP4 bound to the promoters of CXCR3 ligands (**Figure 6D**).

It is known that ICP4 can form a tripartite complex with transcription factor II B (TFIIB) and either TBP or transcription factor II D (TFIID) (35). TBP is required for the initiation of transcription by RNA polymerases I, II, and III, from promoters with or without a TATA box (36–38). TBP associates with a host of factors to form multi-subunit pre-initiation complexes on the core promoter. Through its association with different transcription factors, TBP can initiate transcription from different RNA polymerases (39). Considering that ICP4 induces the phosphorylation of p38 (**Figure 5G**),

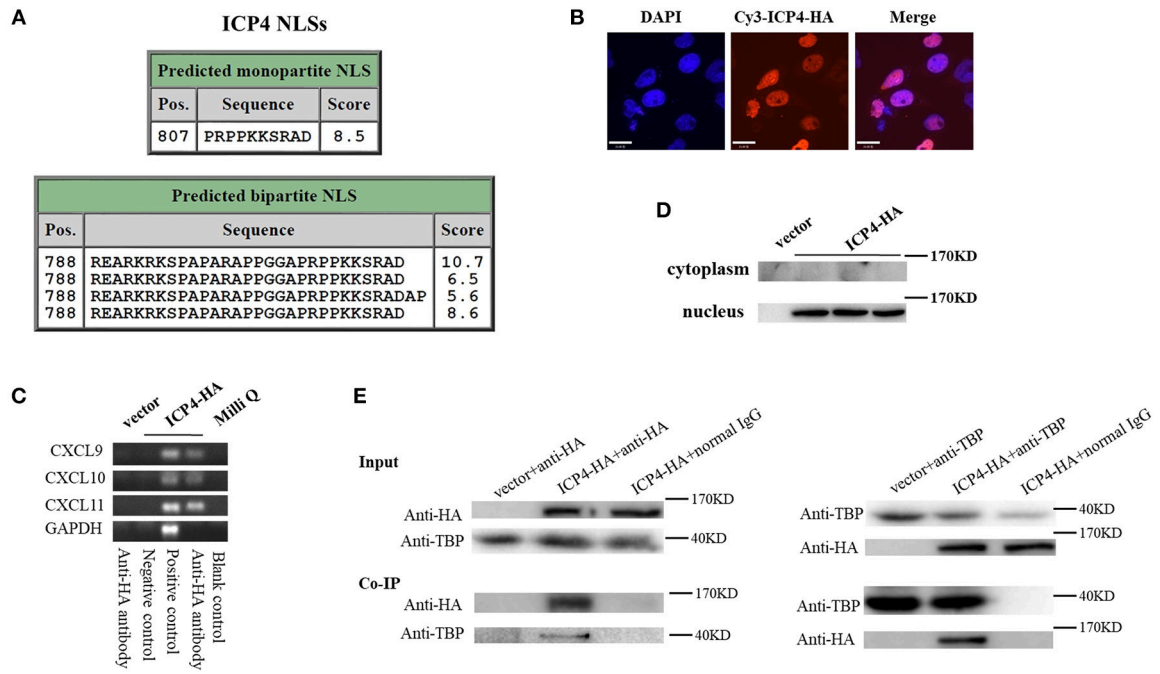


FIGURE 6 | HSV-2 ICP4 binds to the promoters of CXCR3 ligands by interaction with TBP. **(A)** Schematic representation of the predicted NLSs of ICP4 amino acid (AA) sequence. **(B)** ICP4 is located in the nucleus. ME180 cells in 35-mm dishes with glass bottom were transfected with 2 μg empty vector or HA-tagged ICP4 expression plasmid for 24 h. Cells were stained with mouse anti-HA mAb, followed by Cy3-conjugated goat anti-mouse (red) as the secondary Ab. Cell nuclei (blue) were stained with DAPI. The images were obtained by fluorescence microscopy using 60× objective. The scale bar indicates 21 μm. **(C)** The expression of ICP4 was stained using anti-HA mAb. **(D)** ICP4 binds to the promoters of CXCR3 ligands. ME180 cells were transfected with 3 μg empty vector or HA-tagged ICP4 expression plasmid for 24 h. Cells were lysed and subjected to ChIP assay using mouse anti-HA mAb, mouse anti-RNA polymerase II mAb (positive control) or mouse normal IgG (negative control) for immunoprecipitation. **(E)** ICP4 interacts with TBP. ME180 cells in 6-well plates were transfected with 3 μg empty vector or HA-tagged ICP4 expression plasmid for 24 h. Cells were lysed and subjected to co-immunoprecipitation (IP) using rabbit anti-HA or anti-TBP Ab. Rabbit normal IgG was used as a negative control. IP products and 5% input samples were examined using rabbit anti-HA and rabbit anti-TBP Abs by western blot. One representative out of three independent experiments is shown **(B–E)**.

and that the transcriptional activation of TBP requires the activation of p38 MAPK signaling pathway (40, 41), ICP4-induced activation of p38 likely contributes to the transcriptional activation of TBP. To verify the interaction of ICP4 with TBP, ME180 cells were transfected with HA-tagged ICP4 for 24 h. Co-immunoprecipitation assays were performed to detect the interaction of ICP4 with TBP. The results indicated that ICP4 interacts with TBP as evidenced by using an anti-HA antibody to pulldown TBP or an anti-TBP antibody to pulldown ICP4 (**Figure 6E**). These data collectively indicated that HSV-2 ICP4 binds to the promoters of CXCR3 ligands by interaction with TBP, leading to the promoter activation of CXCR3 ligands.

DISCUSSION

Recruitment of $CD4^+$ T cells, irrespective of their specificity, may significantly increase the chance of HIV-1 transmission (42, 43). Our previous study found that CXCL9 levels in cervical mucus from HSV-2–positive women were significantly increased and that HSV-2 infection induced CXCL9 expression in cervical epithelial cells (14). In addition, the expression of CXCL9 and CXCL10 was shown to be increased in the cervical tissues of mice infected by HSV-2 in studies to understand the contribution of recruited $CD8^+$ T cells in control of HSV-2 infection (20), while CXCL9 induced by HSV-1 infection has been shown to recruit $CD4^+$ T cells into the cornea (26). Moreover, circulating memory $CD4^+$ T cells could migrate to the genital mucosa in mice challenged with HSV-2 (44). However, how HSV-2 infection affects the migration of $CD4^+$ T cells at mucosal sites and the biological consequences remain to be fully determined.

In this study, we observed that, following vaginal challenge with HSV-2, mouse CXCR3 ligands CXCL9, CXCL10, and CXCL11 were all upregulated in the vaginal fluids of infected mice. In addition, $CD4^+$ T cells migrated to the vaginal foci of infected mice, while the number of $CD4^+$ T cells was significantly decreased after administration of CXCL9 neutralizing antibody to the vagina of mice. These indicate that HSV-2 infection can promote $CD4^+$ T cell migration and this is mainly due to the induced CXCL9 expression. Although HSV-2 infection likely induces the expression of many other chemokines, our results showed that the migration of $CD4^+$ T cells was significantly reduced after administration of a combination of neutralizing Abs against CXCL9, CXCL10, and CXCL11 into the vaginal

tissue. In human cervical epithelial cells, we demonstrated that HSV-2 infection induced not only the production of CXCL9 but also that of CXCL10 and CXCL11. The common receptor for CXCL9, CXCL10, and CXCL11 is CXCR3, which can be rapidly induced on activated naive cells and sustain high level expression on Th1-type $CD4^+$ T cells and effector $CD8^+$ T cells (15). The other two ligands of CXCR3, CXCL4, and CXCL4L1, are released by platelets and have been implicated in atherogenesis and acute coronary syndrome (16). Therefore, we mainly focused on how HSV-2 infection enhances the production of CXCR3 ligands CXCL9, CXCL10, and CXCL11.

Early studies on transmitted/founder (T/F) HIV-1 have suggested that $CD4^+$ T cells serve as the main target cells in the establishment of HIV-1 early infection (9, 45). Although CXCR3 ligands induced by HSV-2 can activate and recruit $CD8^+$ T cells, these cells are specific for HSV-2 and may have an impact on the control of HSV-2 replication (20, 46, 47). In the current study, we mainly assessed the biological function of CXCR3 ligands induced by HSV-2 on $CD4^+$ T recruitment. We found that chemokines induced by HSV-2 can mediate the migration of $CD4^+$ T cells. Following experiments using neutralizing antibodies, the results indicated that the induced CXCL9 plays a crucial role in recruiting $CD4^+$ T cells, which was further confirmed by using recombinant CXCL9, CXCL10, or CXCL11 at a concentration similar to that induced by HSV-2. Chemokines CXCL10 and CXCL11 at the concentrations around or higher than 300 pg/mL and 350 pg/mL, respectively, have been shown to have chemotactic activity for $CXCR3^+$ cells (48–50), whereas CXCL9 has the same capability at a much lower concentration (14). We did not see significant reduction of $CD4^+$ T cell migration when CXCL10 or CXCL11 was neutralized by the corresponding neutralizing antibody. One reasonable explanation is that the concentrations of CXCL10 and CXCL11 required for $CD4^+$ T cell migration are much higher than that of CXCL9. In our study, the concentrations of CXCL10 and CXCL11 induced by ICP4 or HSV-2 were around or lower than 55 pg/mL and 175 pg/mL, respectively, which was unable to have an impact on $CD4^+$ T cell migration as evidenced by the chemotaxis assay using recombinant CXCL10 and CXCL11. The recombinant CXCL10 at the concentration of 55 pg/mL had a marginal effect on $CD4^+$ T cell migration, whereas the recombinant CXCL11 at the concentration of 175 pg/mL had no effect on the recruitment of $CD4^+$ T cell. Compared to those induced by HSV-2 or ICP4, recombinant CXCL10 and CXCL11 at much higher concentrations chemotracted $CD4^+$ T cells in a dose-dependent manner. Although beyond the scope of this current study, it will be important to address the roles of CXCR3 ligands in mediating $CD4^+$ T cell migration and HIV-1 mucosal transmission when an animal model become available to study HSV-2 and HIV-1 co-infection.

We found that UV-inactivated HSV-2 did not significantly induce the transcriptional activation of CXCL9 and CXCL10. Although CXCL11 appeared to be induced by UV-inactivated HSV-2, the level of induction was low. These results indicate that productive infection of HSV-2 is essential for the induced production of CXCR3 ligands. HSV-2 genome contains over 70 genes (27, 28). Following screening a range of HSV-2 ORFs, we identified the immediate-early protein ICP4 as the key viral component in inducing the expression of CXCR3 ligands. ICP4 was barely detectable when cells were treated with UV-inactivated HSV-2, further suggesting the importance of ICP4 in inducing CXCR3 ligand expression. In agreement, we observed the co-localization of ICP4 with CXCL9, CXCL10 or CXCL11 in the mouse vaginal epithelial layer by immunofluorescence histochemistry assay (**Supplementary Material Figure 8).** ICP4-induced CXCL9 played a crucial role in the chemotaxis of $CD4^+$ T cells, which is in accordance with that induced by HSV-2. We previously found that HSV-2 infection-induced CXCL9 expression is regulated by the transcriptional factor C/EBP-β (14). In the current study, we found that ICP4 did not affect the phosphorylation of C/EBP-β, although ICP4 induced the production of all the three CXCR3 ligands via p38 MAPK signaling pathway. These together indicate a novel mechanism underlying ICP4-induced CXCR3 ligand production, and that other viral component(s) is likely to be involved in the phosphorylation of C/EBP-β. It is known that ICP4 is a major transcriptional activator and essential for progression beyond the immediate-early phase of infection (28). Indeed, we successfully constructed a ICP4-null HSV-2 bacterial artificial chromosome (BAC) but were unable to rescue the ICP4-null HSV-2 (data not shown), further strengthening the essential role of ICP4 in the regulation of viral gene expression. ICP4 can function as a transcriptional activator in some cases (31–34). It may act as a transcriptional activator to induce the activation of CXCR3 ligand promoter. To function as a transcriptional factor, ICP4 needs to be in the nucleus where it can bind to the promoters of CXCR3 ligands. We found that ICP4 is indeed located in the nucleus and can bind to the promoters of CXCR3 ligands, resulting in the expression of corresponding chemokines. ICP4 was also located in the nucleus in the context of HSV-2 infection. We also observed the interaction of ICP4 with TBP, which could contribute to the binding of ICP4 to the promoters of CXCR3 ligands. Nevertheless, ICP4 seems not to serve as a consensus transcription factor to activate gene expression, as ICP4 did not activate the promoters of other cytokines including TNF, IL-6 (**Supplementary Material Figure 7**).

In conclusion, we first found that the expression of CXCR3 ligands CXCL9, CXCL10, and CXCL11 was induced following mice vaginally challenged with HSV-2, which was associated with the increased number of $CD4^+$ T cells in the vaginal foci of infected mice as well as CXCL9-mediated cell migration. We further observed that HSV-2 infection induced the production of CXCL10 and CXCL11 in addition to CXCL9 in human cervical epithelial cells. Although CXCL10 and CXCL11 could be induced by HSV-2, HSV-2-induced CXCL9 played a critical role in recruitment of $CD4^+$ T cells. Mechanistically, after identifying HSV-2 ICP4 as a vital viral component in inducing CXCR3 ligands, we demonstrated the contribution of ICP4-induced CXCL9 in recruiting $CD4^+$ T cells and a critical role played by p38 MAPK signaling pathway in HSV-2 infection- or ICP4–induced CXCR3 ligand expression. HSV-2 ICP4 binds to the corresponding promoters of CXCR3 ligands by interaction with TBP to activate their transcription. Our study together reveals the molecular mechanism underlying HSV-2-induced

$CD4^+$ T cell accumulation in mucosal infection sites, which may be crucial for understanding HSV-2 infection-enhanced HIV-1 sexual transmission and the development of intervention strategies.

AUTHOR CONTRIBUTIONS

MZ and QH conceived the study. MZ, XD, XG, BZ, RC, DZ, and MF conducted experiments. MZ conducted experiments in **Figures 1**–**6**. XG extracted the cytoplasmic and nuclear proteins in **Figure 5**. BZ and RC conducted western blot experiment in **Figures 5**, **6**, respectively. XD, DZ, and MF provided help in conducting mouse experiments in **Figure 1**. LG, KH, ML, and YL offered advices and technical assistance. HH provided help in the construction of Flag-tagged plasmids. MZ, SG, and QH analyzed the data. MZ and QH wrote the manuscript. All authors reviewed the manuscript.

FUNDING

This work was supported by the National Natural Science Foundation of China (81572009) and the State Key Laboratory of Virology (klv-2016-02).

ACKNOWLEDGMENTS

We thank Juan Min and Ding Gao at the Center for Instrumental Analysis and Metrology, Wuhan Institute of Virology, Chinese Academy of Sciences for technical assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02932/full#supplementary-material

REFERENCES

1. Looker KJ, Magaret AS, Turner KM, Vickerman P, Gottlieb SL, Newman LM. Global estimates of prevalent and incident herpes simplex virus type 2 infections in 2012. *PLoS ONE* (2015) 10:e114989. doi: 10.1371/journal.pone.0114989
2. Shukla D, Spear PG. Herpesviruses and heparan sulfate: an intimate relationship in aid of viral entry. *J Clin Invest.* (2001) 108:503–10. doi: 10.1172/JCI200113799
3. Akhtar J, Shukla D. Viral entry mechanisms: cellular and viral mediators of herpes simplex virus entry. *FEBS J.* (2009) 276:7228–36. doi: 10.1111/j.1742-4658.2009.07402.x
4. Hensel MT, Peng T, Cheng A, De Rosa SC, Wald A, Laing KJ, et al. Selective expression of CCR10 and CXCR3 by circulating human herpes simplex virus-specific CD8 T cells. *J Virol.* (2017) 91:e00810-17. doi: 10.1128/JVI.00810-17
5. Garland SM, Steben M. Genital herpes. *Best Pract Res Clin Obstet Gynaecol.* (2014) 28:1098–110. doi: 10.1016/j.bpobgyn.2014.07.015
6. Grossman Z, Meier-Schellersheim M, Paul WE, Picker LJ. Pathogenesis of HIV infection: what the virus spares is as important as what it destroys. *Nat Med.* (2006) 12:289–95. doi: 10.1038/nm1380
7. UNAIDS (2017). *UNAIDS DATA 2017*. UNAIDS.
8. Rubens M, Ramamoorthy V, Saxena A, Shehadeh N, Appunni S. HIV vaccine: recent advances, current roadblocks, and future directions. *J Immunol Res.* (2015) 2015:560347. doi: 10.1155/2015/560347
9. Salazar-Gonzalez JF, Salazar MG, Keele BF, Learn GH, Giorgi EE, Li H, et al. Genetic identity, biological phenotype, and evolutionary pathways of transmitted/founder viruses in acute and early HIV-1 infection. *J Exp Med.* (2009) 206:1273–89. doi: 10.1084/jem.20090378
10. Freeman EE, Weiss HA, Glynn JR, Cross PL, Whitworth JA, Hayes RJ. Herpes simplex virus 2 infection increases HIV acquisition in men and women: systematic review and meta-analysis of longitudinal studies. *AIDS* (2006) 20:73–83. doi: 10.1097/01.aids.0000198081.09337.a7
11. Horbul JE, Schmechel SC, Miller BR, Rice SA, Southern PJ. Herpes simplex virus-induced epithelial damage and susceptibility to human immunodeficiency virus type 1 infection in human cervical organ culture. *PLoS ONE* (2011) 6:e22638. doi: 10.1371/journal.pone.0022638
12. Posavad CM, Zhao L, Mueller DE, Stevens CE, Huang ML, Wald A, et al. Persistence of mucosal T-cell responses to herpes simplex virus type 2 in the female genital tract. *Mucosal Immunol.* (2015) 8:115–26. doi: 10.1038/mi.2014.47
13. Suazo PA, Tognarelli EI, Kalergis AM, Gonzalez PA. Herpes simplex virus 2 infection: molecular association with HIV and novel microbicides to prevent disease. *Med Microbiol Immunol.* (2015) 204:161–76. doi: 10.1007/s00430-014-0358-x
14. Huang W, Hu K, Luo S, Zhang M, Li C, Jin W, et al. Herpes simplex virus type 2 infection of human epithelial cells induces CXCL9 expression and CD4+ T cell migration via activation of p38-CCAAT/enhancer-binding protein-beta pathway. *J Immunol.* (2012) 188:6247–57. doi: 10.4049/jimmunol.1103706
15. Groom JR, Luster AD. CXCR3 ligands: redundant, collaborative and antagonistic functions. *Immunol Cell Biol.* (2011) 89:207–15. doi: 10.1038/icb.2010.158
16. Groom JR, Richmond J, Murooka TT, Sorensen EW, Sung JH, Bankert K, et al. CXCR3 chemokine receptor-ligand interactions in the lymph node optimize CD4+ T helper 1 cell differentiation. *Immunity* (2012) 37:1091–103. doi: 10.1016/j.immuni.2012.08.016
17. Van Raemdonck K, Van den Steen PE, Liekens S, Van Damme J, and Struyf S. CXCR3 ligands in disease and therapy. *Cytokine Growth Factor Rev.* (2015) 26:311–27. doi: 10.1016/j.cytogfr.2014.11.009
18. Thapa M, Welner RS, Pelayo R, Carr DJJ. CXCL9 and CXCL10 expression are critical for control of genital herpes simplex virus type 2 infection through mobilization of HSV-specific CTL and NK cells to the nervous system. *J Immunol.* (2008) 180:1098–106. doi: 10.4049/jimmunol.180.2.1098
19. Thapa M, Carr DJ. Herpes simplex virus type 2-induced mortality following genital infection is blocked by anti-tumor necrosis factor alpha antibody in CXCL10-deficient mice. *J Virol.* (2008) 82:10295–301. doi: 10.1128/JVI.00931-08
20. Nakanishi Y, Lu B, Gerard C, Iwasaki A. CD8(+) T lymphocyte mobilization to virus-infected tissue requires CD4(+) T-cell help. *Nature* (2009) 462:510–3. doi: 10.1038/nature08511
21. Lind L, Studahl M, Persson Berg L, Eriksson K. CXCL11 production in cerebrospinal fluid distinguishes herpes simplex meningitis from herpes simplex encephalitis. *J Neuroinflamm.* (2017) 14:134. doi: 10.1186/s12974-017-0907-5
22. Zhang M, Liu Y, Wang P, Guan X, He S, Luo S, et al. HSV-2 immediate-early protein US1 inhibits IFN-beta production by suppressing association of IRF-3 with IFN-beta promoter. *J Immunol.* (2015) 194:3102–15. doi: 10.4049/jimmunol.1401538
23. Valera MC, Noirrit-Esclassan E, Dupuis M, Buscato M, Vinel A, Guillaume M, et al. Effect of chronic estradiol plus progesterone treatment on experimental arterial and venous thrombosis in mouse. *PLoS ONE* (2017) 12:e0177043. doi: 10.1371/journal.pone.0177043
24. Hu K, Luo S, Tong L, Huang X, Jin W, Huang W, et al. CCL19 and CCL28 augment mucosal and systemic immune responses to HIV-1 gp140 by mobilizing responsive immunocytes into secondary lymph nodes and mucosal tissue. *J Immunol.* (2013) 191:1935–47. doi: 10.4049/jimmunol.1300120
25. Yan Y, Hu K, Deng X, Guan X, Luo S, Tong L, et al. Immunization with HSV-2 gB-CCL19 fusion constructs protects mice against lethal

vaginal challenge. *J Immunol.* (2015) 195:329–38. doi: 10.4049/jimmunol.1500198
26. Wuest T, Farber J, Luster A, Carr DJ. CD4+ T cell migration into the cornea is reduced in CXCL9 deficient but not CXCL10 deficient mice following herpes simplex virus type 1 infection. *Cell Immunol.* (2006) 243:83–9. doi: 10.1016/j.cellimm.2007.01.001
27. Lilley CE, Groutsi F, Han Z, Palmer JA, Anderson PN, Latchman DS, et al. Multiple immediate-early gene-deficient herpes simplex virus vectors allowing efficient gene delivery to neurons in culture and widespread gene delivery to the central nervous system *in vivo*. *J Virol.* (2001) 75:4343–56. doi: 10.1128/JVI.75.9.4343-4356.2001
28. Dolan A, Jamieson FE, Cunningham C, Barnett BC, McGeoch DJ. The genome sequence of herpes simplex virus type 2. *J Virol.* (1998) 72:2010–21.
29. Rasmussen SB, Jensen SB, Nielsen C, Quartin E, Kato H, Chen ZJ, et al. Herpes simplex virus infection is sensed by both Toll-like receptors and retinoic acid-inducible gene- like receptors, which synergize to induce type I interferon production. *J Gen Virol.* (2009) 90(Pt 1):74–8. doi: 10.1099/vir.0.005389-0
30. Preston CM. Control of herpes simplex virus type 1 mRNA synthesis in cells infected with wild-type virus or the temperature-sensitive mutant tsK. *J Virol.* (1979) 29:275–84.
31. Wyrwicz LS, Rychlewski L. Fold recognition insights into function of herpes ICP4 protein. *Acta Biochim Pol.* (2007) 54:551–9. Available online at: http://psjd.icm.edu.pl/psjd/element/bwmeta1.element.bwnjournal-article-abpv54p551kz?q=e1222a56-6498-4fc8-bf2c-e2ff3aea1619$1&qt=IN_PAGE
32. Quinlan MP, Knipe DM. Stimulation of expression of a herpes simplex virus DNA-binding protein by two viral functions. *Mol Cell Biol.* (1985) 5:957–63. doi: 10.1128/MCB.5.5.957
33. Gelman IH, Silverstein S. Identification of immediate early genes from herpes simplex virus that transactivate the virus thymidine kinase gene. *Proc Natl Acad Sci USA.* (1985) 82:5265–9. doi: 10.1073/pnas.82.16.5265
34. DeLuca NA, Schaffer PA. Activation of immediate-early, early, and late promoters by temperature-sensitive and wild-type forms of herpes simplex virus type 1 protein ICP4. *Mol Cell Biol.* (1985) 5:1997–2008. doi: 10.1128/MCB.5.8.1997
35. Smith CA, Bates P, Rivera-Gonzalez R, Gu B, DeLuca NA. ICP4, the major transcriptional regulatory protein of herpes simplex virus type 1, forms a tripartite complex with TATA-binding protein and TFIIB. *J Virol.* (1993) 67:4676–87.
36. Pugh BF. Control of gene expression through regulation of the TATA-binding protein. *Gene* (2000) 255:1–14. doi: 10.1016/S0378-1119(00)00288-2
37. Hochheimer A, Tjian R. Diversified transcription initiation complexes expand promoter selectivity and tissue-specific gene expression. *Genes Dev.* (2003) 17:1309–20. doi: 10.1101/gad.1099903
38. Louder RK, He Y, Lopez-Blanco JR, Fang J, Chacon P, Nogales E. Structure of promoter-bound TFIID and model of human pre-initiation complex assembly. *Nature* (2016) 531:604–9. doi: 10.1038/nature17394
39. Davidson I. The genetics of TBP and TBP-related factors. *Trends Biochem Sci.* (2003) 28:391–8. doi: 10.1016/S0968-0004(03)00117-8
40. Zhong S, Zhang C, Johnson DL. Epidermal growth factor enhances cellular TATA binding protein levels and induces RNA polymerase I- and III-dependent gene activity. *Mol Cell Biol.* (2004) 24:5119–29. doi: 10.1128/MCB.24.12.5119-5129.2004
41. Carter AB, Knudtson KL, Monick MM, Hunninghake GW. The p38 mitogen-activated protein kinase is required for NF-kappaB-dependent gene expression. The role of TATA-binding protein (TBP). *J Biol Chem.* (1999) 274:30858–63. doi: 10.1074/jbc.274.43.30858
42. Rollenhagen C, Lathrop MJ, Macura SL, Doncel GF, Asin SN. Herpes simplex virus type-2 stimulates HIV-1 replication in cervical tissues: implications for HIV-1 transmission and efficacy of anti-HIV-1 microbicides. *Mucosal Immunol.* (2014) 7:1165–74. doi: 10.1038/mi.2014.3
43. Shannon B, Yi TJ, Thomas-Pavanel J, Chieza L, Janakiram P, Saunders M, et al. Impact of asymptomatic herpes simplex virus type 2 infection on mucosal homing and immune cell subsets in the blood and female genital tract. *J Immunol.* (2014) 192:5074–82. doi: 10.4049/jimmunol.1302916
44. Iijima N, Iwasaki A. T cell memory. A local macrophage chemokine network sustains protective tissue-resident memory CD4 T cells. *Science* (2014) 346:93–8. doi: 10.1126/science.1257530
45. Ochsenbauer C, Edmonds TG, Ding H, Keele BF, Decker J, Salazar MG, et al. Generation of transmitted/founder HIV-1 infectious molecular clones and characterization of their replication capacity in CD4 T lymphocytes and monocyte-derived macrophages. *J Virol.* (2012) 86:2715–28. doi: 10.1128/JVI.06157-11
46. Thapa M, Carr DJ. CXCR3 deficiency increases susceptibility to genital herpes simplex virus type 2 infection: uncoupling of CD8+ T-cell effector function but not migration. *J Virol.* (2009) 83:9486–501. doi: 10.1128/JVI.00854-09
47. Schiffer JT. Mucosal HSV-2 specific CD8+ T-cells represent containment of prior viral shedding rather than a correlate of future protection. *Front Immunol.* (2013) 4:209. doi: 10.3389/fimmu.2013.00209
48. Foley JF, Yu CR, Solow R, Yacobucci M, Peden KWC, Farber JM. Roles for CXC chemokine ligands 10 and 11 in recruiting CD4+ T cells to HIV-1-infected monocyte-derived macrophages, dendritic cells, and lymph nodes. *J Immunol.* (2005) 174:4892–900. doi: 10.4049/jimmunol.174.8.4892
49. Hirota Y, Osuga Y, Koga K, Yoshino O, Hirata T, Morimoto C, et al. The expression and possible roles of chemokine CXCL11 and its receptor CXCR3 in the human endometrium. *J Immunol.* (2006) 177:8813–21. doi: 10.4049/jimmunol.177.12.8813
50. Zeng YJ, Lai W, Wu H, Liu L, Xu HY, Wang J, et al. Neuroendocrine-like cells -derived CXCL10 and CXCL11 induce the infiltration of tumor-associated macrophage leading to the poor prognosis of colorectal cancer. *Oncotarget* (2016) 7:27394–407. doi: 10.18632/oncotarget.8423

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 26 February 2019
doi: 10.3389/fimmu.2019.00290

Herpes Simplex Virus Type 2 Immediate Early Protein ICP27 Inhibits IFN-β Production in Mucosal Epithelial Cells by Antagonizing IRF3 Activation

Xinmeng Guan[1,2], *Mudan Zhang*[3]*, *Ming Fu*[1,2], *Sukun Luo*[4] and *Qinxue Hu*[1,5]*

[1] State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China, [2] University of Chinese Academy of Sciences, Beijing, China, [3] The Joint Center of Translational Precision Medicine, Guangzhou Institute of Pediatrics, Guangzhou Women and Children's Medical Center, Wuhan Institute of Virology, Chinese Academy of Science, Wuhan, China, [4] Wuhan Children's Hospital (Wuhan Maternal and Child Healthcare Hospital), Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, [5] Institute for Infection and Immunity, St George's University of London, London, United Kingdom

OPEN ACCESS

Edited by:
Susmit Suvas,
Wayne State University School of Medicine, United States

Reviewed by:
Marie-Lise Gougeon,
Institut Pasteur, France
Jianzhong Zhu,
Yangzhou University, China

***Correspondence:**
Mudan Zhang
mudan@wh.iov.cn
Qinxue Hu
qhu@wh.iov.cn

Specialty section:
This article was submitted to Viral Immunology, a section of the journal Frontiers in Immunology

Received: 26 October 2018
Accepted: 04 February 2019
Published: 26 February 2019

Citation:
Guan X, Zhang M, Fu M, Luo S and Hu Q (2019) Herpes Simplex Virus Type 2 Immediate Early Protein ICP27 Inhibits IFN-β Production in Mucosal Epithelial Cells by Antagonizing IRF3 Activation. Front. Immunol. 10:290. doi: 10.3389/fimmu.2019.00290

Herpes simplex virus type 2 (HSV-2) is the main cause of genital herpes and infections are common in the lower genital tract. Although neuronal and immune cells can be infected, epithelial cells, and keratinocytes are the primary HSV-2 target cells. HSV-2 establishes latency by evading the host immune system and its infection can also increase the risk of HIV-1 sexual transmission. Our pervious study found that HSV-2 immediate early protein ICP22, inhibited IFN-β production by interfering with the IRF3 pathway. However, ICP22-null HSV-2 did not completely lose the capability of suppressing IFN-β induction, suggesting the involvement of other viral components in the process. In this study, by using an *ex vivo* cervical explant model, we first demonstrated that HSV-2 can indeed inhibit IFN-β induction in human mucosal tissues. We further identified HSV-2 immediate early protein ICP27 as a potent IFN-β antagonist. ICP27 significantly suppresses the Sendai virus or polyinosinic-polycytidylic acid-induced IFN-β production in human mucosal epithelial cells, showing that ICP27 inhibits the IFN-β promoter activation, and IFN-β production at both mRNA and protein levels. Additional studies revealed that ICP27 directly associates with IRF3 and inhibits its phosphorylation and nuclear translocation, resulting in the inhibition of IFN-β induction. Our findings provide insights into the molecular mechanism underlying HSV-2 mucosal immune evasion, and information for the design of HSV-2 mucosal vaccines.

Keywords: HSV-2, ICP27, epithelial cells, IFN-β, IRF3

INTRODUCTION

Herpes simplex virus type 2 (HSV-2) is a large dsDNA virus belonging to the α-Herpesviridae subfamily (1). HSV-2 is mainly transmitted by genital mucosa through sex, causing vesicles, and ulcers after acute infection and can be transported to dorsal root or cranial nerve ganglia to establish life-long latency (2, 3). According to a report by WHO, it is estimated that over 400 million

people were infected with HSV-2 and 19.2 million new infections occurred worldwide in 2012 (4). Due to the greater and more fragile surface of the female reproductive tract, the risk of infection with HSV-2 in females is higher than that in males (5). Epidemiological studies have shown that HSV-2 infection can increase the risk of HIV-1 infection by 3–4 fold (6), with several mechanisms for this increased susceptibility being proposed. For instance, although HSV-2 can infect skin epithelial cells, immune cells and nerve cells, it initially infects mucosal epithelial cells during sexual transmission (7), which may facilitate HIV-1 transmission via perturbation of epithelial integrity. Due to the high positive-incidence of HSV-2 and common routes of transmission with HIV-1, HSV-2 mucosal infection and immune escape has attracted increased attention.

A virus infection initially activates innate immunity through recognition by host pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), RIG-I-like receptors (RLR), and DNA sensors (8–10). These PRRs activate downstream signaling pathways using common components TBK-1 and IKK-ε, leading to the activation of IRF3 signaling (11). IRF3 is an important transcription factor which regulates the expression of type I interferons (IFNs) and IFN stimulate genes (ISGs). IRF3 exists as an inactive monomer in most cells. Upon activation, IRF3 is phosphorylated and assembled into dimers, and then translocated into the nucleus to initialize the transcription of IFN-β (12). Type I IFNs are normally expressed at low levels, and their expression can be enhanced through the JAK/STAT signaling pathway during viral or bacterial infections, resulting in the transcription activation of ISGs (13). The type I IFN family consists of IFN-α, IFN-β, IFN-ε, IFN-κ, and IFN-ω (14), with IFN-β being the most intensively investigated in antiviral innate immunity (15).

During HSV-2 infection, the induction of type I IFNs is extremely low (16), suggesting that HSV-2 has evolved strategies to antagonize IFN production. However, our current understanding of HSV-2 immune evasion is limited, whereas a large number of studies focusing on HSV-1 indicate the involvement of multiple countermeasures in subverting type I IFN production (17, 18). Given that most of these studies address how HSV-1 proteins interfere with IFN production or/and signaling using various cell lines as models (17), whether and how IFN induction is affected in the context of viral infection, at the tissue level, remain elusive. It is known that HSV-2 and HSV-1 exhibit substantial differences in latency and reactivation patterns (19), implying that they may use distinct mechanisms to counteract the host innate immunity. Previous studies by others indicated that HSV-2 virion host shutoff (vhs) protein UL41 suppresses IFN-β expression in human genital epithelial cells (20), while HSV-2 US2 activates NF-κB by binding to TAK1 (21). Our previous study revealed that HSV-2 immediate early protein (IE), ICP22 (US1), inhibits IFN-β production by antagonizing the association of IRF3 with the IFN-β promoter (22). Nevertheless, we observed that ICP22-null HSV-2 did not completely lose the inhibitory activity on IFN-β induction, while other IE proteins including ICP27 (UL54) also appeared to inhibit the activation of the IFN-β promoter, although the underlying mechanism remains to be fully addressed.

Our current study focused on whether and how HSV-2 and its IE protein ICP27, inhibit IFN-β production in mucosal epithelial cells. We found that HSV-2 can inhibit IFN-β induction in human cervical tissues. We further revealed that HSV-2 ICP27 significantly suppresses the Sendai virus or polyinosinic-polycytidylic acid-induced IFN-β production in human mucosal epithelial cells. Mechanistically, we demonstrated that HSV-2 ICP27 directly associates with IRF3 and inhibits its phosphorylation and nuclear translocation, resulting in the inhibition of IFN-β induction.

Abbreviations: HSV-2, herpes simplex virus type 2; SeV, Sendai virus; Poly(I:C), polyinosinic-polycytidylic acid; PEI, Polyethylenimine; DLR, dual luciferase report; Co-IP, co-immunoprecipitation; RT-PCR, real-time quantitative PCR; PRR, pattern recognition receptor; TLR, Toll-like receptor; RLR, RIG-I-like receptor; ISG, IFN stimulate gene; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle medium; ORF, open reading frame.

MATERIALS AND METHODS

Cell Lines and Viruses

HEK 293T, HeLa, and ME180 cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Hyclone), supplemented with 10% fetal bovine serum (FBS) (Gibico), 100 U/ml penicillin and 100 U/ml streptomycin (Genom). All cells were cultured at 37°C in a 5% CO_2 incubator.

HSV-2 (G strain) was obtained from LGC standards and propagated in Vero cells. The Sendai virus (SeV) was propagated in embryonated eggs. Special pathogen-free embryonated eggs (Beijing Merial Vital Laboratory Animal Technology Corporation) were incubated at 37°C for 12 days before inoculation with 300 μl 100 HAU ml^{-1} SeV into the allantoic cavity of 12-day-old embryonated eggs and then incubated at 37°C for 72 h. SeV was collected from allantoic fluids and the titers were measured by hemagglutination (HA) assay using chicken red blood cells.

Isolation of Primary Human Mucosal Epithelial Cells

All protocols involving human subjects were reviewed and approved by the local Research Ethics Committee of Wuhan Institute of Virology, Chinese Academy of Sciences. Informed written consents from the human subjects were obtained in this study, and informed written parental consents were obtained for all participants under the age of 16.

Human cervical or foreskin tissues were obtained from the Wuhan Children's Hospital (Wuhan Maternal and Child Healthcare Hospital), Tongji Medical College, Huazhong University of Science & Technology. Tissues were washed carefully with PBS and then minced into small pieces. Prepared tissue pieces were incubated with 5–10 ml Dispase II Solution (25 U/ml Dispase II in PBS pH 7.4 without Ca/Mg) containing penicillin and streptomycin. 10% FBS was then added to avoid excess damage to cells. Following an incubation at 4°C overnight, peeled off epidermis was rinsed with PBS, and placed into 3–5 ml 0.05% Trypsin with EDTA in a 50 ml conical tube. After an incubation in a 37°C water bath for 15–30 min with agitation

every 5 min, twice the EDTA volume of medium with 10% FBS was added. The epidermal cells were released by inverting the tube several times or by pipetting the suspension. The cell/tissue solution was passed through a sterile sieve followed by centrifugation. Cell pellets were resuspended in EpiLife medium, and the isolated epithelial cells were cultured in 12-well plates at 37°C in a 5% CO_2 incubator.

Construction of Plasmids

Primers used for plasmid construction are listed in **Supplementary Table 1**. The open reading frame (ORF) of HSV-2 ICP27 was amplified from HSV-2 genomic DNA extracted from HSV-2 G strain by PCR. For some constructs, an N-terminal Flag or HA was introduced by PCR. PCR products were cloned into pcDNA3.1(+)/(-) (Invitrogen), and the constructed expression plasmids were named ICP27, ICP27-Flag, ICP27-HA, $ICP27_{(1-138aa)}$, $ICP27_{(1-152aa)}$, and $ICP27_{(1-302aa)}$, respectively. All constructs were verified by DNA sequencing (Sunny Biotechnology). The reporter plasmid $PRD(III-I)_4$-Luc was provided by Dr. Stephan Ludwig (University of Muenster, Muenster, Germany). The reporter plasmid p125-Luc and the internal control plasmid phRL-TK were described previously (23). IRF3 and IRF3-5D expression plasmids pIRES-hrGFP/IRF3-Flag and pIRES-hrGFP/IRF3-5D-Flag (constitutively active mutant of IRF3) were provided by Dr. Yiling Lin (Graduate Institute of Life Sciences, National Defense Medical Center, Taiwan, China). pEF-Flag-RIG-IN (a carboxy-terminally truncated, constitutively active RIG-I mutant) expression plasmid was provided by Dr. Takashi Fujita (Kyoto University, Kyoto, Japan). pcDNA3-MAVS-Flag expression plasmid was provided by Dr. Hanzhong Wang (Wuhan Institute of Virology, Wuhan, China). pcDNA3-TBK1-Flag and pcDNA3-IKKε-Flag expression plasmids were provided by Dr. Katherine Fitzgerald (University of Massachusetts Medical School, Worcester, MA). Plasmids encoding influenza virus PR8/NS1 and HSV-2 ICP22, respectively, were described previously (22).

Dual Luciferase Report (DLR) Assay

HEK 293T cells were seeded in 24-well plates overnight and co-transfected with empty vector or plasmid encoding HSV-2 ICP27, truncated HSV-2 ICP27 or influenza virus NS1, reporter plasmid p125-Luc or $PRD(III-I)_4$-Luc and internal control phRL-TK. Transfections were performed using Lipofectamine 2000 (Life Technology, 11668019) according to the manufacturer's instructions. At 24 h post-transfection, cells were stimulated with or without 100 HAU ml^{-1} SeV for 16 h. Cells were harvested and lysed, and the lysates were used for measuring firefly and Renilla luciferase activities using a Dual-luciferase Reporter Assay System (Promega, E1980) according to the manufacturer's instructions. For some experiments, HEK 293T cells were co-transfected with empty vector or ICP27 expression plasmid, reporter plasmid p125-Luc and internal control phRL-TK, together with plasmid encoding the IRF3 pathway inducer RIG-IN, MAVS, TBK-1, IKK-ε, or IRF3-5D. At 40 h post-transfection, the enzymatic activities of firefly and Renilla luciferase were measured.

Immunoblot Assay

The proteins extracted from transfected or infected cells were prepared using Pierce™ IP Lysis Buffer (ThermoFisher Scientific, 87787) supplemented with protein inhibitor (cOmplete Protease Inhibitor Cocktail, 11697498001). The protein samples were resolved by SDS-PAGE and transferred onto PVDF membranes (0.45 μm, Millipore). Cytoplasmic and nuclear proteins were isolated using the Nuclear-Cytosol Extraction Kit (Applygen, P1200-50).

The antibody (Ab) anti-HSV-1 ICP27+HSV-2 ICP27 was purchased from Abcam (ab31631). Rabbit anti IRF3 Polyclonal Antibody, Rabbit anti PCNA Ab and Mouse mAb anti HA-tag were purchased from Proteintech (11312-1-AP, 10205-2-AP, and 66006-1-Ig). Rabbit mAb against phospho-IRF-3 (Ser396) was purchased from Cell Signaling Technology (4947S). Rabbit mAb against HA-tag and Mouse mAb against Flag-tag were purchased from Sigma-Aldrich (H6908 and F1804). Mouse mAb anti β-actin was purchased from Santa Cruz Biotechnology (sc81178). HRP-conjugated goat anti-rabbit IgG (H+L) and HRP-conjugated goat anti-mouse IgG (H+L) were purchased from ThermoFisher Scientific (ZB-2301 and ZB-2305). HRP-conjugated mouse anti-rabbit IgG (Light Chain) was purchased from Sangon Biotech (D110059-0100). Mouse IgG and Rabbit IgG were purchased from BOSTER (BA1046 and BA1045). Alexa Fluor 488-labeled Goat Anti-Mouse IgG (H+L), Alexa Fluor 647-labeled Goat Anti-Rabbit IgG (H+L) and DAPI Staining Solution were purchased from Beyotime (A0428, A0468, and C1006).

RNA Isolation and Quantitative PCR

The transfected cells were collected and total RNAs were extracted using TRIzol (Invitrogen, 15596-026) according to the manufacturer's instructions. cDNA was synthesized by M-MLV Reverse Transcriptase (Promega, M1705). The newly synthesized cDNA was used as template for the amplification of *IFN-β, ISG15, ISG56, CXCL10,* and *GAPDH*. The primer pairs for *IFN-β*, *ISG15*, *ISG56*, *CXCL0,* and *GAPDH* were described previously (22, 24). Relative real-time quantitative PCR (RT-PCR) was performed on BioRad StepOne apparatus using a TransStart® Tip Green qPCR SuperMix (Transgen, AQ141-02), and GAPDH was used as an internal control with conditions of 95°C for 3 min, followed by 40 cycles of 95°C for 10 s, and 55°C for 30 s. The expression difference was calculated on the basis of $2^{-\Delta\Delta Ct}$ values.

ICP27 Knockdown by siRNA

HSV-2 siRNA sequences were described previously (25), and are listed in the **Supplementary Table 1**. All siRNAs were synthesized by Eurofins Genomics. HeLa or ME180 cells were seeded in 6-well plates overnight. Negative control or siRNAs were transfected into HeLa or ME180 cells using Lipofectamine 2000 (Life Technology, 11668019) according to the manufacturer's instruction. At 4 h post-transfection, HeLa cells were infected with or without HSV-2 at an MOI of 1, or ME180 cells at an MOI of 0.5. At 20 h post-infection, cells were stimulated with or without 100 HAU ml^{-1} SeV for 16 h, and supernatants were harvested for ELISA or cells were lysed for DLR assay.

Poly(I:C) Stimulation

HeLa or ME180 cells were seeded in 6-well plates overnight and transfected with empty vector, HSV-2 ICP27 expression plasmid, HSV-2 ICP22 expression plasmid or influenza virus NS1 expression plasmid. At 24 h post-transfection, cells were transfected with 2 μg/well Poly(I:C) (Sigma; P1530-25MG) using Lipofectamine 2000 (Life Technology, 11668019) or mock-transfected. At 16 h post-transfection, cells were lysed for DLR assay or supernatants were harvested for ELISA.

ELISA for IFN-β

HEK 293T cells were seeded in 6-well plates overnight and transfected with empty vector, HSV-2 ICP27 expression plasmid or influenza virus NS1 expression plasmid. At 24 h post-transfection, cells were stimulated with or without 100 HAU ml^{-1} SeV for 16 h. Cell culture supernatants were collected and centrifuged to remove cell debris. Fifty microliters of supernatants were used for IFN-β detection using a VeriKine™ Human IFN Beta ELISA Kit (PBL Assay Science, 41410) according to the manufacturer's instructions.

Immunofluorescence Assay

HeLa cells were seeded in 35 mm glass-bottom dishes and transfected with an empty vector, HSV-2 ICP27-HA expression plasmid or an influenza virus NS1 expression plasmid. At 24 h post-transfection, HeLa cells were stimulated with or without 100 HAU ml^{-1} SeV for 16 h. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. After three washes with PBS, cells were blocked with PBS containing 5% BSA for 1 h at room temperature, and then incubated with rabbit anti-human IRF3 polyclonal Ab and mouse anti HA-tag mAb at a dilution of 1:100 for 1 h at room temperature. After three washes with PBS, cells were incubated with Alexa Fluor 488-labeled Goat Anti-Mouse IgG (H+L) and Alexa Fluor 647-labeled Goat Anti-Rabbit IgG (H+L) at a dilution of 1:50 for 1 h at room temperature. Cells were subsequently washed and incubated with DAPI solution for 10 min at room temperature. Following the addition of 1 ml PBS into the dishes, cells were observed under a Multiphoton Confocal Microscope (Nikon, A1 MP STORM).

Co-immunoprecipitation Assay

HEK 293T cells were seeded in 6-well plates and transfected with ICP27-Flag plasmid or empty vector. At 24 h post-transfection, cells were stimulated with or without 100 HAU ml^{-1} SeV for 16 h. The proteins extracted from transfected cells were prepared using Pierce™ IP Lysis Buffer (ThermoFisher Scientific, 87787). Three microgram mouse anti-Flag Ab or control mouse IgG was diluted in 200 μl PBS with 0.2% Tween-20 (PBST) and added to fresh Dynabeads protein G (Invitrogen, 10009D). After incubation with rotation at 4°C overnight, Dynabeads-Ab complexes were washed once with 200 μl PBST before mixed with the samples, followed by incubation at 4°C overnight. The complexes were washed three times with PBST, and target Ags were subjected to Western Blot analysis after elution by boiling.

Binding Kinetic Analysis

HEK 293F cells were used for the expression and purification of ICP27-Flag and IRF3-Flag. For every 1×10^6 cells, 1.5 μg expression plasmid was transfected into HEK 293F cells using Polyethylenimine (PEI) transfection reagent (Polysciences, 23966-1). Cells were cultured in FreeStyle 293 Expression Medium (Gibico, 12338018) at 37°C in a 5% CO_2 incubator shaker at 135 rpm. At day 3 post-transfection, cells were harvested and lysed by ultrasonic treatment. The Flag-tagged protein was purified by Anti-DYKDDDDK G1 Affinity Resin (GeneScript, L00432) and eluted with 3 M NaCl. The purified protein was concentrated in PBS using 30 kDa Centrifugal Filter Units (Merck, UFC903008) for binding kinetic study.

The kinetics of binding was performed on a Forte-Bio Octet Red System as described previously (26). This system monitors interference of light reflected from the surface of a sensor to measure the thickness of molecules bound to the sensor surface. IRF3-Flag was conjugated with biotin at a molecular weight ratio of 1:3 at room temperature for 1 h followed by washes, and then concentrated in PBS to remove dissociative biotin. 10 microgram/milliliter biotinylated IRF3-Flag was coupled to Biosenors (Fortebio, 18-5019) and immersed in different concentration of ICP27-Flag (50, 200, 500, or 800 nM) for association and disassociation. The response in nm shift was recorded as a function of time.

Statistical Analysis

All experiments were repeated at least three times and the data are presented as mean ± S.D., unless otherwise specified. Data analyses were performed with GraphPad Prism 7 software (GraphPad). A comparison between the two groups was analyzed using a two tailed unpaired Student's *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

HSV-2 ICP27 Inhibits IFN-β Production in Human Mucosal Epithelial Cells

HSV-2 evades mucosal innate immunity, but the underlying mechanisms remain elusive (16). Our previous study showed that HSV-2 immediate early protein ICP22 strongly inhibited IFN-β production; however, knockout of ICP22 did not fully abolish the inhibitory activity on IFN-β production in the context of HSV-2 infection (22), while other IE proteins including ICP27 (UL54) also appeared to be involved, but the underlying mechanism remains to be fully investigated. In this study, we performed experiments to assess whether HSV-2 ICP27 indeed inhibits IFN-β induction, and if so, what is the underlying mechanism. We first confirmed that IFN-β was expressed at a very low level in HSV-2-infected human cervical tissues **(Figure 1A)**, informing that HSV-2 inhibits IFN-β induction during mucosal infection. To assess the contribution of HSV-2 ICP27 in interfering with IFN-β induction, HEK 293T cells were seeded in 24-well plates overnight and co-transfected with empty vector pcDNA3.1(+), plasmid expressing HSV-2 ICP27 or the positive control influenza virus NS1 together with the reporter plasmid p125-Luc and the internal control phRL-TK. At 24 h

post-transfection, cells were stimulated with or without 100 HAU ml^{-1} SeV for 16 h. As shown in **Figure 1B**, HSV-2 ICP27 strongly inhibited the activation of the IFN-β promoter. We subsequently examined whether HSV-2 ICP27 inhibits the production of IFN-β at mRNA or protein level. HEK 293T cells were seeded in 6-well plates overnight and transfected with pcDNA3.1(+), plasmid expressing HSV-2 ICP27 or the influenza virus NS1. At 24 h post-transfection, cells were stimulated with or without 100 HAU ml^{-1} SeV for 16 h. Total RNAs were extracted and IFN-β mRNA was analyzed by RT-PCR. As shown in **Figure 1C**, HSV-2 ICP27 significantly inhibited the production of IFN-β mRNA. The level of IFN-β proteins in the supernatants was measured by ELISA, showing that HSV-2 ICP27 significantly inhibited the production of INF-β at protein level (**Figure 1D**).

In addition to SeV stimulation, we also conducted experiments under the condition of the IFN-β expression induced by polyinosinic-polycytidylic acid [Poly(I:C)], an artificial dsRNA sequence which can stimulate RIG-I signaling pathway (27). HeLa cells were co-transfected with pcDNA3.1(+), plasmid expressing HSV-2 ICP27, HSV-2 ICP22, or the influenza virus NS1, together with the reporter plasmid p125-Luc and the internal control phRL-TK. At 24 h post-transfection, cells were stimulated with Poly(I:C) for 16 h and lysed for DLR assay. The results revealed that HSV-2 ICP27 strongly inhibited Poly(I:C)-induced activation of the IFN-β promoter (**Figure 1E**). In addition, HeLa cells were transfected with pcDNA3.1(+), plasmid expressing HSV-2 ICP27, HSV-2 ICP22, or the influenza virus NS1. At 24 h post-transfection, cells were stimulated with Poly(I:C) for 16 h, and supernatants were harvested for ELISA. The results revealed that HSV-2 ICP27 strongly inhibited Poly(I:C)-induced IFN-β induction at protein level (**Figure 1F**). In addition, HSV-2 ICP27 also significantly inhibited Poly(I:C) induced activation of the IFN-β promoter in ME180 cells (**Supplementary Figure 1A).**

Impaired expression of type I IFNs leads to reduced expression of the downstream interferons stimulated genes (ISGs) (28, 29). To examine the impact of HSV-2 ICP27 on ISG expression, HEK 293T cells were transfected with empty vector pcDNA3.1(+) or plasmid expressing HSV-2 ICP27, followed by stimulation with SeV for 16 h. Total RNAs were extracted, and the mRNAs of ISGs including *ISG56*, *ISG15,* and *CXCL10* were analyzed by RT-PCR. As shown in **Figures 1G–I**, HSV-2 ICP27 strongly inhibited the expression of *ISG56*, *ISG15,* and *CXCL10* at mRNA level.

Having demonstrated the critical role of HSV-2 ICP27 in inhibiting IFN-β induction, we next conducted experiments to examine the impact of HSV-2 ICP27 on IFN-β induction in the context of HSV-2 infection. HeLa cells were co-transfected with the reporter plasmid p125-Luc and the internal control phRL-TK, together with negative control siRNA, ICP27 siRNA-1 or ICP27 siRNA-2, followed by infection with HSV-2. Cells were then stimulated with or without 100 HAU ml^{-1} SeV for 16 h and lysed for DLR assay. As shown in **Figure 2A**, knockdown of HSV-2 ICP27 by ICP27 siRNA-1 reduced the capability of HSV-2 in inhibiting IFN-β production at promoter activation level. To assess the impact of HSV-2 ICP27 on IFN-β production at protein level, HeLa cells were treated with negative control siRNA, ICP27 siRNA-1 or ICP27 siRNA-2, followed by infection with HSV-2. At 24 h post-infection, supernatants were collected for ELISA and cells were lysed for Western Blot. As shown in **Figure 2B**, knockdown of HSV-2 ICP27 by ICP27 siRNA-1 reduced the capability of HSV-2 in inhibiting IFN-β production at the protein level. Knockdown of HSV-2 ICP27 was detected by a monoclonal antibody against HSV-2 ICP27, while β-actin was used as an internal control (**Figure 2C**). We also found that knockdown of HSV-2 ICP27 reduced the capability of HSV-2 in inhibiting IFN-β promoter activation in ME180 cells (**Supplementary Figure 1B**).

HSV-2 mainly infects epithelial cells and causes genital herpes. In addition to human cervical tissue and cervicovaginal epithelial cell lines, we performed experiments using primary human foreskin epithelial cells. Human foreskin epithelial cells were isolated and transfected with empty vector pcDNA3.1(+), plasmid expressing HSV-2 ICP27 or ICP22, or infected with HSV-2, followed by stimulation with or without 100 HAU ml^{-1} SeV for 16 h. Total RNAs were extracted for RT-PCR and supernatants were collected for ELISA. In primary human foreskin epithelial cells, HSV-2 ICP27 significantly inhibited SeV-induced IFN-β production at both mRNA (**Figure 3A**) and protein (**Figure 3B**) levels. These results indicated that HSV-2 ICP27 can inhibit IFN-β induction in primary mucosal epithelial cells.

Altogether, the above findings inform that HSV-2 ICP27 plays an essential role in interfering with the induction of IFN-β in human cervical tissues, cervicovaginal epithelial cell lines, and primary human mucosal epithelial cells.

HSV-2 ICP27 Inhibits IFN-β Production Through IRF3 Signaling Pathway

HSV-1 ICP27 has recently been reported to inhibit type I IFNs by interacting with STING-TBK1 complex in macrophages (30), whereas our previous study found that HSV-2 can interrupts RIG-I mediated IFN-β signaling pathway in human epithelial cells (22). Given that DNA viruses produce dsRNAs during viral replication, which can be recognized by RNA sensors like RIG-I (31, 32), our current study focused on how HSV-2 ICP27 interferes with dsRNA-mediated induction of type I IFNs. We first determined whether HSV-2 ICP27 affects the IRF3-mediated signaling pathway. HEK 293T cells were seeded in 24-well plates overnight and co-transfected with empty vector pcDNA3.1(+), plasmid expressing HSV-2 ICP27 or the influenza virus NS1, together with $PRD(III\text{-}I)_4$-Luc which contains four repeats of IRF3 responsive domain of the IFN-β promoter and the internal control phRL-TK. At 24 h post-transfection, cells were stimulated with or without 100 HAU ml^{-1} SeV for 16 h and lysed for DLR assay. As shown in **Figure 4A**, HSV-2 ICP27 blocks the activation of the IRF3 responsive promoter induced by SeV.

We next carried out experiments to address whether HSV-2 ICP27 directly affects the IRF3 signaling pathway. HEK 293T cells were seeded in 24-well plates overnight and co-transfected with a plasmid expressing IRF3 signaling pathway component RIG-IN, MAVS, TBK1, IKK-ε, or IRF3-5D and HSV-2 ICP27 expressing plasmid or empty vector pcDNA3.1(+), together with the reporter plasmid p125-Luc and the internal control phRL-TK.

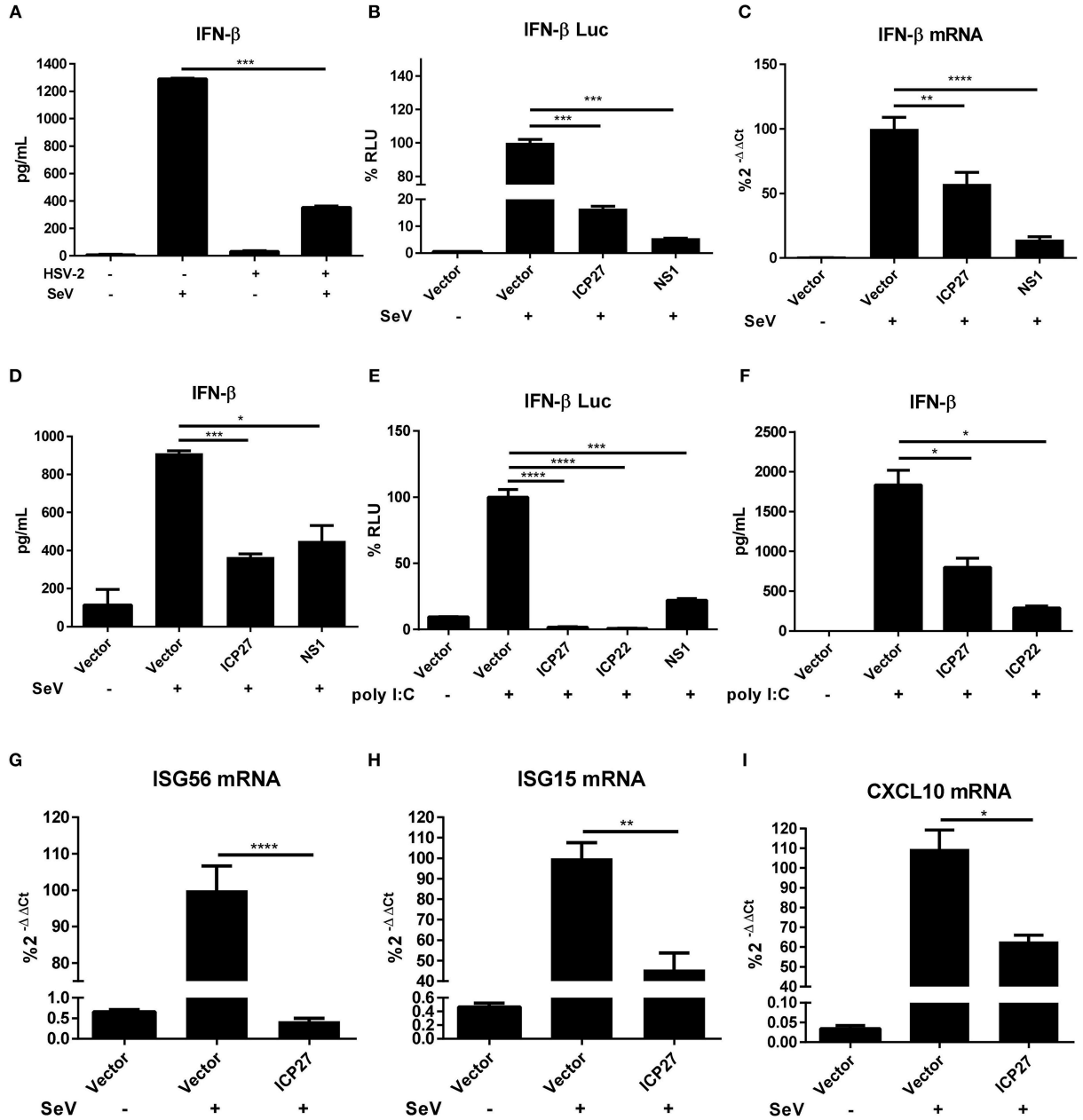


FIGURE 1 | HSV-2 ICP27 inhibits IFN-β production in human mucosal epithelial cells. **(A)** HSV-2 inhibits IFN-β production in human cervical tissues. Human cervical tissues were prepared and infected with or without HSV-2. At 4 h.p.i, the tissues were stimulated with or without 100 HAU ml^{-1} SeV for 16 h, and the supernatants were harvested for ELISA. **(B)** HSV-2 ICP27 inhibits the activation of the IFN-β promoter. HEK 293T cells were seeded in 24-well plates and co-transfected with empty vector pcDNA3.1(+), HSV-2 ICP27 or influenza virus NS1 expressing plasmid, together with the reporter plasmid p125-Luc and the internal control phRL-TK. At 24 h post-transfection, cells were stimulated with or without 100 HAU ml^{-1} SeV for 16 h and lysed for DLR assay. **(C,D)** HSV-2 ICP27 inhibits IFN-β production at both mRNA and protein levels. HEK 293T cells were seeded in 6-well plates and transfected with empty vector pcDNA3.1(+), plasmid expressing HSV-2 ICP27 or influenza virus NS1. At 24 h post-transfection, cells were stimulated with or without 100 HAU ml^{-1} SeV for 16 h and total RNAs were extracted for RT-PCR **(C)**, while the supernatants were harvested for ELISA **(D)**. **(E)** HSV-2 inhibits the Poly(I:C)-induced activation of the IFN-β promoter. HeLa cells were seeded in 6-well plates and co-transfected with empty vector pcDNA3.1(+), plasmid expressing HSV-2 ICP27, ICP22 or the influenza virus NS1, together with the reporter plasmid p125-Luc and the internal control phRL-TK. At 24 h post-transfection, cells were transfected with 2 μg/well Poly(I:C) or mock-transfected for 16 h and lysed for DLR assay. **(F)** HSV-2 ICP27 inhibits Poly(I:C)-induced IFN-β production at protein level. HeLa cells were seeded in 6-well plates and transfected with empty vector pcDNA3.1(+), plasmid expressing HSV-2 ICP27 or ICP22. At 24 h post-transfection, cells were transfected with 2 μg/well Poly(I:C) or mock-transfected for 16 h, and the supernatants were harvested for ELISA. **(G–I)** HSV-2 ICP27 inhibits the production of ISGs. HEK 293T cells were seeded in 6-well plates and transfected with empty vector pcDNA3.1(+) or HSV-2 ICP27 expressing plasmid. At 24 h post-transfection, cells were stimulated with or without 100 HAU ml^{-1} SeV for 16 h and lysed for RT-PCR to measure the expression of *ISG56* **(D)**, *ISG15* **(E)**, and *CXCL10* **(F)** at the mRNA level. The data shown are representative of three independent experiments, with each condition performed in triplicate (mean ± SD) **(A–I)**. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

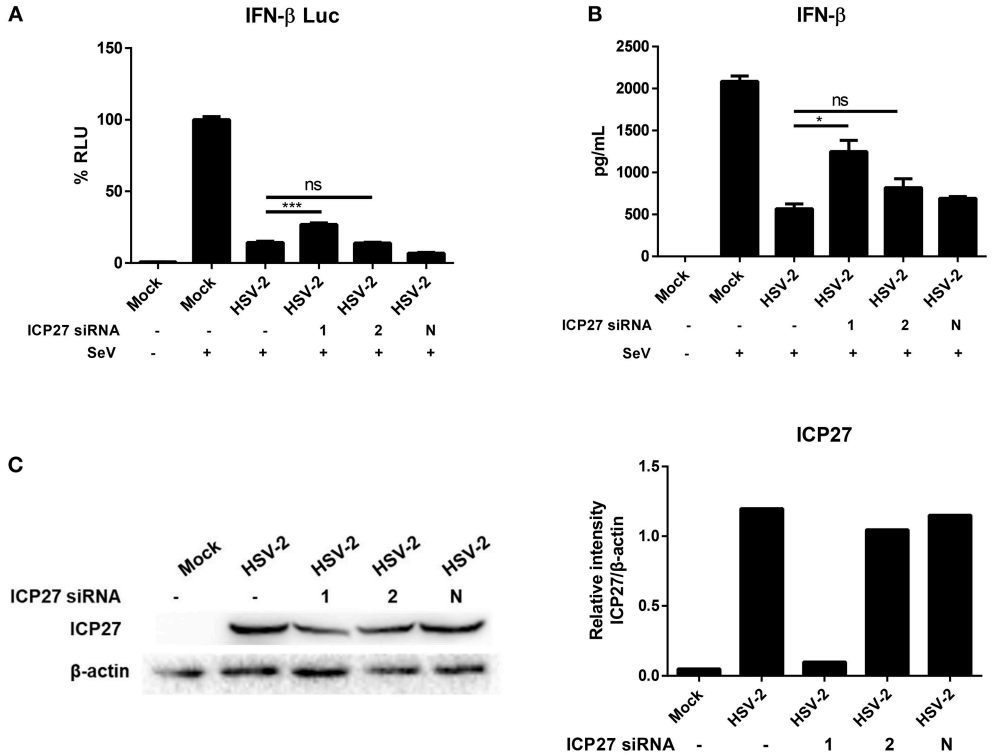


FIGURE 2 | HSV-2 ICP27 inhibits IFN-β production in the context of virus infection. **(A)** Knockdown of ICP27 reduces the capability of HSV-2 in inhibiting the IFN-β promoter activation. HeLa cells were seeded in 6-well plates and co-transfected with HSV-2 ICP27 siRNA-1, siRNA-2, or negative control siRNA (N), together with the reporter plasmid p125-Luc and the internal control phRL-TK. At 4 h post-transfection, cells were infected with HSV-2 at an MOI of 1 or mock-infected. At 20 h.p.i, cells were stimulated with or without 100 HAU ml^{-1} SeV for 16 h and lysed for DLR assay. **(B)** Knockdown of ICP27 reduces the capability of HSV-2 in inhibiting the IFN-β production at the protein level. HeLa cells were seeded in 6-well plates and transfected with HSV-2 ICP27 siRNA-1, siRNA-2, or negative control siRNA (N). At 4 h post-transfection, cells were infected with HSV-2 at an MOI of 1 or mock-infected. At 20 h.p.i, cells were stimulated with or without 100 HAU ml^{-1} SeV for 16 h, and the supernatants were harvested for ELISA. **(C)** Knockdown efficiency of HSV-2 ICP27 siRNA. HeLa cells were seeded in 6-well plates and transfected with HSV-2 ICP27 siRNA-1, siRNA-2, or negative control siRNA (N). At 4 h post-transfection, cells were infected with HSV-2 at an MOI of 1 or mock-infected. At 20 h.p.i, cells were stimulated with or without 100 HAU ml^{-1} SeV for 16 h and lysed for Western Blot. Gray scale scanning was performed by Image J software (version 1.52a). The data shown are representative of three independent experiments, with each condition performed in triplicate (mean ± SD) **(A,B)**. $^{*}P < 0.05$; $^{***}P < 0.001$. One representative experiment out of three is shown **(C)**.

As shown in **Figures 4B–F**, HSV-2 ICP27 blocks the activation of the IFN-β promoter induced by all the tested IRF3 signaling pathway components in a dose-dependent manner.

Altogether, the above results indicate that, unlike HSV-1 ICP27, HSV-2 ICP27 inhibits IFN-β induction through an IRF3 dependent pathway.

HSV-2 ICP27 Blocks IRF3 Activation by Physically Interacting With IRF3

There are two main steps involved in IRF3 activation, phosphorylation and nuclear translocation (12). We first investigated whether HSV-2 ICP27 interferes with IRF3 nuclear translocation. Hela cells were seeded in 35 mm glass-bottom dishes overnight and transfected with plasmid expressing HSV-2 ICP27-HA or influenza virus NS1, or empty vector pcDNA3.1(+). At 24 h post-transfection, cells were stimulated with or without 100 HAU ml^{-1} SeV for 16 h. Indirect immunofluorescence assay was performed to assess IRF3 localization in the presence or absence of HSV-2 ICP27. As shown in **Figure 5A**, IRF3 translocation from the cytoplasm to the nucleus was partially blocked in the presence of HSV-2 ICP27.

Subsequent experiments were conducted to examine whether HSV-2 ICP27 blocks the phosphorylation of IRF3. HEK 293T cells were seeded in 6-well plates overnight and transfected with plasmid expressing HSV-2 ICP27 or influenza virus NS1, or empty vector. At 24 h post-transfection, cells were stimulated with or without 100 HAU ml^{-1} SeV for 16 h. The phosphorylation of IRF3 was detected by an anti-p-IRF3 Ab, showing that HSV-2 ICP27 significantly inhibited IRF3 phosphorylation in cells (**Supplementary Figure 2**), and particularly in the nucleus (**Figure 5B**).

We next asked whether there is an interaction between HSV-2 ICP27 and IRF3. Co-immunoprecipitation (Co-IP) was therefore carried out. HEK 293T cells were seeded in 6-well plates overnight and transfected with plasmid expressing HSV-2 ICP27-Flag or empty vector pcDNA3.1(+). At 24 h post-transfection, cells were stimulated with or without 100 HAU ml^{-1} SeV for 16 h, followed by Co-IP with a control IgG or an anti-Flag Ab. The

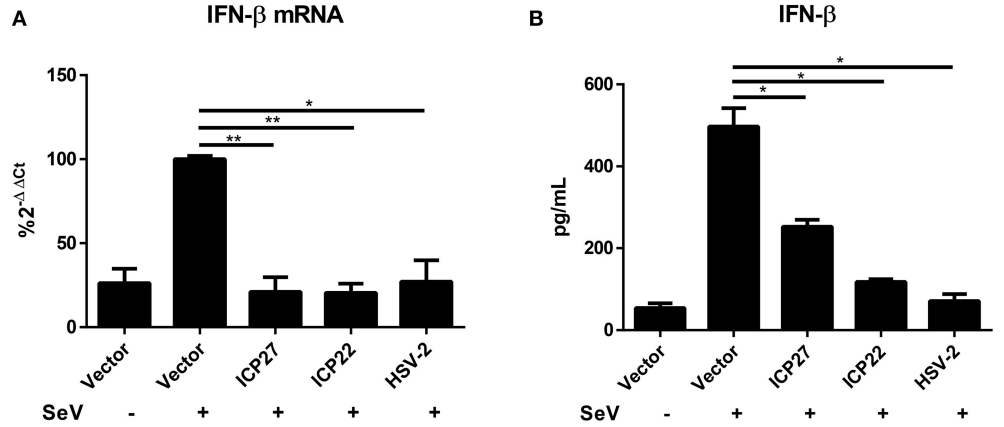


FIGURE 3 | HSV-2 ICP27 inhibits IFN-β production in primary mucosal epithelial cells. **(A,B)** HSV-2 ICP27 inhibits IFN-β production in human foreskin epithelial cells. Primary epithelial cells were isolated from human foreskin tissues and transfected with empty vector pcDNA3.1(+), plasmid expressing HSV-2 ICP27 or ICP22 or infected with HSV-2. At 24 h post-transfection, cells were stimulated with or without 100 HAU ml^{-1} SeV for 16 h, and total RNAs were extracted for RT-PCR **(A)**, while the supernatants were harvested for ELISA **(B)**. The data shown are representative of three independent experiments, with each condition performed in triplicate (mean ± SD) **(A,B)**. *$P < 0.05$; **$P < 0.01$.

precipitates were analyzed by Western Blot using an anti-IRF3 Ab against endogenous IRF3. As shown in **Figure 5C**, HSV-2 ICP27 was able to specifically precipitate the endogenous IRF3, indicating a physical interaction between HSV-2 ICP27 and IRF3. To further confirm the interaction between IRF3 and ICP27, we purified ICP27 and IRF3 proteins, and carried out binding kinetic analyses. A biotinylated IRF3-Flag was coupled to Biosenors and immersed in different concentrations of the ICP27-Flag for association and disassociation. As shown in **Figure 5D**, there was a strong association between IRF3 and ICP27, and a higher concentration of ICP27 resulted in a stronger association.

Altogether these results indicate that HSV-2 ICP27 antagonizes the IRF3 signaling pathway by interacting with IRF3.

The 1-138aa Domain of HSV-2 ICP27 Is Mainly Responsible for the Inhibition of IFN-β Induction

To map the functional region of HSV-2 ICP27 involved in the inhibition of IFN-β production, we constructed several HSV-2 ICP27 truncation mutants according to its structure (https://www.uniprot.org/uniprot/P28276) (**Figure 6A**). HEK 293T cells were co-transfected with empty vector pcDNA3.1(+), truncated or full-length HSV-2 ICP27 expressing plasmid, with the reporter plasmid p125-Luc and the internal control phRL-TK. At 24 h post-transfection, cells were stimulated with or without 100 HAU ml^{-1} SeV for 16 h and lysed for DLR assay. To measure IFN-β at protein level, HEK 293T cells were transfected with empty vector pcDNA3.1(+), truncated or full-length HSV-2 ICP27 expressing plasmid, followed by stimulation with or without 100 HAU ml^{-1} SeV for 16 h, and supernatants were harvested for ELISA. As shown in **Figures 6B**,**C**, the 1-138aa domain of HSV-2 ICP27 significantly inhibited IFN-β induction at both promoter activation and protein levels, and the capability was comparable to that of the full-length HSV-2 ICP27. The expression of ICP27 mutants was confirmed by Western Blot (**Supplementary Figure 3**). In contrast to that of HSV-1 ICP27 (30), we did not observe a direct contribution of the RGG box, which is located in the 138-152aa domain of HSV-2 ICP27, to the inhibited IFN-β induction. Our findings indicate that the 1-138aa domain, rather than the RGG box of HSV-2 ICP27 is mainly responsible for the inhibition of IFN-β induction.

DISCUSSION

HSV-2 is a large enveloped dsDNA virus, and its infections are known to be restricted to mucosal and keratinized epithelia and neuronal ganglia. HSV-2 infection causes genital herpes with sexual transmission being the main route. It is known that HSV-2 can evade the host mucosal innate immunity, but the underlying mechanisms remain to be defined (16). Our current study has demonstrated that HSV-2 immediate early protein, ICP27, interferes with RIG-I-MAVS-IRF3-mediated IFN-β induction in mucosal epithelial cells and HEK 293T cells. Mechanistically, ICP27 directly associates with IRF3 and inhibits its phosphorylation and nuclear translocation, resulting in the inhibition of IFN-β induction. Findings in this study reveal an unconventional strategy exploited by a dsDNA virus to interrupt the type I IFN signaling pathway.

It is generally accepted that DNA viruses are recognized by DNA sensors such as TLR9 and cGAS (33), while RNA viruses are sensed by RNA sensors including TLR3, TLR7/8, RIG-I and MDA5 (34, 35). Indeed, a number of studies on HSV-1 reported that the virus can interfere with the cGAS-STING pathway to inhibit type I IFN induction (30, 36–38). Of interest, we previously found that HSV-2 can also interrupt RIG-I-MAVS-IFN-β pathway in human mucosal epithelial cells (22). One explanation is that, DNA viruses produce dsRNAs during their replication cycles, which can be recognized by RNA sensors like RIG-I (31, 32). In addition, STING has also been reported

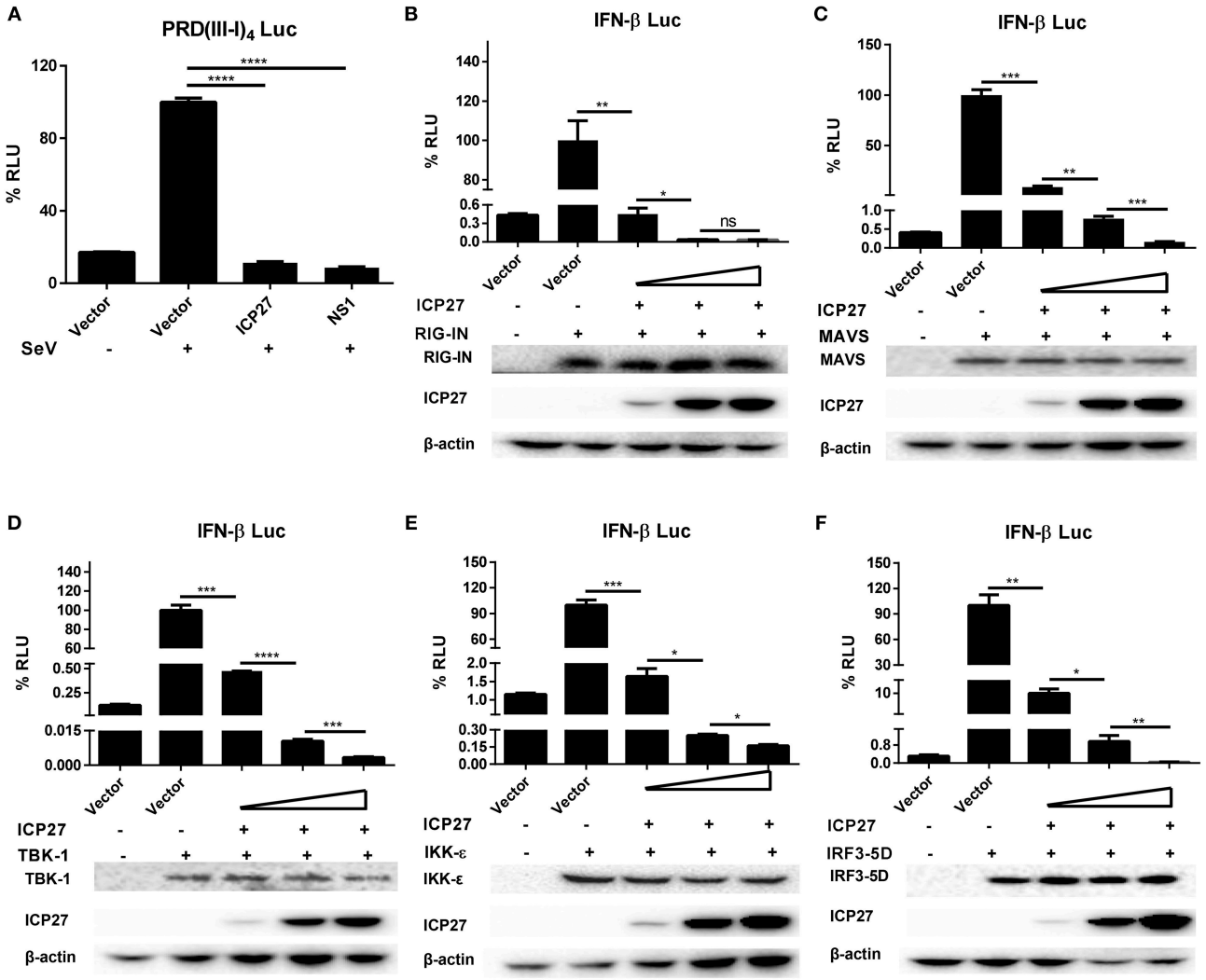


FIGURE 4 | HSV-2 ICP27 inhibits IFN-β production through IRF3 signaling pathway. **(A)** HSV-2 ICP27 inhibits the IRF3 promoter element PRD(III-I)$_4$. HEK 293T cells were seeded in 24-well plates and co-transfected with empty vector pcDNA3.1(+), plasmid expressing HSV-2 ICP27 or influenza virus NS1, together with PRD(III-I)$_4$-Luc and the internal control phRL-TK. At 24 h post-transfection, cells were stimulated with or without 100 HAU ml^{-1} SeV for 16 h and lysed for DLR assay. **(B–F)** HSV-2 ICP27 inhibits RIG-I, MAVS, TBK1, IKK-ε, or IRF3-5D induced IFN-β promoter activation in a dose-dependent manner. HEK 293T cells were seeded in 24-well plates and co-transfected with empty vector pcDNA3.1(+) or HSV-2 ICP27 expressing plasmid, and plasmid expressing IRF3 signaling pathway component RIG-IN, MAVS, TBK1, IKK-ε, or IRF3-5D, together with the reporter plasmid p125-Luc and the internal control phRL-TK. At 40 h post-transfection, cells were lysed for DLR assay. At the same time, cells were lysed for Western Blot. RIG-I, MAVS, TBK1, IKK-ε, and IRF3-5D were detected by Anti-FLAG Ab while actin was detected by Anti-actin Ab. The data shown are representative of three independent experiments, with each condition performed in triplicate (mean ± SD) **(A–F)**. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. For Western Blot, one representative experiment out of three is shown **(B–F)**.

to be involved in RNA virus recognition (39). In the case of HSV-2, its RNAs are rapidly generated during the life cycle of its primary infection (40), which may represent an important alternative source of pathogen-associated molecular patterns to trigger innate immune responses.

HSV-2 and HSV-1 have different initial infection and transmission sites, with HSV-2 mainly resulting in genital infections and HSV-1 normally causing orofacial infections (41). In addition, HSV-2 has a high positive-incidence of infections and common mucosal transmission routes with HIV-1 (42). For instance, HSV-2 predominantly infects mucosal epithelial cells which forms the primary mucosal barriers against HIV-1 infection, and interruption of these barriers may facilitate HIV-1 transmission (5). In the current study, we found that HSV-2 can significantly inhibit IFN-β induction in human cervical tissues. Moreover, by using mucosal epithelial cell lines and primary mucosal epithelial cells as models, we demonstrated that HSV-2 ICP27 contributes to such inhibited IFN-β induction. The significance of HSV-2 ICP27 in inhibiting IFN-β induction was further confirmed in the context of virus infection by the specific siRNA knockdown of HSV-2 ICP27, although knockdown of HSV-2 ICP27 did not fully abolish HSV-2-mediated inhibition of IFN-β induction. Given the complexity of the HSV-2 genome encoding at least 74 proteins, it is highly likely

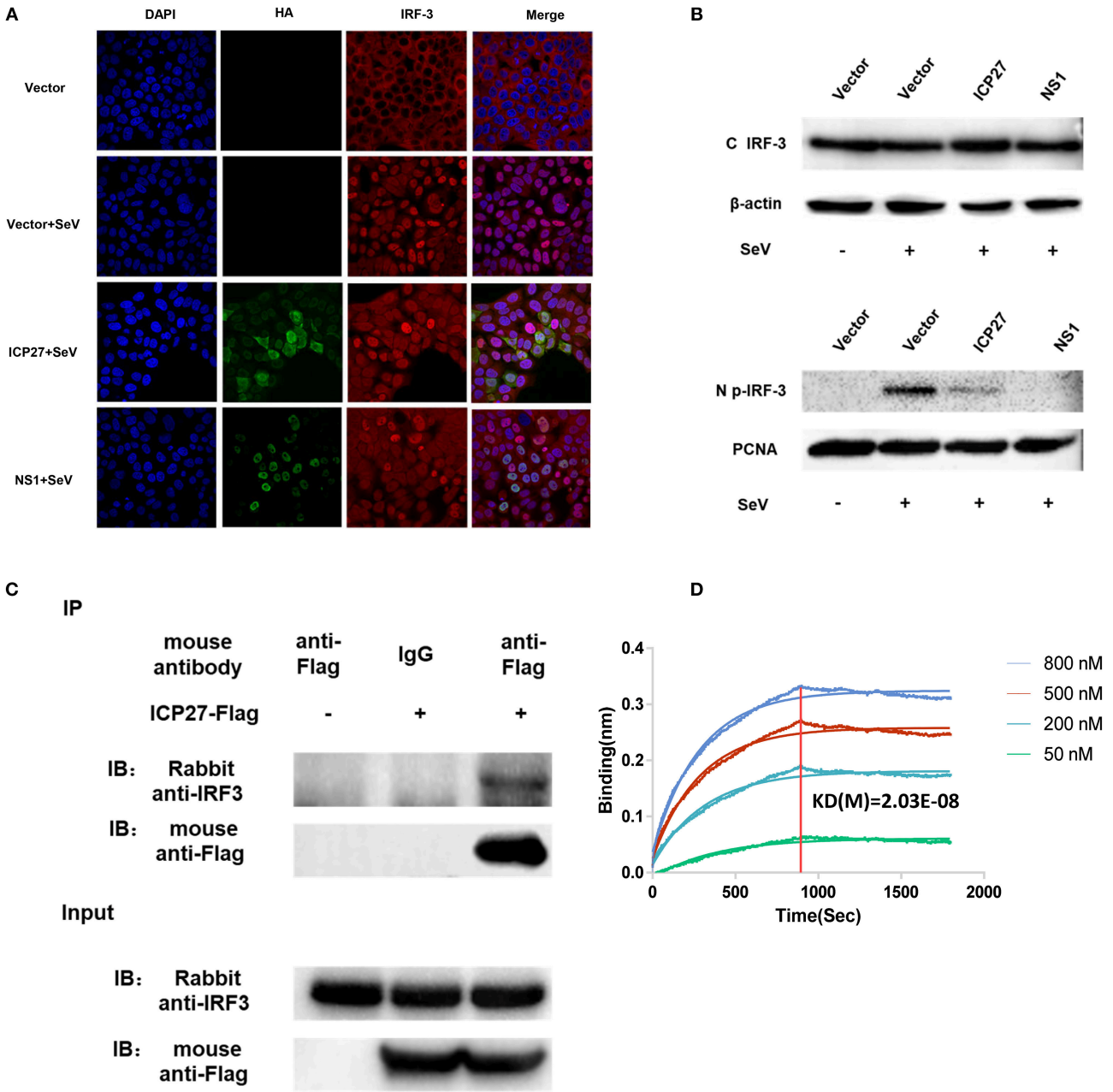


FIGURE 5 | HSV-2 ICP27 blocks IRF3 activation by physical interaction. **(A)** HSV-2 ICP27 interferes with the nuclear translocation of IRF3. HeLa cells were seeded in 35 mm glass-bottom dishes and transfected with empty vector pcDNA3.1(+), plasmid expressing HSV-2 ICP27-HA or influenza virus NS1-HA. At 24 h post-transfection, cells were stimulated with or without 100 HAU ml^{-1} SeV for 16 h and prepared for immunofluorescence assay. IRF3 was detected under 647 nm wavelength, ICP27-HA or NS1-HA under 488 nm wavelength, and nuclei under 405 nm wavelength. **(B)** HSV-2 ICP27 inhibits IRF3 phosphorylation. HEK 293T cells were seeded in 6-well plates and transfected with pcDNA3.1(+), plasmid expressing HSV-2 ICP27 or influenza virus NS1. At 24 h post-transfection, cells were stimulated with or without 100 HAU ml^{-1} SeV for 16 h. Nuclear and cytoplasmic proteins were isolated, and phosphorylated IRF3 was detected with a p-IRF3 mAb. **(C)** HSV-2 ICP27 interacts with endogenous IRF3. HEK 293T cells were seeded in 6-well plates and transfected with pcDNA3.1(+) or HSV-2 ICP27-Flag. At 24 h post-transfection, cells were stimulated with or without 100 HAU ml^{-1} SeV for 16 h and lysed for immunoprecipitation assay. One representative experiment out of three is shown **(A–C)**. **(D)** Kinetics of ICP27-IRF3 binding. The kinetics of binding was performed on a Forte-Bio Octet Red System. Purified IRF3 was conjugated with biotin at a molecular weight ratio of 1:3. Ten microgram/milliliter biotinylated IRF3-Flag was coupled to Biosenors and immersed in different concentration of ICP27-Flag (50, 200, 500, or 800 nM) for association and disassociation. The response in nm shift was recorded as a function of time. KD (M) = 2.03E-08.

that other unidentified HSV-2 proteins may also contribute to the suppression of IFN-β production. Because of the importance of IFN-β in inhibiting SHIV mucosal transmission (43), future studies are warranted to investigate whether HSV-2 infection-mediated IFN-β reduction plays a role in enhancing HIV-1 genital transmission.

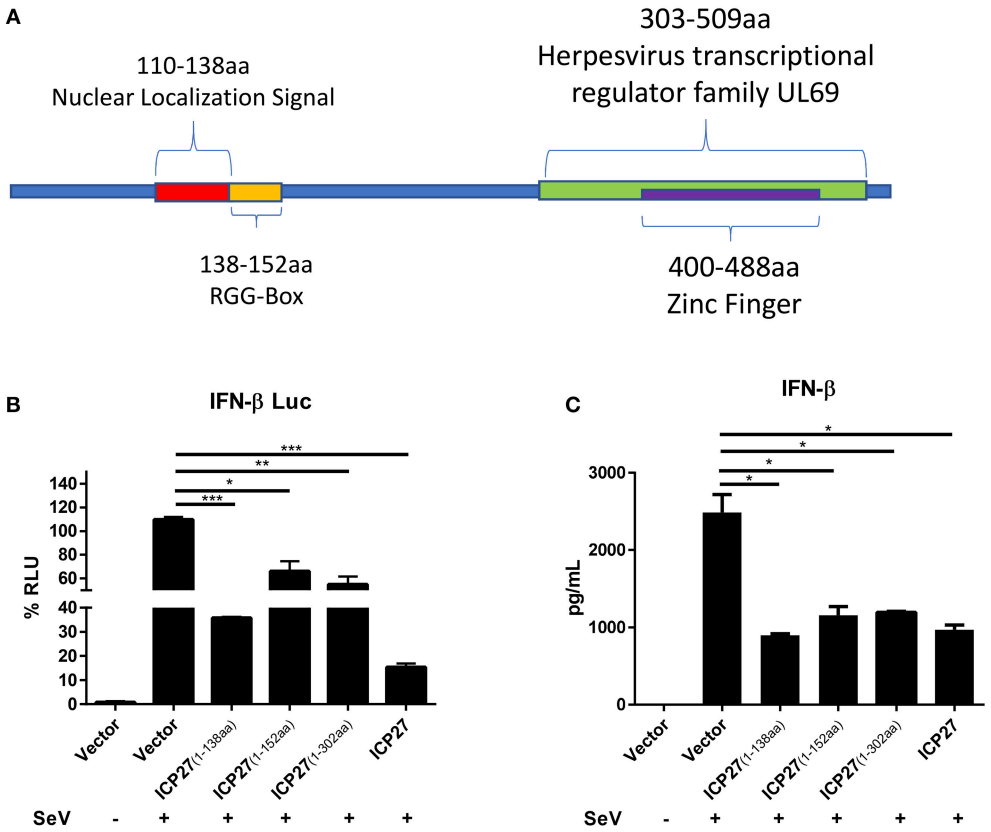


FIGURE 6 | Mapping the key domain of HSV-2 ICP27 that inhibits IFN-β production. **(A)** Illustration of HSV-2 ICP27 structural domain. 110-138aa is the nuclear localization signal of ICP27; 138-152aa is the RGG-box domain; 303-509aa is the Herpesvirus transcriptional regulator family UL69; 400-488aa is the zinc ring finger domain. **(B)** The 1-138aa domain of ICP27 inhibits the IFN-β promoter activation. HEK 293T cells were seeded in 24-well plates and co-transfected with empty vector pcDNA3.1(+), plasmid expressing HSV-2 ICP27 or its truncation mutant, together with the reporter plasmid p125-Luc and the internal control phRL-TK. At 24 h post-transfection, cells were stimulated with or without 100 HAU ml^{-1} SeV for 16 h and lysed for DLR assay. **(C)** The 1-138aa domain of ICP27 inhibits IFN-β induction at protein level. HEK 293T cells were seeded in 24-well plates and transfected with empty vector pcDNA3.1(+), plasmid expressing HSV-2 ICP27 or its truncation mutant. At 24 h post-transfection, cells were stimulated with or without 100 HAU ml^{-1} SeV for 16 h, and supernatants were harvested for ELISA. The data shown are representative of three independent experiments, with each condition performed in triplicate (mean ± SD) **(A,B)**. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

We revealed in the current study that HSV-2 ICP27 can inhibit IFN-β production via the RIG-I-MAVS pathway. HSV-1/2 ICP27 is an essential multifunctional immediate early protein which regulates viral gene expression (44, 45). To date, most of the studies on ICP27 have focused on HSV-1 (44–46). For instance, HSV-1 ICP27 was shown to inhibit the phosphorylation and accumulation of STAT-1 in the nucleus, resulting the interruption of type I IFN signaling (47). In agreement, HSV-1 ICP27 knockout enhanced the activation of IRF3 and NF-κB in macrophages and DCs (48). More recently, HSV-1 ICP27 has been reported to inhibit type I IFN induction by interfering with the cGAS–STING–TBK1 signaling pathway in human macrophages (30). However, HSV-1 ICP27 appeared not to interfere with TBK1 phosphorylation, and the association of HSV-1 ICP27 with TBK1 required STING (30). In agreement, we found that, HSV-2 ICP27 strongly inhibited the production of IFN-β in HEK 293T cell line (22), which does not express STING (49), further strengthening that HSV-2 ICP27 can inhibit IFN production through a cGAS–STING–TBK1 independent pathway.

IRF3 plays a crucial role in type I IFN-mediated antiviral immune response (50). Activation of IRF3 during IFN-β production has several key steps: phosphorylation, dimerization, and cytoplasm-to-nucleus translocation. We previously found that, HSV-2 ICP22 inhibits IFN-β induction by antagonizing the association of IRF3 with the IFN-β promoter without suppressing the phosphorylation and nuclear translocation of IRF3 (22). Unlike the mechanism used by HSV-2 ICP22, we demonstrated in the current study that HSV-2 ICP27 interacts with IRF3 and interferes with IRF3 activation by blocking IRF3 phosphorylation and nuclear translocation, thereby inhibiting the production of IFN-β and ISGs. Given the complexity of HSV-2 genome containing over 70 genes, it is probable that multiple HSV-2 components likely contribute to the suppression of type I IFN induction by HSV-2. Indeed, although our current understanding of HSV-2 immune evasion is limited, work on HSV-1 has revealed multiple countermeasures in subverting type I IFN production (17, 18). By designing and analyzing HSV-2 ICP27 truncation mutants, we found that the 1-138aa region of HSV-2 ICP27 is the key functional domain responsible for HSV-2

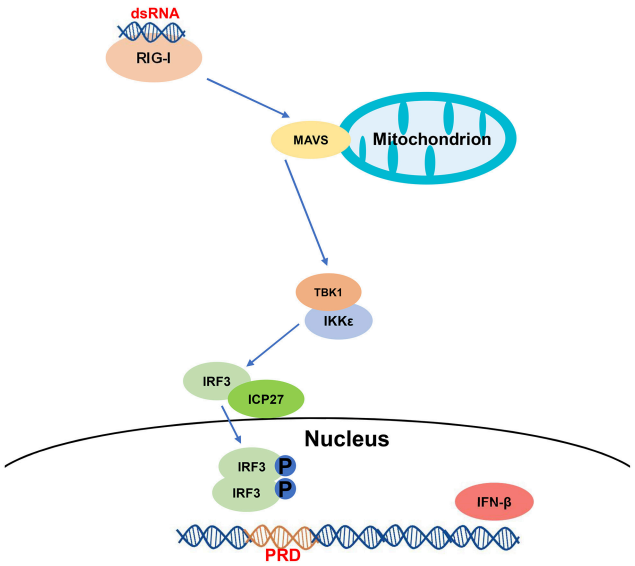


FIGURE 7 | A schematic model of the mechanism by which HSV-2 ICP27 inhibits IFN-β production. HSV-2 dsRNA is detected by RIG-I, which recruits MAVS and downstream TBK1 and IKK-ε complex. HSV-2 ICP27 interacts with IRF3 and interferes with IRF3 activation by blocking IRF3 phosphorylation and nuclear translocation, thereby inhibiting the production of IFN-β and ISGs.

ICP27-mediated IFN-β reduction. In contrast, a study on HSV-1 ICP27 has shown that its RGG box, which is located in the region of 139-152aa, is the main determinant antagonizing the cytosolic DNA-stimulated IFN-β production, by targeting TBK1 and STING (30). Although HSV-2 ICP27 shares 79% of amino acid sequence with HSV-1 ICP27, the key function domain, the N-terminus is only 65% identical, which may explain the differences in biological functions. For instance, compared with HSV-1 ICP27, HSV-2 ICP27 is less efficient in promoting the cytoplasmic localization of ICP4, another important immediate early protein of HSV 1/2 (44). In addition, the capability of HSV-2 ICP27 in inhibiting IFN-β production appeared to be much stronger than that of HSV-1 ICP27 when the ICP27 of HSV-1 was replaced with HSV-2 ICP27 (30), indicating the distinction of HSV-2 ICP27 in inhibiting IFN-β production.

In conclusion, we have demonstrated that HSV-2 ICP27 inhibits IFN-β induction in human cervical tissues, cervicovaginal epithelial cell lines, and primary human mucosal epithelial cells. We further addressed the underlying mechanism and proposed one model based on the RIG-I-MAVS-IRF3 pathway (**Figure 7**). During HSV-2 infection of mucosal epithelial cells, a number of by-products, such as viral dsRNA, are yielded and can be recognized by the RIG-I receptor. RIG-I binds to dsRNA through the helicase domain and signals through caspase activation and recruitment domains to the adaptor MAVS. Engagement of RIG-I initiates signaling through two downstream protein kinase complexes, TBK-1/IKK-ε, leading to the phosphorylation and dimerization of IRF3. IRF3 dimers translocate from the cytoplasm into the nucleus to bind to the IFN-β promoter, and promote IFN-β transcription (51). On the other hand, HSV-2 immediate early protein ICP27 interacts with IRF3, and interferes with IRF3 phosphorylation and nuclear translocation, thereby inhibiting the production of IFN-β and ISGs. Our findings provide important information for understanding how HSV-2 evades mucosal innate immunity and a potential viral target for intervention.

AUTHOR CONTRIBUTIONS

QH conceived the study. XG, MZ, and MF conducted the experiments. SL provided tissues samples. XG, MZ, and QH analyzed the data. XG and QH wrote the manuscript. All authors reviewed the manuscript.

FUNDING

This work was supported by the National Natural Science Foundation of China (81772192 and 81572009).

ACKNOWLEDGMENTS

We thank Dr. Ding Gao at the Core Facility and Technical Support, Wuhan Institute of Virology, Chinese Academy of Sciences, for technical assistance with the Confocal Microscopy and binding kinetic analysis.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00290/full#supplementary-material

REFERENCES

1. Johnston C, Corey L. Current concepts for genital herpes simplex virus infection: diagnostics and pathogenesis of genital tract shedding. *Clin Microbiol Rev.* (2016) 29:149–61. doi: 10.1128/CMR.00043-15
2. Dropulic LK, Cohen JI. The challenge of developing a herpes simplex virus 2 vaccine. *Exp Rev Vacc.* (2012) 11:1429–40. doi: 10.1586/erv.12.129
3. Posavad CM, Zhao L, Dong L, Jin L, Stevens CE, Magaret AS, et al. Enrichment of herpes simplex virus type 2 (HSV-2) reactive mucosal T cells in the human female genital tract. *Mucosal Immunol.* (2017) 10:1259–69. doi: 10.1038/mi.2016.118
4. World Health Organization. *WHO Guidelines for the Treatment of Genital Herpes Simplex Virus* (2016).
5. Lee Y, Dizzell SE, Leung V, Nazli A, Zahoor MA, Fichorova RN, et al. Effects of female sex hormones on susceptibility to HSV-2 in vaginal cells grown in air-liquid interface. *Viruses* (2016) 8:241. doi: 10.3390/v8090241
6. Awasthi S, Hook LM, Shaw CE, Pahar B, Stagray JA, Liu D, et al. An HSV-2 trivalent vaccine is immunogenic in rhesus macaques and highly efficacious in guinea pigs. *PLoS Pathog.* (2017) 13:e1006141. doi: 10.1371/journal.ppat.1006141
7. Iwasaki A. Exploiting mucosal immunity for antiviral vaccines. *Annu Rev Immunol.* (2016) 34:575–608. doi: 10.1146/annurev-immunol-032414-112315

8. Takeuchi O, Akira S. Innate immunity to virus infection. *Immunol Rev.* (2009) 227:75–86. doi: 10.1111/j.1600-065X.2008.00737.x
9. Paludan SR, Bowie AG, Horan KA, Fitzgerald KA. Recognition of herpesviruses by the innate immune system. *Nat Rev Immunol.* (2011) 11:143–54. doi: 10.1038/nri2937
10. Chen Q, Sun L, Chen ZJ. Regulation and function of the cGAS-STING pathway of cytosolic DNA sensing. *Nat Immunol.* (2016) 17:1142–9. doi: 10.1038/ni.3558
11. Fitzgerald KA, McWhirter SM, Faia KL, Rowe DC, Latz E, Golenbock DT, et al. IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. *Nat Immunol.* (2003) 4:491–6. doi: 10.1038/ni921
12. Murphy AA, Rosato PC, Parker ZM, Khalenkov A, Leib DA. Synergistic control of herpes simplex virus pathogenesis by IRF-3, and IRF-7 revealed through non-invasive bioluminescence imaging. *Virology* (2013) 444:71–9. doi: 10.1016/j.virol.2013.05.034
13. McNab F, Mayer-Barber K, Sher A, Wack A, O'Garra A. Type I interferons in infectious disease. *Nat Rev Immunol.* (2015) 15:87–103. doi: 10.1038/nri3787
14. Kalliolias GD, Ivashkiv LB. Overview of the biology of type I interferons. *Arthr Res Therapy* (2010) 12:S1. doi: 10.1186/ar2881
15. Nagarajan UM. Induction and function of IFNβ during viral and bacterial infection. *Crit Rev Immunol.* (2011) 31:459–74. doi: 10.1615/CritRevImmunol.v31.i6.20
16. Peng T, Zhu J, Klock A, Phasouk K, Huang ML, Koelle DM, et al. Evasion of the mucosal innate immune system by herpes simplex virus type 2. *J Virol.* (2009) 83:12559–68. doi: 10.1128/JVI.00939-09
17. Su C, Zhan G, Zheng C. Evasion of host antiviral innate immunity by HSV-1, an update. *Virol J.* (2016) 13:38. doi: 10.1186/s12985-016-0495-5
18. Zheng C. Evasion of cytosolic DNA-stimulated innate immune responses by herpes simplex virus 1. *J Virol.* (2018) 92:e00099–17. doi: 10.1128/JVI.00099-17
19. Ohashi M, Bertke AS, Patel A, Krause PR. Spread of herpes simplex virus to the spinal cord is independent of spread to dorsal root ganglia. *J Virol.* (2011) 85:3030–2. doi: 10.1128/JVI.02426-10
20. Yao XD, Rosenthal KL. Herpes simplex virus type 2 virion host shutoff protein suppresses innate dsRNA antiviral pathways in human vaginal epithelial cells. *J Gen Virol.* (2011) 92:1981–93. doi: 10.1099/vir.0.030296-0
21. Lu X, Huang C, Zhang Y, Lin Y, Wang X, Li Q, et al. The Us2 gene product of herpes simplex virus 2 modulates NF-kappaB activation by targeting TAK1. *Sci Rep.* (2017) 7:8396. doi: 10.1038/s41598-017-08856-4
22. Zhang M, Liu Y, Wang P, Guan X, He S, Luo S, et al. HSV-2 immediate-early protein US1 inhibits IFN-beta production by suppressing association of IRF-3 with IFN-beta promoter. *J Immunol.* (2015) 194:3102–15. doi: 10.4049/jimmunol.1401538
23. Li H, Zheng Z, Zhou P, Zhang B, Shi Z, Hu Q, et al. The cysteine protease domain of porcine reproductive and respiratory syndrome virus non-structural protein 2 antagonizes interferon regulatory factor 3 activation. *J Gen Virol.* (2010) 91:2947–58. doi: 10.1099/vir.0.025205-0
24. Li S, Zhu M, Pan R, Fang T, Cao YY, Chen S, et al. The tumor suppressor PTEN has a critical role in antiviral innate immunity. *Nat Immunol.* (2016) 17:241–9. doi: 10.1038/ni.3311
25. Qing G, Weili W, Fanqin Z, Rongchang Z, Yijin L, Jianqun D. Research of UL54-specific siRNA on herpes simplex virus type II replication. *Int J Dermatol.* (2011) 50:362–6. doi: 10.1111/j.1365-4632.2010.04732.x
26. Du T, Hu K, Yang J, Jin J, Li C, Stieh D, et al. Bifunctional CD4-DC-SIGN fusion proteins demonstrate enhanced avidity to gp120 and inhibit HIV-1 infection and dissemination. *Antimicrob Agents Chemother.* (2012) 56:4640–9. doi: 10.1128/AAC.00623-12
27. Kato H, Takeuchi O, Sato S, Yoneyama M, Yamamoto M, Matsui K, et al. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* (2006) 441:101–5. doi: 10.1038/nature04734
28. Lin R, Noyce RS, Collins SE, Everett RD, Mossman KL. The herpes simplex virus ICP0 RING finger domain inhibits IRF3- and IRF7-mediated activation of interferon-stimulated genes. *J Virol.* (2004) 78:1675–84. doi: 10.1128/JVI.78.4.1675-1684.2004
29. Schneider WM, Chevillotte MD, Rice CM. Interferon-stimulated genes: a complex web of host defenses. *Ann Rev Immunol.* (2014) 32:513–45. doi: 10.1146/annurev-immunol-032713-120231
30. Christensen MH, Jensen SB, Miettinen JJ, Luecke S, Prabakaran T, Reinert LS, et al. HSV-1 ICP27 targets the TBK1-activated STING signalsome to inhibit virus-induced type I IFN expression. *EMBO J.* (2016) 35:1385–99. doi: 10.15252/embj.201593458
31. Chiu YH, Macmillan JB, Chen ZJ. RNA polymerase III detects cytosolic DNA and induces type I interferons through the RIG-I pathway. *Cell* (2009) 138:576–91. doi: 10.1016/j.cell.2009.06.015
32. Ori D, Murase M, Kawai T. Cytosolic nucleic acid sensors and innate immune regulation. *Int Rev Immunol.* (2017) 36:74–88. doi: 10.1080/08830185.2017.1298749
33. Dempsey A, Bowie AG. Innate immune recognition of DNA: a recent history. *Virology* (2015) 480:146–52. doi: 10.1016/j.virol.2015.03.013
34. Beachboard DC, Horner SM. Innate immune evasion strategies of DNA and RNA viruses. *Curr Opin Microbiol.* (2016) 32:113–9. doi: 10.1016/j.mib.2016.05.015
35. Chan YK, Gack MU. Viral evasion of intracellular DNA and RNA sensing. *Nat Rev Microbiol.* (2016) 14:360–73. doi: 10.1038/nrmicro.2016.45
36. Eriksson K, Svensson A, Hait AS, Schluter K, Tunback P, Nordstrom I, et al. Cutting Edge: genetic association between IFI16 single nucleotide polymorphisms and resistance to genital herpes correlates with IFI16 expression levels and HSV-2-induced IFN-beta expression. *J Immunol.* (2017) 199:2613–7. doi: 10.4049/jimmunol.1700385
37. Huang J, You H, Su C, Li Y, Chen S, Zheng C. Herpes simplex virus 1 tegument protein VP22 abrogates cGAS/STING-mediated antiviral innate immunity. *J Virol.* (2018) 92:e00841–18. doi: 10.1128/JVI.00841-18
38. Zhang J, Zhao J, Xu S, Li J, He S, Zeng Y, et al. Species-specific deamidation of cGAS by herpes simplex virus UL37 protein facilitates viral replication. *Cell Host Microbe* (2018) 24:234–48.e5. doi: 10.1016/j.chom.2018.07.004
39. Zevini A, Olagnier D, Hiscott J. Crosstalk between Cytoplasmic RIG-I and STING Sensing Pathways. *Trends Immunol.* (2017) 38:194–205. doi: 10.1016/j.it.2016.12.004
40. Schiffer JT, Corey L. Rapid host immune response and viral dynamics in herpes simplex virus-2 infection. *Nat Med.* (2013) 19:280–90. doi: 10.1038/nm.3103
41. Whitley RJ, Roizman B. Herpes simplex virus infections. *Lancet* (2001) 357:1513–8. doi: 10.1016/S0140-6736(00)04638-9
42. Looker KJ, Elmes JAR, Gottlieb SL, Schiffer JT, Vickerman P, Turner KME, et al. Effect of HSV-2 infection on subsequent HIV acquisition: an updated systematic review and meta-analysis. *Lancet Infect Dis.* (2017) 17:1303–16. doi: 10.1016/S1473-3099(17)30405-X
43. Veazey RS, Pilch-Cooper HA, Hope TJ, Alter G, Carias AM, Sips M, et al. Prevention of SHIV transmission by topical IFN-β treatment. *Mucosal Immunol.* (2016) 9:1528. doi: 10.1038/mi.2015.146
44. Park D, Lalli J, Sedlackova-Slavikova L, Rice SA. Functional comparison of herpes simplex virus 1 (HSV-1) and HSV-2 ICP27 homologs reveals a role for ICP27 in virion release. *J Virol.* (2015) 89:2892–905. doi: 10.1128/JVI.02994-14
45. Tang S, Patel A, Krause PR. Herpes simplex virus ICP27 regulates alternative pre-mRNA polyadenylation and splicing in a sequence-dependent manner. *Proc Natl Acad Sci USA* (2016) 113:12256–61. doi: 10.1073/pnas.1609695113
46. Tunnicliffe RB, Schacht M, Levy C, Jowitt TA, Sandri-Goldin RM, Golovanov AP. The structure of the folded domain from the signature multifunctional protein ICP27 from herpes simplex virus-1 reveals an intertwined dimer. *Sci Rep.* (2015) 5:11234. doi: 10.1038/srep11234

47. Johnson KE, Song B, Knipe DM. Role for herpes simplex virus 1 ICP27 in the inhibition of type I interferon signaling. *Virology* (2008) 374:487–94. doi: 10.1016/j.virol.2008.01.001
48. Melchjorsen J, Siren J, Julkunen I, Paludan SR, Matikainen S. Induction of cytokine expression by herpes simplex virus in human monocyte-derived macrophages and dendritic cells is dependent on virus replication and is counteracted by ICP27 targeting NF-kappaB and IRF-3. *J Gen Virol.* (2006) 87:1099–108. doi: 10.1099/vir.0.81541-0
49. Burdette DL, Monroe KM, Sotelo-Troha K, Iwig JS, Eckert B, Hyodo M, et al. STING is a direct innate immune sensor of cyclic di-GMP. *Nature* (2011) 478:515–8. doi: 10.1038/nature10429
50. Negishi H, Taniguchi T, Yanai H. The interferon (IFN) class of cytokines and the IFN regulatory factor (IRF) transcription factor family. *Cold Spring Harb Perspect Biol.* (2017) 10:a028423. doi: 10.1101/cshperspect.a028423
51. Wang P, Zhao W, Zhao K, Zhang L, Gao C. TRIM26 negatively regulates interferon-beta production and antiviral response through polyubiquitination and degradation of nuclear IRF3. *PLoS Pathog.* (2015) 11:e1004726. doi: 10.1371/journal.ppat.1004726

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 06 March 2019
doi: 10.3389/fimmu.2019.00373

Mechanisms of Immune Control of Mucosal HSV Infection: A Guide to Rational Vaccine Design

Naomi R. Truong[1,2†], *Jacinta B. Smith*[1†], *Kerrie J. Sandgren*[1,2] *and Anthony L. Cunningham*[1,2*]

[1] Centre for Virus Research, The Westmead Institute for Medical Research, Sydney, NSW, Australia, [2] Sydney Medical School, The University of Sydney, Sydney, NSW, Australia

Herpes Simplex Virus (HSV) is a highly prevalent sexually transmitted infection that aside from causing cold sores and genital lesions, causes complications in the immunocompromised and has facilitated a large proportion of HIV acquisition globally. Despite decades of research, there is no prophylactic HSV vaccine ready for use in humans, leaving many questioning whether a prophylactic vaccine is an achievable goal. A previous HSV vaccine trial did have partial success in decreasing acquisition of HSV2–promising evidence that vaccines can prevent acquisition. However, there is still an incomplete understanding of the immune response pathways elicited by HSV after initial mucosal infection and how best to replicate these responses with a vaccine, such that acquisition and colonization of the dorsal root ganglia could be prevented. Another factor to consider in the rational design of an HSV vaccine is adjuvant choice. Understanding the immune responses elicited by different adjuvants and whether lasting humoral and cell-mediated responses are induced is important, especially when studies of past trial vaccines found that a sufficiently protective cell-mediated response was lacking. In this review, we discuss what is known of the immune control involved in initial herpes lesions and reactivation, including the importance of CD4 and CD8 T cells, and the interplay between innate and adaptive immunity in response to primary infection, specifically focusing on the viral relay involved. Additionally, a summary of previous and current vaccine trials, including the components used, immune responses elicited and the feasibility of prophylactic vaccines looking forward, will also be discussed.

Keywords: vaccine development, herpes simplex, antibody, T cells, adjuvants, innate immunity

OPEN ACCESS

Edited by:
Susmit Suvas, Wayne State University School of Medicine, United States

Reviewed by:
Daniel Carr, University of Oklahoma Health Sciences Center, United States
Lbachir Benmohamed, University of California, Irvine, United States

***Correspondence:**
Anthony L. Cunningham
tony.cunningham@sydney.edu.au

†*Co-first authors*

Specialty section:
This article was submitted to Viral Immunology, a section of the journal Frontiers in Immunology

Received: *12 November 2018*
Accepted: *14 February 2019*
Published: *06 March 2019*

Citation:
Truong NR, Smith JB, Sandgren KJ and Cunningham AL (2019) Mechanisms of Immune Control of Mucosal HSV Infection: A Guide to Rational Vaccine Design. Front. Immunol. 10:373. doi: 10.3389/fimmu.2019.00373

1. INTRODUCTION

1.1. The Need for a Herpes Simplex Virus Vaccine

A prophylactic vaccine for herpes simplex virus types 1 and 2 (HSV1 and 2) is a global public health priority for development, as stated by WHO (1, 2) for several reasons: (1) genital herpes caused by HSV1 or 2 is now the commonest sexually transmitted infection; (2) it causes severe disease in neonates; (3) HSV1 is the leading cause of infectious blindness in western countries; (4) prior HSV2 infection leads to a three to six fold increased risk of HIV infection globally (3–5). Up to 50% of HIV transmissions in sub-Saharan Africa are estimated to occur in a setting of HSV2 infection (6, 7) and are more likely to occur soon after HSV2 acquisition (8). Antiviral

therapy for recurrent genital herpes markedly reduces clinical episodes but does not completely suppress viral shedding and does not reduce HIV acquisition (9), probably because of inadequate pharmacokinetics of acyclovir/valaciclovir (10). In contrast a prophylactic HSV vaccine would be likely to reduce HIV spread (11).

1.2. The History of Herpesvirus and HSV Vaccine Development

The development of vaccines for herpesviruses has met with variable success. The only major human successes have been with the vaccines for chicken pox and herpes zoster (shingles), both caused by varicella zoster virus (VZV). Progress with vaccines for herpes simplex virus has been very slow and partial so far.

Both VZV and HSV are alphaherpesviruses and their pathogenesis is similar as both infect skin and nerves and develop latent infection in trigeminal and dorsal root ganglia (TG and DRG), from where they reactivate, but much more frequently for HSV. The live attenuated varicella virus Oka strain (Varivax) was shown to prevent chicken pox in Japan in the 1990s and has been successfully deployed worldwide. Then in 2005, "Zostavax," consisting of a 14-fold more concentrated preparation of the Oka strain, was shown to prevent herpes zoster in 51% of immunized subjects and prolonged pain or postherpetic neuralgia (PHN) in 65% of them. However, vaccine efficacy against the incidence of zoster, although not PHN, is diminished in subjects >70 years of age and markedly declines over 8 years (12, 13).

Unlike live attenuated vaccines, recombinant protein vaccines require combination with an adjuvant to stimulate the immune system. Adjuvants enhance the immune response to an antigen and direct the immune system toward particular arms of the immune response, for example, toward T cell or antibody responses or both. This is usually mediated through antigen presenting cells, particularly dendritic cells (DCs). Recently, a recombinant protein vaccine for herpes zoster (RZV) was shown to be highly effective with >90% efficacy, even in subjects>80 years of age. There was no significant decline in protection over 4 years, with immunogenicity retained for 9 years (14). The vaccine contains a single varicella glycoprotein and the adjuvant system, AS01B, which consists of deacylated monophosphoryl lipid A (dMPL) and QS21, formulated in liposomes. dMPL, a toll-like receptor 4 agonist, is extracted from the cell wall of the bacterium *Salmonella Minnesota* and the saponin QS21 is derived from the bark of the soap bark tree (*Quillaja saponaria*). This adjuvant system stimulates VZV glycoprotein-specific CD4 T cells (and low level CD8 memory T cells) and humoral responses, although primary or naïve CD8 T cells are not stimulated (15).

Thus, very high levels of protection can be induced against herpes zoster by a single recombinant viral protein combined with an adjuvant that induces the appropriate adaptive (T and B cell) immune response by targeting antigen presenting cells. This is a strong improvement over the response induced by the live attenuated vaccine (16).

During 60 years of mostly unsuccessful attempts at development of an HSV vaccine, live attenuated candidates have been avoided because of concerns about potential carcinogenicity (initially as HSV2 was thought to cause cervical cancer) and recombination with clinical strains to produce new, highly virulent strains. However, new live attenuated candidates have been specifically mutated to achieve attenuation, e.g., via deletions of two key proteins, rather than simple point mutations to reduce the likelihood of reversion to virulence, and are currently in clinical trials, such as HSV529 (17). Other vaccine candidates have included DNA vaccines, hybrid recombinant viruses, and recombinant viral proteins.

In the 1990s two recombinant viral protein vaccine candidates were trialled. The Chiron vaccine candidate consisted of HSV2 entry glycoproteins B and D combined with oil in water emulsion adjuvant, MF59. When administered to subjects with recurrent genital herpes it induced high levels of neutralizing antibody but had no persistent or significant effect on frequency of recurrences (18). The GSK vaccine candidate, Simplirix, consisted of just the HSV2 entry glycoprotein D (gD), and the adjuvant system AS04. HSV2 gD is widely recognized by human populations, inducing both neutralizing antibody and CD4 T cells (19). AS04 consists of alum and dMPL. Simplirix showed 74% efficacy but only in HSV1/2 seronegative women with long-term HSV2-infected partners (20). However, the subsequent Herpevac trial of Simplirix in randomly selected HSV1 and 2 seronegative women surprisingly showed significant efficacy against genital herpes caused by HSV1 (58%) but not HSV2 (only 20% and insignificant efficacy) (21). Thus, cross-protection against HSV1 was achieved with recombinant HSV2 gD, which is highly conserved between the two serotypes (22). This protection correlated with HSV1 neutralizing antibody titers whereas HSV2 neutralizing antibody titers were low. The better efficacy of the first trial could be explained by subclinical genital exposure to HSV2 shed by the infected partner, priming a later successful vaccine response. The efficacy of the novel adjuvant dMPL was attributed to induction of CD4 Th1 T cells as well as neutralizing antibody. However, no specific CD8 T cells were induced (23).

1.3. What Can be Learned From Comparison of the Efficacy and Immunogenicity of the Recent Herpes Zoster and Herpes Simplex Vaccines?

Why is there such a marked difference between the remarkable efficacy of the RZV vaccine and the partial success of the Simplirix vaccine given that they are similarly formulated and how can this inform development of a better HSV vaccine? The answer probably lies in understanding the mechanisms of immune control of natural herpes zoster and initial genital herpes. These include (1) "immunotherapy" vs. prophylaxis–the vaccine for herpes zoster seeks to control a reactivation disease, whereas the HSV vaccine seeks to control primary infection and

Abbreviations: HSV1/2, Herpes Simplex Virus 1/2; HIV, Human Immunodeficiency Virus; VZV, Varicella Zoster Virus; DC, Dendritic Cell; dMPL, deacylated Monophosphoryl Lipid A; MHC, Major Histocompatibility Complex; IFN, Interferon; pDC, Plasmacytoid Dendritic Cell; NK, Natural Killer Cell; AS DC, Axl^+ $Siglec6^+$ Dendritic Cell; ILC, Innate Lymphoid Cell; DRG, Dorsal Root Ganglia; T_{RM}, Tissue Resident Memory T cell; Tregs, Regulatory T Cells; LCs, Langerhans cells; MNPs, Mononuclear Phagocytes; PBMCs, Peripheral Blood Mononuclear Cells

disease (2) possible differences in the immune responses required for control, (3) differences in the mechanism of action of the adjuvants and (4) immune evasion strategies of each virus. The latter is reviewed in Abendroth et al. (24) and Su et al. (25).

The distinction between an immunotherapeutic vaccine and prophylactic vaccine is critical. Prophylactic vaccines (such as Simplirix) aim at preventing acquisition of a pathogen and need to stimulate broad and durable immunity at the site of the entry of the pathogen. The Simplirix vaccine stimulated both antibody titers and CD4 T cell responses but antibody correlated best with vaccine efficacy in the Herpevac trial (23). Examination of the immune responses to HSV in nerve ganglia and skin suggest both are important, as well as CD8 T cells. Correlation of immune effectors with vaccine efficacy in trials of candidate immunotherapeutic vaccines from Agenus and Genocea as discussed below, also suggest all three are important–and perhaps are also important for prophylactic HSV vaccines. For a prophylactic vaccine to successfully stimulate the desired antibody, CD4 and CD8 T cell responses, it will need to stimulate dendritic cells (DCs), which are the only immune cell that can stimulate naïve responses.

In contrast, therapeutic vaccines aim to minimize disease severity and duration or reduce recurrences. Herpes zoster is caused by VZV reactivation in the neuronal ganglia so the RZV vaccine is effectively an immunotherapeutic vaccine. It is not intended as and may never be used as a primary prophylactic vaccine although it may effectively protect those previously immunized with Varivax from HZ when this cohort begins to reach the age of 50. This is suggested by the fact that CD4 T cell immunity generated by RZV was unaffected by previous Zostavax administration (26). RZV was demonstrated to activate blood memory T cells into a long-lasting polyfunctional state (27) which may partly explain its increased efficacy compared to Simplirix, although the presence of such T cells in critical tissues–neuronal ganglia or skin/mucosa, is unknown.

From the above discussion it is clear that definition of the innate immune response, in particular the role of critical subsets of DCs, which leads to the required effector response for prevention of infection or disease should make a major contribution to improving vaccine design. To do this it is important to know (1) the type of effector immune responses required (e.g., CD4 and/or CD8 T cells; which cytokines) and especially which pathogen proteins are most immunogenic in this setting; (2) which DCs need to be targeted to stimulate this response and their location; (3) how to target and activate those DCs with adjuvants and (4) how and where these adjuvants work; (5) any "off-target" effects of adjuvants likely to lead to unacceptable toxicity.

2. IMMUNE CONTROL OF HSV

2.1. Innate Immunity

2.1.1. Keratinocytes

Keratinocytes are the first line of defense against HSV infection in the skin and form a formidable barrier to pathogen entry. Keratinocytes also play a key role in innate immunity against pathogens (28). They express many pattern recognition receptors including Toll-like receptors (TLRs), Nod-like receptors (NLRs) and RIG-I-like receptors (RLRs) for the detection of bacterial, fungal, and viral components (29). Keratinocytes produce a vast array of antimicrobial peptides such as LL-37, β-defensins, RNases, and S100 family members (29). Additionally, keratinocytes produce chemokines and cytokines in response to pathogenic stimuli, including the chemokines CCL3, 4, and 5 in response to HSV infection. CCL3 was highly chemotactic for activated CD8 T cells, CCL4 for activated CD4 T cells, and CCL5 for resting and activated CD4 or CD8 T cells (30). Keratinocytes produce pro-inflammatory cytokines such as TNF, IL-1α, IL-1β, IL-6, IL-10, IL-18, and IL-33, which direct the immune response toward a Th1 responses, Th2/Treg responses or have direct antiviral effects (30–32). In addition, keratinocytes have also been shown to be an accessory or "non-professional" antigen presenting cell that upregulate MHC class II in response to IFN-γ produced by T cells (33, 34). In an *in vitro* model of a recurrent herpes simplex lesion, IFN-γ stimulated, HLA-DR expressing human keratinocytes were capable of both presenting HSV antigen to T cells and acting as targets for HSV-specific T cell cytotoxicity (33).

2.1.2. Type I Interferon, Plasmacytoid DCs, and AXL+SIGLEC6+ DCs

Type I Interferons (IFNs) are a key component of innate antiviral immunity. They are produced by antigen presenting cells following detection of a pathogen and activation of pattern recognition receptor signaling, such as the TLR signaling pathway. The Type I IFNs expressed in humans include IFN-α (of which multiple subtypes have been identified), IFN-β, IFN-ε, IFN-ω, and IFN-κ, although the functions of IFN-α and -β have been best characterized (35, 36). Type I IFNs induce the expression of antiviral genes known as IFN stimulated genes (ISGs), which play a role in inhibiting viral replication and promoting degradation of viral mRNA (36). Type I IFNs also activate multiple immune cell types in response to HSV infection, including neutrophils, macrophages, natural killer cells, and DCs (35).

Plasmacytoid dendritic cells (pDC) are extremely potent producers of IFN-α, and thus play an important role in antiviral defense. pDCs can also produce other cytokines and chemokines such as TNF, IL-6, CXCL10, and CCL3, for the recruitment and activation of other immune cells (37). Additionally, pDCs are thought to contribute to adaptive immunity through the activation of T cells. Viral stimulation not only triggers IFN-α, but can also differentiate pDCs into antigen presenting cells, via the upregulation of HLA-DR, CD80, and CD86, that are capable of T cell stimulation and cytokine production (38). In particular, studies of both mouse and human pDCs have demonstrated cross-presentation of exogenous antigens, resulting in the activation of naïve or memory CD8 T cells (39, 40).

In a study of human recurrent genital herpes lesions, pDCs infiltrated at both early (day 4) and late (day 10) phases. They were often found at the dermo-epidermal junction and were closely associated with $CD69^+$ T cells as well as NK cells (41). Despite expressing the HSV entry receptors nectin1, nectin2, and HVEM, pDCs were resistant to HSV infection *in vitro*, but were

able to stimulate virus-specific autologous T cell proliferation, particularly in CD8 T cells, indicating their capacity to cross-present antigens. This study demonstrated specifically in the context of HSV that pDCs are both strong producers of IFN-α and stimulated T cell proliferation in response to the virus. However, more recent studies challenge the notion that T cells proliferation is stimulated by pDCs.

Recently, a new DC sub population with characteristics of both conventional (c)DC and pDC has been described. AS DCs, named for their expression of AXL and SIGLEC6, express markers in common with both pDCs and cDCs (42). Upon cell sorting to obtain pure populations, it was found that pDCs were the producers of Type I IFN with weak ability to stimulate T cell proliferation, while AS DCs had the inverse functional responses. This study also provides evidence that traditional pDC gating is contaminated with AS DCs, suggesting that previous work investigating the role of pDCs in HSV infection would also contain contaminating AS DCs, and that AS DCs may be the true stimulators of T cell proliferation.

No work has as yet been conducted on AS DCs in relation to HSV infection, and it has not been assessed whether they are recruited to the skin during inflammation, as has been shown for pDCs. Therefore, studies investigating the presence of AS DCs in the HSV inflammatory infiltrate need to be conducted. By establishing what role AS DCs play in response HSV infection, it may bifurcate functional roles previously thought to be carried out by pDCs, i.e., Type I IFN and T cell stimulation.

2.1.3. Natural Killer and Innate Lymphoid Cells

Several studies point to an important role for natural killer (NK) cells in response to HSV infection, particularly in controlling the severity of infection. In mouse studies, mice that lack NK cells or are depleted of NK cells have increased susceptibility to HSV2 infection and increased viral titers in the vaginal mucosa, spinal cord, and brain stem (43, 44). Similarly, a more recent study examining the severity of cutaneous HSV infection in mice with atopic dermatitis (AD) compared to normal mice found that AD mice had defective NK cell activity, which correlated with increased severity of skin infection. Furthermore, normal mice that were depleted of NK cells prior to HSV infection also had increased skin inflammation and viral titers compared to those with NK cells present (45). In humans, case studies examining patients with a specific lack of NK cells have correlated this with increased susceptibility to severe HSV infections (46, 47), suggesting an important role for NK cells in control of HSV. Furthermore, enrichment of NK cells has been observed in recurrent herpes lesions (48), interacting with pDCs (41), and CD4 T cells (49). In *in vitro* studies, TLR2-stimulated NK cells could directly activate HSV gD-specific CD4 T cells (49), and their high frequency of contact with CD4 T cells in herpetic lesions suggests they play a role in stimulating CD4 T cells in this setting. These studies indicate that NK cells play a role in controlling HSV infection by restricting viral replication and spread through the early production of IFNγ, and may also be important stimulators of adaptive immunity.However, studies in both mice and humans have not identified a correlation between NK cell activity and viral clearance, which appears to be the role of T lymphocytes (48, 50–52).

In recent years knowledge of the network of innate lymphocytes has become more complex. NK cells are part of a network of innate lymphoid cells (ILCs), whose functions are analogous to T cell subsets (53). NK cells can be considered the innate counterpart of CD8 T cells, while ILC1, ILC2, and ILC3 represent the innate counterparts of CD4 T helper 1 (Th1), Th2 and Th17 cells, identified by the same transcription factors and cytokines: NK/CD8 express Eomes, granzymes and IFN-γ, ILC1/Th1 express Tbet and IFN-γ, ILC2/Th2 express Gata-3 and IL-4, IL-5, and IL-13, and ILC3/Th17 express RORγt or AHR, IL-17, and IL-22 (53). ILCs preferentially localize into barrier tissues such as the skin, lungs and gut (54). Recently, a study examined the *in situ* ILC subset quantities and distribution in human skin (55) and found that there were differences in the proportions of different ILC subsets in normal, AD and psoriasis skin. Additionally there were increased numbers of ILCs in both AD and psoriasis compared to normal skin. However, the location of the ILC subsets was consistent: located in the upper dermis, close to the epidermis, not associated with blood vessels and in close proximity to T cells. Since ILCs were shown to infiltrate into inflamed skin, they are therefore likely to also be present in the inflammatory infiltrate during HSV infection. However, to date, no studies have investigated the presence and role of ILCs in HSV infection.

2.2. Adaptive Immunity

2.2.1. The Role of B Cells and Neutralizing Antibodies in HSV Infection

B cells are the key immune cells of the humoral immune response, producing antibodies, such as IgG and IgA, that protect against many infectious pathogens.Levels of IgG and mucosal IgA are increased in vaginal secretions of mice, guinea pigs, and non-human primates intravaginally vaccinated with HSV2 (56–58), as well as in cervical secretions of women with primary HSV2 infection (56). Antibody responses vary, with IgG present as early as a few days while IgA presents up to 2 weeks post infection, however both persist for weeks after infection. Both antibodies react to various HSV glycoproteins, including gD, gB, and gC (56).However, the role of antibody-mediated protection against HSV2 pathogenesis is unclear (36) and data from vaccine trials is contradictory. In a human *in vitro* model of fetal dorsal root ganglia (DRG) innervating autologous epidermal skin explants, neutralizing antibodies reduced transmission of virus from axons to epidermis by 90% by binding to the virus in the intercellular gaps between axon termini and epidermal cells. It was suggested that antibodies might also be effective in preventing epidermis-to-neuron transmission during primary HSV infection (59). Some murine studies have demonstrated the importance of the antibody response to HSV. One study demonstrated that adoptive transfer of IgG from HSV2 vaccinated mice reduced viral load and pathological signs of disease in the vaginal lumen of naïve mice (60). Later studies showed that antibodies played a role in controlling viral titers and protection with the use of B cell-deficient mice (61, 62). Further studies in mice and guinea pigs have correlated pan-HSV2 antibodies with protection

from vaginal challenge (63). However, other murine studies have shown that humoral immunity alone was unable to control HSV infection and failed to protect against infection. Two studies that compared T cell and B cell depletion found that T cells, rather than B cells, were critical for protection against lethal challenge of HSV2 (64, 65). Additionally, passive transfer of immune serum or anti-HSV antibodies did not protect against vaginal infection (66, 67).

However, most recently, the importance of neutralizing antibodies has once again been demonstrated in studies of a trivalent vaccine containing HSV2 gC, gD, and gE with CpG and alum in rhesus macaques. When the vaccine was administered before virus challenge it induced plasma and mucosae neutralizing antibodies that blocked gD and gE immune evasion activities and stimulated CD4 T cell responses. In guinea pigs, the trivalent group had genital lesions on <1% of days and shedding of virus on 0.2% of days (68). When the vaccine was administered to guinea pigs previously infected with HSV2, the vaccine significantly boosted ELISA and neutralizing antibody titers, reduced the frequency of recurrent lesions and vaginal shedding of HSV2 DNA by approximately 50% and almost completely prevented viral shedding (69). Therefore, neutralizing antibodies were protective against vaginal challenge and contributed significantly to reductions in genital lesions and viral shedding in animal models.

2.2.2. Maternal Immunization: Clues for Protection

Studies of neonatal herpes and the protective effects of maternal immunization also provide some strong evidence for the importance of neutralizing antibodies in protection against HSV infection. Neonatal HSV infections are rare, but cause considerable morbidity and mortality in infants, with an estimated fatality rate of 60% worldwide (70).Globally, there are an estimated 14 000 cases annually, with the highest prevalences in Africa and the Americas (70). The risk of neonatal herpes infection is highest in mothers who have first-episode primary infection at the time of delivery, with transmission rates up to 60%, whereas babies born to mothers with recurrent HSV are only 1–2% likely to develop neonatal herpes (71–73). This is consistent with the hypothesis that maternal immunity provides protection to the neonate.

During pregnancy, antibodies (mostly IgG) are transferred from mother to child across the placenta (74), to ensure the temporary health and survival of the young infant. Low neutralizing antibody titre and avidity have been identified as risk factors for transmission to neonates (71, 72). However, not all pregnant women have protective concentrations of antibodies against pathogens (75), and thus maternal immunization may be an avenue for protection. Maternal immunization has already been shown to provide protection to neonates against tetanus and seasonal influenza (75) and could also be an avenue to protect against neonatal herpes.

Limited studies have investigated the effects of maternal immunization against neonatal herpes in humans, with most work conducted in mice. Murine studies have produced some promising but also conflicting results. In one study that utilized vaccination with a replication defective HSV2 mutant, HSV specific IgG antibodies passively transferred from mother to pup and reduced dissemination of virulent HSV but replication of virus or spread of virus to the CNS in pups was not prevented (76). More recently another study that utilized vaccination with a △gD-2 HSV2 showed that maternal immunization did lead to protection from neuronal involvement of HSV and latency in the pups. Increased antibody levels were also found in the serum of these pups (77). Differences in these findings could be due to the viruses the female mice were immunized with, as the more recent study used a virus that was known to protect adult mice from HSV infection upon re-challenge.

Another recent study found that both mice and humans had HSV specific antibodies in the trigeminal ganglion (TG) during HSV1 latency. Furthermore, in a murine model they demonstrated that maternal IgG accessed and persisted in neonatal TG and was protective not only against disseminated infection but also against neurological disease following neonatal HSV challenge (78). These recent studies provide evidence that maternal immunization could provide protection against neonatal herpes, and that neutralizing antibodies play a critical role in mediating this protection.

Overall, evidence suggests that humoral immunity is likely to play an early beneficial role in primary HSV infection, and may be particularly beneficial in preventing vertical transmission from mother to neonate, but ultimately cell-mediated immunity is necessary for HSV clearance and protection (36).

2.2.3. The Role of T Cells in HSV Infection

CD4 and CD8 T cells are the key components of the cell-mediated immune response. CD4 T cells are critical for the activation of B cells and antibody class-switching, as well as for "licensing" DCs to activate CD8 T cells (79, 80). CD4 T cells also secrete the Type II IFN, IFN-γ, which performs a number of antiviral roles including limiting HSV viral replication and spread (81) through the induction of antiviral genes such as protein kinase RNA-activated (PKR), which inhibits translation within infected cells (82). CD8 T cells have the important role of killing virally infected cells via their cytotoxic components perforin and granzymes, mediated through the engagement of MHC class I molecules on target cells (82). HSV T cell immunity operates at two sites–neuronal ganglia and the mucosa.

In mice, CD4 and CD8 T cells surround the neurons and adherent satellite cells of trigeminal ganglia (TG) and control latency and (some) reactivation. CD8 T cells secrete granzymes which degrade intracellular ICP4 and contribute to this control (83, 84). In the human TG or DRG, there are abundant HSV infected neurons (3% of 27000 neurons per DRG). Effector memory CD4 and CD8 T cells expressing IFN-γ, TNF and CCL5 are found in HSV DNA$^+$ ganglia and occasional clusters of these CD4 and CD8 T cells are found around neurons and are HSV specific and activated (CD69$^+$). The satellite cells surrounding neurons express MHC class II, IL1 and TGF-β which can support (resident) memory T cells. Whether these T cells are truly tissue resident memory (T_{RM}) cells has not been confirmed (85, 86).

From early studies of human recurrent herpes lesions in genital skin and mucosa, we know CD4 T cells infiltrate early and are the predominant T cell subset in the first 12–48 h

post onset (52). CD4 T cells produce IFN-γ, which has been shown to restore HSV-induced MHC class I downregulation and upregulate MHC class II in infected keratinocytes (87). CD4 T cell depletion studies in mice provide evidence of the critical role of CD4 T cells in the immune response to HSV. For example, CD4 deficient or depleted mice fail to recruit CD8 T cells to the vaginal epithelium. CD4 T cell IFN-γ stimulates epithelial cells to secrete CXCL9 and CXCL10, which recruits CD8 T cells to the site of infection (88). In human studies, CD4 T cells have been observed persisting in genital skin at the site of HSV2 reactivation for at least 6 months post-healing (89) and continue to produce IFN-γ early after HSV antigen exposure and lesion healing (90). Similarly, in human recurrent herpetic lesions, CD8 T cells infiltrate later than CD4 T cells (52), and their recruitment into genital lesions is strongly correlated with viral clearance, confirmed by the selective depletion of CD4 T cells (48).

Upon lesion healing, HSV specific CD8 T cells persist at the dermo-epidermal junction adjacent to peripheral nerve endings in small, enriched clusters, and function as sentinels for reactivation in the female genital tract (91, 92). Resident HSV-specific CD8 T cells encounter HSV quite frequently, and as such express genes for antiviral function, chemotaxis, and recruitment (93), as well as a lack of chemokine receptor expression for egress and recirculation, and the ability to produce cytolytic granules during clinical quiescence (94). These findings demonstrate that these cells remain active in immunosurveillance after episode clearance. HSV-specific CD8 T_{RM} located in genital skin and mucosa have also been identified as CD8$\alpha\alpha$ + T cells that express two CD8α chains, instead of an α and β chain. This homodimer expression has been associated with high affinity antiviral effector T cells (94).

Recently, studies of CD8 T cells and HSV have focused on investigating the spatial distribution of CD8 T_{RM} cells. Despite CD8 T_{RM} cells remaining in the genital tract as sentinels to protect against recurrences, shedding continues to occur and at variable rates between individuals. Schiffer and colleagues developed a mathematical model that spatially models the effects of variability of CD8 T_{RM} cells in HSV lesions, as well as HSV replication and spread. The model found that high levels of overall CD8 T cell density did not equate with total control of HSV and that high shedding drove frequent mucosal T cell turnover. HSV was also found to capitalize on the spatial heterogeneity of local immunity, exploiting the gaps and allowing reactivation to occur (95).

Schiffer and colleagues, using a mathematical model, found that HSV infection did not induce sufficient T_{RM} cells in the human genital tract to eliminate reactivation, and that strict spatial distribution is maintained during infection, as was found in murine models. The strict distribution and heterogeneity of T_{RM} cells provide areas for HSV replication to occur upon reactivation. The spatial distribution and heterogeneity of T_{RM} cells calculated from the mathematical model was also confirmed in histological genital biopsies. Understanding how genital tract T_{RM} cells are spaced in the tissue provides insight as to how reactivation continues to occur, even in their presence (96).

Therefore, CD8 T cells have been found to play important roles in HSV infection. They initially clear active lesions, then become T_{RM} cells, immune sentinels, that ensure reactivation is a rare occurrence. These studies on CD8 T cells suggest important insights into why previous vaccines, which have not been able to stimulate CD8 T cell activation, were unsuccessful. New vaccine designs should incorporate a focus on the stimulation of CD8 T cells and induction of a T_{RM} population that remain in the tissue as sentinels, ready for an encounter with HSV. Such vaccines would need to induce high T_{RM} cell numbers in the genital tract to overcome heterogeneous spatial distribution and provide higher killing efficiency and IFN-γ production (96).

Regulatory T cells (Tregs) are a population of CD4 T cells that suppress T cell effector functions. They are characterized by the expression of CD4, CD25, and the transcription factor Foxp3 (97). Tregs are an inherent component of any immunological response as they silence and suppress effector and cytotoxic immune responses to ensure harm does not come to the body. The role Tregs play in HSV lesions is controversial. Some murine studies have found that Tregs are beneficial either in facilitating an effective immune response or suppressing immunopathology. Tregs are essential for promoting the accumulation of HSV specific CD4 T cells in infected tissue and ensuring DCs traffic to the appropriate draining lymph node from the vaginal mucosa, resulting in effective CD4 T cell priming (98). However, other murine studies found that Tregs suppressed T cell effector responses to HSV. Depletion of Tregs before HSV infection significantly enhances HSV-specific CD8 T cell cytotoxicity in neonatal mice, and significantly enhances the IFN-γ responses of CD4 and CD8 T cells in both adult and neonatal mice (97). Furthermore, depletion of Tregs prior to HSV infection significantly decreases skin lesion severity and granulocyte cell numbers at the site of ganglionic spread from flank HSV2 (99). In human genital biopsies from HSV2 recurrent lesions, the density of Tregs directly correlated with HSV2 titers (100) Thus, it may be the balance between effector T cells and Tregs that determines whether Tregs are beneficial or detrimental during HSV infection.

A significant limitation of murine HSV infection models is that HSV does not cause recurrent lesions in mice, and so the role of Tregs in reactivation cannot be assessed. Therefore, it is important to assess the role of human Tregs in response to HSV infection. One study conducted on the peripheral blood of HSV+ patients found that CD4+CD25+ memory Tregs suppressed the proliferation of HSV specific CD4 T cells at times of clinical quiescence (101). It is known that high numbers of Tregs infiltrate the site of viral reactivation in genital skin biopsies and persist in proximity to T cells, specifically during reactivation. There is also a correlation between high Treg numbers and increased viral replication, indicating that Tregs may be suppressing immune effectors and allowing virus to proliferate. This correlates with the observation that Tregs were found to localize with CD4 T cells in the upper dermis (100). Shedding biopsies also had significantly higher ratios of Tregs to other T cells, and this affected the clinical presentation of disease; for example, an increase in Tregs could result in insufficient effector function (100). In the context of human HSV infection, the evidence suggests Tregs could be more detrimental than beneficial, particularly during virus reactivation. Therefore,

an additional consideration for new vaccine designs could be the addition of adjuvants that suppress the activation of Treg responses, particularly in immunotherapeutic vaccines that aim to reduce virus reactivation.

Gamma-delta ($\gamma\delta$) T cells are non-conventional T cells that are uniquely defined by the expression of a $\gamma\delta$ TCR, unlike conventional CD4 and CD8 T cells which express an $\alpha\beta$ TCR. $\gamma\delta$ T cells are enriched in epithelial tissues such as skin, where they maintain epidermal integrity (102). Studies in mice have investigated the role of $\gamma\delta$ T cells in HSV infection. One study found that $\gamma\delta$ T cells were protective, limiting the severity of HSV1 induced epithelial lesions and preventing the development of lethal viral encephalitis (103). They provided evidence that $\gamma\delta$ T cells decrease viral replication and restrict viral progression into the brain. Another murine study found that epidermal $\gamma\delta$ T cells were the first immune effector to encounter HSV and were directly infected, prior to the infection of Langerhans cells (104). However, human $\gamma\delta$ T cells are different to their murine counterparts. Murine $\gamma\delta$ T cells reside in both the epidermis and dermis, whereas human $\gamma\delta$ T cells mainly reside in the dermis and near the dermo-epidermal junction (105). Some reports investigating human blood $\gamma\delta$ T cells suggest that they could play a protective role in antiviral immunity, particularly to HSV (106, 107). However, the role of skin $\gamma\delta$ T cells during HSV infection has not been investigated in human skin or genital mucosa and such studies could reveal important differences between the role of murine and human $\gamma\delta$ T cells in HSV infection and whether they are important targets for vaccine design.

2.2.4. The Role of Dendritic Cells in Stimulating HSV Immunity

Dendritic cells (DCs) are the most important bridge between the innate and adaptive immune system. They patrol blood and tissue compartments to detect pathogens and take up antigens, after which they mature and migrate to lymph nodes where they present the antigens to naïve T cells, thereby activating the adaptive immune response (108). Several subsets of DCs have been identified in various tissue compartments (e.g., blood, skin, liver, brain etc.).

Due to limitations in obtaining tissue-derived human DCs, earlier studies of the role and response of human DCs to HSV infection made use of model DCs generated from monocyte-derived DCs (MDDCs). These studies provided evidence that immature DCs could be productively infected by HSV, HSV induced apoptosis in human MDDCs (a process that HSV normally inhibits) and that uninfected DCs pulsed with apoptotic HSV-infected DCs could cross-present and stimulate HSV specific CD8 T cells (109, 110). These models still must be confirmed in tissue-derived human DCs; a complex process.

2.2.5. The Complexity of the DC/Macrophage Network in Human Skin

It has been known for a long time that Langerhans cells (LCs) are the major DC subtype located in the epidermis. Whether LCs were important in HSV infection was first shown in the 1980s, where mice whose skin was abraded, leading to the fleeing of LCs, and inoculated with HSV1, had an increase in viral pathogenicity due to the absence of LCs. When LCs were present, interactions between LCs and HSV1 were observed as early as 2 h.p.i, and LCs became HSV1 gD positive, indicative of virus uptake (111).

A more recent study of LCs in mice following HSV infection showed that LCs became infected with HSV, however uninfected or bystander LCs were the main emigrant DC subset at 24 h.p.i. Additionally, most infected LCs failed to downregulate E-cadherin (preventing their emigration) and became apoptotic (104). When investigating the role of LCs in humans, one major difference is seen. LCs still become productively infected, mature and become apoptotic, however all infected LCs migrate into the dermis (112). Therefore, unlike murine LCs, where HSV infection seemed to inhibit migration of at least a significant proportion, in human LCs HSV infection induced migration to the dermis. Such differences highlight the importance of examining the immune response to HSV in human skin and in particular the role of subsets of human DCs.

In recent years, the development of technologies such as single cell RNA-sequencing have facilitated the classification of DC subsets. In human dermis, the two main DC subsets are conventional DC type 1 and 2 (cDC1 and cDC2) (113). cDC1s are a minor subset proportionally, but are highly efficient at cross-presentation of exogenous antigen to CD8 T cells (114). They are characterized by the expression of CD141, XCR1, the C-type lectin receptor CLEC9A and TLRs 1, 2, 3, 6, 7, and 8, and are recognized as the equivalent of murine CD103+/CD8α+ cross-presenting DCs (115–117). The major dermal DC subset are cDC2s, which have conventional antigen-presenting capacity to stimulate CD4 T cells, but also have some ability to cross-present to CD8 T cells (117, 118). They express CD1a, CD1c, CD11b, CD11c, and some express langerin (108) and may be the equivalent of murine submucosal CD11c+ and CD11b+ DCs. Single cell analysis has also complicated the definition of what is considered a DC or a macrophage. Dermal CD14+ mononuclear phagocytes (MNPs) were originally classified as DCs due to their ability migrate out of tissue explants, their expression of MHC class II and CD1c, and ability to influence T cells; all properties that dermal DCs have (119, 120). However, there is no known murine equivalent to human CD14+ DCs, yet mice and humans tend to have homologous cells (117, 121). Furthermore, a study found that CD14+ MNPs (which also express DC-SIGN) were transcriptionally and functionally similar to tissue resident monocyte derived macrophages (MDMs). For example their ability to stimulate memory T cells like macrophages but not stimulate naïve T cells, an ability unique to DCs. However, these cells also have DC properties in their ability to migrate out of tissue, making them a MDM with DC-like ability (122).

It has been known for some time that the process by which skin DCs take up HSV and present antigen to CD4 and CD8 T cells leading to the development of memory T cells is complex. Several studies have tried to unravel this complexity and define the process in murine models. It has been shown in mice that LCs take up HSV in the epidermis (111), but they do not present HSV antigen to T cells in lymph nodes. Neither LCs nor lymph node resident DCs present HSV2 antigens to CD4 T cells, but submucosal CD11c+ and CD11b+ DCs (cDC2s) do (123). Furthermore, naive CD8 T cells are primed by CD8α+ DCs and

CD103+ dermal DCs (cDC1s) (124, 125) and the latter are the predominant cells transporting HSV antigens out of murine skin explants (104).

In our recent human study, we investigated the interaction of HSV-infected LCs with dermal cDC1s in human inner foreskin explants and in biopsies of initial herpes simplex virus lesions. HSV1 infected LCs became apoptotic and migrated to the dermis to interact with cDC1s in clusters. LC fragments were detected within some cDC1s, and cDC1s emigrated from HSV1 infected explants, similar to CD103+ dermal DCs in murine models. Additionally, DC-SIGN+ MNPs were also observed in clusters interacting with HSV-infected LCs in the dermis (112). Therefore, this study demonstrated that epidermal LCs take up HSV, become infected and transfer the virus or viral antigens to subsets of dermal DCs/MNPs, facilitating viral relay. This has filled an important gap in knowledge of the immunological processes facilitating HSV antigen presentation to T cells. However, important questions remain: What role do human cDC2s play in interactions with HSV infected LCs? Are there differences in the interactions of different dermal DC/MNP subsets with the LCs that could determine their specific contributions to the activation of CD4 and CD8 T cells? By understanding the roles of specific human DC subsets in response to HSV infection, it should drive vaccine design toward stimulating pathways that induce the same immune responses as natural infection and, in particular, CD8 T cell responses that were not induced by previous vaccine candidates. A summary of the HSV viral relay and localization of immune cell subsets in human skin is shown in **Figure 1**.

3. BUILDING ON KNOWLEDGE OF NATURAL IMMUNITY TO DESIGN A VACCINE

3.1. Challenges of Designing a Protective Vaccine

Prophylactic and immunotherapeutic vaccines have different goals and as such there are challenges to overcome in the development of a successful prophylactic vaccine that are not critical for an immunotherapeutic vaccine. Since prophylactic vaccines aim to prevent acquisition of a pathogen, they need to stimulate effective primary immune responses at the site of pathogen entry. To generate primary immune responses, naïve T cells require two signals to differentiate into effector cells: an antigen-specific signal and a second costimulatory signal (such as CD80/86 ligation of CD27). DCs are the critical cell type for stimulating naïve T and B cells as they provide the second costimulatory signal to T cells that other "secondary" or "non-professional" antigen presenting cells cannot provide. Therefore, a successful prophylactic vaccine needs to stimulate the appropriate DCs. In contrast, immunotherapeutic vaccines aim to reduce morbidity by reducing clinical episodes, and reduce transmission by reducing viral shedding. This may be an easier immunological task than prophylaxis, as it relies on re-stimulating already existing memory T cell responses. Compared to stimulating naïve T cell responses, memory T cells are more abundant and do not require a costimulatory signal for activation. Therefore, a much wider range of immune cells than DCs can act as antigen presenting cells (including keratinocytes and monocytes). This also means memory T cell stimulation is more likely to occur in the periphery (91, 94). It is undetermined yet whether naïve HSV-specific T cell priming occurs in mucosal tissues or only in lymph nodes.

There is now compelling evidence that the presence of T_{RM} cells and neutralizing antibodies in the mucosa are critical for protection against release of virus from the DRG and also likely to prevent virus entry into the DRG during initial infection. T_{RM} cells may also be important in restricting reactivation in the DRG (86). It is therefore important to consider how to design a prophylactic vaccine that will induce the development of local T_{RM}cells and mucosal antibody to prevent infection with HSV as recruitment of B and T cells from the blood may be too slow to prevent viral seeding of the nerves.

3.2. Targeting Key Antigens and Epitopes

HSV1 and 2 consist of double-stranded DNA contained in a capsid, surrounded by a tegument layer and an envelope containing glycoproteins including gB, gC, gD, and gH/gL (126). HSV replication involves the production of rounds of viral proteins for the assembly of the virus, beginning with immediate-early (IE), followed by early (E) then late (L) structural proteins (127). The immune response is capable of targeting many of these viral components and it is important that a vaccine stimulates responses to antigenic epitopes that have been identified as key targets for neutralizing antibodies, CD4 and CD8 T cells.

The late structural proteins gD and gB are dominant targets for HSV neutralizing antibodies, of which multiple epitopes are recognized (128, 129), along with gC and gH/L, specifically seen in human sera directed against HSV1 (19, 128–131). Other glycoproteins, such as gK, have only been investigated in murine models (132). Therefore, gD and gB were used as immunogens in the Chiron trial and gD combined with dMPL (AS04) used in the Simplirix and Herpevac trials. In the Herpevac trial, HSV2 gD was seen to confer protection for genital infection caused by HSV1 (but not HSV2), which correlated with high gD antibody titers, supporting the importance of antibodies in mediating this protection. When investigating the antibody response elicited from this vaccine in guinea pigs, the protection provided against genital disease was due mostly to neutralizing antibodies directed against gD, with various epitopes recognized, such as ID3, DL6, and MC14. The more epitopes the animals recognized, the better protected they were against genital disease. Upon investigating the epitope-specific antibody responses in women from the Herpevac trial, it was found that significantly fewer crucial gD epitopes were recognized compared to the guinea pigs (133). The recently developed trivalent vaccine candidate containing gC, gD ,and gE provided sterilizing immunity in 98% of guinea pigs due to the high levels of plasma and mucosal neutralizing antibodies induced (69). Anti-gE is aimed at preventing cell to cell spread. Human antibody responses to this vaccine have not yet been assessed. Perhaps assessment of the efficacy of this vaccine and any future vaccines should evaluate epitope-specific antibody responses, such as to the gD2 epitopes ID3, DL6, and MC14.

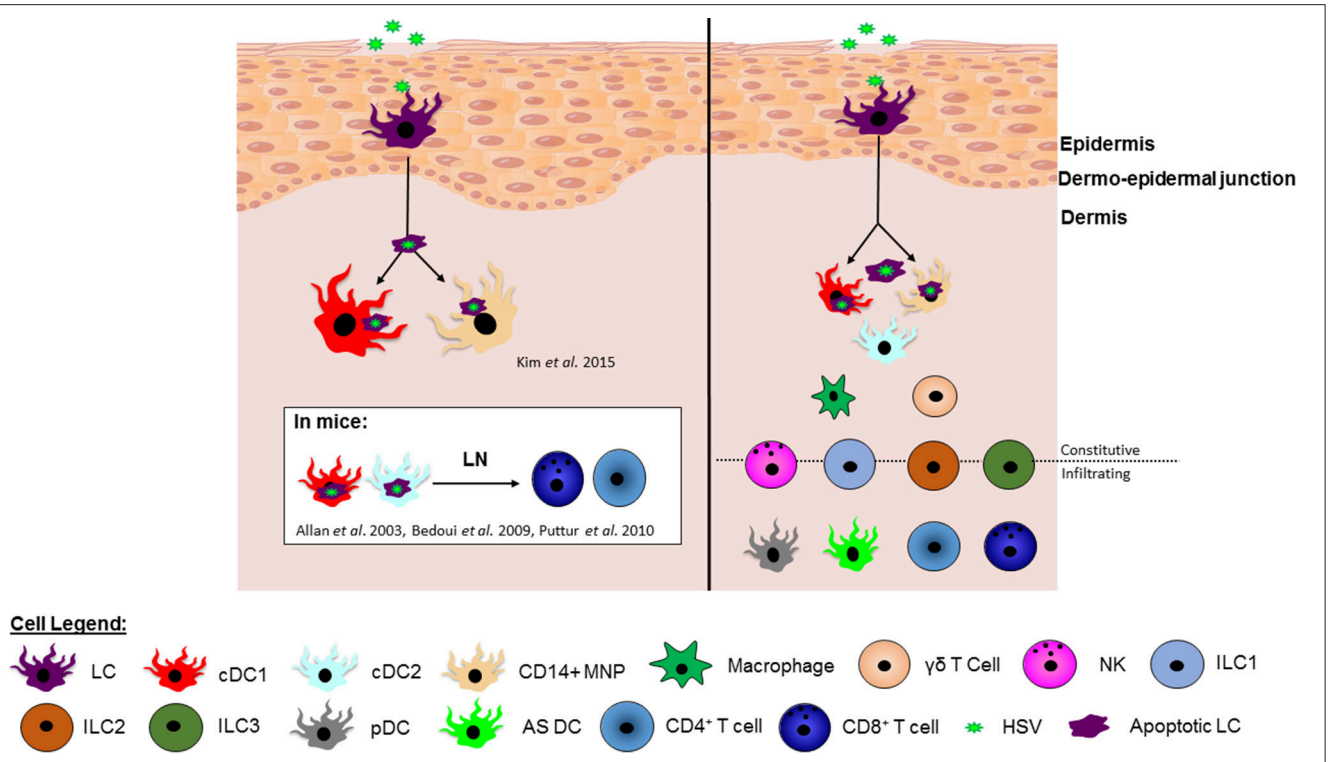


FIGURE 1 | The HSV viral relay and localization of immune cell subsets in human skin. **(Left)** In humans, HSV infects Langerhans cells (LCs) causing them to mature and migrate to the dermis and undergo apoptosis. Once in the dermis, HSV infected apoptotic LCs have been observed in clusters with and taken up by dermal cDC1s and CD14$^+$ MNPs (112), potentially for antigen presentation to T cells. In mice it is known that murine dermal cDC1s and cDC2s present HSV antigen to CD8$^+$ and CD4$^+$ T cells in the lymph node, however this has not yet been shown in human studies.**(Right)** Whilst we have pieced together multiple cellular players in this viral relay, there are an abundance of other innate and adaptive immune cells residing in the dermis, including additional DC subsets, macrophages and $\gamma\delta$ T cells, as well as infiltrating immune cells, such as pDCs and T cells. NK cells and ILCs are found both constitutively in skin in low numbers and also infiltrate into the skin during infection or inflammation. There is increasing evidence that at least some of these additional cell types influence the developing immune response to HSV infection in the skin and further illuminating this complex picture would inform vaccine design.

Interestingly, a live attenuated viral vaccine, with a gD deletion, elicited mucosal antibodies with low neutralization activity but high antibody-dependent cellular cytotoxicity (ADCC) activity, provided sterilizing immunity in murine models and passively transferred immunity against vaginal infection with multiple clinical isolates (134–136). It is noteworthy that they authors did not include complement in their neutralization assays, which should be considered as an alternative mechanism to ADCC. The authors propose that the removal of the immunodominant gD protein may unmask alternative epitopes important in a protective immune response or remove a possible immunosuppressive effect of gD. Furthermore, another live, attenuated vaccine candidate HSV529 (deleted for UL5 and UL29) was shown to induce significant HSV2-specific antibody dependent ADCC, as well as neutralizing antibodies, in humans. (137). ADCC activity may warrant further attention in vaccine evaluations.

Although the above studies provide evidence for the importance of vaccines eliciting strong neutralizing antibody responses, many of the previous human clinical trial vaccines did induce neutralizing antibodies and yet were unsuccessful (138, 139). Although it has been suggested that this may be partially explained by a lack of epitope-specific responses, also neutralizing antibodies may not be sufficient by themselves to provide protection against HSV infection. CD4 and CD8 T cells are also likely to be required. Therefore, the ability to stimulate them in a vaccine needs to be improved from the previous vaccine candidates.

CD4 and CD8 T cells respond to various viral proteins, some of which overlap with those that neutralizing antibodies recognize. CD4 T cells predominately respond to late HSV glycoproteins, such as gD, gB, gC, and gH and the tegument protein VP16 (19, 87, 140). Several immunodominant HSV2 gD epitopes are recognized by CD4 T cells from both HSV1 and HSV2 seropositive patients (141), and such cross-reactive epitopes for HSV1 and HSV2 would be advantageous to use in a vaccine to target both viruses at the same time. CD4 T cells can also recognize the tegument protein UL49 and capsid protein VP5 (140). CD8 T cells recognize a variety of HSV proteins, especially IE and E viral proteins such as ICP27, ICP4, and ICP0 (87, 142), as well as several tegument and capsid proteins (143). Recent studies have found a conserved epitope between VZV, HSV, and EBV that is recognized by CD8 T cells, that has now extended to 13 conserved epitopes between VZV and HSV that

are recognized by both CD4 and CD8 T cells (144, 145). Finally, a study has found that gD is selectively taken up by cDC1s, which can then cross-present to CD8 T cells, meaning that gD may be an important target for both CD4 and CD8 T cells (146), as well as B cells. The identification and use of conserved and cross-reactive epitopes in new vaccine designs may lead to the possibility of targeting multiple immune cells against multiple herpesviruses in the one vaccine.

Many studies that have identified HSV-specific T cell epitopes in humans have investigated the responses of T cells derived from PBMC. However, recent evidence indicates that there may be compartmentalization of T-cell receptor (TCR) repertoires and expansion of particular T cell clones at distinct anatomical sites. This may have important implications for how T cell responses to potential vaccine candidates should be assessed. One study compared the frequency of cervical and PBMC-derived HSV2-reactive CD4 T cells in HSV2 infected women and found there was a 25-fold enrichment of cervical HSV2 reactive CD4 T cells compared to PBMC, demonstrating that there are differences in frequency of HSV-specific CD4 T cells at different anatomical sites in natural HSV2 infection (92). Furthermore, recent data from the same lab presented at the International Herpesvirus Workshop investigated the overlap of TCR sequences between genital skin and PBMCs in HSV2 infected patients and also in response to an immunotherapeutic vaccine. The data suggest there is very little overlap in the TCR repertoires of tissue resident T cells in genital skin and those found in PBMCs. Therefore, it will be important to evaluate tissue-based immune responses in response to vaccines (147, 148).

3.3. Vaccine Delivery

As HSV infects the genital mucosa, vaccine strategies need to be effective at developing protective immunity at mucosal surfaces. One such strategy is direct immunization of the genital tract, a strategy that has been successful in animal models. A group investigating a live attenuated, replication defective HSV2 vaccine candidate (HSV2-gD27) has shown that intravaginal delivery gave the best protection against HSV2 intravaginal challenge compared to intranasal, subcutaneous, or intramuscular delivery (149). However, intravaginal vaccination may be a difficult or impractical strategy to use in human trials. As an alternative approach, Shin and Iwasaki developed the "prime and pull"strategy where systemic T cells were primed by parenteral vaccination then pulled to the genital mucosa by the topical application of CXCL9 and CXCL10 (150). Long term CD8 T_{RM} cells were established in mice, which conferred protection against HSV2 challenge via IFN-γ production (151).

Experimental vaccines using nanoemulsion-based adjuvants are also being investigated for their efficacy in generating mucosal immunity. Intranasally administered nanoemulsiion vaccines have demonstrated the induction of high antibody titers, robust Th1-skewed T cell responses and potent Th17 responses in RSV and TB vaccines (152, 153). A nanoemulsion vaccine for HSV2 is also being developed by BlueWillow Biologics (formerly known as NanoBio Corporation). Preliminary evidence indicates that the intranasal vaccine can protect naïve animals from acute genital HSV2 infection and the establishment of latency, and also significantly reduces lesion recurrence in already infected animals (154). It would be worthwhile to further investigate whether intranasally administered nanoemulsion vaccines generate protective systemic and mucosal HSV immunity without directly immunizing the genital tract. Other experimental vaccines being investigated include the use of peptides as the epitope, either lipopeptides or synthetically designed peptides, or the use of nanoparticle adjuvants. None of these experimental vaccines are currently in Phase I clinical trials but they do hold some promise. Peptide based vaccines are the most developed and promising with these vaccines able to stimulate high titers of polyfunctional cytotoxic CD8 T cells that are found both locally in the genital mucosa and draining lymph nodes, as well as systemically. These CD8 T cells also induced high levels of IFN-γ, IL2, IL12, and TNF, and protected against lethal rechallenge of HSV (155, 156). Work on nanoparticle adjuvants is limited but work on a calcium phosphate based nanoparticle and HSV2 epitope was shown to lead to enhance mucosal and systemic protection. This vaccine was shown to protect against lethal rechallenge with live virus, as well as induce specific IgG and IgA responses. However, no adaptive response was induced by this vaccine (157).

3.4. Vaccine Adjuvants

In contrast to live attenuated vaccines, recombinant protein vaccines are often formulated with an adjuvant to act as antigen carriers (Eg. alum, emulsions such as MF59, liposomes) and as immune stimulants (namely TLR agonists), often combined as "adjuvant systems." Adjuvants can modulate the immune response by activating DCs (replacing endogenous pathogen stimuli), and stimulate the appropriate immune pathway via different patterns of cytokine production. With an expanding pool of chemically well-defined and functionally characterized adjuvants available, there is an opportunity to tune the immune response to the desired outcome.

A protective recombinant protein vaccine will need to induce a combination of robust neutralizing antibody, CD4 and CD8 T cell responses and facilitate the establishment of T_{RM} cells. A number of adjuvants have been shown to induce neutralizing antibody responses including the traditionally used alum, MF59 which was used in the Chiron subunit vaccine (18), dMPL which was used in the Simplirix vaccine (20, 21), and more recently the combination of CpG and alum was used in the recent trivalent vaccine containing HSV2 gC, gD and gE. Notably, although this vaccine was administered intramuscularly in animal models, it elicited mucosal neutralizing antibodies that were protective upon intravaginal challenge (69).

However, for the activation and polarization of T cell responses, there are striking differences in the type of responses stimulated by different adjuvants. Alum adjuvanted vaccines do not elicit strong T cell responses (158, 159). Adjuvants such as MF59 and ISCOMs, as well as TLR2 and TLR5 ligands, enhance T cell responses without altering their Th1/Th2 balance of responses. In contrast, more polarized Th1 cell responses are elicited by adjuvants that incorporate agonists of TLR3, TLR4, TLR7-TLR8, and TLR9. Complete Freund's adjuvant (CFA) and CAF01 induce mixed Th1 and Th17 cell responses. Thus,

selection of an appropriate adjuvant is influenced by the type of CD4+ T cell response required for protection.

Simplirix was the first partially successful HSV vaccine and this was attributable to the Th1 pattern of cytokines (IFN-γ) induced by the adjuvant dMPL, however no CD8 T cell responses were detected. One of the main hurdles in the advancement of vaccine development has been finding adjuvants that enhances cross-presentation, which is necessary for the induction of CD8 T cell responses to soluble antigen. Saponin-based adjuvants have been shown to induce strong T cell responses and in particular memory CD8 T cell responses, and their use in recently trialled immunotherapeutic vaccines has shown some success. The highly successful RZV vaccine for herpes zoster contains dMPL formulated together with QS21, a saponin, in liposomes. RZV induced VZV-specific CD4 T cells as well as memory CD8 T cells, although not naive CD8 T cells (15). Similarly, the Agenus HerpV vaccine contains a patented QS21 stimulon adjuvant and the Genocea vaccine contains a saponin Matrix M2 adjuvant. Both the immunotherapeutic Agenus and Genocea vaccines induced a combination of neutralizing antibody, CD4 and CD8 T cell responses in animal models. In the human clinical trial of the Genocea vaccine, equivalent CD8 T cell responses were induced to both HSV gD and ICP4, confirming that gD contains CD8 T cell epitopes, and that saponin-based adjuvants are able to induce memory CD8 T cell responses through cross presentation (160, 161) .

In order to achieve the breadth of immune responses required (antibody, CD4 and CD8 T cells) in a vaccine for HSV, it may also be important to consider targeting adjuvants to additional immune cells that may assist in enhancing the overall responses. For example in our previous study of the LC-dermal DC viral relay, we suggested that for the targeting of dermal DC subsets by subunit vaccines, adjuvants may need to simulate the immune effects of HSV infected apoptotic LCs (112). Additionally, cell types that have traditionally been overlooked in the design of vaccine candidates, such as NK cells, should also be considered as targets for vaccine adjuvants, especially since NK cells are known to mature DCs and augment CD4 T cell responses (49). NK cells may perhaps also augment CD8 T cell responses as it appears NK cells can stimulate cross-presenting DCs (162, 163).

It is also important to consider whether adjuvants can be used to suppress certain aspects of the immune response that may not be beneficial for an effective response to HSV, such as Tregs. As Tregs are a component of any immune response, they are likely to be recruited in the context of vaccination. A recent study focusing on T cell vaccines for influenza, found that primary and repeated vaccination with viral peptides alone induced antigen specific FoxP3+ Tregs, but that the addition of certain adjuvants, such as CpG, suppressed this phenomenon. This study also found that in the context of influenza, depletion of vaccine induced antigen specific Tregs promoted viral clearance, indicating that Tregs have an inhibitory role *in vivo* (164). Most studies investigating Tregs in the context of HSV vaccination have used mouse models, where Tregs were found to be beneficial (98, 165), however the trend is not carried over into humans. Although not specifically studied in vaccines, Tregs have been shown to decrease effector T cell function in HSV infection as discussed previously (100, 101). Therefore, it is possible that adjuvant suppression of Tregs could be beneficial for a HSV vaccine, and that this is not an influenza specific phenomenon. However, it is also possible that Treg suppression could cause increased inflammation in response to the vaccine and in response to HSV infection. Therefore, the suppression of Tregs would need to be tested to determine whether it is ultimately beneficial or harmful in the context of HSV vaccination.

It is important to note that there are some concerns about potential safety issues in manipulating the immune response with adjuvants e.g., the possibility of inducing or reactivating autoimmune disease. So far, in tens of thousands of subjects immunized with RZV this has not been observed.

TABLE 1 | The developmental status of HSV vaccine candidates.

Vaccine candidate	Company	Vaccine constitution	Developmental stage	References
SUBUNIT/S + ADJUVANTS				
Simplirix/ Herpevac	GlaxoSmithKline	gD2 and AS04 (dMPL)	Ceased after Phase III trials	(21, 166)
GEN-003	Genocea	gD2 and Matrix M2	Ceased after Phase II trials	(167–169)
HerpV	Agenus	Peptide vaccine + QS-21 Stimulon	No development since Phase II trials	(170, 171)
VCL-HB01	Vical	gD2 +/- UL46 and Vaxfectin DNA vaccine	Ceased after Phase II trials	(172, 173)
COR-1	Admedus	gD2 codon optimized DNA vaccine	Phase IIb planned	(174–176)
NE-HSV2	BlueWillow Biologics	Nanoemulsion with gB2 and gD2 antigens	Pre-clinical, clinical trial planned	(154, 177)
HSV2 trivalent vaccine	University of Pennsylvania	gC2, gD2, gE2	Pre-clinical	(68, 178)
G103	Immune Design	HSV2 gD, UL19 and UL25	Pre-clinical	(179)
LIVE-ATTENUATED				
HSV529	Sanofi Pasteur	Replication defective HSV2, UL5, UL29 deletion	Phase I trial ongoing	(17, 180)
RVX201	Rational Vaccines	HSV2 ICP0 deletion mutant	Phase Ib/IIa planned	(181)
VC2	Louisiana State University	HSV1 with mutations in gK and UL20	Pre-clinical	(132, 165, 182)
R2	Thyreos LLC	HSV1 with UL37 R2 region mutation	Pre-clinical	(183)
HSV2 ΔgD2	Albert Einstein College of Medicine	HSV2 with US6 (gD) deletion	Pre-clinical	(134, 136)

However, extensive post-marketing surveillance will be required. Furthermore, the RZV adjuvant QS21 has been shown to elicit a high degree of systemic and local (infection site) reactogenicity as well as efficacy. Efficacy does not necessarily correlate with reactogenicity for individual subjects. However, whether the toxic and immunogenic aspects of such adjuvants can be dissociated, leading to chemical modifications, depends on a detailed understanding of the immunologic mechanisms of each. A summary of the developmental status of current HSV vaccine candidates is provided in **Table 1**.

4. CONCLUDING REMARKS

A new generation of vaccines aim to specifically manipulate the immune response or alternatively attenuate live vaccine candidates through specific mutations. Surprisingly, RZV has a higher degree of efficacy (and also more reactogenicity) than the live attenuated HZ vaccine, Zostavax. RZV is also more immunogenic (26, 27). Whether such higher adjuvant induced efficacy can be extended to vaccines against initial genital herpes infection remains to be proven. More antigens may be needed. These studies demonstrate that the need for a much more detailed understanding of initial protective immune responses and also the need to further analyse partially successful vaccines for immunologic correlates of efficacy (in protected vs unprotected patients) e.g., Genocea, Herpevac.

There remains much to be explored including the role of the microbiome in interacting with mucosal immunity. In sub-Saharan Africa, many women have a "diverse" vaginal microbiome without Lactobacilli which increases the likelihood of HIV and possibly HSV acquisition (86, 184–187). How mucosal immunity is altered and how this might be improved by immunization for HSV are topics for future investigation.

Thus, a successful prophylactic vaccine against initial genital herpes will need to prevent seeding of the neuronal ganglia by both HSV1 and HSV2. In addition to inducing high levels of neutralizing antibodies which are known to penetrate the epidermis, the vaccine would probably need to induce resident immune cells that can quickly migrate into the stratified squamous epidermis or produce rapidly diffusing protective cytokines upon infection and contain/destroy the virus before it enters nerve terminals in the skin. We now know that even if viruses such as HIV obtain a "toehold" in mucosal epidermal cells they can be contained by these mechanisms. More needs to be known about the interaction of key innate and adaptive immune responses. It is becoming clear that multiple innate immune cells such as multiple DC subsets, NK cells, monocytes/macrophages and $\gamma\delta$ T cells are interacting in the mucosae during initial HSV infection and together with antibody and T cells may all have a role in successful control or protection of initial infection.

AUTHOR CONTRIBUTIONS

NT and JS are equal first authors. AC is the corresponding author. NT, JS, KS, and AC all contributed to writing and editing the manuscript.

FUNDING

Our research was funded by the Australian National Health and Medical Research Council (NHMRC) (APP1050909).

REFERENCES

1. Gottlieb SL, Giersing BK, Hickling J, Jones R, Deal C, Kaslow DC. Meeting report: initial World Health Organization consultation on herpes simplex virus (HSV) vaccine preferred product characteristics, March 2017. *Vaccine*. (2017). doi: 10.1016/j.vaccine.2017.10.084. [Epub ahead of print].
2. WHO. *Global Vaccine Action Plan 2011-2020* (2012). Available online at: http://www.who.int/immunization/global_vaccine_action_plan/GVAP_doc_2011_2020/en/.
3. Freeman EE, Weiss HA, Glynn JR, Cross PL, Whitworth JA, Hayes RJ. Herpes simplex virus 2 infection increases HIV acquisition in men and women: systematic review and meta-analysis of longitudinal studies. *Aids*. (2006) 20:73–83. doi: 10.1097/01.aids.0000198081.09337.a7
4. Looker KJ, Elmes JAR, Gottlieb SL, Schiffer JT, Vickerman P, Turner KME, et al. Effect of HSV-2 infection on subsequent HIV acquisition: an updated systematic review and meta-analysis. *Lancet Infect Dis*. (2017) 17:1303–16. doi: 10.1016/s1473-3099(17)30405-x
5. Bradley J, Floyd S, Piwowar-Manning E, Laeyendecker O, Young A, Bell-Mandla N, et al. Sexually transmitted bedfellows: exquisite association between HIV and herpes simplex virus type 2 in 21 communities in Southern Africa in the HIV prevention trials network 071 (PopART) study. *J Infect Dis*. (2018) 218:443–52. doi: 10.1093/infdis/jiy178
6. Wald A, Link K. Risk of human immunodeficiency virus infection in herpes simplex virus type 2-seropositive persons: a meta-analysis. *J Infect Dis*. (2002) 185:45–52. doi: 10.1086/338231
7. Omori R, Nagelkerke N, Abu-Raddad LJ. HIV and herpes simplex virus type 2 epidemiological synergy: misguided observational evidence? A modelling study. *Sex Transm Infect*. (2018) 94:372–6. doi: 10.1136/sextrans-2017-053336
8. Reynolds SJ, Risbud AR, Shepherd ME, Zenilman JM, Brookmeyer RS, Paranjape RS, et al. Recent herpes simplex virus type 2 infection and the risk of human immunodeficiency virus type 1 acquisition in India. *J Infect Dis*. (2003) 187:1513–21. doi: 10.1086/368357
9. Celum C, Wald A, Lingappa JR, Magaret AS, Wang RS, Mugo N, et al. Acyclovir and transmission of HIV-1 from persons infected with HIV-1 and HSV-2. *N Engl J Med*. (2010) 362:427–39. doi: 10.1056/NEJMoa0904849
10. Schiffer JT, Swan DA, Corey L, Wald A. Rapid viral expansion and short drug half-life explain the incomplete effectiveness of current herpes simplex virus 2-directed antiviral agents. *Antimicrob Agents Chemother*. (2013) 57:5820–9. doi: 10.1128/aac.01114-13
11. Spicknall IH, Looker KJ, Gottlieb SL, Chesson HW, Schiffer JT, Elmes J, et al. Review of mathematical models of HSV-2 vaccination: implications for vaccine development. *Vaccine*. (2018). doi: 10.1016/j.vaccine.2018.02.067. [Epub ahead of print].
12. Oxman MN, Levin MJ, Johnson GR, Schmader KE, Straus SE, Gelb LD, et al. A vaccine to prevent herpes zoster and postherpetic neuralgia in older adults. *N Engl J Med*. (2005) 352:2271–84. doi: 10.1056/NEJMoa051016
13. Morrison VA, Johnson GR, Schmader KE, Levin MJ, Zhang JH, Looney DJ, et al. Long-term persistence of zoster vaccine efficacy. *Clin Infect Dis*. (2015) 60:900–9. doi: 10.1093/cid/ciu918
14. Lal H, Cunningham AL, Godeaux O, Chlibek R, Diez-Domingo J, Hwang SJ, et al. Efficacy of an adjuvanted herpes zoster subunit vaccine in older adults. *N Engl J Med*. (2015) 372:2087–96. doi: 10.1056/NEJMoa1501184

15. Leroux-Roels I, Leroux-Roels G, Clement F, Vandepapeliere P, Vassilev V, Ledent E, et al. A phase 1/2 clinical trial evaluating safety and immunogenicity of a varicella zoster glycoprotein e subunit vaccine candidate in young and older adults. *J Infect Dis*. (2012) 206:1280–90. doi: 10.1093/infdis/jis497
16. Weinberg A, Kroehl ME, Johnson MJ, Hammes A, Reinhold D, Lang N, et al. Comparative immune responses to licensed herpes zoster vaccines. *J Infect Dis*. (2018) 218(Suppl. 2):S81–7. doi: 10.1093/infdis/jiy383
17. Bernard MC, Barban V, Pradezynski F, de Montfort A, Ryall R, Caillet C, et al. Immunogenicity, protective efficacy, and non-replicative status of the HSV-2 vaccine candidate HSV529 in mice and guinea pigs. *PLoS ONE*. (2015) 10:e0121518. doi: 10.1371/journal.pone.0121518
18. Corey L, Langenberg AG, Ashley R, Sekulovich RE, Izu AE, Douglas JJ M, et al. Recombinant glycoprotein vaccine for the prevention of genital HSV-2 infection: two randomized controlled trials. Chiron HSV Vaccine Study Group. *JAMA*. (1999) 282:331–40.
19. Mikloska Z, Cunningham AL. Herpes simplex virus type 1 glycoproteins gB, gC and gD are major targets for CD4 T-lymphocyte cytotoxicity in HLA-DR expressing human epidermal keratinocytes. *J Gen Virol*. (1998) 79(Pt 2):353–61.
20. Stanberry LR, Spruance SL, Cunningham AL, Bernstein DI, Mindel A, Sacks S, et al. Glycoprotein-D-adjuvant vaccine to prevent genital herpes. *N Engl J Med*. (2002) 347:1652–61. doi: 10.1056/NEJMoa011915
21. Belshe RB, Leone PA, Bernstein DI, Wald A, Levin MJ, Stapleton JT, et al. Efficacy results of a trial of a herpes simplex vaccine. *N Engl J Med*. (2012) 366:34–43. doi: 10.1056/NEJMoa1103151
22. Lamers SL, Newman RM, Laeyendecker O, Tobian AA, Colgrove RC, Ray SC, et al. Global diversity within and between human herpesvirus 1 and 2 glycoproteins. *J Virol*. (2015) 89:8206–18. doi: 10.1128/jvi.01302-15
23. Belshe RB, Heineman TC, Bernstein DI, Bellamy AR, Ewell M, van der Most R, et al. Correlate of immune protection against HSV-1 genital disease in vaccinated women. *J Infect Dis*. (2014) 209:828–36. doi: 10.1093/infdis/jit651
24. Abendroth A, Kinchington PR, Slobedman B. Varicella zoster virus immune evasion strategies. *Curr Top Microbiol Immunol*. (2010) 342:155–71. doi: 10.1007/82_2010_41
25. Su C, Zhan G, Zheng C. Evasion of host antiviral innate immunity by HSV-1, an update. *Virol J*. (2016) 13:38. doi: 10.1186/s12985-016-0495-5
26. Levin MJ, Kroehl ME, Johnson MJ, Hammes A, Reinhold D, Lang N, et al. Th1 memory differentiates recombinant from live herpes zoster vaccines. *J Clin Invest*. (2018) 128:4429–40. doi: 10.1172/jci121484
27. Cunningham AL, Heineman TC, Lal H, Godeaux O, Chlibek R, Hwang SJ, et al. Immune responses to a recombinant glycoprotein E herpes zoster vaccine in adults aged 50 years or older. *J Infect Dis*. (2018) 217:1750–60. doi: 10.1093/infdis/jiy095
28. Heath WR, Carbone FR. The skin-resident and migratory immune system in steady state and memory: innate lymphocytes, dendritic cells and T cells. *Nat Immunol*. (2013) 14:978–85. doi: 10.1038/ni.2680
29. Kuo IH, Yoshida T, De Benedetto A, Beck LA. The cutaneous innate immune response in patients with atopic dermatitis. *J Allergy Clin Immunol*. (2013) 131:266–78. doi: 10.1016/j.jaci.2012.12.1563
30. Mikloska Z, Danis VA, Adams S, Lloyd AR, Adrian DL, Cunningham AL. *In vivo* production of cytokines and beta (C-C) chemokines in human recurrent herpes simplex lesions–do herpes simplex virus-infected keratinocytes contribute to their production? *J Infect Dis*. (1998) 177:827–38.
31. Albanesi C, Scarponi C, Giustizieri ML, Girolomoni G. Keratinocytes in inflammatory skin diseases. *Curr Drug Targets Inflamm Allergy*. (2005) 4:329–34. doi: 10.2174/1568010054022033
32. Meephansan J, Tsuda H, Komine M, Tominaga S, Ohtsuki M. Regulation of IL-33 expression by IFN-gamma and tumor necrosis factor-alpha in normal human epidermal keratinocytes. *J Invest Dermatol*. (2012) 132:2593–600. doi: 10.1038/jid.2012.185
33. Cunningham AL, Noble JR. Role of keratinocytes in human recurrent herpetic lesions. Ability to present herpes simplex virus antigen and act as targets for T lymphocyte cytotoxicity *in vitro*. *J Clin Invest*. (1989) 83:490–6.
34. Nickoloff BJ, Turka LA. Immunological functions of non-professional antigen-presenting cells: new insights from studies of T-cell interactions with keratinocytes. *Immunol Today*. (1994) 15:464–9.
35. Takaoka A, Yanai H. Interferon signalling network in innate defence. *Cell Microbiol*. (2006) 8:907–22. doi: 10.1111/j.1462-5822.2006.00716.x
36. Chan T, Barra NG, Lee AJ, Ashkar AA. Innate and adaptive immunity against herpes simplex virus type 2 in the genital mucosa. *J Reprod Immunol*. (2011) 88:210–8. doi: 10.1016/j.jri.2011.01.001
37. Baranek T, Zucchini N, Dalod M. Plasmacytoid dendritic cells and the control of herpesvirus infections. *Viruses*. (2009) 1:383–419. doi: 10.3390/v1030383
38. Kadowaki N, Antonenko S, Lau JY, Liu YJ. Natural interferon alpha/beta-producing cells link innate and adaptive immunity. *J Exp Med*. (2000) 192:219–26. doi: 10.1084/jem.192.2.219
39. Hoeffel G, Ripoche AC, Matheoud D, Nascimbeni M, Escriou N, Lebon P, et al. Antigen crosspresentation by human plasmacytoid dendritic cells. *Immunity*. (2007) 27:481–92. doi: 10.1016/j.immuni.2007.07.021
40. Mouries J, Moron G, Schlecht G, Escriou N, Dadaglio G, Leclerc C. Plasmacytoid dendritic cells efficiently cross-prime naive T cells *in vivo* after TLR activation. *Blood*. (2008) 112:3713–22. doi: 10.1182/blood-2008-03-146290
41. Donaghy H, Bosnjak L, Harman AN, Marsden V, Tyring SK, Meng TC, et al. Role for plasmacytoid dendritic cells in the immune control of recurrent human herpes simplex virus infection. *J Virol*. (2009) 83:1952–61. doi: 10.1128/jvi.01578-08
42. Villani AC, Satija R, Reynolds G, Sarkizova S, Shekhar K, Fletcher J, et al. Single-cell RNA-seq reveals new types of human blood dendritic cells, monocytes, and progenitors. *Science*. (2017) 356:eaah4573. doi: 10.1126/science.aah4573
43. Ashkar AA, Rosenthal KL. Interleukin-15 and natural killer and NKT cells play a critical role in innate protection against genital herpes simplex virus type 2 infection. *J Virol*. (2003) 77:10168–71. doi: 10.1128/JVI.77.18.10168-10171.2003
44. Thapa M, Kuziel WA, Carr DJ. Susceptibility of CCR5-deficient mice to genital herpes simplex virus type 2 is linked to NK cell mobilization. *J Virol*. (2007) 81:3704–13. doi: 10.1128/jvi.02626-06
45. Kawakami Y, Ando T, Lee JR, Kim G, Kawakami Y, Nakasaki T, et al. Defective natural killer cell activity in a mouse model of eczema herpeticum. *J Allergy Clin Immunol*. (2017) 139:997–1006.e10. doi: 10.1016/j.jaci.2016.06.034
46. Biron CA, Byron KS, Sullivan JL. Severe herpesvirus infections in an adolescent without natural killer cells. *N Engl J Med*. (1989) 320:1731–5.
47. Dalloul A, Oksenhendler E, Chosidow O, Ribaud P, Carcelain G, Louvet S, et al. Severe herpes virus (HSV-2) infection in two patients with myelodysplasia and undetectable NK cells and plasmacytoid dendritic cells in the blood. *J Clin Virol*. (2004) 30:329–36. doi: 10.1016/j.jcv.2003.11.014
48. Koelle DM, Posavad CM, Barnum GR, Johnson ML, Frank JM, Corey L. Clearance of HSV-2 from recurrent genital lesions correlates with infiltration of HSV-specific cytotoxic T lymphocytes. *J Clin Invest*. (1998) 101:1500–8.
49. Kim M, Osborne NR, Zeng W, Donaghy H, McKinnon K, Jackson DC, et al. Herpes simplex virus antigens directly activate NK cells via TLR2, thus facilitating their presentation to CD4 T lymphocytes. *J Immunol*. (2012) 188:4158–70. doi: 10.4049/jimmunol.1103450
50. Milligan GN, Bernstein DI. Interferon-gamma enhances resolution of herpes simplex virus type 2 infection of the murine genital tract. *Virology*. (1997) 229:259–68.
51. Gill N, Ashkar AA. Overexpression of interleukin-15 compromises CD4-dependent adaptive immune responses against herpes simplex virus 2. *J Virol*. (2009) 83:918–26. doi: 10.1128/jvi.01282-08
52. Cunningham AL, Turner RR, Miller AC, Para MF, Merigan TC. Evolution of recurrent herpes simplex lesions. An immunohistologic study. *J Clin Invest*. (1985) 75:226–33.
53. Eberl G, Colonna M, Di Santo JP, McKenzie AN. Innate lymphoid cells. Innate lymphoid cells: a new paradigm in immunology. *Science*. (2015) 348:aaa6566. doi: 10.1126/science.aaa6566
54. Yang Q, Bhandoola A. The development of adult innate lymphoid cells. *Curr Opin Immunol*. (2016) 39:114–20. doi: 10.1016/j.coi.2016.01.006
55. Bruggen MC, Bauer WM, Reininger B, Clim E, Captarencu C, Steiner GE, et al. *In situ* mapping of innate lymphoid cells in human skin: evidence for remarkable differences between normal and inflamed skin. *J Invest Dermatol*. (2016) 136:2396–405. doi: 10.1016/j.jid.2016.07.017

56. Ashley RL, Corey L, Dalessio J, Wilson P, Remington M, Barnum G, et al. Protein-specific cervical antibody responses to primary genital herpes simplex virus type 2 infections. *J Infect Dis*. (1994) 170:20–6.
57. Gallichan WS, Rosenthal KL. Long-term immunity and protection against herpes simplex virus type 2 in the murine female genital tract after mucosal but not systemic immunization. *J Infect Dis*. (1998) 177:1155–61.
58. Milligan GN, Bernstein DI. Generation of humoral immune responses against herpes simplex virus type 2 in the murine female genital tract. *Virology*. (1995) 206:234–41.
59. Mikloska Z, Sanna PP, Cunningham AL. Neutralizing antibodies inhibit axonal spread of herpes simplex virus type 1 to epidermal cells *in vitro*. *J Virol*. (1999) 73:5934–44.
60. Parr EL, Parr MB. Immunoglobulin G is the main protective antibody in mouse vaginal secretions after vaginal immunization with attenuated herpes simplex virus type 2. *J Virol*. (1997) 71:8109–15.
61. Parr MB, Parr EL. Immunity to vaginal herpes simplex virus-2 infection in B-cell knockout mice. *Immunology*. (2000) 101:126–31. doi: 10.1046/j.1365-2567.2000.00080.x
62. Halford WP, Geltz J, Messer RJ, Hasenkrug KJ. Antibodies are required for complete vaccine-induced protection against herpes simplex virus 2. *PLoS ONE*. (2015) 10:e0145228. doi: 10.1371/journal.pone.0145228
63. Halford WP, Geltz J, Gershburg E. Pan-HSV-2 IgG antibody in vaccinated mice and guinea pigs correlates with protection against herpes simplex virus 2. *PLoS ONE*. (2013) 8:e65523. doi: 10.1371/journal.pone.0065523
64. Dudley KL, Bourne N, Milligan GN. Immune protection against HSV-2 in B-cell-deficient mice. *Virology*. (2000) 270:454–63. doi: 10.1006/viro.2000.0298
65. Harandi AM, Svennerholm B, Holmgren J, Eriksson K. Differential roles of B cells and IFN-gamma-secreting CD4(+) T cells in innate and adaptive immune control of genital herpes simplex virus type 2 infection in mice. *J Gen Virol*. (2001) 82(Pt 4):845–53. doi: 10.1099/0022-1317-82-4-845
66. McDermott MR, Brais LJ, Evelegh MJ. Mucosal and systemic antiviral antibodies in mice inoculated intravaginally with herpes simplex virus type 2. *J Gen Virol*. (1990) 71(Pt 7):1497–504.
67. Morrison LA, Zhu L, Thebeau LG. Vaccine-induced serum immunoglobin contributes to protection from herpes simplex virus type 2 genital infection in the presence of immune T cells. *J Virol*. (2001) 75:1195–204. doi: 10.1128/jvi.75.3.1195-1204.2001
68. Awasthi S, Hook LM, Shaw CE, Pahar B, Stagray JA, Liu D, et al. An HSV-2 trivalent vaccine is immunogenic in rhesus macaques and highly efficacious in guinea pigs. *PLoS Pathog*. (2017) 13:e1006141. doi: 10.1371/journal.ppat.1006141
69. Awasthi S, Hook LM, Shaw CE, Friedman HM. A trivalent subunit antigen glycoprotein vaccine as immunotherapy for genital herpes in the guinea pig genital infection model. *Hum Vaccin Immunother*. (2017) 13:2785–93. doi: 10.1080/21645515.2017.1323604
70. Looker KJ, Magaret AS, May MT, Turner KME, Vickerman P, Newman LM, et al. First estimates of the global and regional incidence of neonatal herpes infection. *Lancet Glob Health*. (2017) 5:e300–9. doi: 10.1016/s2214-109x(16)30362-x
71. Prober CG, Sullender WM, Yasukawa LL, Au DS, Yeager AS, Arvin AM. Low risk of herpes simplex virus infections in neonates exposed to the virus at the time of vaginal delivery to mothers with recurrent genital herpes simplex virus infections. *N Engl J Med*. (1987) 316:240–4.
72. Brown ZA, Wald A, Morrow RA, Selke S, Zeh J, Corey L. Effect of serologic status and cesarean delivery on transmission rates of herpes simplex virus from mother to infant. *JAMA*. (2003) 289:203–9. doi: 10.1001/jama.289.2.203
73. Allen UD, Robinson JL. Prevention and management of neonatal herpes simplex virus infections. *Paediatr Child Health*. (2014) 19:201–12. doi: 10.1093/pch/19.4.201
74. Dancis J, Lind J, Oratz M, Smolens J, Vara P. Placental transfer of proteins in human gestation. *Am J Obstet Gynecol*. (1961) 82:167–71.
75. Marchant A, Sadarangani M, Garand M, Dauby N, Verhasselt V, Pereira L, et al. Maternal immunisation: collaborating with mother nature. *Lancet Infect Dis*. (2017) 17:e197–208. doi: 10.1016/s1473-3099(17)30229-3
76. Evans IA, Jones CA. Maternal immunization with a herpes simplex virus type 2 replication-defective virus reduces visceral dissemination but not lethal encephalitis in newborn mice after oral challenge. *J Infect Dis*. (2002) 185:1550–60. doi: 10.1086/340572
77. Kao C, Burn C, Jacobs WR, Herold BC. Maternal immunization with a single-cycle herpes simplex virus (HSV) candidate vaccine, (delta) gD-2, protects neonatal mice from lethal viral challenge. *Open Forum Infect Dis*. (2017) 4(Suppl. 1):S22. doi: 10.1093/ofid/ofx162.056
78. Jiang Y, Patel CD, Manivanh R, North B, Backes IM, Posner DA, et al. Maternal antiviral immunoglobulin accumulates in neural tissue of neonates to prevent HSV neurological disease. *MBio*. (2017) 8:e00678-17. doi: 10.1128/mBio.00678-17
79. Cella M, Scheidegger D, Palmer-Lehmann K, Lane P, Lanzavecchia A, Alber G. Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. *J Exp Med*. (1996) 184:747–52.
80. Murphy KM, Stockinger B. Effector T cell plasticity: flexibility in the face of changing circumstances. *Nat Immunol*. (2010) 11:674–80. doi: 10.1038/ni.1899
81. Iijima N, Linehan MM, Zamora M, Butkus D, Dunn R, Kehry MR, et al. Dendritic cells and B cells maximize mucosal Th1 memory response to herpes simplex virus. *J Exp Med*. (2008) 205:3041–52. doi: 10.1084/jem.20082039
82. Egan KP, Wu S, Wigdahl B, Jennings SR. Immunological control of herpes simplex virus infections. *J Neurovirol*. (2013) 19:328–45. doi: 10.1007/s13365-013-0189-3
83. Decman V, Kinchington PR, Harvey SA, Hendricks RL. Gamma interferon can block herpes simplex virus type 1 reactivation from latency, even in the presence of late gene expression. *J Virol*. (2005) 79:10339–47. doi: 10.1128/jvi.79.16.10339-10347.2005
84. Knickelbein JE, Khanna KM, Yee MB, Baty CJ, Kinchington PR, Hendricks RL. Noncytotoxic lytic granule-mediated CD8+ T cell inhibition of HSV-1 reactivation from neuronal latency. *Science*. (2008) 322:268–71. doi: 10.1126/science.1164164
85. Ouwendijk WJ, Laing KJ, Verjans GM, Koelle DM. T-cell immunity to human alphaherpesviruses. *Curr Opin Virol*. (2013) 3:452–60. doi: 10.1016/j.coviro.2013.04.004
86. van Velzen M, Jing L, Osterhaus AD, Sette A, Koelle DM, Verjans GM. Local CD4 and CD8 T-cell reactivity to HSV-1 antigens documents broad viral protein expression and immune competence in latently infected human trigeminal ganglia. *PLoS Pathog*. (2013) 9:e1003547. doi: 10.1371/journal.ppat.1003547
87. Mikloska Z, Kesson AM, Penfold ME, Cunningham AL. Herpes simplex virus protein targets for CD4 and CD8 lymphocyte cytotoxicity in cultured epidermal keratinocytes treated with interferon-gamma. *J Infect Dis*. (1996) 173:7–17.
88. Nakanishi Y, Lu B, Gerard C, Iwasaki A. CD8(+) T lymphocyte mobilization to virus-infected tissue requires CD4(+) T-cell help. *Nature*. (2009) 462:510–3. doi: 10.1038/nature08511
89. Zhu J, Hladik F, Woodward A, Klock A, Peng T, Johnston C, et al. Persistence of HIV-1 receptor-positive cells after HSV-2 reactivation is a potential mechanism for increased HIV-1 acquisition. *Nat Med*. (2009) 15:886–92. doi: 10.1038/nm.2006
90. Schiffer JT, Corey L. Rapid host immune response and viral dynamics in herpes simplex virus-2 infection. *Nat Med*. (2013) 19:280–90. doi: 10.1038/nm.3103
91. Zhu J, Koelle DM, Cao J, Vazquez J, Huang ML, Hladik F, et al. Virus-specific CD8+ T cells accumulate near sensory nerve endings in genital skin during subclinical HSV-2 reactivation. *J Exp Med*. (2007) 204:595–603. doi: 10.1084/jem.20061792
92. Posavad CM, Zhao L, Dong L, Jin L, Stevens CE, Magaret AS, et al. Enrichment of herpes simplex virus type 2 (HSV-2) reactive mucosal T cells in the human female genital tract. *Mucosal Immunol*. (2017) 10:1259–69. doi: 10.1038/mi.2016.118
93. Peng T, Zhu J, Phasouk K, Koelle DM, Wald A, Corey L. An effector phenotype of CD8+ T cells at the junction epithelium during clinical quiescence of herpes simplex virus 2 infection. *J Virol*. (2012) 86:10587–96. doi: 10.1128/jvi.01237-12

94. Zhu J, Peng T, Johnston C, Phasouk K, Kask AS, Klock A, et al. Immune surveillance by CD8alphaalpha+ skin-resident T cells in human herpes virus infection. *Nature*. (2013) 497:494–7. doi: 10.1038/nature12110
95. Schiffer JT. Mucosal HSV-2 specific CD8+ T-cells represent containment of prior viral shedding rather than a correlate of future protection. *Front Immunol.* (2013) 4:209. doi: 10.3389/fimmu.2013.00209
96. Schiffer JT, Swan DA, Roychoudhury P, Lund JM, Prlic M, Zhu J, et al. A fixed spatial structure of CD8(+) T cells in tissue during chronic HSV-2 infection. *J Immunol.* (2018) 201:1522–35. doi: 10.4049/jimmunol.1800471
97. Fernandez MA, Puttur FK, Wang YM, Howden W, Alexander SI, Jones CA. T regulatory cells contribute to the attenuated primary CD8+ and CD4+ T cell responses to herpes simplex virus type 2 in neonatal mice. *J Immunol.* (2008) 180:1556–64. doi: 10.4049/jimmunol.180.3.1556
98. Soerens AG, Da Costa A, Lund JM. Regulatory T cells are essential to promote proper CD4 T-cell priming upon mucosal infection. *Mucosal Immunol.* (2016) 9:1395–406. doi: 10.1038/mi.2016.19
99. Fernandez MA, Yu U, Zhang G, White R, Sparwasser T, Alexander SI, et al. Treg depletion attenuates the severity of skin disease from ganglionic spread after HSV-2 flank infection. *Virology*. (2013) 447:9–20. doi: 10.1016/j.virol.2013.08.027
100. Milman N, Zhu J, Johnston C, Cheng A, Magaret A, Koelle DM, et al. *In situ* detection of regulatory T cells in human genital herpes simplex virus type 2 (HSV-2) reactivation and their influence on spontaneous HSV-2 reactivation. *J Infect Dis*. (2016) 214:23–31. doi: 10.1093/infdis/jiw091
101. Diaz GA, Koelle DM. Human CD4+ CD25 high cells suppress proliferative memory lymphocyte responses to herpes simplex virus type 2. *J Virol*. (2006) 80:8271–3. doi: 10.1128/jvi.00656-06
102. Ciofani M, Zúñiga-Pflücker JC. Determining gammaDelta versus alphaBeta T cell development. *Nat Rev Immunol*. (2010) 10:657. doi: 10.1038/nri2820
103. Sciammas R, Kodukula P, Tang Q, Hendricks RL, Bluestone JA. T cell receptor-gamma/delta cells protect mice from herpes simplex virus type 1-induced lethal encephalitis. *J Exp Med.* (1997) 185:1969–75.
104. Puttur FK, Fernandez MA, White R, Roediger B, Cunningham AL, Weninger W, et al. Herpes simplex virus infects skin gamma delta T cells before Langerhans cells and impedes migration of infected Langerhans cells by inducing apoptosis and blocking E-cadherin downregulation. *J Immunol.* (2010) 185:477–87. doi: 10.4049/jimmunol.0904106
105. Bos JD, Teunissen MB, Cairo I, Krieg SR, Kapsenberg ML, Das PK, et al. T-cell receptor gamma delta bearing cells in normal human skin . *J Invest Dermatol.* (1990) 94:37–42.
106. Maccario R, Revello MG, Comoli P, Montagna D, Locatelli F, Gerna G. HLA-unrestricted killing of HSV-1-infected mononuclear cells. Involvement of either gamma/delta+ or alpha/beta+ human cytotoxic T lymphocytes. *J Immunol.* (1993) 150:1437–45.
107. Bukowski JF, Morita CT, Brenner MB. Recognition and destruction of virus-infected cells by human gamma delta CTL. J Immunol. (1994) 153:5133–40.
108. Kashem SW, Haniffa M, Kaplan DH. Antigen-presenting cells in the skin. *Annu Rev Immunol*. (2017) 35:469–99.
109. Mikloska Z, Bosnjak L, Cunningham AL. Immature monocyte-derived dendritic cells are productively infected with herpes simplex virus type 1. *J Virol.* (2001) 75:5958–64. doi: 10.1128/jvi.75.13.5958-5964.2001
110. Bosnjak L, Miranda-Saksena M, Koelle DM, Boadle RA, Jones CA, Cunningham AL. Herpes simplex virus infection of human dendritic cells induces apoptosis and allows cross-presentation via uninfected dendritic cells. *J Immunol.* (2005) 174:2220–7. doi: 10.4049/jimmunol.174.4.2220
111. Sprecher E, Becker Y. Skin Langerhans cells play an essential role in the defense against HSV-1 infection. *Arch Virol.* (1986) 91:341–9.
112. Kim M, Truong NR, James V, Bosnjak L, Sandgren KJ, Harman AN, et al. Relay of herpes simplex virus between Langerhans cells and dermal dendritic cells in human skin. *PLoS Pathog*. (2015) 11:e1004812. doi: 10.1371/journal.ppat.1004812
113. Guilliams M, Ginhoux F, Jakubzick C, Naik SH, Onai N, Schraml BU, et al. Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny. *Nat Rev Immunol*. (2014) 14:571–8. doi: 10.1038/nri3712
114. Theisen D, Murphy K. The role of cDC1s *in vivo*: CD8 T cell priming through cross-presentation. *F1000Res*. (2017) 6:98. doi: 10.12688/f1000research.9997.1
115. Bachem A, Guttler S, Hartung E, Ebstein F, Schaefer M, Tannert A, et al. Superior antigen cross-presentation and XCR1 expression define human CD11c+CD141+ cells as homologues of mouse CD8+ dendritic cells. *J Exp Med.* (2010) 207:1273–81. doi: 10.1084/jem.20100348
116. Poulin LF, Salio M, Griessinger E, Anjos-Afonso F, Craciun L, Chen JL, et al. Characterization of human DNGR-1+ BDCA3+ leukocytes as putative equivalents of mouse CD8alpha+ dendritic cells. *J Exp Med*. (2010) 207:1261–71. doi: 10.1084/jem.20092618
117. Haniffa M, Shin A, Bigley V, McGovern N, Teo P, See P, et al. Human tissues contain CD141hi cross-presenting dendritic cells with functional homology to mouse CD103+ nonlymphoid dendritic cells. *Immunity*. (2012) 37:60–73. doi: 10.1016/j.immuni.2012.04.012
118. Fehres CM, Bruijns SC, Sotthewes BN, Kalay H, Schaffer L, Head SR, et al. Phenotypic and functional properties of human steady state CD14+ and CD1a+ antigen presenting cells and epidermal langerhans cells. *PLoS ONE*. (2015) 10:e0143519. doi: 10.1371/journal.pone.0143519
119. Nestle FO, Zheng XG, Thompson CB, Turka LA, Nickoloff BJ. Characterization of dermal dendritic cells obtained from normal human skin reveals phenotypic and functionally distinctive subsets. *J Immunol.* (1993) 151:6535–45.
120. Klechevsky E, Morita R, Liu M, Cao Y, Coquery S, Thompson-Snipes L, et al. Functional specializations of human epidermal Langerhans cells and CD14+ dermal dendritic cells. *Immunity*. (2008) 29:497–510. doi: 10.1016/j.immuni.2008.07.013
121. Reynolds G, Haniffa M. Human and mouse mononuclear phagocyte networks: a tale of two species?. *Front Immunol.* (2015) 6:330. doi: 10.3389/fimmu.2015.00330
122. McGovern N, Schlitzer A, Gunawan M, Jardine L, Shin A, Poyner E, et al. Human dermal CD14(+) cells are a transient population of monocyte-derived macrophages. *Immunity*. (2014) 41:465–77. doi: 10.1016/j.immuni.2014.08.006
123. Zhao X, Deak E, Soderberg K, Linehan M, Spezzano D, Zhu J, et al. Vaginal submucosal dendritic cells, but not Langerhans cells, induce protective Th1 responses to herpes simplex virus-2. *J Exp Med.* (2003) 197:153–62. doi: 10.1084/jem.20021109
124. Allan RS, Smith CM, Belz GT, van Lint AL, Wakim LM, Heath WR, et al. Epidermal viral immunity induced by CD8alpha+ dendritic cells but not by Langerhans cells. Science. (2003) 301:1925–8. doi: 10.1126/science.1087576
125. Bedoui S, Whitney PG, Waithman J, Eidsmo L, Wakim L, Caminschi I, et al. Cross-presentation of viral and self antigens by skin-derived CD103+ dendritic cells. *Nat Immunol.* (2009) 10:488–95. doi: 10.1038/ni.1724
126. Cunningham AL, Mikloska Z. The Holy Grail: immune control of human herpes simplex virus infection and disease. *Herpes*. (2001) 8(Suppl. 1):6–10a.
127. Taylor TJ, Brockman MA, McNamee EE, Knipe DM. Herpes simplex virus. *Front Biosci*. (2002) 7:d752–64. doi: 10.2741/A809
128. Cairns TM, Huang ZY, Gallagher JR, Lin Y, Lou H, Whitbeck JC, et al. Patient-specific neutralizing antibody responses to herpes simplex virus are attributed to epitopes on gD, gB, or both and can be type specific. *J Virol*. (2015) 89:9213–31. doi: 10.1128/jvi.01213-15
129. Cairns TM, Huang ZY, Whitbeck JC, Ponce de Leon M, Lou H, Wald A, et al. Dissection of the antibody response against herpes simplex virus glycoproteins in naturally infected humans. *J Virol*. (2014) 88:12612–22. doi: 10.1128/jvi.01930-14
130. Berman PW, Gregory T, Crase D, Lasky LA. Protection from genital herpes simplex virus type 2 infection by vaccination with cloned type 1 glycoprotein D. *Science*. (1985) 227:1490–2.
131. Eisenberg RJ, Long D, Ponce de Leon M, Matthews JT, Spear PG, Gibson MG, et al. Localization of epitopes of herpes simplex virus type 1 glycoprotein D. *J Virol.* (1985) 53:634–44.
132. Stanfield BA, Stahl J, Chouljenko VN, Subramanian R, Charles AS, Saied AA, et al. A single intramuscular vaccination of mice with the HSV-1 VC2 virus with mutations in the glycoprotein K and the membrane protein UL20 confers full protection against lethal intravaginal challenge with virulent HSV-1 and HSV-2 strains. *PLoS ONE*. (2014) 9:e109890. doi: 10.1371/journal.pone.0109890
133. Hook LM, Cairns TM, Awasthi S, Brooks BD, Ditto NT, Eisenberg RJ, et al. Vaccine-induced antibodies to herpes simplex virus glycoprotein D epitopes involved in virus entry and cell-to-cell spread correlate with protection

against genital disease in guinea pigs. *PLoS Pathog.* (2018) 14:e1007095. doi: 10.1371/journal.ppat.1007095
134. Petro CD, Weinrick B, Khajoueinejad N, Burn C, Sellers R, Jacobs J W R, et al. HSV-2 DeltagD elicits FcgammaR-effector antibodies that protect against clinical isolates. *JCI Insight*. (2016) 1:e88529. doi: 10.1172/jci.insight.88529
135. Petro C, Gonzalez PA, Cheshenko N, Jandl T, Khajoueinejad N, Benard A, et al. Herpes simplex type 2 virus deleted in glycoprotein D protects against vaginal, skin and neural disease. *Elife*. (2015) 4:e06054. doi: 10.7554/eLife.06054
136. Burn C, Ramsey N, Garforth SJ, Almo S, Jacobs J W R, Herold BC. A herpes simplex virus (HSV)-2 single-cycle candidate vaccine deleted in glycoprotein D protects male mice from lethal skin challenge with clinical isolates of HSV-1 and HSV-2. *J Infect Dis.* (2018) 217:754–8. doi: 10.1093/infdis/jix628
137. Dropulic L, Wang K, Oestreich M, Pietz H, Garabedian D, Jegaskanda S, et al. A replication-defective herpes simplex virus (HSV)-2 vaccine, HSV529, is safe and well-tolerated in adults with or without HSV infection and induces significant HSV-2-Specific antibody responses in HSV seronegative individuals. *Open Forum Infect Dis*. (2017) 4(Suppl. 1):S415–6. doi: 10.1093/ofid/ofx163
138. Mertz GJ, Ashley R, Burke RL, Benedetti J, Critchlow C, Jones CC, et al. Double-blind, placebo-controlled trial of a herpes simplex virus type 2 glycoprotein vaccine in persons at high risk for genital herpes infection . *J Infect Dis.* (1990) 161:653–60.
139. Terhune SS, Coleman KT, Sekulovich R, Burke RL, Spear PG. Limited variability of glycoprotein gene sequences and neutralizing targets in herpes simplex virus type 2 isolates and stability on passage in cell culture. *J Infect Dis.* (1998) 178:8–15.
140. Koelle DM, Schomogyi M, McClurkan C, Reymond SN, Chen HB. CD4 T-cell responses to herpes simplex virus type 2 major capsid protein VP5: comparison with responses to tegument and envelope glycoproteins . *J Virol.* (2000) 74:11422–5. doi: 10.1128/JVI.74.23.11422-11425.2000
141. Kim M, Taylor J, Sidney J, Mikloska Z, Bodsworth N, Lagios K, et al. Immunodominant epitopes in herpes simplex virus type 2 glycoprotein D are recognized by CD4 lymphocytes from both HSV-1 and HSV-2 seropositive subjects. *J Immunol.* (2008) 181:6604–15. doi: 10.4049/jimmunol.181.9.6604
142. Mikloska Z, Ruckholdt M, Ghadiminejad I, Dunckley H, Denis M, Cunningham AL. Monophosphoryl lipid A and QS21 increase CD8 T lymphocyte cytotoxicity to herpes simplex virus-2 infected cell proteins 4 and 27 through IFN-gamma and IL-12 production. *J Immunol.* (2000) 164:5167–76. doi: 10.4049/jimmunol.164.10.5167
143. Hosken N, McGowan P, Meier A, Koelle DM, Sleath P, Wagener F, et al. Diversity of the CD8+ T-cell response to herpes simplex virus type 2 proteins among persons with genital herpes. *J Virol.* (2006) 80:5509–15. doi: 10.1128/jvi.02659-05
144. Chiu C, McCausland M, Sidney J, Duh FM, Rouphael N, Mehta A, et al. Broadly reactive human CD8 T cells that recognize an epitope conserved between VZV, HSV and EBV. *PLoS Pathog.* (2014) 10:e1004008. doi: 10.1371/journal.ppat.1004008
145. Jing L, Laing KJ, Dong L, Russell RM, Barlow RS, Haas JG, et al. Extensive CD4 and CD8 T cell cross-reactivity between alphaherpesviruses. *J Immunol.* (2016) 196:2205–18. doi: 10.4049/jimmunol.1502366
146. Porchia B, Moreno ACR, Ramos RN, Diniz MO, de Andrade L, Rosa DS, et al. Herpes simplex virus glycoprotein D targets a specific dendritic cell subset and improves the performance of vaccines to human papillomavirus-associated tumors. *Mol Cancer Ther*. (2017) 16:1922–33. doi: 10.1158/1535-7163.Mct-17-0071
147. Corey L, Johnston C, Posavad C, Vigneault F, Koelle D, Ford F, et al. Disparities in TCR repertoires between anatomic sites: implications for harnessing the tissue memory T-cell response to chronic viral infections. In: *International Herpesvirus Workshop*. Vancouver, BC (2018).
148. Ford E, Li A, Dong L, Jing L, Laing K, Sun S, et al. Expansion of the tissue-based T-cell receptor repertoire is distinct from the PBMC response after immunotherapeutic HSV-2 vaccine. In: *International Herpesvirus Workshop*. Vancouver, BC (2018).
149. Wang K, Goodman KN, Li DY, Raffeld M, Chavez M, Cohen JI. A herpes simplex virus 2 (HSV-2) gD mutant impaired for neural tropism is superior to an HSV-2 gD subunit vaccine to protect animals from challenge with HSV-2. *J Virol.* (2016) 90:562–74. doi: 10.1128/jvi.01845-15
150. Shin H, Iwasaki A. A vaccine strategy that protects against genital herpes by establishing local memory T cells. *Nature*. (2012) 491:463–7. doi: 10.1038/nature11522
151. Shin H, Kumamoto Y, Gopinath S, Iwasaki A. CD301b+ dendritic cells stimulate tissue-resident memory CD8+ T cells to protect against genital HSV-2. *Nat Commun*. (2016) 7:13346. doi: 10.1038/ncomms13346
152. O'Konek JJ, Makidon PE, Landers JJ, Cao Z, Malinczak CA, Pannu J, et al. Intranasal nanoemulsion-based inactivated respiratory syncytial virus vaccines protect against viral challenge in cotton rats. *Hum Vaccin Immunother*. (2015) 11:2904–12. doi: 10.1080/21645515.2015.1075680
153. Ahmed M, Smith DM, Hamouda T, Rangel-Moreno J, Fattom A, Khader SA. A novel nanoemulsion vaccine induces mucosal Interleukin-17 responses and confers protection upon *Mycobacterium tuberculosis* challenge in mice. *Vaccine*. (2017) 35:4983–89. doi: 10.1016/j.vaccine.2017.07.073
154. Biologics B. *HSV2 Vaccine* (2018). Available online at: http://www.bluewillow.com/vaccine-pipeline/hsv-2-vaccine/.
155. Zhang X, Dervillez X, Chentoufi AA, Badakhshan T, Bettahi I, Benmohamed L. Targeting the genital tract mucosa with a lipopeptide/recombinant adenovirus prime/boost vaccine induces potent and long-lasting CD8+ T cell immunity against herpes: importance of MyD88. *J Immunol.* (2012) 189:4496–509. doi: 10.4049/jimmunol.1201121
156. Zhang X, Chentoufi AA, Dasgupta G, Nesburn AB, Wu M, Zhu X, et al. A genital tract peptide epitope vaccine targeting TLR-2 efficiently induces local and systemic CD8+ T cells and protects against herpes simplex virus type 2 challenge. *Mucosal Immunol.* (2009) 2:129–43. doi: 10.1038/mi.2008.81
157. He Q, Mitchell A, Morcol T, Bell SJ. Calcium phosphate nanoparticles induce mucosal immunity and protection against herpes simplex virus type 2. *Clin Diagn Lab Immunol*. (2002) 9:1021–4. doi: 10.1128/CDLI.9.5.1021-1024.2002
158. Marrack P, McKee AS, Munks MW. Towards an understanding of the adjuvant action of aluminium. *Nat Rev Immunol.* (2009) 9:287–93. doi: 10.1038/nri2510
159. Di Pasquale A, Preiss S, Tavares Da Silva F, Garcon N. Vaccine Adjuvants: from 1920 to 2015 and Beyond. *Vaccines*. (2015) 3:320–43. doi: 10.3390/vaccines3020320
160. den Brok MH, Bull C, Wassink M, de Graaf AM, Wagenaars JA, Minderman M, et al. Saponin-based adjuvants induce cross-presentation in dendritic cells by intracellular lipid body formation. *Nat Commun.* (2016) 7:13324. doi: 10.1038/ncomms13324
161. Flechtner JB, Long D, Larson S, Clemens V, Baccari A, Kien L, et al. Immune responses elicited by the GEN-003 candidate HSV-2 therapeutic vaccine in a randomized controlled dose-ranging phase 1/2a trial. *Vaccine*. (2016) 34:5314–20. doi: 10.1016/j.vaccine.2016.09.001
162. Ferlazzo G, Morandi B. Cross-talks between natural killer cells and distinct subsets of dendritic cells. *Front Immunol*. (2014) 5:159. doi: 10.3389/fimmu.2014.00159
163. Deauvieau F, Ollion V, Doffin AC, Achard C, Fonteneau JF, Verronese E, et al. Human natural killer cells promote cross-presentation of tumor cell-derived antigens by dendritic cells. *Int J Cancer*. (2015) 136:1085–94. doi: 10.1002/ijc.29087
164. Lin PH, Wong WI, Wang YL, Hsieh MP, Lu CW, Liang CY, et al. Vaccine-induced antigen-specific regulatory T cells attenuate the antiviral immunity against acute influenza virus infection. *Mucosal Immunol*. (2018) 11:1239–53. doi: 10.1038/s41385-018-0004-9
165. Stanfield BA, Rider PJF, Caskey J, Del Piero F, Kousoulas KG. Intramuscular vaccination of guinea pigs with the live-attenuated human herpes simplex vaccine VC2 stimulates a transcriptional profile of vaginal Th17 and regulatory Tr1 responses. *Vaccine*. (2018) 36:2842–9. doi: 10.1016/j.vaccine.2018.03.075
166. Cohen J. Immunology. Painful failure of promising genital herpes vaccine . *Science*. (2010) 330:304. doi: 10.1126/science.330.6002.304
167. Bernstein DI, Wald A, Warren T, Fife K, Tyring S, Lee P, et al. Therapeutic vaccine for genital herpes simplex virus-2 infection: findings from a randomized trial. *J Infect Dis*. (2017) 215:856–64. doi: 10.1093/infdis/jix004
168. Van Wagoner N, Fife K, Leone PA, Bernstein DI, Warren T, Panther L, et al. Effects of different doses of GEN-003, a therapeutic vaccine for genital HSV-2, on viral shedding and lesions: results of a randomized placebo-controlled trial. *J Infect Dis.* (2018) 218:1890–9. doi: 10.1093/infdis/jiy415

169. Genocea. Press Releases: Genocea Announces Strategic Shift to Immunooncology and the Development of Neoantigen Cancer Vaccines (2017). Available online at: https://ir.genocea.com/news-releases/news-release-details/genocea-announces-strategic-shift-immuno-oncology-and.
170. Wald A, Koelle DM, Fife K, Warren T, Leclair K, Chicz RM, et al. Safety and immunogenicity of long HSV-2 peptides complexed with rhHsc70 in HSV-2 seropositive persons. *Vaccine*. (2011) 29:8520–9. doi: 10.1016/j.vaccine.2011.09.046
171. AgenusBio. *Agenus Vaccine Shows Significant Reduction in Viral Burden after HerpV Generated Immune Activation* (2014). Available online at: http://investor.agenusbio.com/2014-06-26-Agenus-Vaccine-Shows-Significant-Reduction-in-Viral-Burden-after-HerpV-Generated-Immune-Activation.
172. Veselenak RL, Shlapobersky M, Pyles RB, Wei Q, Sullivan SM, Bourne N. A Vaxfectin((R))-adjuvanted HSV-2 plasmid DNA vaccine is effective for prophylactic and therapeutic use in the guinea pig model of genital herpes. *Vaccine*. (2012) 30:7046–51. doi: 10.1016/j.vaccine.2012.09.057
173. Vical. *Vical Reports Phase 2 Trial of HSV-2 Therapeutic Vaccine Did Not Meet Primary Endpoint* (2018). Available online at: http://www.vical.com/investors/news-releases/News-Release-Details/2018/Vical-Reports-Phase-2-Trial-of-HSV-2-Therapeutic-Vaccine-Did-Not-Meet-Primary-Endpoint/default.aspx.
174. Dutton JL, Woo WP, Chandra J, Xu Y, Li B, Finlayson N, et al. An escalating dose study to assess the safety, tolerability and immunogenicity of a Herpes Simplex Virus DNA vaccine, COR-1 [Journal Article]. *Hum Vaccin Immunother*. (2016) 12:3079–88. doi: 10.1080/21645515.2016.1221872
175. Admedus. *Admedus HSV 2 Phase IIA Results* (2017). Available online at: https://www.admedus.com/uncategorized/08052017/admedus-hsv-2-phase-iia-results-may-2017/.
176. Investors P. *Admedus Meets Primary Endpoint for Herpes Vaccine Study*. (2017). Available online at: https://www.proactiveinvestors.com.au/companies/news/177275/admedus-meets-primary-endpoint-for-herpes-vaccine-study-177275.html.
177. Biologics B. *NanoBio Receives SBIR Grant For Genital Herpes Vaccine* (2017). Available online at: http://www.bluewillow.com/nanobio-receives-sbir-grant-for-genital-herpes-vaccine/.
178. Awasthi S, Huang J, Shaw C, Friedman HM. Blocking herpes simplex virus 2 glycoprotein E immune evasion as an approach to enhance efficacy of a trivalent subunit antigen vaccine for genital herpes. *J Virol.* (2014) 88:8421–32. doi: 10.1128/jvi.01130-14
179. Odegard JM, Flynn PA, Campbell DJ, Robbins SH, Dong L, Wang K, et al. A novel HSV-2 subunit vaccine induces GLA-dependent CD4 and CD8 T cell responses and protective immunity in mice and guinea pigs [Journal Article]. *Vaccine*. (2016) 34:101–9. doi: 10.1016/j.vaccine.2015.10.137
180. NIH. *HSV529 Vaccine in HSV-2 Seropositive Adults* (2018). Available online at: https://clinicaltrials.gov/ct2/show/record/NCT02571166?view=record
181. Halford WP, Puschel R, Gershburg E, Wilber A, Gershburg S, Rakowski B. A live-attenuated HSV-2 ICP0 virus elicits 10 to 100 times greater protection against genital herpes than a glycoprotein D subunit vaccine. *PLoS ONE*. (2011) 6:e17748. doi: 10.1371/journal.pone.0017748
182. Stanfield BA, Pahar B, Chouljenko VN, Veazey R, Kousoulas KG. Vaccination of rhesus macaques with the live-attenuated HSV-1 vaccine VC2 stimulates the proliferation of mucosal T cells and germinal center responses resulting in sustained production of highly neutralizing antibodies. *Vaccine*. (2017) 35:536–43. doi: 10.1016/j.vaccine.2016.12.018
183. Richards AL, Sollars PJ, Pitts JD, Stults AM, Heldwein EE, Pickard GE, et al. The pUL37 tegument protein guides alpha-herpesvirus retrograde axonal transport to promote neuroinvasion. *PLoS Pathog*. (2017) 13:e1006741. doi: 10.1371/journal.ppat.1006741
184. Chohan V, Baeten JM, Benki S, Graham SM, Lavreys L, Mandaliya K, et al. A prospective study of risk factors for herpes simplex virus type 2 acquisition among high-risk HIV-1 seronegative women in Kenya. *Sex Transm Infect*. (2009) 85:489–92. doi: 10.1136/sti.2009.036103
185. Masese L, Baeten JM, Richardson BA, Bukusi E, John-Stewart G, Jaoko W, et al. Incident herpes simplex virus type 2 infection increases the risk of subsequent episodes of bacterial vaginosis. *J Infect Dis*. (2014) 209:1023–7. doi: 10.1093/infdis/jit634
186. Masese L, Baeten JM, Richardson BA, Bukusi E, John-Stewart G, Graham SM, et al. Changes in the contribution of genital tract infections to HIV acquisition among Kenyan high-risk women from 1993 to 2012. *Aids*. (2015) 29:1077–85. doi: 10.1097/qad.0000000000000646
187. Gosmann C, Anahtar MN, Handley SA, Farcasanu M, Abu-Ali G, Bowman BA, et al. Lactobacillus-deficient cervicovaginal bacterial communities are associated with increased HIV acquisition in Young South African Women. *Immunity*. (2017) 46:29–37. doi: 10.1016/j.immuni.2016.12.013

Conflict of Interest Statement: AC reports other funding to his institution from GSK, Merck and BioCSL/Sequiris, outside the submitted work.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 19 March 2019
doi: 10.3389/fimmu.2019.00512

The Role of T Cells in Herpes Stromal Keratitis

*Naveen K. Rajasagi and Barry T. Rouse**

Biomedical and Diagnostic Sciences, College of Veterinary Medicine, The University of Tennessee, Knoxville, TN, United States

The blinding inflammatory lesion stromal keratitis (SK), which occurs in some patients in response to ocular herpes simplex virus (HSV) infection, represents mainly an immune cell mediated inflammatory response to the virus infection. The principal orchestrators of the immunopathological lesions are T cells although additional events participate that include the extent of recruitment of non-lymphoid cells, the extent of neoangiogenesis, and the extent of damage to nerve function. This review focuses on evidence that the balance of the functional subsets of T cells has a major impact on lesion severity and duration. Accordingly, if proinflammatory Th1 and Th17 CD4 T cells, and perhaps in some cases CD8 T cells, predominate lesions occur earlier and are more severe. Lesions are diminished when cells with regulatory function predominate. Moreover, when regulatory cells acquire the property to produce Amphiregulin this may facilitate lesion resolution. An objective to controlling lesions is to learn how to manipulate the balance of T cells to favor the representation and function of regulatory T cells and their products over proinflammatory cells. In this review we emphasize how exploiting the differential metabolic requirements of immune cells could be a valuable approach to control SK.

Keywords: herpes stromal keratitis, CD4 T cells, metabolism, regulatory T cells, plasticity

OPEN ACCESS

Edited by:
Susmit Suvas,
Wayne State University, United States

Reviewed by:
Paul R. Kinchington,
University of Pittsburgh, United States
Homayon Ghiasi,
Cedars-Sinai Medical Center, United States

****Correspondence:***
Barry T. Rouse
btr@utk.edu

Specialty section:
This article was submitted to Viral Immunology, a section of the journal Frontiers in Immunology

Received: *09 November 2018*
Accepted: *26 February 2019*
Published: *19 March 2019*

Citation:
Rajasagi NK and Rouse BT (2019) The Role of T Cells in Herpes Stromal Keratitis. Front. Immunol. 10:512. doi: 10.3389/fimmu.2019.00512

BACKGROUND

Herpes simplex virus (HSV) type 1 is a major human pathogen worldwide. It is estimated that around 67% of people worldwide (under age 50) are infected with HSV-1 (1). HSV-1 establishes a lifelong, latent infection for which no effective vaccine is currently available (2). Primary infection with HSV-1 is usually mild or subclinical and most individuals remain asymptomatic (3). However, HSV-1 infection can cause several complications in humans. Among these, corneal infection can lead to blinding immunopathological lesions in the eye referred to as herpes stromal keratitis (SK) (4, 5). Epidemiology studies outside of the United States have estimated incidence rates of HSV eye disease range from ∼4 to 13 new cases per 100,000 per year. A previous study from Rochester, Minnesota, estimated an incidence of 8.4 new cases per 100,000 and 20.7 total episodes per 100,000 people per year. Extrapolating these data to the US population census in 2000, the study predicted an estimated incidence of ∼24,000 new cases and 58,000 total episodes per year (6). Moreover, a study published in 2014, estimated an incidence of 6.8 new cases/100,000 in Northern California (7). Thus, herpes keratitis represents a clinically relevant syndrome and the SK form is a frequent cause of vision damage.

Primary ocular infection most likely occurs by the direct infection of the eye with HSV-1. Upon infection, the virus replicates in the corneal epithelial cells and can causes epithelial lesions. These primary lesions can last up to 2 weeks and usually resolve with minimal damage and the virus is efficiently cleared by the immune system (8). However, one of the consequences of HSV ocular infection is the establishment of latency in the trigeminal ganglia (TG) (9). Some of the HSV virions can enter the sensory nerve endings which innervate the infected cells and traffic via retrograde transport mechanisms to the sensory ganglia where the virus can persist in a latent stage (10). Sometimes the latent virus reactivates by disturbances caused by environmental or physiological stress and the reactivated HSV replicates in the TG. The virus can then travel by anterograde axonal transport to the peripheral tissues and cause recurrent lesions either in the corneal or orofacial tissues often resulting in clinical consequences (11). In humans, recurrent virus infections of the cornea are usually confined to the epithelial layer, but in some individuals such frequent recurrent infections could affect the deeper corneal stroma leading to an immunopathological disease referred to as herpes stromal keratitis (SK). This chronic inflammatory response in the corneal stroma is mediated by both innate and adaptive immune cells in response to virus infection and can lead to progressive corneal scarring and vision loss. The local corneal epithelial lesions and virus infections are usually treated using antivirals such as acyclovir, but SK lesions are often treated with a combination of an antiviral and a corticosteroid (12).

Most of our current understanding of the pathogenesis of SK in humans comes from studies done animal models (5, 13). HSV-1 corneal infection in mice is the most widely used animal model to study SK as it offers several advantages and the inflammatory lesions in the corneal stroma mimic SK lesions observed in humans (14). However, one limitation of the mice model is that it is mainly a primary infection model, but not a reactivation model of disease as mostly occurs in humans. The immune response to HSV-1 ocular infection occurs in a bi-phasic manner and involves both innate and adaptive components of the immune system (8). During the pre-clinical or acute phase, the first wave of immune cells mainly consisting of neutrophils, natural killer cells, and macrophages enter into the corneal stroma and help to clear the replicating virus (5). In the later clinical or chronic phase of the disease, CD4 T cells start to appear in the cornea around day 6–7 post-infection, a stage when virus is usually already cleared from the cornea (8). The CD4 T cells are considered to be the primary orchestrators of SK lesions as they facilitate the influx of the second wave of neutrophils (15). The massive cellular infiltration especially neutrophils coupled with the inflammatory mediators secreted by the immune cells are primarily responsible for the swelling and destruction of the cornea (16, 17).

ROLE OF Th1, Th17, AND CD8 T CELLS IN SK LESIONS

Stromal keratitis (SK) is an immunopathological disease orchestrated by T cells (14). This view is supported by findings which show that mice depleted of T cells are less susceptible to HSV-1 induced corneal stromal disease. In both humans and mice, there is a predominance of CD4 T cells in the ocular tissues during SK and their functional activities are often associated with the tissue damage in the corneal stroma. In mice, CD4 T cells appear in the corneas around day 6 post-ocular infection with HSV-1 and their numbers continue to increase during the latter stage of SK development. Among the CD4 T cell population, there is a preferential accumulation of CD4 T helper (Th1) subset in the eye (18). Th1 cells express the transcription factor, T-bet, and produce various immune-modulatory mediators which play a role in SK lesion expression. The Th1 cells secrete the cytokines IFN-γ and IL-2 which are capable of inducing corneal inflammation and neovascularization (19, 20). In addition, these cytokines also modulate chemokine factors, and in doing so could facilitate the massive influx of neutrophils and macrophages into the cornea during the latter phase of SK development (21, 22). Another CD4 subset which gained recent prominence in inflammation and autoimmunity are the Th17 cells (23). These cells express the transcription factor ROR-γt and produce cytokines such as IL-17, IL-21, and IL-22. They preferentially produce IL-17 which is a potent inducer of additional pro-inflammatory cytokines, chemokines, and metalloproteinases (24, 25). Th17 cells accumulate in the HSV infected cornea during the later stages of SK pathogenesis and help sustain and expand the disease (26, 27). Moreover, HSV-1 ocular infection of IL-17R knock-out mice or neutralization of IL-17 using monoclonal antibodies delayed disease progression and reduced the severity of HSK (26). Importantly, IL-17 was expressed in corneas of patients with SK (28). In addition, the human corneal fibroblasts constitutively express the IL-17R. The data from these studies suggest that IL-17 strongly induces the production of key inflammatory mediators such as IL-6, IL-8, and matrix mettalloproteinase-1 in the human corneal fibroblast cultures (28). Thus, Th17 cells through the production of IL-17 modulate the levels of chemotactic factors such as CXCL-1 and IL-8 and influence the migration of neutrophils into the inflamed corneal tissues (26).

Although, CD4 T cells are considered to be the chief perpetuators of SK, the data presented in some experimental models implicate CD8 T cells in the pathogenesis of SK. The outcome depends to a large extent on the virus strain used for the studies. Some studies found that ocular infection of mice with the HSV-1 RE strain mainly induces SK mediated by CD4 T cells, whereas infection of the same stain of mice with HSV-1 KOS show SK which is dependent on CD8 T cells (29). In mice infected with a recombinant stain of HSV-1 (HSV-gK), the corneal scaring and the corneal disease were mainly mediated by CD8 T cells (30, 31). Results from these studies suggest that gK strongly induces CD8 T cell responses leading to exacerbation of SK lesions. Of note, the recombinant HSV-gK strain used in these studies contains three copies of glycoprotein K (gk) (a protein essential for virus replication) compared to one copy in the wild type HSV-1 McKrae strain (30). The HSV-1 mutant strains which lack gK were found to be defective in infectivity and failed to establish latency in the neurons in mouse models which suggests that gK expression is crucial for virus replication (32). Thus, the respective roles of different CD4 and CD8 subsets in SK is not clear and remains an unresolved issue. Additionally, some

evidence shows that CD8 T cells mainly play more of a protective role (33). Observations in both mice and humans show that HSV-1 specific CD8 T cells are selectively retained in the TG and might help control HSV reactivation (34–36). These tissue resident CD8 T cells appear to use IFN-γ and non-cytolytic mechanisms to block virus reactivation in the TG (37, 38).

ROLE OF REGULATORY T CELLS (TREG) IN SK PATHOGENESIS

A beneficial subset of CD4 T cells in SK are regulatory T cells (Treg) (39, 40). Treg express the master transcription factor, Foxp3 which controls their development, and function (41). Treg are either produced as a functionally mature T cell sub population in the thymus (natural Treg) or are induced in the periphery from naive CD4 T cells (induced Treg). Treg mainly function to maintain tolerance to self-antigens and prevent autoimmune diseases (42). They also constrain excessive immune responses to non-self-antigens or infectious agents and help to maintain peripheral tolerance and immune homeostasis (41). Treg use several mechanisms to suppress aberrant immune responses and these include immunomodulatory cytokines (IL-10, TGF-β, IL-35) or contact dependent suppression (granzyme/perforin) (41, 43, 44). In addition, Tregs also exert their function on effector T cells through inhibitory molecules such as CTLA-4. Treg also condition dendritic cells to secrete indoleamine 2,3-dioxygenase, a molecule which suppresses the activation of effector T cells (44).

During microbial infections, a major function of Treg is to control the excessive inflammatory responses to prevent collateral tissue damage and limit injury to the host. In HSV-1 ocular infection, Treg were shown to be crucial to control HSV induced corneal immunopathology. SK lesions were more severe if mice were depleted of Treg before infection using monoclonal antibody treatment, whereas adoptive transfer of *in vitro* converted Treg suppressed HSK severity (45, 46). Furthermore, findings using the depletion of regulatory T cells (DEREG) transgenic mice showed that lesions became more severe even when depletion was begun in the later phases (clinical/chronic phase) of the disease (47). The DEREG mice carry the diphtheria toxin receptor-enhanced green fluorescent protein (DTR-eGFP) transgene under the control of an additional Foxp3 promoter, which facilitates specific depletion of Treg by application of diphtheria toxin at any chosen point of time (48). Thus, measures to expand the representation of Treg by the administration of various reagents have been useful in reducing the severity of SK lesions in the mouse model. One such approach used was galectin-9 which induces apoptosis of pathogenic CD4 Th1 cells and increases the representation of the anti-inflammatory Treg population (49). In addition, a combination treatment using a tumor necrosis factor receptor superfamily member 25 (TNFRSF25) agonist antibody which expands Treg numbers along with galectin-9 was particularly effective in diminishing HSV-1 induced corneal immunopathology (50). Other approaches that were successful in expanding Treg population and reducing SK lesions included the use of IL-2/anti-IL-2 mAb complexes and the fungal metabolite drug, fingolimod hydrochloride (FTY720) (51, 52). In addition, phosphorylated FTY720 also targets sphingosine-1-phosphate receptor and perhaps diminishes inflammation by modulating lymphocyte trafficking (53).

Although increasing the representation of Treg in lesions is a valuable approach to minimize lesion severity, it has become evident that the Treg population is functionally heterogeneous. Accordingly, some functions are more valuable to achieve control than others. For example, our group recently observed that a function of Treg valuable for resolving SK lesions is their ability to produce amphiregulin (AMP) (54). This molecule acts to facilitate tissue repair by binding to the epidermal growth factor receptor expressed mainly on epithelial cells and stem cells and its binding can result in the activation of downstream signaling kinases resulting in growth, proliferation, and migration of cells (55). Treg that produce AMP are relatively infrequent in the early stages of SK, but their representation is most evident in later stages. The change of Treg function to become AMP producers appears to be driven by the cytokines IL-12 and IL-18. In fact, exposure of AMP negative Treg cells *in vitro* to these cytokines can induce them to become AMP producers. In addition, if animals were treated *in vivo* with a plasmid which expresses IL-18, this led to the reduced expression of SK lesions, an effect that correlated with a higher frequency of Treg that were AMP producers (54). Finding practical approaches to induce cells in SK to become AMP producers could represent a useful approach to therapy, an issue that merits further investigation.

PLASTICITY OF REGULATORY T CELL POPULATIONS

Some recent observations suggest that Treg might become unstable in certain highly inflammatory environments and lose their regulatory activity (56). Under such conditions, Treg that downregulate Foxp3 expression might even take up an effector phenotype and start producing pro-inflammatory cytokines such as IFN-γ and IL-17 Treg, a phenomenon commonly referred to as plasticity (57–59). In recent times, plasticity in T cells has been a matter of debate as it has biological implications especially in therapeutic regimens which use Treg (60, 61). Factors which influence Treg stability are as yet not clear and remains an active area of research. Although multiple mechanism might be involved in the stability and plasticity of Treg, most evidence indicates that Treg stability and Foxp3 expression is controlled by epigenetic mechanisms, namely DNA methylation in the non-coding region (CNS2) of the Foxp3 gene locus, also known as Treg-specific demethylation region (TSDR) (62). Any changes or modifications in the DNA methylation status in the TSDR region tend to have an effect on Foxp3 expression and stability of Treg populations (63). Most Treg populations are generally resistant to destabilization and reprogramming and maintain their transcriptional expression of regulatory genes and functional phenotype (61). Some of the Tregs generated *in vitro* or *in vivo* which have incomplete demethylation status in the cytosine-phospho-guanine (CpG) sites in the TSDR region are more prone to instability when exposed to cytokine milieu

containing IL-6, IL-12, IL-21, or IL-23 (57, 64). The Bluestone group, using Foxp3-Cre reporter mice in an Experimental autoimmune encephalomyelitis (EAE) model observed that some of the Treg cells downregulated Foxp3 expression and these were referred to as exFoxp3 cells (59). Such exFoxp3 cells isolated from the CNS at the peak of the response produced IFN- γ when stimulated with cognate antigen (59). Our group using fate mapping mice showed that Treg plasticity can occur in HSV-1-induced inflammatory environment and such Treg may contribute to SK lesion severity by secreting the proinflammatory cytokine IFN-γ (65). In particular, Treg cells showing low expression of the IL-2R (CD25) could exhibit instability, in part due to the exposure to the pro-inflammatory cytokine IL-12 in the cornea (65). In such circumstances, drugs such as azacytidine, retinoic acid, and vitamin C which maintain demethylation of the TSDR region of Foxp3, can be helpful in promoting the stability and improving the functionality of Treg especially under chronic inflammatory conditions (65). In fact, in a recent study, Treg generated *in vitro* in the presence of Azacytidine expressed a fully demethylated TSDR and these cells displayed enhanced suppressive activity (66). Moreover, administration of 5-Azacytidine reduced the incidence of SK lesions in mice infected ocularly with HSV-1 (66).

MANIPULATING METABOLISM TO CONSTRAIN SK LESIONS

In the previous section, we have argued that the clinical expression of SK is affected by the representation of different participants in lesions. When the T cell participants were dominated by Treg, lesions will be less severe and may even resolve. Hence, a potentially valuable approach to therapy is to use maneuvers that can shift the balance of events away from dominance by proinflammatory components. This therapeutic challenge is also faced by those working with other in other chronic inflammatory diseases, especially autoimmune diseases (AID). In the AID field, some are considering using approaches such as adoptive cell transfer to enrich the population of Treg (67). However, such an approach, which is most effective when the Treg are antigen specific, would likely fail to adequately gain access to the eye. Other approaches include administering reagents that expand the Treg population as we discussed previously. A potentially more useful therapeutic option would be to exploit the accumulating knowledge that cells involved in immune function may differ in the major metabolic pathways they use to provide them with energy and other events that maintain of their various functions (68, 69). For example, proinflammatory and Treg cells use different pathways to provide energy with the former mainly use extracellular glucose and Treg rely on fatty acid oxidation (68). Rathmell's group reported that effector T cells (both CD4 and CD8) express high levels of the glucose transporter Glut1 and utilize the mammalian target of rapamycin (mTOR) pathway to increase glycolysis to support their function (70). In contrast, Treg primarily use AMP-activated protein kinase and rely upon lipid oxidation for their energy. The activated AMPK pathway in Treg acts to inhibit mTOR by suppressing mTOR signaling and promotes mitochondrial oxidative metabolism rather than glycolysis and is considered to be anti-inflammatory (70). In our own studies, we have begun to exploit the differences by which proinflammatory and Treg cells derive their energy needs. We have shown that if glucose utilization is inhibited, as can be achieved by the use of 2 deoxy glucose administration from the initial time of lesion development, that lesions are significantly reduced (71). The outcome occurred because the activity of proinflammatory cells such as Th1 and Th17 cells were inhibited, but Treg were unaffected. Thus, the representation of the two populations changed with Treg becoming enriched (71). Findings from another group demonstrated the importance of hypoxia associated glycolytic molecules in SK pathogenesis (72). Besides glycolytic metabolism, T effectors, and Treg also show differences in amino acid metabolism. Amino acids, particularly glutamine, plays a key role in fueling effector T cell differentiation, whereas Treg are less dependent on amino acids for their energy (68). In addition, microbial metabolites such as short chain fatty acids or diets rich in vitamin A promote Treg differentiation and function in the gut (73, 74). Additional metabolic differences are also under investigation such as the differential use of lipid oxidation and synthesis pathways. Thus, manipulating metabolic pathways to influence inflammatory lesions is in the early stages of investigation but the approach has great potential and could be more affordable than many of the alternatives. However, the strategy will need considerable scrutiny especially if used for long term therapy. Indeed, our own studies have already documented some untoward consequences when glucose metabolism is compromised during the time when virus is actively replicating.

CONTRIBUTION OF CORNEAL NERVE DAMAGE TO SK PATHOLOGY

Following corneal infection, HSV-1 replicates in the epithelial cells and gains access to the sensory nerve endings which drain the corneal tissues and can travel up (retrograde) to the TG where the virus establishes latency. The virus travels back (anterograde) from the TG to the cornea through the sensory nerves after reactivation. HSV-1 corneal infection can result in destruction of corneal nerve endings resulting in loss of corneal sensitivity (75). Such loss of corneal sensation and nerve function is one of the hall marks of SK in humans and is commonly referred to as neurotrophic keratopathy (76). Evidence from recent studies in mice have shown that sympathetic nerves innervate the cornea and replace the sensory nerve endings lost after HSV-1 corneal infection (75). These sympathetic nerves enhance the infiltration of immune cells resulting in severe corneal inflammation and pathology. A surgical procedure called superior cervical ganglionectomy (SCGx) that removes sympathetic nerves from the cornea helped to alleviate SK severity. Of note, after the SCGx procedure, the sensory nerves reinnervated the cornea resulting in the restoration of corneal sensitivity (75). The exact mechanisms involved in sympathetic corneal innervation are not known and this aspect requires

further examination. It is likely that immune cells such as CD4 T cells could play a key role, as their depletion resulted in reversing nerve damage (77). Findings from another study suggest that the molecule involved in cell migration, semaphorin 7A might play a role in the corneal nerves degeneration and regeneration process in HSV-1 infected mice (78). The cytokine IL-6 produced during the inflammatory response to HSV-1 infection in the cornea might also be responsible for causing corneal sensory nerve damage (79).

CONCLUDING REMARKS

Stromal keratitis (SK) caused by HSV-1 corneal infection is a debilitating disease and one of the major causes of vision loss due to an infectious agent. As T cells are the primary orchestrators of SK, steps to improve the host environment which favors Treg over pathogenic Th1/Th17 cells is likely to help ease the severity of SK lesions. In addition, it is becoming increasingly clear from recent developments that metabolism plays a key role in immune function. Thus, as discussed in this review, understanding the events involved in pathogenesis along with key molecules and metabolic pathways involved in inflammation and applying this knowledge to develop better therapies might help control SK in the future.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

Research was supported by National Institutes of Health Grant R01EY005093 to BR.

ACKNOWLEDGMENTS

We also thank the department of Biomedical and Diagnostic Sciences, University of Tennessee, Knoxville for supporting the research work through the COE funds to NR.

REFERENCES

1. Looker KJ, Magaret AS, May MT, Turner KM, Vickerman P, Gottlieb SL, et al. Global and regional estimates of prevalent and incident herpes simplex virus type 1 infections in 2012. *PLoS ONE.* (2015) 10:e0140765. doi: 10.1371/journal.pone.0140765
2. Johnston C, Gottlieb SL, Wald A. Status of vaccine research and development of vaccines for herpes simplex virus. *Vaccine.* (2016) 34:2948–52. doi: 10.1016/j.vaccine.2015.12.076
3. Whitley RJ, Roizman B. Herpes simplex virus infections. *Lancet.* (2001) 357:1513–8. doi: 10.1016/S0140-6736(00)04638-9
4. Liesegang TJ. Herpes simplex virus epidemiology and ocular importance. *Cornea.* (2001) 20:1–13. doi: 10.1097/00003226-200101000-00001
5. Rowe AM, St Leger AJ, Jeon S, Dhaliwal DK, Knickelbein JE, Hendricks RL. Herpes keratitis. *Prog Retin Eye Res.* (2013) 32:88–101. doi: 10.1016/j.preteyeres.2012.08.002
6. Young RC, Hodge DO, Liesegang TJ, Baratz KH. Incidence, recurrence, and outcomes of herpes simplex virus eye disease in olmsted county, minnesota, 1976-2007: the effect of oral antiviral prophylaxis. *Arch Ophthalmol.* (2010) 128:1178–83. doi: 10.1001/archophthalmol.2010.187
7. Stanzel TP, Diaz JD, Mather R, Wong IG, Margolis TP, Gritz DC. The epidemiology of herpes simplex virus eye disease in Northern California. *Ophthalmic Epidemiol.* (2014) 21:370–7. doi: 10.3109/09286586.2014.966848
8. Biswas PS, Rouse BT. Early events in HSV keratitis–setting the stage for a blinding disease. *Microbes Infect.* (2005) 7:799–810. doi: 10.1016/j.micinf.2005.03.003
9. Roizman B, Whitley RJ. An inquiry into the molecular basis of HSV latency and reactivation. *Annu Rev Microbiol.* (2013) 67:355–74. doi: 10.1146/annurev-micro-092412-155654
10. Koyuncu OO, MacGibeny MA, Enquist LW. Latent versus productive infection: the alpha herpesvirus switch. *Future Virol.* (2018) 13:431–43. doi: 10.2217/fvl-2018-0023
11. Koyuncu OO, Hogue IB, Enquist LW. Virus infections in the nervous system. *Cell Host Microbe.* (2013) 13:379–93. doi: 10.1016/j.chom.2013.03.010
12. Knickelbein JE, Hendricks RL, Charukamnoetkanok P. Management of herpes simplex virus stromal keratitis: an evidence-based review. *Surv Ophthalmol.* (2009) 54:226–34. doi: 10.1016/j.survophthal.2008.12.004
13. Gimenez F, Suryawanshi A, Rouse BT. Pathogenesis of herpes stromal keratitis–a focus on corneal neovascularization. *Prog Retin Eye Res.* (2013) 33:1–9. doi: 10.1016/j.preteyeres.2012.07.002
14. Rajasagi NK, Rouse BT. Application of our understanding of pathogenesis of herpetic stromal keratitis for novel therapy. *Microbes Infect.* (2018) 20:526–30. doi: 10.1016/j.micinf.2017.12.014
15. Doymaz MZ, Rouse BT. Herpetic stromal keratitis: an immunopathologic disease mediated by CD4+ T lymphocytes. *Invest Ophthalmol Vis Sci.* (1992) 33:2165–73.
16. Thomas J, Gangappa S, Kanangat S, Rouse BT. On the essential involvement of neutrophils in the immunopathologic disease: herpetic stromal keratitis. *J Immunol.* (1997) 158:1383.
17. Daheshia M, Kanangat S, Rouse BT. Production of key molecules by ocular neutrophils early after herpetic infection of the cornea. *Exp Eye Res.* (1998) 67:619–24. doi: 10.1006/exer.1998.0565
18. Niemialtowski MG, Rouse BT. Predominance of Th1 cells in ocular tissues during herpetic stromal keratitis. *J Immunol.* (1992) 149:3035–9.
19. Epstein RJ, Hendricks RL, Stulting RD. Interleukin-2 induces corneal neovascularization in A/J mice. *Cornea.* (1990) 9:318–23. doi: 10.1097/00003226-199010000-00009
20. Hendricks RL, Tumpey TM, Finnegan A. IFN-gamma and IL-2 are protective in the skin but pathologic in the corneas of HSV-1-infected mice. *J Immunol.* (1992) 149:3023–8.
21. Tang Q, Hendricks RL. Interferon gamma regulates platelet endothelial cell adhesion molecule 1 expression and neutrophil infiltration into herpes simplex virus-infected mouse corneas. *J Exp Med.* (1996) 184:1435–47. doi: 10.1084/jem.184.4.1435
22. Tang Q, Chen W, Hendricks RL. Proinflammatory functions of IL-2 in herpes simplex virus corneal infection. *J Immunol.* (1997) 158:1275–83.
23. Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 Cells. *Annu Rev Immunol.* (2009) 27:485–517. doi: 10.1146/annurev.immunol.021908.132710
24. Damsker JM, Hansen AM, Caspi RR. Th1 and Th17 cells: adversaries and collaborators. *Ann N Y Acad Sci.* (2010) 1183:211–21. doi: 10.1111/j.1749-6632.2009.05133.x
25. Xu S, Cao X. Interleukin-17 and its expanding biological functions. *Cell Mol Immunol.* (2010) 7:164–74. doi: 10.1038/cmi.2010.21
26. Suryawanshi A, Veiga-Parga T, Rajasagi NK, Reddy PB, Sehrawat S, Sharma S, et al. Role of IL-17 and Th17 cells in herpes simplex virus-induced corneal immunopathology. *J Immunol.* (2011) 187:1919–30. doi: 10.4049/jimmunol.1100736
27. Suryawanshi A, Veiga-Parga T, Reddy PB, Rajasagi NK, Rouse BT. IL-17A differentially regulates corneal vascular endothelial growth factor (VEGF)-A and soluble VEGF receptor 1 expression and promotes corneal angiogenesis

after herpes simplex virus infection. *J Immunol.* (2012) 188:3434–46. doi: 10.4049/jimmunol.1102602
28. Maertzdorf J, Osterhaus AD, Verjans GM. IL-17 expression in human herpetic stromal keratitis: modulatory effects on chemokine production by corneal fibroblasts. *J Immunol.* (2002) 169:5897–903. doi: 10.4049/jimmunol.169.10.5897
29. Hendricks RL, Tumpey TM. Contribution of virus and immune factors to herpes simplex virus type I-induced corneal pathology. *Invest Ophthalmol Vis Sci.* (1990) 31:1929–39.
30. Mott KR, Perng GC, Osorio Y, Kousoulas KG, Ghiasi H. A recombinant herpes simplex virus type 1 expressing two additional copies of gK is more pathogenic than wild-type virus in two different strains of mice. *J Virol.* (2007) 81:12962–72. doi: 10.1128/JVI.01442-07
31. Jaggi U, Wang S, Tormanen K, Matundan H, Ljubimov AV, Ghiasi H. Role of Herpes Simplex Virus Type 1 (HSV-1) Glycoprotein K (gK) Pathogenic CD8(+) T Cells in Exacerbation of Eye Disease. *Front Immunol.* (2018) 9:2895. doi: 10.3389/fimmu.2018.02895
32. Saied AA, Chouljenko VN, Subramanian R, Kousoulas KG. A replication competent HSV-1(McKrae) with a mutation in the amino-terminus of glycoprotein K (gK) is unable to infect mouse trigeminal ganglia after cornea infection. *Curr Eye Res.* (2014) 39:596–603. doi: 10.3109/02713683.2013.855238
33. Zhu J, Peng T, Johnston C, Phasouk K, Kask AS, Klock A, et al. Immune surveillance by CD8alphaalpha+ skin-resident T cells in human herpes virus infection. *Nature.* (2013) 497:494–7. doi: 10.1038/nature12110
34. Khanna KM, Lepisto AJ, Decman V, Hendricks RL. Immune control of herpes simplex virus during latency. *Curr Opin Immunol.* (2004) 16:463–9. doi: 10.1016/j.coi.2004.05.003
35. Verjans GM, Hintzen RQ, van Dun JM, Poot A, Milikan JC, Laman JD, et al. Selective retention of herpes simplex virus-specific T cells in latently infected human trigeminal ganglia. *Proc Natl Acad Sci U.S.A.* (2007) 104:3496–501. doi: 10.1073/pnas.0610847104
36. St Leger AJ, Hendricks RL. CD8+ T cells patrol HSV-1-infected trigeminal ganglia and prevent viral reactivation. *J Neurovirol.* (2011) 17:528–34. doi: 10.1007/s13365-011-0062-1
37. Liu T, Khanna KM, Carriere BN, Hendricks RL. Gamma interferon can prevent herpes simplex virus type 1 reactivation from latency in sensory neurons. *J Virol.* (2001) 75:11178–84. doi: 10.1128/JVI.75.22.11178-11184.2001
38. Knickelbein JE, Khanna KM, Yee MB, Baty CJ, Kinchington PR, Hendricks RL. Noncytotoxic lytic granule-mediated CD8+ T cell inhibition of HSV-1 reactivation from neuronal latency. *Science.* (2008) 322:268–71. doi: 10.1126/science.1164164
39. Rouse BT, Sehrawat S. Immunity and immunopathology to viruses: what decides the outcome? *Nat Rev Immunol.* (2010) 10:514–26. doi: 10.1038/nri2802
40. Veiga-Parga T, Sehrawat S, Rouse BT. Role of regulatory T cells during virus infection. *Immunol Rev.* (2013) 255:182–96. doi: 10.1111/imr.12085
41. Josefowicz SZ, Lu LF, Rudensky AY. Regulatory T cells: mechanisms of differentiation and function. *Annu Rev Immunol.* (2012) 30:531–64. doi: 10.1146/annurev.immunol.25.022106.141623
42. Ohkura N, Kitagawa Y, Sakaguchi S. Development and maintenance of regulatory T cells. *Immunity.* (2013) 38:414–23. doi: 10.1016/j.immuni.2013.03.002
43. Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. *Nat Rev Immunol.* (2008) 8:523–32. doi: 10.1038/nri2343
44. Shevach EM. Mechanisms of foxp3+ T regulatory cell-mediated suppression. *Immunity.* (2009) 30:636–45. doi: 10.1016/j.immuni.2009.04.010
45. Suvas S, Azkur AK, Kim BS, Kumaraguru U, Rouse BT. CD4+CD25+ regulatory T cells control the severity of viral immunoinflammatory lesions. *J Immunol.* (2004) 172:4123–32. doi: 10.4049/jimmunol.172.7.4123
46. Sehrawat S, Suvas S, Sarangi PP, Suryawanshi A, Rouse BT. *In vitro*-generated antigen-specific CD4+ CD25+ Foxp3+ regulatory T cells control the severity of herpes simplex virus-induced ocular immunoinflammatory lesions. *J Virol.* (2008) 82:6838–51. doi: 10.1128/JVI.00697-08
47. Veiga-Parga T, Suryawanshi A, Mulik S, Gimenez F, Sharma S, Sparwasser T, et al. On the role of regulatory T cells during viral-induced inflammatory lesions. *J Immunol.* (2012) 189:5924–33. doi: 10.4049/jimmunol.1202322
48. Lahl K, Sparwasser T. *In vivo* depletion of FoxP3+ Tregs using the DEREG mouse model. *Methods Mol Biol.* (2011) 707:157–72. doi: 10.1007/978-1-61737-979-6_10
49. Sehrawat S, Suryawanshi A, Hirashima M, Rouse BT. Role of Tim-3/galectin-9 inhibitory interaction in viral-induced immunopathology: shifting the balance toward regulators. *J Immunol.* (2009) 182:3191–201. doi: 10.4049/jimmunol.0803673
50. Reddy PBJ, Schreiber TH, Rajasagi NK, Suryawanshi A, Mulik S, Veiga-Parga T, et al. TNFRSF25 agonistic antibody and galectin-9 combination therapy controls herpes simplex virus-induced immunoinflammatory lesions. *J Virol.* (2012) 86:10606–20. doi: 10.1128/JVI.01391-12
51. Sehrawat S, Rouse BT. Anti-inflammatory effects of FTY720 against viral-induced immunopathology: role of drug-induced conversion of T cells to become Foxp3+ regulators. *J Immunol.* (2008) 180:7636–47. doi: 10.4049/jimmunol.180.11.7636
52. Gaddipati S, Estrada K, Rao P, Jerome AD, Suvas S. IL-2/anti-IL-2 antibody complex treatment inhibits the development but not the progression of herpetic stromal keratitis. *J Immunol.* (2015) 194:273–82. doi: 10.4049/jimmunol.1401285
53. Brinkmann V. Sphingosine 1-phosphate receptors in health and disease: mechanistic insights from gene deletion studies and reverse pharmacology. *Pharmacol Ther.* (2007) 115:84–105. doi: 10.1016/j.pharmthera.2007.04.006
54. Varanasi SK, Rajasagi NK, Jaggi U, Rouse BT. Role of IL-18 induced Amphiregulin expression on virus induced ocular lesions. *Mucosal Immunol.* (2018). doi: 10.1038/s41385-018-0058-8
55. Zaiss DMW, Gause WC, Osborne LC, Artis D. Emerging functions of amphiregulin in orchestrating immunity, inflammation, and tissue repair. *Immunity.* (2015) 42:216–26. doi: 10.1016/j.immuni.2015.01.020
56. Bailey-Bucktrout SL, Bluestone JA. Regulatory T cells: stability revisited. *Trends Immunol.* (2011) 32:301–6. doi: 10.1016/j.it.2011.04.002
57. Zhou L, Chong MM, Littman DR. Plasticity of CD4+ T cell lineage differentiation. *Immunity.* (2009) 30:646–55. doi: 10.1016/j.immuni.2009.05.001
58. Zhou X, Bailey-Bucktrout SL, Jeker LT, Penaranda C, Martinez-Llordella M, Ashby M, et al. Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells *in vivo*. *Nat Immunol.* (2009) 10:1000–7. doi: 10.1038/ni.1774
59. Bailey-Bucktrout SL, Martinez-Llordella M, Zhou X, Anthony B, Rosenthal W, Luche H, et al. Self-antigen-driven activation induces instability of regulatory T cells during an inflammatory autoimmune response. *Immunity.* (2013) 39:949–62. doi: 10.1016/j.immuni.2013.10.016
60. Hori S. Regulatory T cell plasticity: beyond the controversies. *Trends Immunol.* (2011) 32:295–300. doi: 10.1016/j.it.2011.04.004
61. Sakaguchi S, Vignali DA, Rudensky AY, Niec RE, Waldmann H. The plasticity and stability of regulatory T cells. *Nat Rev Immunol.* (2013) 13:461–7. doi: 10.1038/nri3464
62. Kitagawa Y, Sakaguchi S. Molecular control of regulatory T cell development and function. *Curr Opin Immunol.* (2017) 49:64–70. doi: 10.1016/j.coi.2017.10.002
63. Lal G, Bromberg JS. Epigenetic mechanisms of regulation of Foxp3 expression. *Blood.* (2009) 114:3727–35. doi: 10.1182/blood-2009-05-219584
64. Hori S. Lineage stability and phenotypic plasticity of Foxp3(+) regulatory T cells. *Immunol Rev.* (2014) 259:159–72. doi: 10.1111/imr.12175
65. Bhela S, Varanasi SK, Jaggi U, Sloan SS, Rajasagi NK, Rouse BT. The plasticity and stability of regulatory T cells during viral-induced inflammatory lesions. *J Immunol.* (2017) 199:1342–52. doi: 10.4049/jimmunol.1700520
66. Varanasi SK, Reddy PB, Bhela S, Jaggi U, Gimenez F, Rouse BT. Azacytidine treatment inhibits the progression of herpes stromal keratitis by enhancing regulatory T cell function. *J Virol.* (2017) 91:e02367-16. doi: 10.1128/JVI.02367-16
67. Wright GP, Notley CA, Xue S-A, Bendle GM, Holler A, Schumacher TN, et al. Adoptive therapy with redirected primary regulatory T cells results in antigen-specific suppression of arthritis. *Proc Natl Acad Sci USA.* (2009) 106:19078. doi: 10.1073/pnas.0907396106
68. O'Neill LAJ, Kishton RJ, Rathmell J. A guide to immunometabolism for immunologists. *Nat Rev Immunol.* (2016) 16:553. doi: 10.1038/nri.2016.70

69. Bettencourt IA, Powell JD. Targeting metabolism as a novel therapeutic approach to autoimmunity, inflammation, and transplantation. *J Immunol.* (2017) 198:999. doi: 10.4049/jimmunol.1601318
70. Michalek RD, Gerriets VA, Jacobs SR, Macintyre AN, MacIver NJ, Mason EF, et al. Cutting edge: distinct glycolytic and lipid oxidative metabolic programs are essential for effector and regulatory CD4+ T cell subsets. *J. Immunol.* (2011) 186:3299–303. doi: 10.4049/jimmunol.1003613
71. Varanasi SK, Donohoe D, Jaggi U, Rouse BT. Manipulating glucose metabolism during different stages of viral pathogenesis can have either detrimental or beneficial effects. *J Immunol.* (2017) 199:1748–61. doi: 10.4049/jimmunol.1700472
72. Rao P, Suvas S. Development of inflammatory hypoxia and prevalence of glycolytic metabolism in progressing herpes stromal keratitis lesions. *J Immunol.* (2019) 202:514–26. doi: 10.4049/jimmunol.1800422
73. Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly-Y, M, et al. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. *Science.* (2013) 341:569–73. doi: 10.1126/science.1241165
74. Zeng H, Chi H. Metabolic control of regulatory T cell development and function. *Trends Immunol.* (2015) 36:3–12. doi: 10.1016/j.it.2014.08.003
75. Yun H, Lathrop KL, Hendricks RL. A central role for sympathetic nerves in herpes stromal keratitis in mice. *Invest Ophthalmol Vis Sci.* (2016) 57:1749–56. doi: 10.1167/iovs.16-19183
76. Hamrah P, Cruzat A, Dastjerdi MH, Zheng L, Shahatit BM, Bayhan HA, et al. Corneal sensation and subbasal nerve alterations in patients with herpes simplex keratitis: an *in vivo* confocal microscopy study. *Ophthalmology.* (2010) 117:1930–6. doi: 10.1016/j.ophtha.2010.07.010
77. Yun H, Rowe AM, Lathrop KL, Harvey SA, Hendricks RL. Reversible nerve damage and corneal pathology in murine herpes simplex stromal keratitis. *J Virol.* (2014) 88:7870–80. doi: 10.1128/JVI.01146-14
78. Chucair-Elliott AJ, Zheng M, Carr DJ. Degeneration and regeneration of corneal nerves in response to HSV-1 infection. *Invest Ophthalmol Vis Sci.* (2015) 56:1097–107. doi: 10.1167/iovs.14-15596
79. Chucair-Elliott AJ, Jinkins J, Carr MM, Carr DJ. IL-6 contributes to corneal nerve degeneration after herpes simplex virus type I infection. *Am J Pathol.* (2016) 186:2665–78. doi: 10.1016/j.ajpath.2016.06.007

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 20 March 2019
doi: 10.3389/fimmu.2019.00500

An Intra-Vaginal Zinc Oxide Tetrapod Nanoparticles (ZOTEN) and Genital Herpesvirus Cocktail Can Provide a Novel Platform for Live Virus Vaccine

Alex Agelidis[1,2†], *Lulia Koujah*[1,2†], *Rahul Suryawanshi*[1], *Tejabhiram Yadavalli*[1], *Yogendra Kumar Mishra*[3], *Rainer Adelung*[3] *and Deepak Shukla*[1,2*]

[1] Department of Ophthalmology and Visual Sciences, University of Illinois, Chicago, IL, United States, [2] Department of Microbiology and Immunology, University of Illinois, Chicago, IL, United States, [3] Institute for Materials Science, Kiel University, Kiel, Germany

OPEN ACCESS

Edited by:
Susmit Suvas,
Wayne State University, United States

Reviewed by:
Ashok Kumar,
Wayne State University, United States
Lbachir Benmohamed,
University of California, Irvine,
United States

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

[†] These authors have contributed equally to this work

Specialty section:
This article was submitted to Viral Immunology, a section of the journal Frontiers in Immunology

Received: 06 November 2018
Accepted: 25 February 2019
Published: 20 March 2019

Citation:
Agelidis A, Koujah L, Suryawanshi R, Yadavalli T, Mishra YK, Adelung R and Shukla D (2019) An Intra-Vaginal Zinc Oxide Tetrapod Nanoparticles (ZOTEN) and Genital Herpesvirus Cocktail Can Provide a Novel Platform for Live Virus Vaccine. Front. Immunol. 10:500. doi: 10.3389/fimmu.2019.00500

Herpes simplex virus type-2 (HSV-2) is a common cause of genital infections throughout the world. Currently no prophylactic vaccine or therapeutic cure exists against the virus that establishes a latent infection for the life of the host. Intravaginal microbivac is a developing out-of-the-box strategy that combines instant microbicidal effects with future vaccine-like benefits. We have recently shown that our uniquely designed zinc oxide tetrapod nanoparticles (ZOTEN) show strong microbivac efficacy against HSV-2 infection in a murine model of genital infection. In our attempts to further understand the antiviral and immune bolstering effects of ZOTEN microbivac and to develop ZOTEN as a platform for future live virus vaccines, we tested a ZOTEN/HSV-2 cocktail and found that prior incubation of HSV-2 with ZOTEN inhibits the ability of the virus to infect vaginal tissue in female Balb/c mice and blocks virus shedding as judged by plaque assays. Quite interestingly, the ZOTEN-neutralized virions elicit a local immune response that is highly comparable with the HSV-2 infection alone with reduced inflammation and clinical manifestations of disease. Information provided by our study will pave the way for the further development of ZOTEN as a microbivac and a future platform for live virus vaccines.

Keywords: herpes simplex virus, genital herpes, immunotherapy, live virus vaccine, viral infection

INTRODUCTION

Herpes simplex virus-2 (HSV-2) is a neurotropic double stranded DNA virus capable of lytic infection in multiple host cell types as well as latent infection in neuronal cells (1). The viral DNA genome is encased in an icosadeltahedral protein capsid which is surrounded by tegument proteins (2). The capsid and tegument are enveloped in a lipid bilayer composed of multiple viral proteins and glycoproteins on the surface of the virus particle (3). HSV-2 entry into the host cell primarily involves the interaction of the viral entry glycoproteins with various cell surface receptors that facilitate virion envelope fusion with the plasma membrane of the host cell causing capsid penetration into the cytoplasm (4). Once the genome reaches the nucleus, viral protein production occurs in a sequential manner beginning with immediate early gene products that promote immune evasion and neurovirulence (5). Early proteins are then synthesized which are required for viral

DNA replication. This is followed by production of late proteins, providing structural components of the capsid that are necessary for viral egress. HSV spreads rapidly to neighboring cells as well as the dorsal root ganglia where it establishes latency (6).

Primary infection of HSV-2 results in a variety of prolonged clinical manifestations, ranging from genital ulcerations to more severe cases like meningitis (7). While HSV-2 infection most commonly occurs in the genitalia, it may also result in oral, ocular and neurologic infections (8). In addition, genital ulcerations caused by HSV-2 and viral shedding have been definitively linked to an increased risk for acquisition of human immunodeficiency virus (HIV) infection (9, 10). HSV-2 infects over 400 million people worldwide and is one of the most common sexually transmitted infections (11). Despite its high prevalence, no cure or vaccination has been developed. Acyclovir, a nucleoside analog, is widely used to treat primary HSV-2 infection and has shown to be an efficacious therapeutic in most cases. However, acyclovir resistant strains have also evolved and treatment options are limited in those cases (12–14).

A traditional antiviral may not be the best choice since diverse response from infected patients has been observed with variations in episodes of viral shedding due to the varying degrees of localized immune response among individuals. Roughly 80% of HSV-2 seroprevalent persons are asymptomatic and report no genital lesions even with detection of viral genomes at the site of infection (15). Upon infection in immunocompetent individuals, the virus is rapidly contained by a prompt innate immune response and further suppressed by resident memory HSV-specific T cells (16–18). For the large majority of HSV-2 infected individuals, cell-mediated immune responses are able to control and protect against clinical recurrences and genital lesion development (19). While the majority of currently prescribed antivirals target the virus itself, the development of an antiviral or immunotherapeutic that inhibits infection and at the same time, facilitates a protective immune response can better guard the host against the deleterious effects of primary HSV-2 infection as well as recurrences (20–22). Alternatively, since subunit vaccines have failed to show real promise in clinical trials, a safe live virus vaccine may provide a better solution (23).

Previously, our group discovered a novel microbicidal and vaccine-like (or microbivac) platform against primary and secondary female genital herpes infections (24). The dual microbivac platform was demonstrated through the ability of uniquely designed zinc oxide tetrapod nanoparticles (ZOTEN) with engineered oxygen vacancies to strongly trap HSV-2 virion, neutralize the virus and prevent cell entry in the vaginal epithelium (25, 26). ZOTEN showed to be an effective suppressor of HSV-2 genital infection in female BALB/c mice with apparent reduction of clinical signs of vaginal infection and decreased animal mortality. ZOTEN therapy ultimately was found to create a platform for viral antigen presentation and therefore was presented as a novel microbivac with the potential to prevent primary infection and viral shedding (27). Interestingly, treatment of ZOTEN was found to have adjuvant-like properties, enhancing immunity against the virus in mice (24). The proposed mechanism for this is that ZOTEN acts to capture the virus, allowing for detection by immune cells which in turn results in enhanced T cell-mediated and antibody-mediated responses to infection and thereby suppressing a reinfection. ZOTEN's ability to target the virus particle and manipulate the host immune system demonstrates its novel and multifunctional antiviral properties with promising prophylactic and therapeutic effects (28).

In this article, we aim to better understand the vaginal immune responses and antiviral benefits of a short-term acute infection in female BALB/c mice using a ZOTEN/HSV-2 cocktail. Such a cocktail could provide more information on the microbivac benefits of ZOTEN while demonstrating its promise as a unique platform for live virus vaccine development. Our tissue specific analyses show that the cocktail inhibits infection but generates a local immune response that is highly comparable to the infection with the virus alone. It also shows the promise that ZOTEN can be given alongside to reduce the possibility of infection via any live virus vaccine.

MATERIALS AND METHODS

Mouse Model of Genital Herpes Infection

Animal care and procedures were performed in accordance with institutional and NIH guidelines and approved by the Animal Care Committee at the University of Illinois at Chicago. Six to Eight-weeks-old female BALB/c mice obtained from Charles River Laboratories were injected with 0.1 mL medroxyprogesterone acetate (Depo-Provera) (Greenstone) to synchronize estrous cycles. Seven days after injection, mice were inoculated with HSV-2, or mock infected, with or without ZOTEN. HSV-2 strain 333 was used for all experiments. Synthesis and use of ZOTEN in antiviral assays have been described previously (24, 26, 29). ZOTEN cocktail treatment consisted of preincubating HSV-2 (or mock) in PBS for 30 min at room temperature with or without 0.1 mg/mL ZOTEN and then inoculating female mice genitals with respective solution. Each infected mouse received a viral inoculum of 5×10^5 pfu in a 10 μL volume. Untreated mice received virus that was similarly incubated at room temperature.

Synthesis of ZOTEN (Tetrapod-Form ZnO Micro-Nanoparticles)

Nanoparticles were synthesized and characterized according to our previously published studies (24). Spherical zinc microparticles, polyvinyl butyral (PVB) powder, and ethanol were obtained commercially. A mixture using these materials is prepared and burned together in the furnace at 900°C. Zn microparticles (in the form of Zn atoms, Zn dimers, Zn trimers, etc.) are generated in the flame that results from the burning of polymer PVB. In the presence of oxygen from the surrounding environment, the unstable atomic variants of Zn microparticles participate in nucleation and growth processes. Initially, Zn and O combine to form a primary cluster and once the stable nucleus has been formed, further available Zn and O atoms contribute to conventional 1D spike growth which results in growth of tetrapod-type structures. The process continues as PVB decomposes completely into CO_2 and O_2, resulting in an actual yield of 99.9% of ZOTEN. The formation

of uniform ZnO tetrapods (ZOTEN) has been confirmed by electron microscopy; as well as the size and shape by scanning electron microscopy (30). Identical ZOTENs were used for all experiments demonstrated in this article.

Mouse Vaginal Swabs and Detection of Virus Shedding

At days 2 and 4 post infection, mouse vaginal canal was sampled using calcium alginate swabs (Puritan, 25–800) previously dipped in OptiMEM for approximately 2 min. Swabs were performed by gently streaking vaginal canal in a circular motion 5 times and then dipping the swab into 500 μL OptiMEM. This process was performed twice for each mouse. Collected washes were briefly vortexed and centrifuged then plated on confluent monolayers of Vero cells in a plaque assay.

Plaque Assay

Monolayers of Vero cells grown in DMEM + 10% FBS + 1% penicillin/streptomycin were washed once with PBS, then overlaid with vaginal swab washes freshly collected from mice. After incubation for 2 h, inocula were aspirated, and Vero cells were overlaid with DMEM containing 5% methylcellulose. 72 h later, cells were fixed with methanol for 10 min, media was removed, and cells were then incubated with crystal violet staining solution for 30 min to visualize plaques.

Flow Cytometry

Mouse vaginal tissue was dissected and dissociated by incubating in 100 μL of 2 mg/mL collagenase in PBS for 4 h at 37°C. The resulting mixture was triturated with a pipet tip, suspended in an additional 1 mL of FACS buffer (5% FBS in PBS) and passed through a 70 μm filter. Cells were aliquoted into 96-well round bottom plates for staining. F_c receptors were blocked using TruStain FcX (101319, Biolegend) according to the manufacturer's protocol, and cells were then stained with the following antibodies from BioLegend: APC anti-mouse Gr-1 (108411), FITC anti-mouse CD45 (103107) APC anti-mouse CD3e (100311), FITC anti-mouse CD49b (103503) APC anti-mouse CD11c (117309) and PE anti-mouse F4/80 (123109). Cells were incubated with fluor conjugated primary antibodies for 1 h on ice, washed twice with FACS buffer, and analyzed with a BD Accuri C6 Plus flow cytometer. 10,000 singlet non-debris events were collected for each sample, and FlowJo X was used to process and analyze the data.

Quantitative Polymerase Chain Reaction

Mouse vaginal tissue was dissected and dissociated by incubating in 100 μL of 2 mg/mL collagenase in PBS for 4 h at 37°C. The resulting mixture was triturated with a pipet tip, suspended in 1 mL of Trizol, and frozen at −80°C until processing. RNA extraction was performed according to Trizol manufacturer's guidelines. 2 μg of total RNA was reverse transcribed to cDNA using High Capacity cDNA Reverse Transcription kit (Thermo Fisher). Real time qPCR was performed with Fast SYBR Green Master Mix (Thermo Fisher) with the QuantStudio 7 Flex system (Life Technologies). The following mouse specific primers were used in this study:

β-actin fwd 5′-GACGGCCAGGTCATCACTATTG-3′
β-actin rev 5′-AGG AAGGCTGGAAAAGAGCC-3′
IFN-α fwd 5′-CCTGCTGGCTGTGAAAT-3′
IFN-α rev 5′-GACAGGGCTCTCCAGACTTC-3′
IFN-β fwd 5′-TGTCCTCAACTGCTCTCCAC-3′
IFN-β rev 5′-CATCCAGGCGCTGTTGT-3′
IL-1β fwd 5′-GTGGCTGTGGAGAAGCTGTG-3′
IL-1β rev 5′-GAAGGTCCACGGGAAAGACAC-3′
IL-6 fwd 5′-ACGGCCTTCCCTACTTCACA-3′
IL-6 rev 5′-CATTTCCACGATTTCCGAGA-3′
TNF-α fwd 5′-GCCTCTTCTCATTCCTGCTTG-3′
TNF-α rev 5′-CTGATGAGAGGGAGGCCATT-3′

Mouse Tissue Histology and Staining

Mouse vaginal tissue was dissected and embedded in Tissue-Plus O.C.T. (Fisher HealthCare) then frozen on dry ice and kept at −80°C until processing. 10 μm sections were cut with a Cryostar NX50 microtome (Thermo Scientific). Sections were air dried at room temperature, fixed in ice-cold acetone for 5 min, and washed under running water for 2 min. Slides were then incubated in Mayer's Hemalum solution (EMD Millipore, 109249) for 1 min and then washed under running water for 1 min. Slides were dipped in 70% ethanol for 2 min, then in 100% ethanol for 1 min, and incubated with eosin Y alcoholic, with phloxine (Sigma, HT110316) for 1 min. Slides were then dipped in 70% ethanol for 1 min, then in 100% ethanol for 1 min, then xylene for 1 min, and coverslipped with Permount mounting medium (Thermo Fisher). Sections were visualized and photographed using a Zeiss Axioskop 2 plus microscope.

Draining inguinal lymph nodes were excised from mice at time of euthanasia and placed in 24-well plates. Lymph nodes were then photographed using a desktop scanner at 1,200 dots per inch. Lymph node areas in pixels were quantified using Adobe Photoshop CC 2018.

Statistical Analysis

Errors bars denote SEM ($n = 5$ mice per group) unless specified otherwise. Asterisks denote significant difference by two-tailed unpaired Student's t-test, $^*p < 0.05$, ns or unlabeled, not significant.

RESULTS

Antiviral Effects of ZOTEN/HSV-2 Cocktail at the Primary Site of HSV-2 Infection

In order to maximize the virus neutralization potential of ZOTEN and study its antiviral and immune benefits we decided to generate a ZOTEN/HSV-2 cocktail by incubating the virus [5×10^5 PFU of HSV-2 (strain 333)] with ZOTEN for 30 min. ZOTEN/HSV-2 was then used for the intravaginal infection of BALB/c mice. To study the effects of the cocktail we created 4 treatment groups of mice: HSV-2 infected, mock infected, ZOTEN/HSV-2 infected and ZOTEN/mock infected (**Figure 1**). The animals were monitored daily and the antiviral effects were measured for the next 7 days. To determine the presence of productive virus at the primary site of infection and local shedding of infectious virions, vaginal swabs were collected

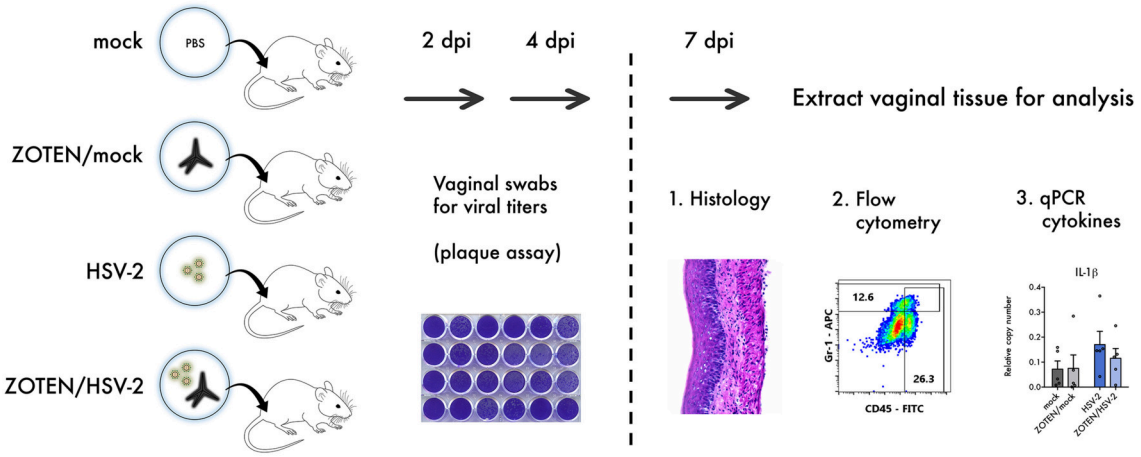


FIGURE 1 | Study design. 6–8 weeks old female BALB/c mice were infected with HSV-2 or mock infected in the presence or absence of ZOTEN. At 2 and 4 days post infection (dpi), mice genitals were swabbed to detect viral shedding using a plaque assay. At 7 dpi, mice were euthanized, and vaginal tissues were extracted and analyzed by histology, flow cytometry, and quantitative PCR (qPCR) to appreciate differences in cellular infiltration and local inflammation.

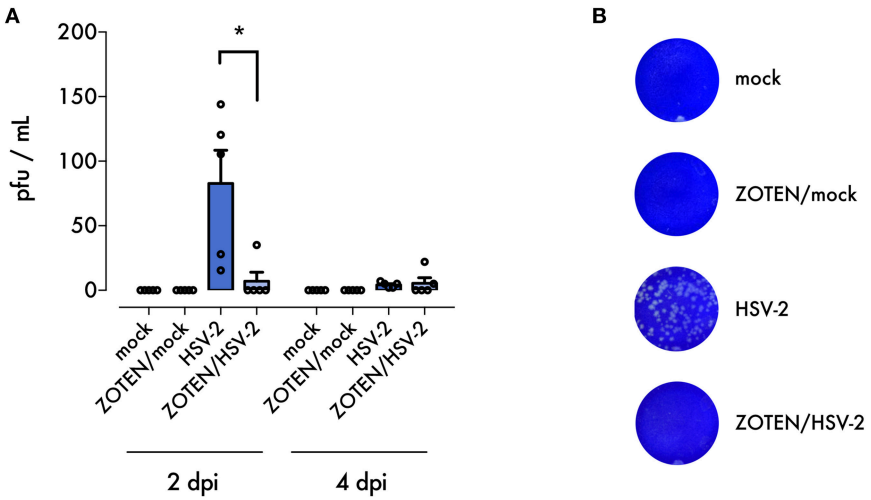


FIGURE 2 | ZOTEN treatment reduces viral shedding. **(A)** Plaque assay results from vaginal swabs at 2 and 4 dpi. Error bars indicate SEM ($n = 5$ per group). Asterisk denotes significant difference by two-tailed unpaired Student's *t*-test, $^*p < 0.05$, ns, or unlabeled, not significant. **(B)** Representative images of crystal violet stained plaque assay results. Zones of clearing are noted in samples from untreated HSV-2 infected mice, indicating presence of replicating virus.

following genital infection with the 4 groups mentioned above. As shown in **Figure 2**, the viral titers recovered from these vaginal swabs were significantly lower in ZOTEN/HSV-2 group at 2 days post infection, with 4 out of 5 mice displaying no detectable virus. These findings confirm the potent antiviral activity displayed by ZOTEN and its ability to neutralize virus and decrease viral shedding as early as 2 days post infection (**Figures 2A,B**).

ZOTEN/HSV-2 Infection Restricts Local Inflammation and Cell Infiltration in Vaginal Tissue

To assess disease development, tissue inflammation or damage at the primary site of infection, vaginal tissue was excised at 7 days post infection and analyzed by three methods: histology, quantitative polymerase chain reaction (qPCR) and flow cytometry (**Figure 1**). Hematoxylin and Eosin (H&E) staining of the vaginal tissue was performed to quantify the phenotypic development of infection as well as activation of innate immune response (**Figure 3A**). It is evident that ZOTEN treated mice exhibit decreased signs of immune cell infiltration and inflammation, developing low or no apparent levels of acute HSV-2 infection. The thickness of the epithelium in ZOTEN/HSV-2 treated vaginal tissue is comparable to mock infected, as opposed to the apparently inflamed epithelium and increased cell infiltration in HSV-2 infected tissue. Looking beyond the primary site of infection, draining lymph nodes were also isolated to give an indication of the extent of the systemic immune response generated in each group. Lymph nodes isolated from HSV-2 infected mice were apparently larger than those of

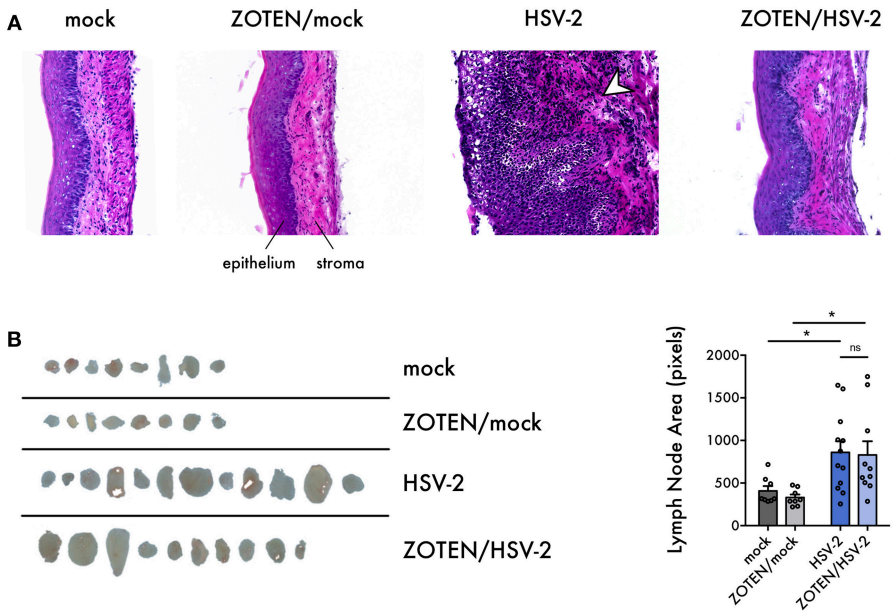


FIGURE 3 | Histological characterization of HSV-2 infection and ZOTEN treatment. **(A)** Representative images of hematoxylin and eosin stained vaginal tissue sections harvested from mice at 7 days post infection. Tissue epithelium and stroma are indicated. Arrowhead in HSV-2 panel indicates tissue infiltration and inflammation observed in infection. Images taken at 20X magnification. **(B)** Draining lymph nodes extracted at 7 days post infection. Areas of lymph nodes in pixels are quantified for each group at right. Error bars indicate SEM. Asterisks denote significant difference by two-tailed unpaired Student's *t*-test, $^*p < 0.05$, ns, or unlabeled, not significant.

mock infected mice, regardless of whether they received ZOTEN treatment (**Figure 3B**).

ZOTEN/HSV-2 Infected Female Mouse Genitalia Show Signs of Reduced Local Immune Response

Previously published work by our lab demonstrated ZOTEN's ability to exert adjuvant properties by showing increased levels of CD4 and $CD8^+$ T cells in isolated splenocytes in response to ZOTEN treatment of HSV-2 infection (24). In this study, we sought to understand the nature of the elicited immune response at the primary site of infection and further identify acute disease development. The isolated vaginal tissues of varying treatment groups were subjected to flow cytometry and the presence of various immune cells were detected (**Figure 4**). The tissue was stained for CD45, Gr-1, CD3, CD49b, CD11c, and F4/80 positive cells. $CD45^+$, $Gr\text{-}1^+$, and $F4/80^+$ cells showed trends of heightened levels in the presence of infection and interestingly displayed a similar trend of decreased levels with ZOTEN/HSV-2 treatment. $CD45^+$ cells were significantly higher in HSV-2 infected mice, in comparison to mock infected, as well as ZOTEN/HSV-2 infected mice, in comparison to ZOTEN/mock treatment group. Similarly, $Gr\text{-}1^+$ cells were detected at significantly higher levels in HSV-2 infected mice when compared to mock infected. A decrease in infiltration of $Gr\text{-}1^+$ cells was observed between HSV-2 infected and ZOTEN/HSV-2 infected mice and the amount of $Gr\text{-}1^+$ cells in the vaginal tissue of ZOTEN/HSV-2 infected cells were comparable to mock and ZOTEN/mock infected mice. $CD49b^+$ and $CD11c^+$ cells increased upon HSV-2 infection but remained at basal levels in the ZOTEN/HSV-2 group. Finally, relatively similar levels of $CD3^+$ cells were observed in the four treatment groups. qPCR was also performed on the vaginal tissue to assess levels of pro-inflammatory cytokine transcripts at the local site of infection (**Figure 5**). While no discernible trends were observed among IFN-α, IFN-β, TNF-α, and IL-6, there was a slight decrease in IL-1β transcript levels in ZOTEN/HSV-2 infected vaginal tissues further supporting the observation of decreased local inflammation (**Figure 5**).

DISCUSSION

HSV-2 infection causes significant disease worldwide, putting over 400 million people at risk of increased genital herpes and lifelong viral persistence in latently infected cells (11). HSV-2 most commonly results in painful ulcerations of genital mucosa and skin as well as increased psychological distress among carriers (19). HSV is also capable of infecting the central nervous system resulting in more severe disease development such as meningitis and encephalitis, which in some cases may be fatal (31). More recently, HSV-2 has received more attention as it has been associated with increased risk of HIV acquisition, making it a more relevant and critical virus to study (10, 32, 33). Current HSV-2 treatment options are not optimal as they exhibit problematic features such as developed drug resistance, toxicities and recurrences of infection. The majority of FDA approved drugs target the virus itself and are efficacious in restricting productive viral replication, however they lack the ability to entirely eliminate quiescent viral genomes and therefore cannot prevent

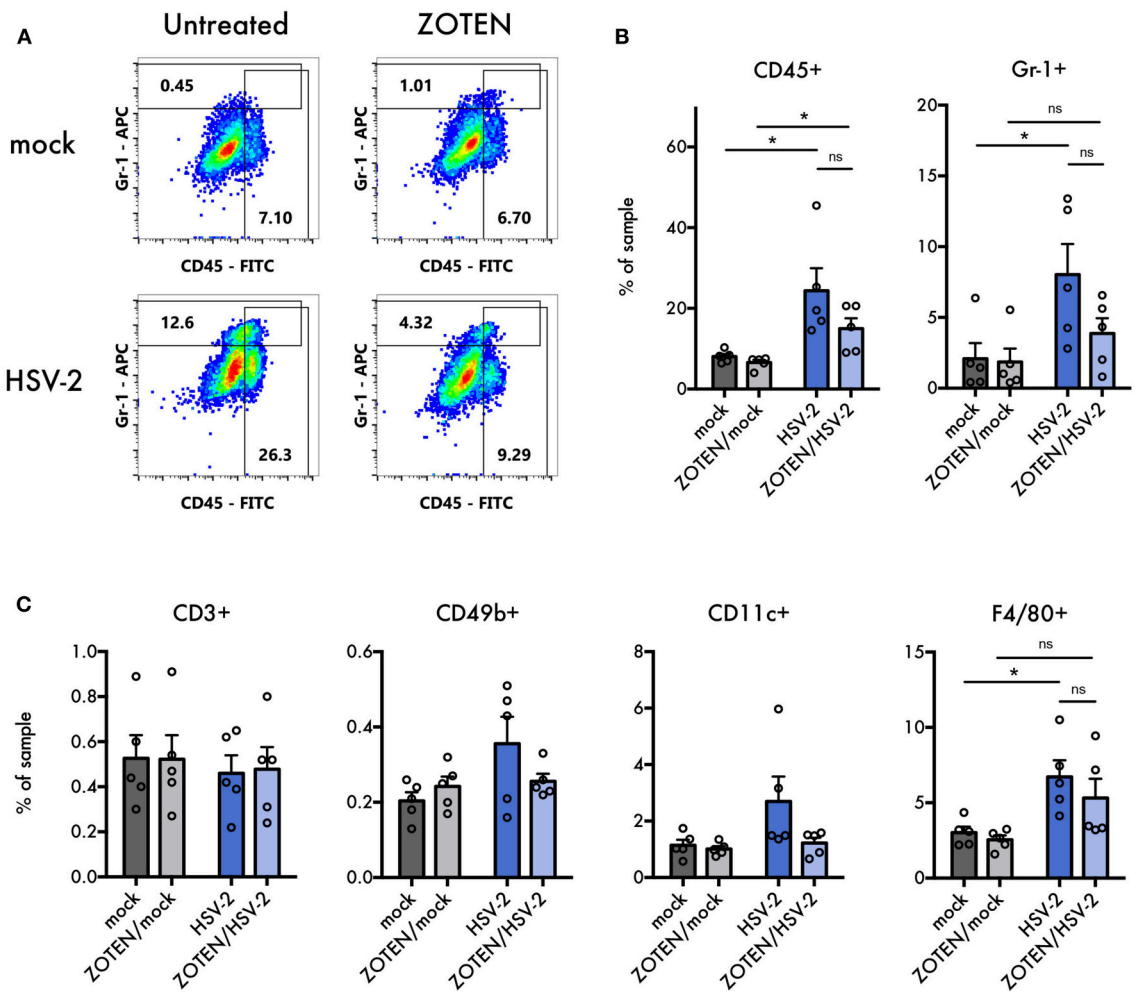


FIGURE 4 | Flow cytometry analysis of immune cell infiltration into vaginal tissues. **(A)** Representative flow cytometry plots of APC anti-Gr-1 vs. FITC anti-CD45. Values in gated regions indicate percentages of singlet non-debris events. **(B)** Quantification of flow cytometry analysis for $CD45^+$ cells and $Gr\text{-}1^+$ cells in vaginal tissues at 7 dpi. Error bars indicate SEM ($n = 5$ per group). **(C)** Quantification of flow cytometry analysis for $CD3^+$, $CD49b^+$, $CD11c^+$, and $F4/80^+$ cells in vaginal tissues at 7 dpi. Error bars indicate SEM ($n = 5$ per group). Asterisks denote significant difference by two-tailed unpaired Student's *t*-test, $^*p < 0.05$, ns, or unlabeled, not significant.

reactivation from latency. Evidently, there is a critical need for a protective vaccine or an immunotherapeutic with a novel antiviral mechanism.

Viral survival in the host relies on the ability of the virus to evade host detection of viral determinants, block immediate host antiviral responses and induce responses favorable for its replication and shedding (16, 17). HSV is known to subvert various pathways in the cell such as DNA repair process, type I interferon (IFN) signaling, cell death and proliferation (17). Highly dynamic interactions between replicating HSV-2 and host mediated processes, like local immune responses in genital tissue, contribute to observed disease manifestations and viral persistence. An example observed is the host enzyme, heparanase, which has been identified as a key host protein that drives tissue destruction and viral pathogenesis (34–37). Exploiting tactics used by the virus in the host can provide an effective anti-HSV microbicide.

A microbivac like ZOTEN demonstrates unique and diverse antiviral mechanism that make it a great candidate for further development into a treatment/vaccination for HSV-2 genital infection. We have previously shown that ZOTEN traps the virus, inhibiting viral entry into the cell and simultaneously allowing for detection by immune cells such as antigen presenting cells. ZOTEN enhances anti-HSV-2 immunity and T cell responses and facilitates the development of memory T cells as well as neutralizing antibody response, acting as an immune booster (24, 38).

In this proof-of-concept study, we sought to elucidate short-term tissue specific antiviral efficacy and immune effects of a ZOTEN/HSV-2 cocktail. The cocktail helps to address two important questions. It sheds light on the virus neutralization potential of ZOTEN and more innovatively, shows its promise as a live virus vaccine platform, which reduces infection without compromising local immune responses. We studied

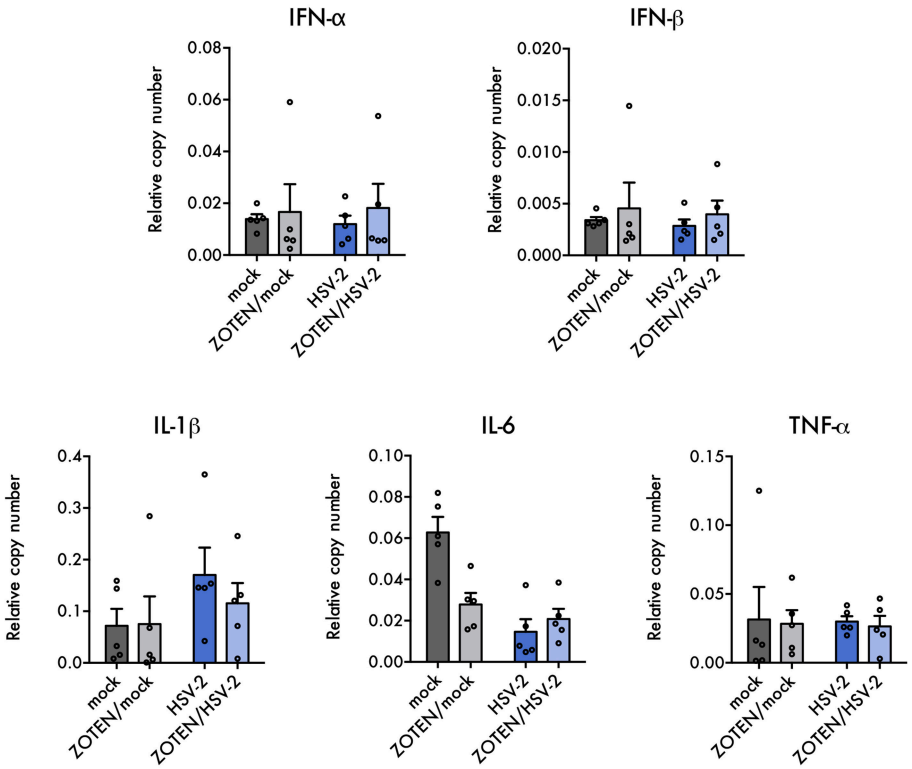


FIGURE 5 | Local cytokine expression in infection and ZOTEN treatment. Quantification of key antiviral type I interferon and pro-inflammatory cytokine transcripts in mice vaginal tissues. Copy numbers relative to b-actin are shown. Error bars indicate SEM ($n = 5$ per group).

the phenotype of infection, pathogenesis and resulting local immune response following genital infection of female BALB/c mice. The elicited immune response by ZOTEN/HSV-2 acts to decrease local cell infiltration and inflammation and therefore results in a global decrease of pathogenesis. To confirm this, we created 4 treatment groups of mice: HSV-2 infected, mock infected, ZOTEN/HSV-2 infected and ZOTEN/mock infected. To maximize our understanding of the events occurring at the primary site of infection, the excised vaginal tissue at 7 days post infection was divided in to 3 equal pieces and each section was subjected to different analysis.

First, we looked at the phenotype of infection by H&E staining (**Figure 3A**). Representative images of each animal group are shown with evident increased levels of cell infiltration, tissue inflammation and damage in HSV-2 infected mice as opposed to the other treatment groups. The vaginal epithelium of ZOTEN/HSV-2 infected mice was comparable in thickness and morphology as mock infected mice. Interestingly, ZOTEN/HSV-2 infected mice exhibited significantly larger draining lymph nodes than mock infected, comparable to HSV-2 infected mice without treatment, leading us to believe that presence of ZOTEN mediates an immune response similar to non-treated infection. However, ZOTEN treatment more so triggers the development of adaptive immunity and memory against the pathogen (**Figure 3B**). While further studies are needed, it appears that ZOTEN equips the host with heightened immune surveillance against the virus, allowing it to fight off the infection while minimizing the inevitable side effect of disease development by innate immunity.

In hopes of better understanding the key players contributing to the changes in local immune response upon ZOTEN treatment, we looked at the different types of cells infiltrating the vaginal tissue by flow cytometry. CD45, Gr-1, CD3, CD49b, CD11c, and F4/80 were used as markers for leukocytes, neutrophils, T lymphocytes, natural killer cells, dendritic cells, and macrophages, respectively. A trend of decreased infiltration of $CD45^+$, $Gr\text{-}1^+$, and $F4/80^+$ cells was observed in the vaginal tissue upon ZOTEN/HSV-2 genital infection. ZOTEN also restored basal levels of $CD49b^+$ and $CD11c^+$ cells. It is understood that neutrophils (expressing Gr-1) are a major component of the innate inflammatory infiltrate at the primary site of herpes infection (18). The observed trends of decreased $CD45^+$ and $Gr\text{-}1^+$ infiltrating cells in the vaginal epithelium in addition to lower levels of proinflammatory IL-1β transcripts further demonstrates the decreased local inflammation observed in ZOTEN/HSV-2 infected mice (**Figures 4A,B**, **5**).

Tissue specific analysis of the application of ZOTEN has allowed for better understanding of how the infection is processed in the local tissue environment. HSV-2 infected mice, in the presence or absence of a prior ZOTEN treatment, demonstrate similar levels of activation of the immune system

however differ drastically in phenotype of local infection. This leads us to believe that while the immune response is activated in the presence of ZOTEN, local inflammation is limited and therefore clinical manifestations of infection are suppressed. Therefore, ZOTEN acts to bolster the immune system and equip the host with a better response to infection. ZOTEN shows to be a practical solution for instant benefit as a microbicide and future development of vaccine against HSV. In addition, our studies show the promise that ZOTEN can be developed as a live virus vaccine platform whereby the viral candidates for the vaccine can be preincubated with ZOTEN and then delivered via intravaginal or other routes. An optimized combination will not cause infection but elicit a protective and/or therapeutic immune response. While more studies are definitely needed, ZOTEN as a live virus vaccine platform is another out-of-the-box strategy, which may lead to new and more effective vaccine strategies.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

ETHICS STATEMENT

All animal experiments were reviewed by the UIC Animal Care Committee and the experiments were performed in adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision research.

AUTHOR CONTRIBUTIONS

AA, LK, RS, TY, and YM performed the experiments. AA, LK, RS, and TY analyzed the results from the biological experiments and YM and RA analyzed the ZOTEN synthesis data. AA, LK, and DS conceived the study and wrote the manuscript.

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health grants AI133557 and AI139768 (to DS) and a core grant (EY001792). Kiel authors would like to acknowledge the support from Deutsche Forschungsgemeinschaft Germany for the Grant GRK2ˆ54 (Materials4Brain).

REFERENCES

1. Wald A, Corey L. *Persistence in the Population: Epidemiology, transmission. Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis*. Cambridge: Cambridge University Press (2007).
2. Whitley RJ, Roizman B. Herpes simplex virus infections. *Lancet.* (2001) 357:1513–8. doi: 10.1016/S0140-6736(00)04638-9
3. Campadelli-Fiume G, Cocchi F, Menotti L, Lopez M. The novel receptors that mediate the entry of herpes simplex viruses and animal alphaherpesviruses into cells. *Rev Med Virol.* (2000) 10:305–19. doi: 10.1002/1099-1654(200009/10)10:5<305::AID-RMV286>3.0.CO;2-T
4. Agelidis AM, Shukla D. Cell entry mechanisms of HSV: what we have learned in recent years. *Fut Virol.* (2015) 10:1145–54. doi: 10.2217/fvl.15.85
5. Roizman B, Taddeo B. The strategy of herpes simplex virus replication and takeover of the host cell. In: Arvin A, Campadelli-Fiume G, Mocarski E, editors. *Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis*. Cambridge: Cambridge University Press (2007). doi: 10.1017/CBO9780511545313.014
6. Nicoll MP, Proença JT, Efstathiou S. The molecular basis of herpes simplex virus latency. *FEMS Microbiol Rev.* (2012) 36:684–705. doi: 10.1111/j.1574-6976.2011.00320.x
7. Jaishankar D, Shukla D. Genital herpes: insights into sexually transmitted infectious disease. *Microb Cell.* (2016) 3:438–50. doi: 10.15698/mic2016.09.528
8. Chayavichitsilp P, Buckwalter JV, Krakowski AC, Friedlander SF. Herpes simplex. *Pediatr Rev.* (2009) 30:29; quiz 130. doi: 10.1542/pir.30-4-119
9. Zhu J, Hladik F, Woodward A, Klock A, Peng T, Johnston C, et al. Persistence of HIV-1 receptor-positive cells after HSV-2 reactivation is a potential mechanism for increased HIV-1 acquisition. *Nat Med.* (2009) 15:886–92. doi: 10.1038/nm.2006
10. Wald A, Link K. Risk of human immunodeficiency virus infection in herpes simplex virus type 2-seropositive persons: a meta-analysis. *J Infect Dis.* (2002) 185:45–52. doi: 10.1086/338231
11. Looker KJ, Magaret AS, Turner KM, Vickerman P, Gottlieb SL, Newman LM. Global estimates of prevalent and incident herpes simplex virus type 2 infections in 2012. *PLoS ONE.* (2015) 10:e114989. doi: 10.1371/journal.pone.0114989
12. Elion GB. Acyclovir: discovery, mechanism of action, and selectivity. *J Med Virol.* (1993) 41(Suppl. 1):2–6.
13. Englund JA, Zimmerman ME, Swierkosz EM, Goodman JL, Scholl DR, Balfour HH Jr. Herpes simplex virus resistant to acyclovir. A study in a tertiary care center. *Ann Intern Med.* (1990) 112:416–22. doi: 10.7326/0003-4819-76-3-112-6-416
14. Elion GB. Mechanism of action and selectivity of acyclovir. *Am J Med.* (1982) 73:7–13. doi: 10.1016/0002-9343(82)90055-9
15. Xu F, Sternberg MR, Kottiri BJ, McQuillan GM, Lee FK, Nahmias AJ, et al. Trends in herpes simplex virus type 1 and type 2 seroprevalence in the United States. *JAMA.* (2006) 296:964–73. doi: 10.1001/jama.296.8.964
16. Takeuchi O, Akira S. Innate immunity to virus infection. *Immunol Rev.* (2009) 227:75–86. doi: 10.1111/j.1600-065X.2008.00737.x
17. Melchjorsen J, Matikainen S, Paludan S. Activation and evasion of innate antiviral immunity by herpes simplex virus. *Viruses.* (2009) 1:737–59. doi: 10.3390/v1030737
18. Wang JP, Bowen GN, Zhou S, Cerny A, Zacharia A, Knipe DM, et al. Role of specific innate immune responses in herpes simplex virus infection of the central nervous system. *J Virol.* (2012) 86:2273. doi: 10.1128/JVI.06010-11
19. Joshua T Schiffer, Lawrence Corey. Rapid host immune response and viral dynamics in herpes simplex virus-2 infection. *Nat Med.* (2013) 19:280–8. doi: 10.1038/nm.3103
20. Richman DD. *Antiviral Drug Resistance*. Chichester: Wiley (1996).
21. Endy D, Yin J. Toward antiviral strategies that resist viral escape. *Antimicrob Agents Chemother.* (2000) 44:1097. doi: 10.1128/AAC.44.4.1097-1099.2000
22. Domingo E, Menendez-Arias L, Quinones-Mateu ME, Holguin A, Gutierrez-Rivas M, Martinez MA, et al. Viral quasispecies and the problem of vaccine-escape and drug-resistant mutants. *Prog Drug Res.* (1997) 48:99–128. doi: 10.1007/978-3-0348-8861-5_4
23. Stanberry LR, Spruance SL, Cunningham AL, Bernstein DI, Mindel A, Sacks S, et al. Glycoprotein-D-adjuvant vaccine to prevent genital herpes. *N Engl J Med.* (2002) 347:1652–61. doi: 10.1056/NEJMoa011915
24. Antoine TE, Hadigal SR, Yakoub AM, Mishra YK, Bhattacharya P, Haddad C, et al. Intravaginal zinc oxide tetrapod nanoparticles as novel immunoprotective agents against genital herpes. *J Immunol.* (2016) 196:4566–75. doi: 10.4049/jimmunol.1502373
25. Mishra YK, Adelung R. ZnO tetrapod materials for functional applications. *Mater Today.* (2018) 21:631–51. doi: 10.1016/j.mattod.2017.11.003
26. Antoine TE, Mishra YK, Trigilio J, Tiwari V, Adelung R, Shukla D. Prophylactic, therapeutic and neutralizing effects of zinc oxide tetrapod

structures against herpes simplex virus type-2 infection. *Antiviral Res.* (2012) 96:363–75. doi: 10.1016/j.antiviral.2012.09.020
27. Coleman JL, Shukla D. Recent advances in vaccine development for herpes simplex virus types I and II. *Hum Vaccin Immunother.* (2013) 9:729–35. doi: 10.4161/hv.23289
28. Yadavalli T, Shukla D. Could zinc oxide tetrapod nanoparticles be used as an effective immunotherapy against HSV-2? *Nanomedicine.* (2016) 11:2239–42. doi: 10.2217/nnm-2016-0249
29. Adelung R, Kaps S, Mishra YK, Claus M, Preusse T, Wolpert C inventors. AnonymousGerman Patent. WO2011–116751. (2011).
30. Mishra YK, Modi G, Cretu V, Postica V, Lupan O, Reimer T, et al. Direct growth of freestanding ZnO tetrapod networks for multifunctional applications in photocatalysis, UV photodetection, and gas sensing. *ACS Appl Mater Interfaces.* (2015) 7:14303–16. doi: 10.1021/acsami.5b02816
31. Tyler KL. Herpes simplex virus infections of the central nervous system: encephalitis and meningitis, including Mollaret's. *Herpes.* (2004) 11(Suppl. 2):64A.
32. Looker KJ, Elmes JAR, Gottlieb SL, Schiffer JT, Vickerman P, Turner KME, et al. Effect of HSV-2 infection on subsequent HIV acquisition: an updated systematic review and meta-analysis. *Lancet Infectious Dis.* (2017) 17:1303–16. doi: 10.1016/S1473-3099(17)30405-X
33. Barnabas RV, Wasserheit JN, Huang Y, Janes H, Morrow R, Fuchs J, et al. Impact of herpes simplex virus type 2 on HIV-1 acquisition and progression in an HIV vaccine trial (the Step study). *J Acquir Immune Defic Syndr.* (2011) 57:238–44. doi: 10.1097/QAI.0b013e31821acb5
34. Hopkins J, Yadavalli T, Agelidis AM, Shukla D. Host enzymes heparanase and cathepsin L promote herpes simplex virus-2 release from cells. *J Virol.* (2018) 92:e01179–18. doi: 10.1128/JVI.01179-18
35. Agelidis AM, Hadigal SR, Jaishankar D, Shukla D. Viral activation of heparanase drives pathogenesis of herpes simplex virus-1. *Cell Rep.* (2017) 20:439–50. doi: 10.1016/j.celrep.2017.06.041
36. Hadigal SR, Agelidis AM, Karasneh GA, Antoine TE, Yakoub AM, Ramani VC, et al. Heparanase is a host enzyme required for herpes simplex virus-1 release from cells. *Nat Comm.* (2015) 6:6985. doi: 10.1038/ncomms7985
37. Lobo AM, Agelidis AM, Shukla D. Pathogenesis of herpes simplex keratitis: the host cell response and ocular surface sequelae to infection and inflammation. *Ocul Surf.* (2018). 17:40–9. doi: 10.1016/j.jtos.2018.10.002
38. Mishra YK, Adelung R, Rohl C, Shukla D, Spors F, Tiwari V. Virostatic potential of micro-nano filopodia-like ZnO structures against herpes simplex virus-1. *Antiviral Res.* (2011) 92:305–12. doi: 10.1016/j.antiviral.2011.08.017

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 11 April 2019
doi: 10.3389/fimmu.2019.00732

SOCS and Herpesviruses, With Emphasis on Cytomegalovirus Retinitis

Christine I. Alston[1,2] *and Richard D. Dix*[1,2]*

[1] Department of Biology, Viral Immunology Center, Georgia State University, Atlanta, GA, United States, [2] Department of Ophthalmology, Emory University School of Medicine, Atlanta, GA, United States

Suppressor of cytokine signaling (SOCS) proteins provide selective negative feedback to prevent pathogeneses caused by overstimulation of the immune system. Of the eight known SOCS proteins, SOCS1 and SOCS3 are the best studied, and systemic deletion of either gene causes early lethality in mice. Many viruses, including herpesviruses such as herpes simplex virus and cytomegalovirus, can manipulate expression of these host proteins, with overstimulation of SOCS1 and/or SOCS3 putatively facilitating viral evasion of immune surveillance, and SOCS suppression generally exacerbating immunopathogenesis. This is particularly poignant within the eye, which contains a diverse assortment of specialized cell types working together in a tightly controlled microenvironment of immune privilege. When the immune privilege of the ocular compartment fails, inflammation causing severe immunopathogenesis and permanent, sight-threatening damage may occur, as in the case of AIDS-related human cytomegalovirus (HCMV) retinitis. Herein we review how SOCS1 and SOCS3 impact the virologic, immunologic, and/or pathologic outcomes of herpesvirus infection with particular emphasis on retinitis caused by HCMV or its mouse model experimental counterpart, murine cytomegalovirus (MCMV). The accumulated data suggests that SOCS1 and/or SOCS3 can differentially affect the severity of viral diseases in a highly cell-type-specific manner, reflecting the diversity and complexity of herpesvirus infection and the ocular compartment.

Keywords: suppressor of cytokine signaling, SOCS1, SOCS3, herpesvirus, cytomegalovirus, retinitis

OPEN ACCESS

Edited by:
Aurelio Cafaro,
Istituto Superiore di Sanità (ISS), Italy

Reviewed by:
François J. M. A. Meurens,
INRA UMR703 Ecole Nationale Vétérinaire, Agroalimentaire et de l'alimentation de Nantes-Atlantique, France
Deepak Shukla,
University of Illinois at Chicago, United States
Homayon Ghiasi,
Cedars-Sinai Medical Center, United States

***Correspondence:**
Richard D. Dix
rdix@gsu.edu

Specialty section:
This article was submitted to Viral Immunology, a section of the journal Frontiers in Immunology

Received: *26 October 2018*
Accepted: *19 March 2019*
Published: *11 April 2019*

Citation:
Alston CI and Dix RD (2019) SOCS and Herpesviruses, With Emphasis on Cytomegalovirus Retinitis. Front. Immunol. 10:732. doi: 10.3389/fimmu.2019.00732

INTRODUCTION

Herpesviruses skillfully manipulate their hosts by various mechanisms while viral lytic and latent cycles maintain a lifelong, Sisyphean struggle with host innate, and adaptive immune systems. Cells of innate and adaptive immunity are efficient producers of pro-inflammatory cytokines, chemokines, and cell surface receptors, and they rely heavily on cell-type-specific intracellular signaling pathways to differentiate and function properly. Upon infection, herpesviruses are recognized by circulating innate cells such as monocytes, macrophages, dendritic cells (DC), neutrophils, or natural killer (NK) cells (1), and by local resident innate cell types specialized in certain tissues, such as Müller cells and microglia (2) of the retina. Interactions between receptors and pathogens begin signaling cascades that result in progressively amplified, harmonious transcriptional stimulation of hundreds of downstream gene products, many of them cytokines released extracellularly to function in autocrine or paracrine positive feedback capacities.

of homeostasis being paramount for biological systems, this signaling also induces negative feedback agents such as suppressor of cytokine signaling (SOCS) proteins to aid in the prevention of damaging immunopathologies. The eight known SOCS members comprise a family of host proteins which, among their other functions, negatively regulate signaling pathways induced by antiviral and inflammatory cytokines, effectively increasing tolerance for specific cytokines signaling within specific cells [for reviews, see (3–6)]. Once activated, innate immune cells such as DCs or microglia can become professional antigen presenting cells, which instruct and activate adaptive immune cells such as B cells and $CD4^+$ and $CD8^+$ T lymphocytes to produce their effector functions against pathogens and pathogen-infected cells. During primary and lytic infection, herpesviruses nimbly evade sufficient aspects of innate and adaptive immunity to avoid complete clearance. Eventually they enter or are forced by the immune system into a state of latency during which the virus continues to modulate host immunity despite only a small subset of viral genes being detectable. Reactivation from latency to lytic infection then back to latency may then occur periodically throughout the life of the host [for reviews, see (1, 7–10)].

Despite the relatively large number of virus-encoded gene products contained within herpesviruses compared with other viruses, they remain obligate intracellular pathogens and therefore still rely on host-encoded gene products for survival and propagation. SOCS proteins are one such example of host-encoded proteins that are manipulated by many different types of viruses and other pathogens, as reviewed by others (5, 6). In addition to the viruses featured in these reviews, more herpesviruses also are now known to stimulate SOCS1 and/or SOCS3 during *in vitro* or *in vivo* infection. These include the human herpesviruses herpes simplex type 1 (HSV-1), varicella zoster virus (VZV), human cytomegalovirus (HCMV), Epstein-Barr virus (EBV), and Kaposi's sarcoma-associated herpesvirus (KSHV), as well as the animal herpesviruses *gallid alphaherpesvirus 2* (GaHV-2, or Marek's disease virus, MDV), *suid alphaherpesvirus 1* (SuHV-1, or pseudorabies virus, PRV), murine cytomegalovirus (MCMV), and murine gammaherpesvirus-68 (MHV-68) (11–23).

Herein we discuss these human and animal herpesviruses currently known to affect SOCS proteins in various *in vitro* and *in vivo* model systems, with particular emphasis on SOCS1 and SOCS3 expression during experimental MCMV retinitis, a mouse model used to study AIDS-related HCMV retinitis (24). AIDS-related HCMV retinitis is a blinding, degenerative disease of the retina that once threatened the bilateral vision of ∼30% of AIDS patients (25). Despite the advent of antiretroviral therapies (ART) in the developed world, HCMV remains a significant opportunistic pathogen of AIDS patients worldwide. As with humans and AIDS, mice with murine AIDS (MAIDS) experience retrovirus-induced immune suppression and become susceptible to diseases of opportunistic pathogens (26). For many years our laboratory has used MAIDS-related MCMV retinitis as a clinically relevant mouse model with high face validity and predictive validity [per (27, 28)] to AIDS-related HCMV retinitis to elucidate the role of potential candidates contributing to this disease (29), including host SOCS proteins (21, 23). Thus, the purposes of this review are to explore briefly the model systems under which herpesviruses manipulate SOCS proteins and to review the effects of SOCS manipulation on virologic, immunologic, or pathologic outcomes, with a focus on experimental cytomegalovirus retinitis. Specialized therapeutic inhibition or mimicry of SOCS proteins, perhaps combined with immunotherapies or antiviral drugs, may become a viable tactic for more effectively combating herpesvirus pathologies.

SUPPRESSOR OF CYTOKINE SIGNALING (SOCS) FAMILY

Innate and adaptive immune cells secrete cytokines and chemokines to orchestrate a coherent, integrated immune response to protect the host against pathogens. During infection, cytokines initiate, execute, and resolve inflammatory responses, such that cytokine signaling is the crucial control switch between the initiation of the immune response and the maintenance of homeostasis in the periphery. Therefore, cellular negative feedback loops play an important role in maintaining the tight balance of cytokine secretion and cytokine inhibition, and SOCS proteins function in such a capacity.

SOCS Structure, Function, and Expression

SOCS proteins were first discovered in the mid-1990s as cytokine-induced inhibitors of signal transducers and activators of transcription (STAT) cell signaling pathways (30–33). The SOCS protein family currently contains eight known members: SOCS1 through SOCS7 and the cytokine-inducible Src homology 2 (SH2)-containing domain protein (CIS). These proteins are selectively upregulated in response to various cell signaling pathways (34) and subsequently act intracellularly as negative regulators of cell signaling (4). All SOCS proteins characteristically contain a C-terminal SOCS box, an internal SH2 domain, and a variable-length N-terminal region (4) (**Figure 1**). SH2 domains are conserved throughout most eukarya, excluding single-celled fungi, and they recognize and bind to specific phosphorylated tyrosine motifs on their target proteins (37). At least 110 unique human proteins contain SH2 domains (38), and specificity to their targets is achieved by primary and secondary binding sites within these SH2 domains (39). Immediately upstream of the SH2 domain is the extended SH2 sequence (ESS) which increases binding affinity to phosphotyrosine residues (40–42). The SOCS box is also a conserved sequence found within more than 70 different human proteins (43). This motif primarily functions to recruit cellular ubiquitination machinery, thus allowing such proteins to flag their specific substrates for proteasomal degradation (43). It achieves this by binding cellular Elongin B, Elongin C, Cullin5, and RING-box-2, thus forming an E3 ubiquitin ligase complex (4–6, 43). SOCS1 and SOCS3 additionally possess an N-terminal kinase inhibitory region (KIR) which can act as a pseudosubstrate to block the kinase activity of such proteins as Janus kinases (JAKs) (32, 44, 45). These SOCS proteins negatively regulate intracellular signaling pathways by several mechanisms,

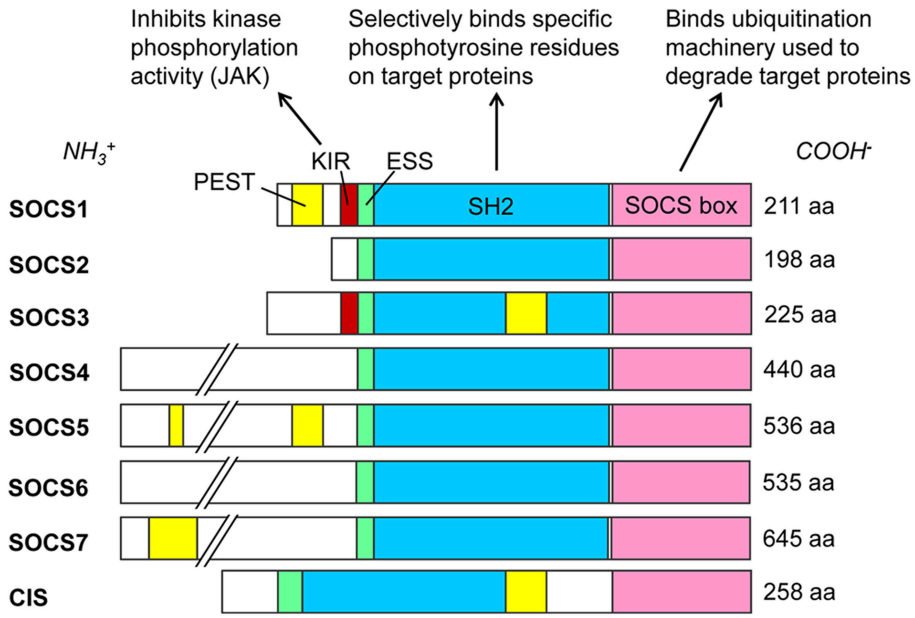


FIGURE 1 | SOCS family proteins and their domains. Src homology 2 (SH2) domains (blue) govern target protein specificity by recognizing phosphorylated tyrosine residues flanked by specific sequences such as those on cytoplasmic residues of cytokine receptors. SOCS1 and SOCS3 exclusively contain kinase inhibitory regions (KIR, red), which bind and inhibit JAK proteins. Extended SH2 sequences (ESS, green) enhance binding specificity and affinities to phosphotyrosine residues. SOCS box domains (pink) recruit cellular Elongin BC, Cullin5, and RING-box-2 to form an E3 ubiquitin ligase complex, ubiquitinating target proteins for proteasomal degradation. PEST motifs (yellow) greatly decrease the half-lives of the proteins; see (35, 36) for predicted PEST domain locations. Amino acid (aa) lengths for *Homo sapiens* SOCS proteins are from the National Center for Biotechnology Information (NCBI) database (February 2019).

including competitive binding of phosphotyrosine residues with various recruited STAT proteins, inhibition of JAK activity via KIR domains, or ubiquitination of SOCS-bound elements by the SOCS box, marking them for degradation (4, 5). In addition to these domains, SOCS1, SOCS3, SOCS5, SOCS7, and CIS each contain a sequence rich in proline (P), glutamic acid (E), serine (S), and threonine (T) known as a PEST motif (46), which decreases the half-life of the entire protein to about 2 h (42). The predicted locations for these PEST motifs vary, and to our knowledge no such predicted sequence has yet been found for SOCS2, SOCS4, or SOCS6 (35, 36).

Several different types of cell signaling pathways are capable of inducing SOCS (47–50), with JAK/STAT signaling driven by cytokines such as interferons (IFN) and interleukins (IL) being one of the best studied SOCS-inducing pathways (4). When transmembrane cytokine receptors on a cell surface recognize their cognate extracellular cytokines, they initiate intracellular phosphorylation cascades via specific combinations of JAK and STAT proteins, transcriptionally stimulating scores of gene products (51–53), including negative-feedback SOCS family proteins. Well-described cytokine receptor-JAK/STAT-gene target combinations are reviewed and summarized elsewhere (54, 55). Intracellular SOCS proteins then selectively inhibit components of JAK/STAT and other cell signaling pathways, within the specific cells expressing them (4, 33, 56–58) (**Figure 2**). Although some crosstalk occurs between individual SOCS members and their targets, the variations between SOCS protein SH2 domains equip them with preferential affinity to their respective substrates, as listed elsewhere (50). Receptor expression, cytokine milieus, and signaling pathways tend to differ greatly between cell types, even within the contexts of different tissues or microenvironments.

Many different cell types in various organs are capable of producing SOCS family proteins (33), and they are most amply produced by hematopoietic cells (59) of the innate and adaptive immune systems (4, 58). Some of these SOCS-expressing cell types include monocytes (60), macrophages (32, 61), DCs (62, 63), microglia (64), neutrophils (65), NK cells (66), $CD4^+$, and $CD8^+$ T cells (67, 68), and ocular Müller cells (69). SOCS proteins primarily function within the very cells which transcriptionally produce them, although cell-to-cell vesicular transport of SOCS proteins has been demonstrated from alveolar macrophages to adjacent epithelial cells (70).

SOCS1 and SOCS3

The importance of SOCS1 and SOCS3 in modulating immune responses is emphasized in knockout mice, as SOCS1-deficient mice die within 3–4 weeks of birth from massive IFN-related inflammation (71–73), and deletion of the SOCS3 gene is embryonically lethal (74). SOCS1 proteins are able to limit the surface expression of molecules that mediate the immune response, suppress inflammation by dampening expression of cytokines and chemokines, inhibit pathogen infiltration and replication, and prevent central nervous system demyelination. SOCS1 is quickly induced by IFN signaling and inhibits the specific JAK and STAT proteins involved during IFN signaling

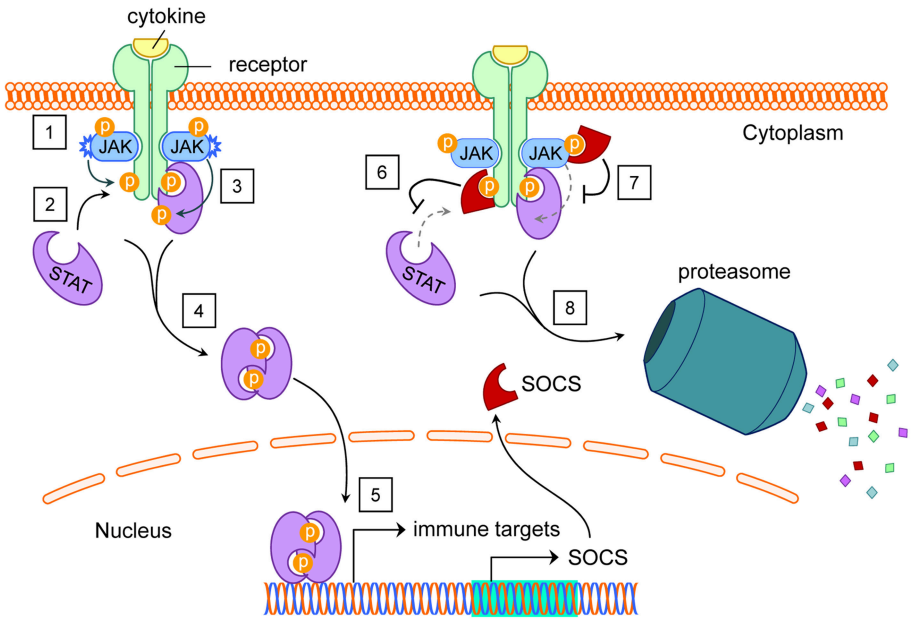


FIGURE 2 | SOCS induction by and inhibition of the JAK/STAT pathway. **(1)** Extracellular cytokines cause dimerization of their cognate transmembrane receptors. This brings intracellular receptor-associated JAK proteins into proximity to cross-phosphorylate each other and tyrosine residues on the receptors. **(2)** STAT proteins dock at phosphotyrosines on intracellular receptor subunits. **(3)** JAK proteins phosphorylate STAT proteins, activating them. **(4)** Activated STAT proteins undock from their receptors, dimerize, and translocate to the nucleus. **(5)** STAT proteins act as transcription factors for dozens of immune targets, including SOCS. **(6)** Functioning in the cytoplasm, SOCS proteins can bind various phosphotyrosines on intracellular receptors, blocking STATs from their native docking sites. **(7)** With their KIR domains, SOCS1 and SOCS3 can inhibit the kinase activity of JAK proteins, preventing tyrosine phosphorylation of STAT proteins. **(8)** SOCS boxes facilitate ubiquitination of SOCS-bound protein targets for proteasomal degradation. Abbreviations: suppressor of cytokine signaling (SOCS), Janus kinase (JAK), signal transducers and activators of transcription (STAT), kinase inhibitory region (KIR). See Akhtar and Benveniste (5).

(75, 76). In addition to its primary role in the regulation of components of the JAK/STAT pathway, SOCS1 is capable of regulating other cellular signaling pathways such as toll-like receptor (TLR) signaling and macrophage activation (47). Whereas inactivated macrophages produce low baseline levels of SOCS1 and SOCS3, induction of SOCS1 generally drives macrophages toward an M2 phenotype, and SOCS3 toward M1 (77, 78). SOCS1 also plays a dual role in $CD4^+$ T-helper (T_H) cell differentiation (67, 79–81). As a key attenuator of type II IFN (IFN-γ) signaling, SOCS1 can inhibit IFN-γ-mediated STAT1 activation by targeting JAK2, thus suppressing the differentiation of the T_H1 lineage in $CD4^+$ T cells (75, 82). SOCS1 is alternatively able to inhibit IL-4 signaling, thereby driving differentiation toward a T_H1 phenotype (67, 83). By comparison, SOCS3 is classically upregulated as a consequence of signaling by the IL-6 family of cytokines (33). Once induced, a major function of SOCS3 is then to inhibit the signaling of IL-6 family cytokines by targeting their common gp130 receptor (58, 84, 85). Furthermore, SOCS3 is a key regulator of IL-23-mediated STAT3 (79, 86) and of IL-12-mediated STAT4 activation (85), such that SOCS3 is also able to inhibit the development of $CD4^+$ T_H1 and T_H17 cells (87), thereby promoting differentiation to the T_H2 lineage.

Both SOCS1 and SOCS3 have demonstrated transcriptional induction by type I IFNs, key immune regulators in mounting an antiviral response (88, 89). These cytokines play a role in the activation of NK and T cells, and they induce cell death in virus-infected cells (90, 91). The type I IFN family consists of the many subtypes of IFN-α, as well as IFN-β, IFN-ε, IFN-κ, and IFN-ω (92). Almost all cell types are capable of producing type I IFNs in response to various stimuli (89, 90, 93). Plasmacytoid DCs (pDC) in particular are one of the highest contributors to the secretion of type I IFNs (90). Type I IFNs signal through the heterodimerization of the type I IFN receptor (IFNAR)-1 and IFNAR-2, which signal through the JAK/STAT pathway, mediated specifically by the JAKs Tyk2 and JAK1, and by STAT1, and STAT2 (90, 94). Unlike most dimerized STATs, the STAT1/STAT2 heterodimer must bind to an additional protein, interferon regulatory factor 9 (IRF9), and form the interferon-stimulated gene factor 3 (ISGF3), before they are able to recognize the interferon-stimulated response element (ISRE) and begin transcription of ISGs (90). The more than 300 ISGs that have been identified to date (95) include SOCS proteins, particularly SOCS1, and, to a lesser extent, SOCS3.

In addition to this classical induction by cytokine signaling via the JAK/STAT pathway, SOCS proteins have also shown to be stimulated by alternative cell signaling pathways. Among these pathways are nuclear factor κB (NF-κB) and mitogen activated protein kinase (MAPK) signaling pathways through phosphorylation of c-Jun N-terminal kinases (JNKs) (96, 97). SOCS proteins can also be induced by stimulation of TLRs (48, 98, 99), which are expressed by many cell types, including

the retinal pigment epithelium (RPE) (100, 101) and Müller cells (102) of the eye. In macrophages and DCs, non-TLR sensor dectin-1 induces SOCS1 by MAPK/ERK, and SOCS1 modulates TLR9 signaling by inhibiting NF-κB (103). Stimulation of these pathways therefore may trigger the production of SOCS proteins directly or indirectly by the production of SOCS-inducing cytokines such as type I IFN.

SOCS2

Although the rest of the SOCS family (CIS, SOCS2, and SOCS4—SOCS7) remains less studied than SOCS1 and SOCS3, ever more research on these accumulates over time. SOCS2, briefly discussed below in the context of alphaherpesviruses, is stimulated within different cell types in response to signals from various hormones or cytokines, including growth hormone, insulin, IFN-α, and IL-6, possibly through STAT5 [reviewed in (6, 104)]. It is believed that SOCS2 and CIS primarily bind to phosphotyrosines on intracellular receptor residues to block STAT binding in a competitive manner (5). Among its other functions, SOCS2 negatively regulates the growth hormone receptor, and SOCS2-knockout mice are significantly (∼40%) larger than wild type mice (105). Like most other SOCS members, SOCS2 is also implicated in some types of cancer, albeit less abundantly so than other SOCS members.

HERPESVIRUSES

Herpesviridae Classification and Characteristics

Admittance into the *Herpesviridae* family of the taxonomic order *Herpesvirales* traditionally is based upon the virus structure: dsDNA within an icosahedral capsid surrounded by an amorphous tegument between the host cell-derived envelope encrusted with viral glycoproteins. Members of this family share the biological characteristics of replication within host cell nuclei, the establishment of latency, and ultimate destruction of lytically infected host cells. With notable exceptions, it is generally rare that herpesviruses cause severe disease in immunocompetent, endogenous hosts, with the majority of morbidities or mortalities occurring in the very young, very old, immune compromised, or non-native host. To date, there are nine known herpesviruses that infect humans; these are designated human herpesvirus (HHV)-1 through HHV-8, with a ninth member in the division of HHV-6 into HHV-6A and HHV-6B (106) as distinct herpesvirus species. The *Herpesviridae* family contains three subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae*. Members of these subfamilies are phylogenetically classified based on genetic sequence homology but can also be generally distinguished by their respective cell or tissue preference for establishing latency, relative rate of replication cycle, and/or natural or experimental host restriction [reviewed in (1, 107–110)]. Classifications of select herpesviruses pertinent to this review are organized in **Table 1**.

TABLE 1 | Taxonomic classifications of select members of the *Herpesviridae* family.

	Genus	Species name	Common name
α	*Mardivirus*	*Gallid alphaherpesvirus 2* (GaHV-2)	Marek's disease virus (MDV)
	Simplexvirus	*Human herpesvirus 1* (HHV-1)	Herpes simplex virus type 1 (HSV-1)
		Human herpesvirus 2 (HHV-2)	Herpes simplex virus type 2 (HSV-2)
	Varicellovirus	*Bovine herpesvirus 1* (BoHV-1)	
		Bovine herpesvirus 5 (BoHV-5)	
		Human herpesvirus 3 (HHV-3)	Varicella zoster virus (VZV)
		Suid herpesvirus 1 (SuHV-1)	Pseudorabies virus (PRV)
β	*Cytomegalovirus*	*Human herpesvirus 5* (HHV-5)	Human cytomegalovirus (HCMV)
	Muromegalovirus	*Murid herpesvirus 1* (MuHV-1)	Murine cytomegalovirus (MCMV)
	Roseolavirus	*Human herpesvirus 6A* (HHV-6A) *Human herpesvirus 6B* (HHV-6B) *Human herpesvirus 7* (HHV-7)	
γ	*Lymphocryptovirus*	*Human herpesvirus 4* (HHV-4)	Epstein-Barr virus (EBV)
	Rhadinovirus	*Human herpesvirus 8* (HHV-8)	Kaposi's sarcoma-associated herpesvirus (KSHV)
		Murid herpesvirus 4 (MuHV-4), isolate MHV-68	Murine gammaherpesvirus 68 (MHV-68)

Herpesviridae subfamilies: Alphaherpesvirinae (α), Betaherpesvirinae (β), Gammaherpesvirinae (γ). Classifications from the July 2017 International Committee on Taxonomy of Viruses (ICTV) and Pellett and Roizman (1) and Davison et al. (109).

Alphaherpesvirinae

The α-herpesviruses are characterized by their ability to establish latency in neurons, to infect a variety of host species, to replicate and spread relatively quickly, and to destroy infected host cells. This subfamily currently consists of five genera, two of which infect mammals: *Simplexvirus* and *Varicellovirus*. Pathologies of *Simplexvirus* HSV-1 include oropharyngeal lesions (cold sores), herpetic epithelial or stromal keratitis, herpes simplex encephalitis, and genital herpes (111), with the latter more frequently caused by HSV-2, another *Simplexvirus*. VZV of the *Varicellovirus* genus is the etiological agent of varicella (chickenpox) and herpes zoster (shingles). Also in this genus is *suid alphaherpesvirus 1* (SuHV-1), or PRV, which causes fatal disease following natural infection of swine as well as a wide range of mammalian host species. In addition, *bovine herpesvirus 1* (BoHV-1) and BoHV-5 are highly similar varicelloviruses (112) which cause significant infections of cattle (113, 114). The genus *Mardivirus* of the *Alphaherpesvirinae* subfamily contains *gallid alphaherpesvirus 2* (GaHV-2), or MDV, which infects chickens and is responsible for significant losses in the poultry industry (115, 116).

Betaherpesvirinae

The β-herpesviruses generally replicate more slowly than other herpesviruses and display host species specificity, with a propensity to establish latency in lymphoid cells of hematopoietic origin. The genus *Roseolavirus* comprises HHV-6 and HHV-7, of which HHV-6B and HHV-7 have been shown to cause exanthem *subitum* (roseola, sixth disease) (106, 117). Of particular importance to this review are the genera *Cytomegalovirus*, which contains HCMV, and *Muromegalovirus*, which includes *murid herpesvirus 1* (MuHV-1), or MCMV. HCMV and MCMV represent a central focus of this report and are discussed more thoroughly in following sections.

Gammaherpesvirinae

The γ-herpesvirus subfamily contains viruses that are species-specific, generally prefer B or T lymphocytes for replication, and establish latency within lymphoid tissue. This subfamily contains four genera, of which *Lymphocryptovirus* contains EBV, and *Rhadinovirus* includes KSHV (HHV-8) and MHV-68, an isolate of *murid herpesvirus 4* that is widely used in experimental model systems (1).

Herpesvirus Immune Evasion: HCMV and MCMV

The balance between virulence and the host immune response sways the outcome of any viral infection. Just as the host has an arsenal of mechanisms for sensing, stopping, and clearing viral infection, viruses have as many mechanisms for evading, escaping, and producing productive infections in the host. Herpesviruses undergo lytic and latent life cycles for the lifetime of their hosts, and they are particularly adept at manipulating the innate and adaptive immune responses by a multitude of mechanisms. As HSV-1 is a quintessential example of the α-herpesviruses, HCMV and its mouse counterpart MCMV are well-studied examples of the β-herpesviruses. HCMV and MCMV, like many herpesviruses, modulate their host cells by interfering with signaling pathways important to the innate or adaptive immune response (110). As HCMV and MCMV represent a major focus of this review, they are depicted in this section as examples of herpesvirus immune evasion.

Integral to the first-responding cells of innate immunity is the vast family of pattern recognition receptors (PRR) which are capable of detecting common non-self, pathogen-associated molecular patterns (PAMPs) (118). PAMPs are highly-conserved molecules which are usually indispensable to the pathogens with which they are associated (91, 118, 119). Many types of PRRs have been identified so far, including TLRs, retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), nucleotide oligomerization domain (NOD)-like receptors (NLRs), C-type lectin receptors (CLRs), and absent in melanoma 2 (AIM2)-like receptors (120, 121). In general, activation of any of these PRRs leads to one or more well-characterized cell signaling pathways responsible for the upregulation of pro-inflammatory cytokines, including type I IFNs (120). Among these pathways are NF-κB and MAPK signaling pathways through phosphorylation of JNKs (96, 97), as well as inflammasome/caspase-1-dependent IL-1β maturation (122). Infection with herpesviruses such as HCMV or MCMV has the capacity to stimulate and/or to modulate several of these PRRs (110). For instance, MCMV infection of monocytes and other cell types stimulates TLR2/myeloid differentiation primary response 88 (MyD88) (123), TLR3/TIR-domain-containing adapter-inducing interferon-β (TRIF), and TLR9/MyD88 (124) signaling. Macrophages and their progenitor cells (monocytes, bone marrow cells) highly express PRRs and are major players during systemic HCMV or MCMV dissemination and latency (125–133).

As major players in the innate immune response, macrophages exhibit divergent activation phenotypes in response to various stimuli. These have very generally been categorized into M1 classically-activated macrophages and M2 alternatively-activated macrophages (134), so called for their association with $CD4^+$ T_H1 or T_H2 polarization, respectively. In general, M1 macrophages are activated via exposure to IFN-γ alone or together with tumor necrosis factor (TNF)-α, PAMPs such as TLR4-recognized lipopolysaccharide (LPS). They express TNF-α, IL-6, IL-1, and IL-12 upon activation, and through production of these pro-inflammatory cytokines and nitric oxide, they exhibit a pro-inflammatory phenotype (135). Alternatively-activated M2 macrophages have grown to include all non-classically-activated macrophages and therefore display a diverse range of activation phenotypes. An M2 phenotype is generally induced by exposure to IL-4 or corticosteroids, results in the production of anti-inflammatory IL-10 and IL-1 receptor antagonist, and participates in anti-inflammatory or pro-angiogenic activities (135). These macrophage polarizations exhibit extreme plasticity, however, and are not as clearly defined as originally thought. Monocytes infected with HCMV, for instance, display a hybrid M1/M2 activation phenotype, simultaneously showing pro-inflammatory and pro-angiogenic properties, but with a propensity mostly toward the M1 phenotype (136–139).

Also integral in early control of herpesvirus infection are NK cells. These granulocytic cells are highly effective at destroying cells that fail to display sufficient amounts of major histocompatibility complex (MHC) class I (MHC-I), which presents intracellularly-derived antigens to MHC-I-restricted immune cells such as $CD8^+$ T cells (140). The cytotoxic effector function of NK cells also requires signaling by activating receptors and/or signaling by cytokines such as type I IFN or IL-12 (141). Activated NK cells produce high amounts of IFN-γ and use an arsenal of cytotoxic molecules like perforin or granzyme B to fulfill their cytotoxic functions (142). NK cells play a protective role in response to systemic HCMV and MCMV infections (110, 143) and are primarily responsible for immediate control of infection.

In addition to the immediate response of NK cells of the innate immune system, large numbers of MHC-II-restricted $CD4^+$ T cells as well as MHC-I-restricted $CD8^+$ T cells of the adaptive immune system specifically target HCMV or MCMV antigens during viral infection (110, 142, 144). More so than the HCMV- or MCMV-specific antibody response of B cells, T cells keep the virus in check throughout the life of the host and play a role in the balance between persistent infection and latency (141, 142). The importance of $CD4^+$ and $CD8^+$ T cells in controlling lifelong

HCMV or MCMV infection is underscored by the profound susceptibility to cytomegalovirus-derived pathologies that occur during depletion or dysfunction of these cells (24, 25, 107, 108, 110, 145–152).

HERPESVIRUSES AFFECTING HOST SOCS PROTEINS

Because of the immunomodulatory effects of SOCS proteins, it is not surprising that infectious microbes may take advantage of host SOCS expression. Indeed, SOCS1 and/or SOCS3 exploitation by such viruses as human immunodeficiency virus (HIV) (153–156), hepatitis B virus (157), hepatitis C virus (158, 159), Semliki forest virus (56), respiratory syncytial virus (160), coxsackievirus (161), Ebola virus (162), influenza A virus (163), HSV-1 (164–166), and EBV (12) has been beautifully reviewed elsewhere (5, 6). As Akhtar and Benveniste foresaw, more viruses affecting SOCS proteins have been discovered, many of them herpesviruses. In addition to HSV-1, these include the human herpesviruses VZV (17), HCMV (14), and KSHV (13), as well as the animal herpesviruses MDV (15, 19), PRV (20), MCMV (11, 16, 18, 21, 23), and MHV-68 (22) (**Table 2**). In addition to these, recent reports discuss the effects of SOCS2 gene knockout during infection with HSV-1 (171), HSV-2 (172), or BHV-5 (173). It is likely that still more viruses affecting SOCS proteins will be discovered in the future.

TABLE 2 | Herpesviruses that manipulate host SOCS expression.

Virus	SOCS	Cell/tissue type	Effect	References
HSV-1	↑SOCS1	HEL-30 (*not L929*), J774A.1 at M0, FL	↓IFN-γ signaling, ↑viral replication	(166–168)
	↑SOCS3	FL, TALL-1, CCRF-CEM (*not U937, THP-1, AKATA*)	↓IFN-α/β signaling, ↑viral replication	(164, 165)
VZV	↑SOCS1	MRC-5, HaCaT		(17)
	↑SOCS3	MRC-5, HaCaT, THP-1	↓IL-6 production, ↑viral gene expression	
HCMV	↑SOCS1 ↑SOCS3	Human MoDC		(14)
EBV	↑SOCS1 ↑SOCS3	HK-1, NP69 PBMC	↓JAK/STAT ↓IFN-α/β positive feedback signaling	(12, 169)
KSHV	↑SOCS3	Primary human endothelial cells	↓neutrophil recruitment ↓IFN-γ/STAT1 signaling, ↓MHC II, CIITA	(13, 170)
MDV	↑SOCS1 ↑SOCS3	Thymus, spleen, and skin of chickens	Unknown	(15, 19)
PRV	↑SOCS3	RAW264.7		(20)
MCMV	↑SOCS1 ↑SOCS3	BMM, IC-21, MEF, mouse eyes during experimental MCMV retinitis	↑Severity retinitis correlation	(11, 18, 21, 23)
MHV-68	↑SOCS1	BMM, RAW264.7 (*but not MLE-12, NIH3T3*)	↓IFN-γ signaling ↑viral replication	(22)

↑ increases; ↓ decreases. Cells: HEL-30 mouse keratinocytes, L929 mouse fibroblasts, J774A.1 mouse macrophages, FL human amnion cell line, TALL-1 T-cell leukemia cell line CCRF-CEM T-lymphoblastoid cell line, U937 and THP-1 human monocytes, AKATA EBV-negative clone of the Burkitt's lymphoma B-cell line, MRC-5 human lung fibroblasts, HaCaT human keratinocytes, monocyte-derived dendritic cell (MoDC), HK-1 and NP69 human nasopharyngeal epithelial cell lines, primary human peripheral blood mononuclear cells (PBMC), RAW264.7 mouse (BALB/c strain) macrophages, primary mouse bone marrow macrophages (BMM), IC-21 mouse (C57BL/6 strain) macrophages, MLE-12 mouse lung epithelial cells, NIH3T3 mouse fibroblasts.

Human Herpesviruses and SOCS1 or SOCS3

The consequences of virally manipulated SOCS1 and SOCS3 expression during HSV-1 infection are probably thus far the best studied among herpesviruses. After hepatitis C virus, HSV-1 is the second virus reported to stimulate host SOCS3 (164). In the human amnion cell line FL (174), this SOCS3 induction occurs very early, within 1 h post-infection (hpi) and coincides with reduction in type I IFN signaling downstream of JAK phosphorylation (164). The same group soon after reported that this is cell-type-specific, as SOCS3 is upregulated within 1 hpi (HSV-1 strain VR3) in the human T-cell leukemia cell line TALL-1 and the T-lymphoblastoid cell line CCRF-CEM, but not in human U937 or THP-1 monocytic cell lines, nor in an EBV-negative clone of the Burkitt's lymphoma B-cell line AKATA (165). This SOCS3 stimulation in FL cells is partly dependent on activation of JAK3 (165). Furthermore, siRNA-targeted suppression of SOCS3 results in lower HSV-1 virus titers in FL cells. Taken together, these studies provide strong evidence that during HSV-1 infection of FL cells, JAK3 signaling stimulates SOCS3, which then modulates the antiviral effects of IFN-α/β signaling, thus facilitating greater viral replication (165). Although this group found no stimulation of SOCS1 within 1 hpi in these cell types with HSV-1 strain VR3, they later detected both SOCS1 and SOCS3 transcriptional stimulation by RT-qPCR in FL cells at 4 hpi that is dependent on the HSV-1 UL13 protein kinase (167). Still others (166) later reported that HSV-1 strain syn17$^+$ stimulates SOCS1 expression between 1 and 6 hpi in HEL-30 keratinocytes but not L929 fibroblasts, cell lines derived from mouse strain C3H. Importantly, this correlates with the ability of IFN-γ to protect L929 cells but not HEL-30 cells from HSV-1-induced cell death, with inhibition of STAT1α activation downstream of IFN-γ signaling, and with transcriptional activation of the SOCS1 promoter (166). In primary human astrocytes and neurons, SOCS1 expression during HSV-1 infection is significantly reduced by exposure to type III IFN (IFN-λ) in primary human astrocytes and neurons (175). This cell type specificity for virologic and/or immunologic outcomes is a common theme with herpesviruses, with some outcomes even limited to specific cell activation phenotypes. For instance, HSV-1 infection stimulates SOCS1 in unactivated (M0) J774A.1 mouse macrophages (BALB/cN strain), but not in M1 nor M2 activated macrophages (168).

The α-herpesvirus VZV of the *Varicellovirus* genus initially infects the lungs then disseminates through the blood to cause skin lesions characteristic of varicella (chicken pox). The virus

establishes lifelong latency in dorsal root ganglia, where it may reactivate to cause herpes zoster (shingles) (176). Primary infection elicits an innate immune response characterized by stimulation of IFN-α and IFN-γ (17, 176) that is kept in check by multiple viral mechanisms (177). In immunocompetent individuals, adaptive immunity follows, and although anti-VZV antibodies are abundantly produced by B cells, an effective T-cell response is more important for control of severe disease (178), as with many herpesviruses. During experimental *in vitro* infection of permissive cell lines, VZV stimulates SOCS1 and, to a greater extent, SOCS3 in HaCaT human keratinocytes and MRC-5 human lung fibroblasts, and it also stimulates SOCS3 but not SOCS1 in THP-1 human monocytes (17). Suppression of SOCS3 by siRNA significantly reduces viral gene expression and greatly increases IL-6 production during VZV infection of MRC-5 cells (17).

The β-herpesvirus HCMV persistently infects about 80% of the worldwide population without usually causing disease in immunocompetent individuals (110, 179). As with most herpesviruses, most severe HCMV pathologies present only during immune suppression, as in HIV/AIDS patients or solid organ recipients, or underdevelopment of immunity (congenital cytomegalovirus) rather than in immunocompetent hosts. AIDS-related HCMV retinitis, for instance, causes vision loss and blindness in ∼30% of untreated AIDS patients (110, 152, 180–183). Upon primary infection, HCMV disseminates via the blood to various organs and establishes latency in circulating monocytes and bone marrow cells (129). Monocyte-derived DCs infected with HCMV (TB40/E or VHLE strains with endothelial cell tropism) stimulate SOCS1 and SOCS3 compared with uninfected cells (14). SOCS3 upregulation in these cells occurs via HCMV stimulation of IL-6/STAT3 signaling, and once stimulated, SOCS3 but not SOCS1 inhibits STAT5 activation downstream of the granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor (14). GM-CSF/STAT5 signaling in monocytic cells drives differentiation toward DCs, and inhibition of this pathway in already-differentiated DCs by HCMV-driven SOCS3 changes their phenotype from $CD1a^+$ to $CD1a^-$, rendering them inefficient at presenting lipid antigens to T cells (14). Genome sequence analysis of human epithelial HEK293 cells stably expressing the HCMV viral protein US27 showed stimulation of SOCS2 and SOCS5, but not SOCS3, compared with nontransfected HEK293 cells (184), suggesting that the HCMV-encoded G-protein coupled receptor protein US27 may not contribute to SOCS3 stimulation. Like many other herpesviruses, the HCMV genome contains homologs presumably purloined from their hosts (185), such as HCMV-encoded vIL10 (186). HCMV vIL10 stimulates SOCS3 in HeLa cells (187) and monocytes (188). These studies demonstrate pathways whereby HCMV indirectly stimulates SOCS1 and/or SOCS3 in various cell types, which then functionally change host and/or bystander cells to contribute to viral immune evasion.

EBV is a γ-herpesvirus in the genus *Lymphocryptovirus* that ubiquitously infects most of the world's population, frequently without symptoms, and establishes latency in B cells (189). Along with causing most cases of infectious mononucleosis, EBV also is associated with many types of cancer such as nasopharyngeal carcinoma and Burkitt's lymphoma (189, 190). Although the virus efficiently infects B-cell lines *in vitro*, experimental infection of epithelial cells has been more difficult, requiring innovative strategies to develop such model systems (191–193). During persistent EBV infection of the HK-1 and NP69 human nasopharyngeal epithelial cell lines, signaling pathways including STAT3 and NF-κB are activated compared with uninfected cells, resulting in transcriptional upregulation of downstream targets, including SOCS1 and SOCS3 (169). During EBV infection of human PBMCs, the viral Zta or ZEBRA protein stimulates SOCS3, thereby downregulating JAK/STATs involved with IFN-α/β positive feedback signaling (12).

KSHV (HHV-8), an oncogenic γ-herpesvirus, is the etiological agent of Kaposi's sarcoma (194), a neoplasm of endothelial cells that is characterized by dysregulated angiogenesis and massive inflammation, found primarily in patients with HIV/AIDS (195). During latency, KSHV expresses latency-associated nuclear antigen (LANA) that contains a virally-encoded SOCS box motif, which binds to host cell ubiquitination machinery and flags target proteins including tumor suppressor p53 for proteasome degradation (196). Not only does KSHV encode its own SOCS box-containing protein, it also indirectly induces host SOCS3 in endothelial cells. When infected with KSHV, immortalized human TIME dermal microvascular endothelial cells (DMVECs) significantly induce SOCS3 over uninfected cells or cells infected with UV-inactivated virus at 24, 48, and 96 hpi (197). Like other herpesviruses, KSHV also encodes many proteins homologous with host proteins as well as its own viral-encoded microRNA sequences (195). KSHV-encoded microRNA miR-K12-3 and miRK-12-7 stimulate IL-6 and IL-10 in RAW264.7 mouse macrophages and human myelomonocytic leukemia MM6 cells (198). KSHV-infected primary human endothelial cells repress neutrophil recruitment through stimulation of host IL-6 and SOCS3 (13), and SOCS3 stimulation also suppresses MHC II expression on these cells by suppression of IFN-γ/STAT1 signaling and the downstream class II transactivator (CIITA) (170). Therefore, KSHV and other herpesviruses contain multiple strategies to evade immune surveillance, including stimulation of host SOCS1 and/or SOCS3 by multiple mechanisms.

Animal Herpesviruses and SOCS1 or SOCS3

MDV (GaHV-2) in the *Mardivirus* genus is an oncogenic α-herpesvirus of chickens. MDV is the etiological agent of Marek's disease, characterized by immunosuppression, neurological disorders, and $CD4^+$ T-cell lymphoma with subsequent solid tumors (115, 116). Transmission occurs through inhalation or ingestion of contaminated dust and dander from feather follicle epithelium (199) of the skin of infected chickens. The virus infects many cell types, including lymphocytes, which disseminate through the blood to various organs, including the thymus and spleen (115, 116). Analyses of whole genome arrays have shown that 2–4 days following systemic MDV infection of chickens, SOCS1 and SOCS3 are stimulated in thymus and spleen tissues, with greater upregulation occurring in chicken strains that are

more susceptible to MDV (15). Transcriptional stimulation of host SOCS1 and SOCS3 was also found in skin samples of MDV-infected chickens at 20 and 30 days post-infection (19). The specific effects of SOCS1 and/or SOCS3 stimulation during MDV infection are yet unknown.

PRV (SuHV-1) is a *Varicellovirus* endogenous to swine but can infect many different animal and cell types. It therefore has been widely used in various animal model systems, including as a neural tracer (200). In a recent study using PRV infection of RAW264.7 mouse macrophages as an oxidative stress model to measure the antioxidant qualities of *Dunaliella salina* alga extract, it was incidentally reported that PRV induces expression of SOCS3 in these cells at 12 and 24 hpi (20). To our knowledge, thus far the impact of SOCS3 stimulation on PRV infection or pathology remains unknown, as does the effect of PRV on SOCS1 expression.

Mouse-specific salivary gland virus (201, 202), now called MCMV, is in the *Muromegalovirus* genus of the β-herpesvirus subfamily. It frequently is used in experimental mouse models and has contributed greatly to our understanding of infection and pathogenesis of its human-specific counterpart, HCMV (108, 203). HCMV and MCMV both establish latency in circulating monocytes and bone marrow cells (129). SOCS1 and SOCS3 are stimulated very early after *in vitro* MCMV infection of bone marrow macrophages (BMM) (11) as well as IC-21 mouse macrophages and mouse embryonic fibroblast (MEF) cells (18). This stimulation and its temporal patterns are dependent on host cell type and on the mouse strain (C57BL/6 or BALB/c) used for propagation of the MCMV stocks (18). In addition to these *in vitro* models, we have observed in our laboratory that after intraocular (subretinal) MCMV inoculation of immunocompromised mice during experimental MCMV retinitis, SOCS1 and SOCS3 mRNA (16, 23) and protein (21) are upregulated in retinitis-susceptible eyes. As a major topic of focus in this review, the effects of SOCS1 and/or SOCS3 stimulation in this model are discussed in greater detail in a subsequent section of this review.

MHV-68 (or γHV-68) of the *Rhadinovirus* genus natively infects rodents such as mice and voles (204, 205). Because of its genomic and physiologic similarities with both EBV and KSHV, MHV-68 infection of mice is a useful animal model to study pathogen-host interactions of these human γ-herpesviruses (206, 207). It persistently infects lung epithelial cells and establishes latency in B cells, macrophages, and DCs (208). In yet another demonstration of cell type specificity, SOCS1 mRNA and protein are induced upon MHV-68 infection of mouse BMMs and RAW264.7 mouse macrophages, but not MLE-12 mouse lung epithelial cells, NIH3T3 fibroblasts, or MEF cells (22). Transcription of viral genes is likely required for SOCS1 stimulation as UV-inactivation of the virus abrogates this effect. Viral induction of the TLR3/NF-κB pathway induces SOCS1, which then inhibits the antiviral effects of IFN-γ through inhibition of pSTAT1, resulting in increased viral titers (22). Suppression of SOCS1 during MHV-68 infection restores the antiviral qualities of IFN-γ signaling (22). None of these cell types produced SOCS3 stimulation during MHV-68 infection.

Alphaherpesviruses and SOCS2

In addition to these findings with SOCS1 and SOCS3, a few studies also explore the effects of SOCS2 during α-herpesvirus infection. Following intracranial injection with HSV-1, SOCS2-deficient mice are more resistant to HSV-1 encephalitis, neuroinflammation, and immune cell infiltration to the brain compared with wild type C57BL/6 mice (171), suggesting that SOCS2 contributes to the severity of this disease. HSV-2, the causative agent of genital herpes, has long been debated to have a putative involvement in oncogenesis, particularly as a cofactor in cervical cancer, but this remains unproven (209). In LTEP-α-2 and SPC-α-1 human lung cancer cell lines experimentally infected with HSV-2, the virally-encoded microRNA Hsv2-miR-H9-5p targets and inhibits SOCS2, thereby driving experimental tumor metastasis in these cell lines (172). BHV-5 in the *Varicellovirus* genus natively infects cattle but can establish productive infection in rabbits and mice, which are frequently used as animal models to study neurological disease caused by this virus (173). Unlike HSV-1 infection, infection with BHV-5 exacerbates meningoencephalitis in SOCS2-knockout mice compared with wild type animals (173), suggesting a protective role during intracranial BHV-5 infection. Although it remains unknown whether HSV-1 or BHV-5 stimulates or dampens host SOCS2 expression in these models, SOCS2 nevertheless plays a multivariate role in the pathologies of these herpesviruses.

CYTOMEGALOVIRUS RETINITIS AND SOCS

Despite the development of antiretroviral therapies to treat HIV infection, AIDS-related HCMV retinitis remains a major sight-threatening disease worldwide (110, 152, 180–183). Understanding the pathogenesis of this disease is essential for developing new, safe, and effective treatments for its prevention or management in the clinical setting, yet much remains unknown about the virologic and immunologic mechanisms contributing to its pathology. The pathogenesis of AIDS-related HCMV retinitis involves the complex orchestration of cytomegalovirus infection during AIDS-mediated progressive destruction of the immune system, within the context of retinal cells in the eye.

Vision is facilitated by a complex system whose gross anatomy, microanatomy, biophysical, and biochemical properties are critical to its function. Disruption of any one of thousands of components of this system could lead to visual impairment or blindness. Light first encounters the cornea, which acts as a powerful lens to focus light through the liquid-filled anterior chamber, through the aperture of the pupil, and into the crystalline lens. The lens focuses light with greater precision through the viscous vitreous gel and onto the parfait-like layers of the neurosensory retina at the back of the eye. Photoreceptors in the retina detect photons of light and transmit signals through first-order, second-order, and third-order neurons into ganglion cell axons that exit the eye as the optic nerve. The specialized neuronal cells of the retina are supported by networks of Müller cells, astrocytes, and microglia, as well as by the RPE,

a specialized layer of phagocytic, multifunctional epithelial cells (210). As part of the posterior segment of the eye and an extension of the brain, the retina is considered an immune-privileged site (211) primarily because it does not elicit a typical inflammatory immune response to the introduction of antigens (212, 213). Thus, irreplaceable neuronal tissue is somewhat protected from the damaging effects of inflammation and immunopathogenesis.

AIDS-Related HCMV Retinitis

When the immune privilege of the ocular compartment fails, inflammation causing severe immunopathogeneses and permanent, sight-threatening damage may occur, as in the case of AIDS-related HCMV retinitis. Prior to the era of antiretroviral therapies, this progressive necrosis of the retina is estimated to have occurred in ∼30% of HIV/AIDS patients with $CD4^+$ T-cell counts fewer than 50 cells/μL blood (25, 180, 181, 214–216). Antiretroviral therapies targeting HIV have greatly reduced the number of new cases of AIDS-related HCMV retinitis in developed countries (151, 180) but have failed to eliminate them completely (215). This disease therefore remains a significant clinical problem worldwide.

Although HCMV is ubiquitous in the population and relatively mild as an infectious disease of immunocompetent individuals, it can become a severe opportunistic pathogen during the immune suppression that occurs when HIV infection progresses to AIDS. It is likely that during AIDS-related HCMV retinitis, HCMV reactivates from latency and travels to the eye hematogenously within monocytes or macrophages, as ophthalmoscopic examination of the retina reveals the characteristic foci of dense retinal whitening that follow retinal blood vessels and may be accompanied by hemorrhage (151). Failure to treat AIDS-related HCMV retinitis results in blindness of most or all of the affected eye, usually followed within 1 year by vision loss in the contralateral eye (110, 152, 180–183). The mechanisms of blindness involve destruction of the retina itself, retinal detachment, or a uveitis that can occur with reconstitution of the immune system associated with well-tolerated antiretroviral therapies (immune recovery uveitis, IRU) (151, 180). Current treatment strategies for HIV/AIDS patients presenting with HCMV retinitis target HCMV replication through lifelong administration of antiviral drugs such as ganciclovir, cidofovir, or foscarnet that can control but not eradicate the virus, slowing but not reversing HCMV-induced ocular damage (217–221). Unfortunately, frequent administration of these drugs has led to an increase in drug-resistant strains of HCMV (222). Vaccination has been one of the most effective methods for controlling other problematic infectious diseases, but attempts to engineer a suitably efficacious vaccine against HCMV thus far have been unsuccessful (223, 224).

Mouse Models of Experimental Cytomegalovirus Retinitis

Because the species-specificity of HCMV precludes its ability to establish productive infection in animal models or cells (225), MCMV is commonly substituted in research laboratories to investigate cytomegalovirus infection and pathogenesis in mouse models (108, 203) because of high face validity and predictive validity (27). Such research with MCMV has significantly improved our collective understanding of HCMV characteristics and pathogeneses, including the involvement of immune cell types such as $CD8^+$ T cells and NK cells in controlling infection (110).

As with humans and HCMV, immunologically normal mice are generally resistant to MCMV retinitis (24, 147, 226, 227), depending on mouse strain (228, 229), viral load, and route of viral inoculum (230–232). Establishment of an immune-suppressed state together with delivery of a substantial amount (10^4 plaque forming units, pfu) of MCMV directly into the subretinal space of the eye overcomes this resistance, consistently manifesting high frequencies (75–100%) of experimental MCMV retinitis (29, 150, 230) in a manner dependent upon viral load (230) and mouse strain (24, 150, 228–233). Two successful immunosuppression strategies to achieve susceptibility to MCMV retinitis include systemic delivery of corticosteroid drugs (150, 230, 234) or a mixture of mouse-specific retroviruses designated lymphoproliferative-bone marrow 5 (LP-BM5) (235, 236) that induces MAIDS after 8–10 weeks in C57BL/6 mice (26, 237, 238).

The strain of mouse used during experimental MCMV retinitis studies impacts susceptibility to MCMV infection and to the MAIDS-producing LP-BM5 retrovirus mixture. BALB/c mice are more susceptible than C57BL/6 mice to systemic MCMV infection (228, 231, 239–242), and this appears to affect the incidence of experimental retinitis in the corticosteroid model. During corticosteroid-induced immune suppression, the frequency of MCMV retinitis in BALB/c mice is about 90% (150), compared with 50% in C57BL/6 mice (23, 233). BALB/c mice, however, are more resistant than C57BL/6 mice to the induction of MAIDS by LP-BM5 (26, 243), as C57BL/6 mice reach late-phase MAIDS within 10 weeks whereas a year or longer is required for BALB/c mice to progress to late-stage MAIDS. For this reason, although BALB/c mice are generally used for experimental MCMV retinitis models with corticosteroid-induced immune suppression, C57BL/6 mice are used for MAIDS models. Importantly, the frequency of experimental MCMV retinitis after subretinal MCMV injection in C57BL/6 mice with MAIDS is 80–100% (24, 226, 227), comparable with the frequency in drug-immunosuppressed BALB/c mice (150).

Just as later stages of AIDS in humans correlates with greater susceptibility to HCMV retinitis, so mice with late-stage MAIDS at 10 weeks (MAIDS-10) are more susceptible to MCMV retinitis than mice with early- or mid-stage MAIDS around 4 weeks (MAIDS-4). Importantly, SOCS1 and SOCS3 are highly stimulated following subretinal MCMV infection in the retinitis-susceptible eyes of MAIDS-10 mice, but not in the MCMV-infected retinitis-resistant eyes of MAIDS-4 mice (16, 21). In C57BL/6 mice with corticosteroid-induced immune suppression, however, subretinal MCMV infection does not significantly alter SOCS1 or SOCS3 protein expression and only mildly stimulates SOCS3 mRNA (23). To our knowledge, the effect of subretinal MCMV infection on SOCS1 and SOCS3 expression in the

eyes of BALB/c mice during corticosteroid-induced immune suppression has not been reported to date.

In the absence of MCMV infection, these two different techniques to accomplish immune suppression also differ in their types of dysfunctional immune cells and the timing of immune cell demise (23). One of the major differences between these models is the number and function of macrophages. MAIDS, without MCMV infection, causes reduced $Mac1^+$ ($CD11b^+$) macrophage population percentages and activation frequencies at MAIDS-4 (237, 244), with increased macrophage numbers between MAIDS-8 and MAIDS-12 (245). Macrophage populations in MAIDS mice are driven toward an alternatively-activated proangiogenic phenotype that is between classically-activated M1 and alternatively-activated M2. They have decreased TNF-α and IFN-α production but increased IL-1β and IL-6 production in response to LPS (246, 247). By contrast, corticosteroids such as methylprednisolone acetate, in the absence of MCMV infection, very quickly suppress or destroy most of both the innate and adaptive immune systems, including macrophages (248). Whatever macrophages remain tend to be driven toward the M2 alternatively-activated phenotype, in a similar manner as macrophages exposed to IL-4, and they avidly produce IL-10, but not TNF-α, IL-1, or IL-6 (134, 135). Therefore, whereas MAIDS mice experience a functional change in macrophage phenotype after weeks (245–247), drug-induced immune suppression decreases macrophage populations within days (248). Corticosteroids also decrease the overall number and function of $CD4^+$ and $CD8^+$ T cells [∼93% depletion, (234, 248, 249) and generally dampen the immune response by suppressing the expression, release, and/or function of inflammatory cytokines such as IFN-γ TNF-α, and IL-2 (249). This rapid, acute decline of the immune system is not observed during MAIDS, which slowly progresses through distinct phases of immune cell dysfunction. Whereas corticosteroid treatment causes apoptosis in leukocytes and lymphocytes therefore decreasing the overall number of these populations (248, 249), MAIDS causes aberrant proliferation of B and T lymphocytes (250, 251) that results in increases in these cell populations coupled with retrovirus-induced cellular dysfunction (26, 251, 252). By late-stage MAIDS, NK cells (253), and neutrophils (254) are also dysfunctional, and macrophage phenotypes are irregular (245–247).

Throughout the many years that these mouse models have been studied, both drug-induced and retrovirus-induced immune suppression strategies during subretinal MCMV infection have contributed to our collective theoretical knowledge of MCMV retinitis and our clinical knowledge of HCMV retinitis. While the drug-induced immune suppression model yields relatively faster results, it bypasses the many nuances and complexities of retroviral immune suppression that the MAIDS model alone bridges to clinical relevance.

MAIDS-Related MCMV Retinitis and SOCS

AIDS of humans and MAIDS of mice are both caused by species-specific retroviruses and share many immunologic and pathologic features (26, 237). Both syndromes are characterized by progressive generalized lymphadenopathy, polyclonal B-cell activation (250), diminished $CD4^+$ T-cell and $CD8^+$ T-cell functions (251), and a cytokine shift from a T_H1 origin to T_H2-associated cytokines (236, 255, 256). Although profound splenomegaly also occurs in MAIDS mice, this overall increase in splenic cell counts is associated with dysfunctional immune cells (257). By MAIDS-10, B cells (247, 258), $CD4^+$ and $CD8^+$ T cells (245, 251, 259), NK cells (253), and neutrophils (254) are dysfunctional, and macrophage phenotypes are irregular (245–247). Mice with late-stage MAIDS (8–12 weeks) develop a retinitis at 8–10 days following subretinal MCMV injection that exhibits histopathologic features similar to those found in AIDS-related HCMV retinitis (24, 260), including full-thickness retinitis, cytomegalic cells, and transition zones of histologically normal to necrotic retina. **Table 3** summarizes the similarities and differences between the retroviruses causing AIDS or MAIDS, and between HCMV retinitis and MCMV retinitis during each, respectively.

TABLE 3 | AIDS-related HCMV retinitis vs. MAIDS-related MCMV retinitis.

	AIDS-related HCMV retinitis	MAIDS-related MCMV retinitis
Retrovirus-Induced Immune Suppression		
Macrophages among targeted cell types	Yes	Yes
Polyclonal B-cell activation	Yes	Yes
Hypergammaglobunemia	Yes	Yes
Splenomegaly	No	Yes
T_H1-to-T_H2 cytokine shift	Yes	Yes
Diminished $CD4^+$ and $CD8^+$ T-Cell:		
Numbers	Yes	No
Functions	Yes	Yes
Cytomegalovirus Retinitis Histologic Characteristics		
Foci of cytomegalic cells	Yes	Yes
Hemorrhage	Yes	Yes
Transition zones between normal and necrotic retina	Yes	Yes
Full-thickness retinal necrosis	Yes	Yes

Reviewed in Jolicoeur (26) and Watson (237).

Immunologically normal C57BL/6 mice and MAIDS-4 C57BL/6 mice are resistant to MCMV retinitis (0% frequency). Mice with MAIDS-8 to MAIDS-12, however, are susceptible (80–100%) to MCMV retinitis following subretinal (24, 226, 227), but not systemic (232), MCMV inoculation. Importantly, retinitis susceptibility does not correlate with ocular viral titers, because MCMV replication in the ocular compartment at 6–10 days after subretinal inoculation reaches equivalently high levels ($\sim 3 \times 10^4$ pfu/eye) in retinitis-resistant MAIDS-4 mice as those in retinitis-susceptible MAIDS-10 mice (227, 261). By comparison, immunologically normal mice receiving the same amount of subretinally-injected MCMV typically produce only $\sim 10^2$ pfu/eye (24). Thus, high intraocular MCMV titers alone are insufficient for retinitis, and susceptibility to intraocular MCMV replication precedes susceptibility to retinitis in this model (227).

Thus far mechanisms of humoral immunity (262), cellular immunity (263, 264), cell death pathways (261), and several

cytokines have been studied during onset and development of retinal disease in the MAIDS model of MCMV retinitis. Among the putative SOCS-inducing cytokines examined in this model are TNF-α (227, 261), IFN-α/β and IL-6 (21), IFN-γ (21, 227), IL-2 (265, 266), IL-12 (266), IL-4 (226, 267), IL-10 (267), and IL-17 (16). In addition, SOCS1 and SOCS3 are highly stimulated following MCMV infection in retinitis-susceptible MAIDS-10 eyes, but not MCMV infected retinitis-resistant MAIDS-4 eyes (16, 21). In MAIDS-10 eyes with MCMV retinitis, SOCS1 and SOCS3 are produced by infiltrating macrophages and granulocytes, as well as resident microglia and Müller cells (21). Uninfected bystander cells as well as MCMV-infected cells of the retina also abundantly produce SOCS1 and SOCS3 (21), a phenomenon that also has been reported in MCMV-infected IC-21 macrophages (18) and in HCMV-infected monocyte-derived DCs (14). Systemic MCMV in immunocompetent mice without MAIDS moderately stimulates splenic SOCS1 transcripts and SOCS-inducing cytokines IFN-γ and IL-6, but this stimulation decreases in amplitude as MAIDS progresses (21). Furthermore, there is a decreased intraocular stimulation of SOCS1 and SOCS3 during experimental MCMV retinitis during corticosteroid-induced immune suppression that correlates with reduced severity of retinitis (23). Thus, during *in vivo* MCMV infection, substantial and extended SOCS1 and SOCS3 stimulation appears only in the eye (21) and is correlated with more severe MCMV retinitis (23). Stimulation of pro-inflammatory and antiviral cytokines such as TNF-α and IFN-γ in the eyes of mice with severe MAIDS-related MCMV retinitis fails to control viral replication, but concurrent stimulation of anti-inflammatory cytokines like IL-10 and IL-4 is not sufficient for protection against ocular immunopathogenesis in this disease model (21). Although many questions remain, SOCS1 and/or SOCS3 may play promising roles in the balance of this phenomenon, potentially revealing themselves as novel therapeutic targets to improve the management and/or prevention of AIDS-related HCMV retinitis.

SOCS1 or SOCS3 as Potential Therapeutic Targets During Cytomegalovirus Retinitis

Several strategies for inhibiting or enhancing SOCS1 or SOCS3 gene expression or protein activity in the context of infectious or inflammatory diseases, including over-expression or inhibition gene therapies via viral vectors, have been developed and tested *in vitro* and *in vivo* with promising results, as summarized elsewhere (6). One attractive approach to control the functions SOCS1 and/or SOCS3 includes therapeutic use of small-molecule protein antagonists or mimetics of SOCS1 and/or SOCS3 proteins.

Although stimulation of SOCS1 and SOCS3 during experimental MAIDS-related MCMV retinitis suggests that one or both of these contribute to the severity of the disease, at this time it remains unknown whether SOCS1 and/or SOCS3 inhibition or overexpression would improve the clinical outcome of AIDS-related HCMV retinitis. If SOCS1 and/or SOCS3 contribute to the pathogenesis of this disease, then their inhibition in HIV/AIDS patients with HCMV retinitis could prevent further damage to affected eyes and/or protect the contralateral eye from vision loss. One such SOCS-sequestering small synthetic peptide is pJAK2[1001–1013] (LPQDKEYYKVKEP), which includes the phosphorylated activation loop of JAK2 (44, 268) and antagonizes both SOCS1 and SOCS3. This peptide has shown efficacy against HSV-1 infection in keratinocytes (166) and protects against lethal doses of vaccinia virus, encephalomyocarditis virus, and influenza A virus in mice (269, 270). Because SOCS1 and SOCS3 dampen the ability of cytokines to propagate effective signals within their target cells, inhibition of SOCS1 and/or SOCS3 coupled with immunotherapy treatments such as antiviral IFNs (271) could improve the efficacy of such treatments.

It remains a possibility that the immunosuppressive effect of SOCS1 and/or SOCS3 may play a protective role against a potential immunopathology of experimental MCMV retinitis or AIDS-related HCMV retinitis. If overexpression of SOCS1 and/or SOCS3 reduces retinitis severity, SOCS1 and/or SOCS3 mimetic peptides or overexpression treatment strategies could be efficacious against this disease, as with experimental autoimmune uveitis (EAU) (272, 273). This seems to be the case for HSV-1 infection in the eye, where the role of SOCS1 during HSV-1 infection appears to be protective despite *in vitro* HSV-1 infection stimulating SOCS1 and SOCS3 very early to increase viral load and cytopathology in different cell types (166, 175). In transgenic rats overexpressing SOCS1 in the retina, however, intraocular HSV-1 (McKrae strain) infection is reduced or delayed compared with wild type rats (274). These SOCS1-overexpressing rats bred to a Lewis strain background also display reduced severity during interphotoreceptor retinoid binding protein (IRBP) antigen-induced (retina-specific) EAU (275). In a mouse model of IRBP antigen-induced EAU, treatment with the cell-penetrating SOCS1-KIR-derived peptide (272, 273) reduces severity of disease. EAU is also less severe in mice containing a conditional SOCS3 knockout in $CD4^+$ T-cells (276). The anti-inflammatory role of SOCS1 and/or SOCS3 functioning with cell-type-specificity within the complexity of the eye may therefore protect the precious cells of the retina during immunopathologies such as intraocular HSV-1 infection or autoimmune uveitis. Further studies utilizing knockdown or overexpression of SOCS1 or SOCS3 would elucidate this possibility for experimental MCMV retinitis and/or AIDS-related HCMV retinitis.

CONCLUDING REMARKS

Host manipulation strategies among herpesviruses, diverse and redundant, share many similarities, such as stimulation of host SOCS1 and/or SOCS3. The virologic, immunologic, and pathologic effects of SOCS1 or SOCS3 stimulation during herpesvirus infection frequently depend on cell type, virus strain, and host or host organ system. Such parameters reflect the complexities of the diverse cells and organ systems directly or indirectly involved with herpesvirus infection, disease, and latency. Although it remains unclear whether viral stimulation of SOCS1 and/or SOCS3 is protective or pathogenic in the

eye during AIDS-related cytomegalovirus retinitis, these host proteins may yet prove useful therapeutic targets for treatment or prevention of this sight-threatening disease, as well as other disease of herpesvirus etiology.

AUTHOR CONTRIBUTIONS

CA composed and RD conceptualized this review. Both authors contributed to manuscript revision and approved the submitted version.

ACKNOWLEDGMENTS

This work was supported in part by NIH/NEI grants EY010568 and EY024630, NIH/NEI Core Grant P30EY006360, Emory Eye Center Vision Training Grant NIH/NEI T32-EY007092, and Fight for Sight, Inc. Part of this review was reproduced from the dissertation of CA (277). Illustrations were assembled using the Motifolio Biology Illustration Toolkit (motifolio.com). We sincerely apologize to any authors whose work may be relevant to this review but is not included.

REFERENCES

1. Pellett PE, Roizman B. Herpesviridae. In: Knipe DM, Howley PM, editors. *Fields Virology*. 6th ed. Philadelphia, PA: Lippencott Williams and Wilkins (2013). p. 1802–22.
2. Matsubara T, Pararajasegaram G, Wu G-S, Rao NA. Retinal microglia differentially express phenotypic markers of antigen-presenting cells *in vitro*. *Invest Ophthalmol Vis Sci*. (1999) 40:3186–93.
3. Alexander WS, Starr R, Metcalf D, Nicholson SE, Farley A, Elefanty AG, et al. Suppressors of cytokine signaling (SOCS): negative regulators of signal transduction. *J Leukoc Biol*. (1999) 66:588–92. doi: 10.1002/jlb.66.4.588
4. Yoshimura A, Naka T, Kubo M. SOCS proteins, cytokine signalling and immune regulation. *Nat Rev Immunol*. (2007) 7:454–65. doi: 10.1038/nri2093
5. Akhtar LN, Benveniste EN. Viral exploitation of host SOCS protein functions. *J Virol*. (2011) 85:1912–21. doi: 10.1128/JVI.01857-10
6. Delgado-Ortega M, Marc D, Dupont J, Trapp S, Berri M, Meurens F. SOCS proteins in infectious diseases of mammals. *Vet Immunol Immunopathol*. (2013) 151:1–19. doi: 10.1016/j.vetimm.2012.11.008
7. Griffin BD, Verweij MC, Wiertz EJ. Herpesviruses and immunity: the art of evasion. *Vet Microbiol*. (2010) 143:89–100. doi: 10.1016/j.vetmic.2010.02.017
8. Paludan SR, Bowie AG, Horan KA, Fitzgerald KA. Recognition of herpesviruses by the innate immune system. *Nat Rev Immunol*. (2011) 11:143–54. doi: 10.1038/nri2937
9. White DW, Suzanne Beard R, Barton ES. Immune modulation during latent herpesvirus infection. *Immunol Rev*. (2012) 245:189–208. doi: 10.1111/j.1600-065X.2011.01074.x
10. Grinde B. Herpesviruses: latency and reactivation – viral strategies and host response. *J Oral Microbiol*. (2013) 5:22766. doi: 10.3402/jom.v5i0.22766
11. Strobl B, Bubic I, Bruns U, Steinborn R, Lajko R, Kolbe T, et al. Novel functions of tyrosine kinase 2 in the antiviral defense against murine cytomegalovirus. *J Immunol*. (2005) 175:4000–8. doi: 10.4049/jimmunol.175.6.4000
12. Michaud F, Coulombe F, Gaudreault E, Paquet-Bouchard C, Rola-Pleszczynski M, Gosselin J. Epstein-Barr virus interferes with the amplification of IFNalpha secretion by activating suppressor of cytokine signaling 3 in primary human monocytes. *PLoS ONE*. (2010) 5:e11908. doi: 10.1371/journal.pone.0011908
13. Butler LM, Jeffery HC, Wheat RL, Rae PC, Townsend K, Alkharsah KR, et al. Kaposi's sarcoma-associated herpesvirus infection of endothelial cells inhibits neutrophil recruitment through an interleukin-6-dependent mechanism: a new paradigm for viral immune evasion. *J Virol*. (2011) 85:7321–32. doi: 10.1128/JVI.00021-11
14. Carlier J, Martin H, Mariame B, Rauwel B, Mengelle C, Weclawiak H, et al. Paracrine inhibition of GM-CSF signaling by human cytomegalovirus in monocytes differentiating to dendritic cells. *Blood*. (2011) 118:6783–92. doi: 10.1182/blood-2011-02-337956
15. Smith J, Sadeyen JR, Paton IR, Hocking PM, Salmon N, Fife M, et al. Systems analysis of immune responses in Marek's disease virus-infected chickens identifies a gene involved in susceptibility and highlights a possible novel pathogenicity mechanism. *J Virol*. (2011) 85:11146–58. doi: 10.1128/JVI.05499-11
16. Blalock EL, Chien H, Dix RD. Murine cytomegalovirus downregulates interleukin-17 in mice with retrovirus-induced immunosuppression that are susceptible to experimental cytomegalovirus retinitis. *Cytokine*. (2013) 61:862–75. doi: 10.1016/j.cyto.2013.01.009
17. Choi EJ, Lee CH, Shin OS. Suppressor of cytokine signaling 3 expression induced by varicella-zoster virus infection results in the modulation of virus replication. *Scand J Immunol*. (2015) 82:337–44. doi: 10.1111/sji.12323
18. Alston CI, Dix RD. Murine cytomegalovirus infection of mouse macrophages stimulates early expression of suppressor of cytokine signaling (SOCS)1 and SOCS3. *PLoS ONE*. (2017) 12:e0171812. doi: 10.1371/journal.pone.0171812
19. Heidari M, Delekta PC. Transcriptomic analysis of host immune response in the skin of chickens infected with Marek's disease virus. *Viral Immunol*. (2017) 30:377–87. doi: 10.1089/vim.2016.0172
20. Lin HW, Liu CW, Yang DJ, Chen CC, Chen SY, Tseng JK, et al. Dunaliella salina alga extract inhibits the production of interleukin-6, nitric oxide, and reactive oxygen species by regulating nuclear factor-kappaB/Janus kinase/signal transducer and activator of transcription in virus-infected RAW264.7 cells. *J Food Drug Anal*. (2017) 25:908–18. doi: 10.1016/j.jfda.2016.11.018
21. Chien H, Alston CI, Dix RD. Suppressor of cytokine signaling 1 (SOCS1) and SOCS3 are stimulated within the eye during experimental murine cytomegalovirus retinitis in mice with retrovirus-induced immunosuppression. *J Virol*. (2018) 92:e00526-18. doi: 10.1128/JVI.00526-18
22. Shen Y, Wang S, Sun F, Zheng G, Wu T, Du Y, et al. Inhibition of murine herpesvirus-68 replication by IFN-gamma in macrophages is counteracted by the induction of SOCS1 expression. *PLoS Pathog*. (2018) 14:e1007202. doi: 10.1371/journal.ppat.1007202
23. Alston CI, Dix RD. Reduced frequency of murine cytomegalovirus retinitis in C57BL/6 mice correlates with low levels of suppressor of cytokine signaling (SOCS)1 and SOCS3 expression within the eye during corticosteroid-induced immunosuppression. *Cytokine*. (2017) 97:38–41. doi: 10.1016/j.cyto.2017.05.021
24. Dix RD, Cray C, Cousins SW. Mice immunosuppressed by murine retrovirus infection (MAIDS) are susceptible to cytomegalovirus retinitis. *Curr Eye Res*. (1994) 13:587–95. doi: 10.3109/02713689408999892
25. Jabs DA, Enger C, Bartlett JG. Cytomegalovirus retinitis and acquired immunodeficiency syndrome. *Arch Ophthalmol*. (1989) 107:75–80. doi: 10.1001/archopht.1989.01070010077031
26. Jolicoeur P. Murine acquired immunodeficiency syndrome (MAIDS): an animal model to study the AIDS pathogenesis. *FASEB J*. (1991) 5:2398–405. doi: 10.1096/fasebj.5.10.2065888
27. Denayer T, Stöhr T, Van Roy M. Animal models in translational medicine: validation and prediction. *New Horiz Transl Med*. (2014) 2:5–11. doi: 10.1016/j.nhtm.2014.08.001
28. Käser T, Renois F, Wilson HL, Cnudde T, Gerdts V, Dillon J-AR, et al. Contribution of the swine model in the study of human sexually transmitted infections. *Infect Genet Evol*. (2018) 66:346–60. doi: 10.1016/j.meegid.2017.11.022

29. Dix RD, Cousins SW. AIDS-related cytomegalovirus retinitis: lessons from the laboratory. *Curr Eye Res.* (2004) 29:91–101. doi: 10.1080/02713680490504641
30. Yoshimura A, Ohkubo T, Kiguchi T, Jenkins NA, Gilbert DJ, Copeland NG, et al. A novel cytokine-inducible gene CIS encodes an SH2-containing protein that binds to tyrosine-phosphorylated interleukin 3 and erythropoietin receptors. *EMBO J.* (1995) 14:2816–26. doi: 10.1002/j.1460-2075.1995.tb07281.x
31. Endo TA, Masuhara M, Yokouchi M, Suzuki R, Sakamoto H, Mitsui K, et al. A new protein containing an SH2 domain that inhibits JAK kinases. *Nature*. (1997) 387:921–4. doi: 10.1038/43213
32. Naka T, Narazaki M, Hirata M, Matsumoto T, Minamoto S, Aono A, et al. Structure and function of a new STAT-induced STAT inhibitor. *Nature*. (1997) 387:924–9. doi: 10.1038/43219
33. Starr R, Willson TA, Viney EM, Murray LJ, Rayner JR, Jenkins BJ, et al. A family of cytokine-inducible inhibitors of signalling. *Nature*. (1997) 387:917–21. doi: 10.1038/43206
34. Krebs DL, Hilton DJ. SOCS proteins: negative regulators of cytokine signaling. *Stem Cells*. (2001) 19:378–87. doi: 10.1634/stemcells.19-5-378
35. Babon JJ, Yao S, DeSouza DP, Harrison CF, Fabri LJ, Liepinsh E, et al. Secondary structure assignment of mouse SOCS3 by NMR defines the domain boundaries and identifies an unstructured insertion in the SH2 domain. *FEBS J.* (2005) 272:6120–30. doi: 10.1111/j.1742-4658.2005.05010.x
36. Williams JJL, Alotaiq N, Mullen W, Burchmore R, Liu L, Baillie GS, et al. Interaction of suppressor of cytokine signalling 3 with cavin-1 links SOCS3 function and cavin-1 stability. *Nat Commun*. (2018) 9:168. doi: 10.1038/s41467-017-02585-y
37. Pawson T, Nash P. Protein–protein interactions define specificity in signal transduction. *Genes Dev*. (2000) 14:1027–47.
38. Liu BA, Jablonowski K, Raina M, Arcé M, Pawson T, Nash PD. The human and mouse complement of SH2 domain proteins—establishing the boundaries of phosphotyrosine signaling. *Mol Cell*. (2006) 22:851–68. doi: 10.1016/j.molcel.2006.06.001
39. Bae JH, Lew ED, Yuzawa S, Tomé F, Lax I, Schlessinger J. The selectivity of receptor tyrosine kinase signaling is controlled by a secondary SH2 domain binding site. *Cell*. (2009) 138:514–24. doi: 10.1016/j.cell.2009.05.028
40. Sasaki A, Yasukawa H, Suzuki A, Kamizono S, Syoda T, Kinjyo I, et al. Cytokine-inducible SH2 protein-3 (CIS3/SOCS3) inhibits Janus tyrosine kinase by binding through the N-terminal kinase inhibitory region as well as SH2 domain. *Genes Cells*. (1999) 4:339–51. doi: 10.1046/j.1365-2443.1999.00263.x
41. Yasukawa H, Misawa H, Sakamoto H, Masuhara M, Sasaki A, Wakioka T, et al. The JAK-binding protein JAB inhibits Janus tyrosine kinase activity through binding in the activation loop. *EMBO J.* (1999) 18:1309–20. doi: 10.1093/emboj/18.5.1309
42. Babon JJ, McManus EJ, Yao S, DeSouza DP, Mielke LA, Sprigg NS, et al. The structure of SOCS3 reveals the basis of the extended SH2 domain function and identifies an unstructured insertion that regulates stability. *Mol Cell*. (2006) 22:205–16. doi: 10.1016/j.molcel.2006.03.024
43. Piessevaux J, Lavens D, Peelman F, Tavernier J. The many faces of the SOCS box. *Cytokine Growth Factor Rev*. (2008) 19:371–81. doi: 10.1016/j.cytogfr.2008.08.006
44. Waiboci LW, Ahmed CM, Mujtaba MG, Flowers LO, Martin JP, Haider MI, et al. Both the suppressor of cytokine signaling 1 (SOCS-1) kinase inhibitory region and SOCS-1 mimetic bind to JAK2 autophosphorylation site: implications for the development of a SOCS-1 antagonist. *J Immunol.* (2007) 178:5058–68. doi: 10.4049/jimmunol.178.8.5058
45. Mallette FA, Calabrese V, Ilangumaran S, Ferbeyre G. SOCS1, a novel interaction partner of p53 controlling oncogene-induced senescence. *Aging*. (2010) 2:445–52. doi: 10.18632/aging.100163
46. Rogers S, Wells R, Rechsteiner M. Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. *Science*. (1986) 234:364–8. doi: 10.1126/science.2876518
47. Kinjyo I, Hanada T, Inagaki-Ohara K, Mori H, Aki D, Ohishi M, et al. SOCS1/JAB is a negative regulator of LPS-induced macrophage activation. *Immunity*. (2002) 17:583–91. doi: 10.1016/S1074-7613(02)00446-6
48. Alexander WS, Hilton DJ. The role of suppressors of cytokine signaling (SOCS) proteins in regulation of the immune response. *Annu Rev Immunol*. (2004) 22:503–29. doi: 10.1146/annurev.immunol.22.091003.090312
49. Mansell A, Smith R, Doyle SL, Gray P, Fenner JE, Crack PJ, et al. Suppressor of cytokine signaling 1 negatively regulates Toll-like receptor signaling by mediating Mal degradation. *Nat Immunol*. (2006) 7:148–55. doi: 10.1038/ni1299
50. Dimitriou ID, Clemenza L, Scotter AJ, Chen G, Guerra FM, Rottapel R. Putting out the fire: coordinated suppression of the innate and adaptive immune systems by SOCS1 and SOCS3 proteins. *Immunol Rev*. (2008) 224:265–83. doi: 10.1111/j.1600-065X.2008.00659.x
51. Darnell JE Jr, Kerr IM, Stark GR. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science*. (1994) 264:1415–21. doi: 10.1126/science.8197455
52. Watanabe S, Arai K. Roles of the JAK-STAT system in signal transduction via cytokine receptors. *Curr Opin Genet Dev*. (1996) 6:587–96. doi: 10.1016/S0959-437X(96)80088-8
53. Aaronson DS, Horvath CM. A road map for those who don't know JAK-STAT. *Science*. (2002) 296:1653–5. doi: 10.1126/science.1071545
54. Yoshimura A, Nishinakamura H, Matsumura Y, Hanada T. Negative regulation of cytokine signaling and immune responses by SOCS proteins. *Arthritis Res Ther*. (2005) 7:100–10. doi: 10.1186/ar1741
55. Tamiya T, Kashiwagi I, Takahashi R, Yasukawa H, Yoshimura A. Suppressors of cytokine signaling (SOCS) proteins and JAK/STAT pathways: regulation of T-cell inflammation by SOCS1 and SOCS3. *Arterioscler Thromb Vasc Biol*. (2011) 31:980–5. doi: 10.1161/ATVBAHA.110.207464
56. Fenner JE, Starr R, Cornish AL, Zhang JG, Metcalf D, Schreiber RD, et al. Suppressor of cytokine signaling 1 regulates the immune response to infection by a unique inhibition of type I interferon activity. *Nat Immunol*. (2006) 7:33–9. doi: 10.1038/ni1287
57. Qin H, Niyongere SA, Lee SJ, Baker BJ, Benveniste EN. Expression and functional significance of SOCS-1 and SOCS-3 in astrocytes. *J Immunol*. (2008) 181:3167–76. doi: 10.4049/jimmunol.181.5.3167
58. Baker BJ, Akhtar LN, Benveniste EN. SOCS1 and SOCS3 in the control of CNS immunity. *Trends Immunol*. (2009) 30:392–400. doi: 10.1016/j.it.2009.07.001
59. Metcalf D, Alexander WS, Elefanty AG, Nicola NA, Hilton DJ, Starr R, et al. Aberrant hematopoiesis in mice with inactivation of the gene encoding SOCS-1. *Leukemia*. (1999) 13:926–34. doi: 10.1038/sj.leu.2401440
60. Dickensheets HL, Venkataraman C, Schindler U, Donnelly RP. Interferons inhibit activation of STAT6 by interleukin 4 in human monocytes by inducing SOCS-1 gene expression. *Proc Natl Acad Sci USA*. (1999) 96:10800–5. doi: 10.1073/pnas.96.19.10800
61. Stoiber D, Kovarik P, Cohney S, Johnston JA, Steinlein P, Decker T. Lipopolysaccharide induces in macrophages the synthesis of the suppressor of cytokine signaling 3 and suppresses signal transduction in response to the activating factor IFN-gamma. *J Immunol*. (1999) 163:2640–7.
62. Hanada T, Yoshida H, Kato S, Tanaka K, Masutani K, Tsukada J, et al. Suppressor of cytokine signaling-1 is essential for suppressing dendritic cell activation and systemic autoimmunity. *Immunity*. (2003) 19:437–50. doi: 10.1016/S1074-7613(03)00240-1
63. Starr R, Hilton DJ. Defining control: regulation of dendritic cell activation and immune homeostasis by SOCS1. *Immunity*. (2003) 19:308–9. doi: 10.1016/S1074-7613(03)00243-7
64. O'Keefe GM, Nguyen VT, Ping Tang LL, Benveniste EN. IFN-gamma regulation of class II transactivator promoter IV in macrophages and microglia: involvement of the suppressors of cytokine signaling-1 protein. *J Immunol*. (2001) 166:2260–9. doi: 10.4049/jimmunol.166.4.2260
65. Ratthé C, Pelletier M, Chiasson S, Girard D. Molecular mechanisms involved in interleukin-4-induced human neutrophils: expression and regulation of suppressor of cytokine signaling. *J Leukoc Biol*. (2007) 81:1287–96. doi: 10.1189/jlb.0306209
66. Zhao D, Zhang Q, Liu Y, Li X, Zhao K, Ding Y, et al. H3K4me3 demethylase Kdm5a is required for NK cell activation by associating with p50 to suppress SOCS1. *Cell Rep*. (2016) 15:288–99. doi: 10.1016/j.celrep.2016.03.035
67. Egwuagu CE, Yu CR, Zhang M, Mahdi RM, Kim SJ, Gery I. Suppressors of cytokine signaling proteins are differentially expressed in Th1 and Th2 cells:

implications for Th cell lineage commitment and maintenance. *J Immunol*. (2002) 168:3181–7. doi: 10.4049/jimmunol.168.7.3181
68. Fletcher J, Starr R. The role of suppressors of cytokine signalling in thymopoiesis and T cell activation. *Int J Biochem Cell Biol*. (2005) 37:1774–86. doi: 10.1016/j.biocel.2005.04.005
69. Liu X, Mameza MG, Lee YS, Eseonu CI, Yu CR, Kang Derwent JJ, et al. Suppressors of cytokine-signaling proteins induce insulin resistance in the retina and promote survival of retinal cells. *Diabetes*. (2008) 57:1651–8. doi: 10.2337/db07-1761
70. Bourdonnay E, Zasłona Z, Penke LRK, Speth JM, Schneider DJ, Przybranowski S, et al. Transcellular delivery of vesicular SOCS proteins from macrophages to epithelial cells blunts inflammatory signaling. *J Exp Med*. (2015) 212:729–42. doi: 10.1084/jem.20141675
71. Naka T, Matsumoto T, Narazaki M, Fujimoto M, Morita Y, Ohsawa Y, et al. Accelerated apoptosis of lymphocytes by augmented induction of Bax in SSI-1 (STAT-induced STAT inhibitor-1) deficient mice. *Proc Natl Acad Sci USA*. (1998) 95:15577–82. doi: 10.1073/pnas.95.26.15577
72. Starr R, Metcalf D, Elefanty AG, Brysha M, Willson TA, Nicola NA, et al. Liver degeneration and lymphoid deficiencies in mice lacking suppressor of cytokine signaling-1. *Proc Natl Acad Sci USA*. (1998) 95:14395–9. doi: 10.1073/pnas.95.24.14395
73. Marine JC, Topham DJ, McKay C, Wang D, Parganas E, Stravopodis D, et al. SOCS1 deficiency causes a lymphocyte-dependent perinatal lethality. *Cell*. (1999) 98:609–16. doi: 10.1016/S0092-8674(00)80048-3
74. Marine JC, McKay C, Wang D, Topham DJ, Parganas E, Nakajima H, et al. SOCS3 is essential in the regulation of fetal liver erythropoiesis. *Cell*. (1999) 98:617–27. doi: 10.1016/S0092-8674(00)80049-5
75. Alexander WS, Starr R, Fenner JE, Scott CL, Handman E, Sprigg NS, et al. SOCS1 is a critical inhibitor of interferon gamma signaling and prevents the potentially fatal neonatal actions of this cytokine. *Cell*. (1999) 98:597–608. doi: 10.1016/S0092-8674(00)80047-1
76. Gil MP, Bohn E, O'Guin AK, Ramana CV, Levine B, Stark GR, et al. Biologic consequences of Stat1-independent IFN signaling. *Proc Natl Acad Sci USA*. (2001) 98:6680–5. doi: 10.1073/pnas.111163898
77. Wilson HM. SOCS proteins in macrophage polarization and function. *Front Immunol*. (2014) 5:357. doi: 10.3389/fimmu.2014.00357
78. Zhou D, Chen L, Yang K, Jiang H, Xu W, Luan J. SOCS molecules: the growing players in macrophage polarization and function. *Oncotarget*. (2017) 8:60710–22. doi: 10.18632/oncotarget.19940
79. Tanaka K, Ichiyama K, Hashimoto M, Yoshida H, Takimoto T, Takaesu G, et al. Loss of suppressor of cytokine signaling 1 in helper T cells leads to defective Th17 differentiation by enhancing antagonistic effects of IFN-gamma on STAT3 and Smads. *J Immunol*. (2008) 180:3746–56. doi: 10.4049/jimmunol.180.6.3746
80. Yu CR, Mahdi RM, Liu X, Zhang A, Naka T, Kishimoto T, et al. SOCS1 regulates CCR7 expression and migration of CD4+ T cells into peripheral tissues. *J Immunol*. (2008) 181:1190–8. doi: 10.4049/jimmunol.181.2.1190
81. Palmer DC, Restifo NP. Suppressors of cytokine signaling (SOCS) in T cell differentiation, maturation, and function. *Trends Immunol*. (2009) 30:592–602. doi: 10.1016/j.it.2009.09.009
82. Diehl S, Anguita J, Hoffmeyer A, Zapton T, Ihle JN, Fikrig E, et al. Inhibition of Th1 differentiation by IL-6 is mediated by SOCS1. *Immunity*. (2000) 13:805–15. doi: 10.1016/S1074-7613(00)00078-9
83. Losman JA, Chen XP, Hilton D, Rothman P. Cutting edge: SOCS-1 is a potent inhibitor of IL-4 signal transduction. *J Immunol*. (1999) 162:3770–4.
84. Croker BA, Krebs DL, Zhang JG, Wormald S, Willson TA, Stanley EG, et al. SOCS3 negatively regulates IL-6 signaling *in vivo*. *Nat Immunol*. (2003) 4:540–5. doi: 10.1038/ni931
85. Seki Y, Inoue H, Nagata N, Hayashi K, Fukuyama S, Matsumoto K, et al. SOCS-3 regulates onset and maintenance of T(H)2-mediated allergic responses. *Nat Med*. (2003) 9:1047–54. doi: 10.1038/nm896
86. Chen Z, Laurence A, Kanno Y, Pacher-Zavisin M, Zhu BM, Tato C, et al. Selective regulatory function of Socs3 in the formation of IL-17-secreting T cells. *Proc Natl Acad Sci USA*. (2006) 103:8137–42. doi: 10.1073/pnas.0600666103
87. Taleb S, Romain M, Ramkhelawon B, Uyttenhove C, Pasterkamp G, Herbin O, et al. Loss of SOCS3 expression in T cells reveals a regulatory role for interleukin-17 in atherosclerosis. *J Exp Med*. (2009) 206:2067–77. doi: 10.1084/jem.20090545
88. Honda K, Takaoka A, Taniguchi T. Type I interferon [corrected] gene induction by the interferon regulatory factor family of transcription factors. *Immunity*. (2006) 25:349–60. doi: 10.1016/j.immuni.2006.08.009
89. Gonzalez-Navajas JM, Lee J, David M, Raz E. Immunomodulatory functions of type I interferons. *Nat Rev Immunol*. (2012) 12:125–35. doi: 10.1038/nri3133
90. Leonard WJ. Type I cytokines and interferons and their receptors. In: Paul WE, editor. *Fundamental Immunology*. 6th ed. Philadelphia, PA: Wolters Kluwer/Lippincott Williams and Wilkins (2008). p. 706–48.
91. Takeuchi O, Akira S. MDA5/RIG-I and virus recognition. *Curr Opin Immunol*. (2008) 20:17–22. doi: 10.1016/j.coi.2008.01.002
92. Pestka S, Krause CD, Walter MR. Interferons, interferon-like cytokines, and their receptors. *Immunol Rev*. (2004) 202:8–32. doi: 10.1111/j.0105-2896.2004.00204.x
93. Fensterl V, Sen GC. Interferons and viral infections. *Biofactors*. (2009) 35:14–20. doi: 10.1002/biof.6
94. Platanias LC. Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nat Rev Immunol*. (2005) 5:375–86. doi: 10.1038/nri1604
95. de Veer MJ, Holko M, Frevel M, Walker E, Der S, Paranjape JM, et al. Functional classification of interferon-stimulated genes identified using microarrays. *J Leukoc Biol*. (2001) 69:912–20. doi: 10.1189/jlb.69.6.912
96. Akira S. Toll-like receptor signaling. *J Biol Chem*. (2003) 278:38105–8. doi: 10.1074/jbc.R300028200
97. Jensen S, Thomsen AR. Sensing of RNA viruses: a review of innate immune receptors involved in recognizing RNA virus invasion. *J Virol*. (2012) 86:2900–10. doi: 10.1128/JVI.05738-11
98. Dalpke A, Heeg K, Bartz H, Baetz A. Regulation of innate immunity by suppressor of cytokine signaling (SOCS) proteins. *Immunobiology*. (2008) 213:225–35. doi: 10.1016/j.imbio.2007.10.008
99. Strebovsky J, Walker P, Dalpke AH. Suppressor of cytokine signaling proteins as regulators of innate immune signaling. *Front Biosci*. (2012) 17:1627–39. doi: 10.2741/4008
100. Kumar MV, Nagineni CN, Chin MS, Hooks JJ, Detrick B. Innate immunity in the retina: Toll-like receptor (TLR) signaling in human retinal pigment epithelial cells. *J Neuroimmunol*. (2004) 153:7–15. doi: 10.1016/j.jneuroim.2004.04.018
101. Ebihara N, Chen L, Tokura T, Ushio H, Iwatsu M, Murakami A. Distinct functions between Toll-like receptors 3 and 9 in retinal pigment epithelial cells. *Ophthalmic Res*. (2007) 39:155–63. doi: 10.1159/000103235
102. Kumar A, Shamsuddin N. Retinal Muller glia initiate innate response to infectious stimuli via toll-like receptor signaling. *PLoS ONE*. (2012) 7:e29830. doi: 10.1371/journal.pone.0029830
103. Eberle ME, Dalpke AH. Dectin-1 stimulation induces suppressor of cytokine signaling 1, thereby modulating TLR signaling and T cell responses. *J Immunol*. (2012) 188:5644–54. doi: 10.4049/jimmunol.1103068
104. Rico-Bautista E, Flores-Morales A, Fernandez-Perez L. Suppressor of cytokine signaling (SOCS) 2, a protein with multiple functions. *Cytokine Growth Factor Rev*. (2006) 17:431–9. doi: 10.1016/j.cytogfr.2006.09.008
105. Greenhalgh CJ, Rico-Bautista E, Lorentzon M, Thaus AL, Morgan PO, Willson TA, et al. SOCS2 negatively regulates growth hormone action *in vitro* and *in vivo*. *J Clin Invest*. (2005) 115:397–406. doi: 10.1172/JCI200522710
106. Yamanishi K, Okuno T, Shiraki K, Takahashi M, Kondo T, Asano Y, et al. Identification of human herpesvirus-6 as a causal agent for exanthem subitum. *Lancet*. (1988) 1:1065–7. doi: 10.1016/S0140-6736(88)91893-4
107. Mocarski ES, Courcelle CT. Cytomegaloviruses and their replication. In: DM Knipe, PM Howley, editors. *Fields Virology*. Philadelphia, PA: Lippincott Williams and Wilkins (2001). 2629 p.
108. Mocarski ES Jr, Shenk T, Pass RF. Cytomegalovirus. In: DM Knipe, PM Howley, editors. *Fields Virology*. Philadelphia, PA: Lippincott Williams and Wilkins (2007). p. 2702–72.
109. Davison AJ, Eberle R, Ehlers B, Hayward GS, McGeoch DJ, Minson AC, et al. The order Herpesvirales. *Arch Virol*. (2009) 154:171–7. doi: 10.1007/s00705-008-0278-4
110. Mocarski ES Jr, Shenk T, Griffiths PD, Pass RF. Cyotmegaloviruses. In: DM Knipe, PM Howley, editors. *Fields Virology*. 6th ed. Philadelphia, PA: Lippencott Williams and Wilkins (2013). p. 1960–2014.

111. Koujah L, Suryawanshi RK, Shukla D. Pathological processes activated by herpes simplex virus-1 (HSV-1) infection in the cornea. *Cell Mol Life Sci*. (2019) 76:405–19. doi: 10.1007/s00018-018-2938-1
112. Meurens F, Keil GM, Muylkens B, Gogev S, Schynts F, Negro S, et al. Interspecific recombination between two ruminant alphaherpesviruses, bovine herpesviruses 1 and 5. *J Virol*. (2004) 78:9828–36. doi: 10.1128/JVI.78.18.9828-9836.2004
113. Thiry J, Keuser V, Muylkens B, Meurens F, Gogev S, Vanderplasschen A, et al. Ruminant alphaherpesviruses related to bovine herpesvirus 1. *Vet Res*. (2006) 37:169–90. doi: 10.1051/vetres:2005052
114. Rissi DR, Pierezan F, Sa e Silva M, Flores E.F, de Barros CS. Neurological disease in cattle in southern Brazil associated with Bovine herpesvirus infection. *J Vet Diagn Invest*. (2008) 20:346–9. doi: 10.1177/104063870802000315
115. Osterrieder N, Kamil JP, Schumacher D, Tischer BK, Trapp S. Marek's disease virus: from miasma to model. *Nat Rev Microbiol*. (2006) 4:283–94. doi: 10.1038/nrmicro1382
116. Boodhoo N, Gurung A, Sharif S, Behboudi S. Marek's disease in chickens: a review with focus on immunology. *Vet Res*. (2016) 47:119. doi: 10.1186/s13567-016-0404-3
117. Tanaka K, Kondo T, Torigoe S, Okada S, Mukai T, Yamanishi K. Human herpesvirus 7: another causal agent for roseola (exanthem subitum). *J Pediatr*. (1994) 125:1–5. doi: 10.1016/S0022-3476(94)70113-X
118. Meylan E, Tschopp J, Karin M. Intracellular pattern recognition receptors in the host response. *Nature*. (2006) 442:39–44. doi: 10.1038/nature04946
119. Brown GD. Dectin-1: a signalling non-TLR pattern-recognition receptor. *Nat Rev Immunol*. (2006) 6:33–43. doi: 10.1038/nri1745
120. Chen GY, Nunez G. Sterile inflammation: sensing and reacting to damage. *Nat Rev Immunol*. (2010) 10:826–37. doi: 10.1038/nri2873
121. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. *Cell*. (2010) 140:805–20. doi: 10.1016/j.cell.2010.01.022
122. Aoshi T, Koyama S, Kobiyama K, Akira S, Ishii KJ. Innate and adaptive immune responses to viral infection and vaccination. *Curr Opin Virol*. (2011) 1:226–32. doi: 10.1016/j.coviro.2011.07.002
123. Barbalat R, Lau L, Locksley RM, Barton GM. Toll-like receptor 2 on inflammatory monocytes induces type I interferon in response to viral but not bacterial ligands. *Nat Immunol*. (2009) 10:1200–7. doi: 10.1038/ni.1792
124. Tabeta K, Georgel P, Janssen E, Du X, Hoebe K, Crozat K, et al. Toll-like receptors 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection. *Proc Natl Acad Sci USA*. (2004) 101:3516–21. doi: 10.1073/pnas.0400525101
125. Jordan MC, Mar VL. Spontaneous activation of latent cytomegalovirus from murine spleen explants. Role of lymphocytes and macrophages in release and replication of virus. *J Clin Invest*. (1982) 70:762–8. doi: 10.1172/JCI110672
126. Yamaguchi T, Shinagawa Y, Pollard RB. Relationship between the production of murine cytomegalovirus and interferon in macrophages. *J Gen Virol*. (1988) 69 (Pt 12):2961–71. doi: 10.1099/0022-1317-69-12-2961
127. Stoddart CA, Cardin RD, Boname JM, Manning WC, Abenes GB, Mocarski ES. Peripheral blood mononuclear phagocytes mediate dissemination of murine cytomegalovirus. *J Virol*. (1994) 68:6243–53.
128. Heise MT, Virgin HWT. The T-cell-independent role of gamma interferon and tumor necrosis factor alpha in macrophage activation during murine cytomegalovirus and herpes simplex virus infections. *J Virol*. (1995) 69:904–9.
129. Hahn G, Jores R, Mocarski ES. Cytomegalovirus remains latent in a common precursor of dendritic and myeloid cells. *Proc Natl Acad Sci USA*. (1998) 95:3937–42. doi: 10.1073/pnas.95.7.3937
130. Heise MT, Pollock JL, O'Guin A, Barkon ML, Bormley S, Virgin HWT. Murine cytomegalovirus infection inhibits IFN gamma-induced MHC class II expression on macrophages: the role of type I interferon. *Virology*. (1998) 241:331–44. doi: 10.1006/viro.1997.8969
131. Hanson LK, Slater JS, Karabekian Z, Virgin HWT, Biron CA, Ruzek MC, et al. Replication of murine cytomegalovirus in differentiated macrophages as a determinant of viral pathogenesis. *J Virol*. (1999) 73:5970–80.
132. Hanson LK, Slater JS, Karabekian Z, Ciocco-Schmitt G, Campbell AE. Products of US22 genes M140 and M141 confer efficient replication of murine cytomegalovirus in macrophages and spleen. *J Virol*. (2001) 75:6292–302. doi: 10.1128/JVI.75.14.6292-6302.2001
133. Kropp KA, Robertson KA, Sing G, Rodriguez-Martin S, Blanc M, Lacaze P, et al. Reversible inhibition of murine cytomegalovirus replication by gamma interferon (IFN-gamma) in primary macrophages involves a primed type I IFN-signaling subnetwork for full establishment of an immediate-early antiviral state. *J Virol*. (2011) 85:10286–99. doi: 10.1128/JVI.00373-11
134. Mantovani A, Sica A, Locati M. Macrophage polarization comes of age. *Immunity*. (2005) 23:344–6. doi: 10.1016/j.immuni.2005.10.001
135. Mosser DM. The many faces of macrophage activation. *J Leukoc Biol*. (2003) 73:209–12. doi: 10.1189/jlb.0602325
136. Smith MS, Bentz GL, Alexander JS, Yurochko AD. Human cytomegalovirus induces monocyte differentiation and migration as a strategy for dissemination and persistence. *J Virol*. (2004) 78:4444–53. doi: 10.1128/JVI.78.9.4444-4453.2004
137. Chan G, Bivins-Smith ER, Smith MS, Smith PM, Yurochko AD. Transcriptome analysis reveals human cytomegalovirus reprograms monocyte differentiation toward an M1 macrophage. *J Immunol*. (2008) 181:698–711. doi: 10.4049/jimmunol.181.1.698
138. Chan G, Bivins-Smith ER, Smith MS, Yurochko AD. NF-κB and phosphatidylinositol 3-kinase activity mediates the HCMV-induced atypical M1/M2 polarization of monocytes. *Virus Res*. (2009) 144:329–33. doi: 10.1016/j.virusres.2009.04.026
139. Alfano M, Graziano F, Genovese L, Poli G. Macrophage polarization at the crossroad between HIV-1 infection and cancer development. *Arterioscler Thromb Vasc Biol*. (2013) 33:1145–52. doi: 10.1161/ATVBAHA.112.300171
140. Gumperz JE, Parham P. The enigma of the natural killer cell. *Nature*. (1995) 378:245–8. doi: 10.1038/378245a0
141. Andrews DM, Andoniou CE, Scalzo AA, van Dommelen SL, Wallace ME, Smyth MJ, et al. Cross-talk between dendritic cells and natural killer cells in viral infection. *Mol Immunol*. (2005) 42:547–55. doi: 10.1016/j.molimm.2004.07.040
142. Vieira Braga FA, Hertoghs KM, van Lier RA, van Gisbergen KP. Molecular characterization of HCMV-specific immune responses: Parallels between CD8(+) T cells, CD4(+) T cells, and NK cells. *Eur J Immunol*. (2015) 45:2433–45. doi: 10.1002/eji.201545495
143. Bukowski JF, Woda BA, Welsh RM. Pathogenesis of murine cytomegalovirus infection in natural killer cell-depleted mice. *J Virol*. (1984) 52:119–28.
144. Terrazzini N, Kern F. Cell-mediated immunity to human CMV infection: a brief overview. *F1000Prime Rep*. (2014) 6:28. doi: 10.12703/P6-28
145. Jonjic S, Mutter W, Weiland F, Reddehase MJ, Koszinowski UH. Site-restricted persistent cytomegalovirus infection after selective long-term depletion of CD4+ T lymphocytes. *J Exp Med*. (1989) 169:1199–212. doi: 10.1084/jem.169.4.1199
146. Bale JF Jr, O'Neil ME, Lyon B, Perlman S. The pathogenesis of murine cytomegalovirus ocular infection. Anterior chamber inoculation. *Invest Ophthalmol Vis Sci*. (1990) 31:1575–81.
147. Holland GN, Fang EN, Glasgow BJ, Zaragoza AM, Siegel LM, Graves MC, et al. Necrotizing retinopathy after intraocular inoculation of murine cytomegalovirus in immunosuppressed adult mice. *Invest Ophthalmol Vis Sci*. (1990) 31:2326–34.
148. Mizota A, Hamasaki DI, Atherton SS. Physiologic and morphologic retinal changes induced by murine cytomegalovirus in BALB/c and severe combined immune deficient mice. *Invest Ophthalmol Vis Sci*. (1991) 32:1479–91.
149. Welsh RM, Brubaker JO, Vargas-Cortes M, O'Donnell CL. Natural killer (NK) cell response to virus infections in mice with severe combined immunodeficiency. The stimulation of NK cells and the NK cell-dependent control of virus infections occur independently of T and B cell function. *J Exp Med*. (1991) 173:1053–63. doi: 10.1084/jem.173.5.1053
150. Atherton SS, Newell CK, Kanter MY, Cousins SW. T cell depletion increases susceptibility to murine cytomegalovirus retinitis. *Invest Ophthalmol Vis Sci*. (1992) 33:3353–60.
151. van der Meer JT, Drew WL, Bowden RA, Galasso GJ, Griffiths PD, Jabs DA, et al. Summary of the International Consensus Symposium on Advances in the Diagnosis, Treatment and Prophylaxis and Cytomegalovirus Infection. *Antiviral Res*. (1996) 32:119–40. doi: 10.1016/S0166-3542(96)01006-6

152. Jabs DA. Cytomegalovirus retinitis and the acquired immunodeficiency syndrome–bench to bedside: LXVII Edward Jackson Memorial Lecture. *Am J Ophthalmol.* (2011) 151:198–216 e191. doi: 10.1016/j.ajo.2010.10.018
153. Song XT, Evel-Kabler K, Rollins L, Aldrich M, Gao F, Huang XF, et al. An alternative and effective HIV vaccination approach based on inhibition of antigen presentation attenuators in dendritic cells. *PLoS Med.* (2006) 3:e11. doi: 10.1371/journal.pmed.0030011
154. Ryo A, Tsurutani N, Ohba K, Kimura R, Komano J, Nishi M, et al. SOCS1 is an inducible host factor during HIV-1 infection and regulates the intracellular trafficking and stability of HIV-1 Gag. *Proc Natl Acad Sci USA*. (2008) 105:294–9. doi: 10.1073/pnas.0704831105
155. Akhtar LN, Qin H, Muldowney MT, Yanagisawa LL, Kutsch O, Clements JE, et al. Suppressor of cytokine signaling 3 inhibits antiviral IFN-beta signaling to enhance HIV-1 replication in macrophages. *J Immunol.* (2010) 185:2393–404. doi: 10.4049/jimmunol.0903563
156. Miller RC, Schlaepfer E, Baenziger S, Crameri R, Zeller S, Byland R, et al. HIV interferes with SOCS-1 and−3 expression levels driving immune activation. *Eur J Immunol.* (2011) 41:1058–69. doi: 10.1002/eji.201041198
157. Koeberlein B, zur Hausen A, Bektas N, Zentgraf H, Chin R, Nguyen LT, et al. Hepatitis B virus overexpresses suppressor of cytokine signaling-3 (SOCS3) thereby contributing to severity of inflammation in the liver. *Virus Res.* (2010) 148:51–9. doi: 10.1016/j.virusres.2009.12.003
158. Bode JG, Ludwig S, Ehrhardt C, Albrecht U, Erhardt A, Schaper F, et al. IFN-alpha antagonistic activity of HCV core protein involves induction of suppressor of cytokine signaling-3. *FASEB J.* (2003) 17:488–90. doi: 10.1096/fj.02-0664fje
159. Zhang Y, Ma CJ, Ni L, Zhang CL, Wu XY, Kumaraguru U, et al. Cross-talk between programmed death-1 and suppressor of cytokine signaling-1 in inhibition of IL-12 production by monocytes/macrophages in hepatitis C virus infection. *J Immunol.* (2011) 186:3093–103. doi: 10.4049/jimmunol.1002006
160. Zhao DC, Yan T, Li L, You S, Zhang C. Respiratory syncytial virus inhibits interferon-alpha-inducible signaling in macrophage-like U937 cells. *J Infect.* (2007) 54:393–8. doi: 10.1016/j.jinf.2006.06.005
161. Yasukawa H, Yajima T, Duplain H, Iwatate M, Kido M, Hoshijima M, et al. The suppressor of cytokine signaling-1 (SOCS1) is a novel therapeutic target for enterovirus-induced cardiac injury. *J Clin Invest.* (2003) 111:469–78. doi: 10.1172/JCI16491
162. Okumura A, Pitha PM, Yoshimura A, Harty RN. Interaction between Ebola virus glycoprotein and host toll-like receptor 4 leads to induction of proinflammatory cytokines and SOCS1. *J Virol.* (2010) 84:27–33. doi: 10.1128/JVI.01462-09
163. Pauli EK, Schmolke M, Wolff T, Viemann D, Roth J, Bode JG, et al. Influenza A virus inhibits type I IFN signaling via NF-kappaB-dependent induction of SOCS-3 expression. *PLoS Pathog.* (2008) 4:e1000196. doi: 10.1371/journal.ppat.1000196
164. Yokota S, Yokosawa N, Okabayashi T, Suzutani T, Miura S, Jimbow K, et al. Induction of suppressor of cytokine signaling-3 by herpes simplex virus type 1 contributes to inhibition of the interferon signaling pathway. *J Virol.* (2004) 78:6282–6. doi: 10.1128/JVI.78.12.6282-6286.2004
165. Yokota S, Yokosawa N, Okabayashi T, Suzutani T, Fujii N. Induction of suppressor of cytokine signaling-3 by herpes simplex virus type 1 confers efficient viral replication. *Virology.* (2005) 338:173–81. doi: 10.1016/j.virol.2005.04.028
166. Frey KG, Ahmed CM, Dabelic R, Jager LD, Noon-Song EN, Haider SM, et al. HSV-1-induced SOCS-1 expression in keratinocytes: use of a SOCS-1 antagonist to block a novel mechanism of viral immune evasion. *J Immunol.* (2009) 183:1253–62. doi: 10.4049/jimmunol.0900570
167. Sato Y, Koshizuka T, Ishibashi K, Hashimoto K, Ishioka K, Ikuta K, et al. Involvement of herpes simplex virus type 1 UL13 protein kinase in induction of SOCS genes, the negative regulators of cytokine signaling. *Microbiol Immunol.* (2017) 61:159–67. doi: 10.1111/1348-0421.12483
168. Reichard AC, Cheemarla NR, Bigley NJ. SOCS1/3 expression levels in HSV-1-infected, cytokine-polarized and -unpolarized macrophages. *J Interferon Cytokine Res.* (2015) 35:32–41. doi: 10.1089/jir.2013.0070
169. Lo AK, Lo KW, Tsao SW, Wong HL, Hui JW, To KF, et al. Epstein-Barr virus infection alters cellular signal cascades in human nasopharyngeal epithelial cells. *Neoplasia.* (2006) 8:173–80. doi: 10.1593/neo.05625
170. Butler LM, Jeffery HC, Wheat RL, Long HM, Rae PC, Nash GB, et al. Kaposi's sarcoma-associated herpesvirus inhibits expression and function of endothelial cell major histocompatibility complex class II via suppressor of cytokine signaling 3. *J Virol.* (2012) 86:7158–66. doi: 10.1128/JVI.06908-11
171. da Cunha Sousa LF, Rachid MA, Lima GK, de Miranda AS, de Carvalho Vilela M, Lacerda Queiroz N, et al. Suppressor of cytokine signaling 2 (SOCS2) contributes to encephalitis in a model of Herpes infection in mice. *Brain Res Bull.* (2016) 127:164–70. doi: 10.1016/j.brainresbull.2016.09.011
172. Wang X, Liu S, Zhou Z, Yan H, Xiao J. A herpes simplex virus type 2-encoded microRNA promotes tumor cell metastasis by targeting suppressor of cytokine signaling 2 in lung cancer. *Tumour Biol.* (2017) 39:1010428317701633. doi: 10.1177/1010428317701633
173. Aparecida Silva Barbosa A, Freitas Versiani A, Fonseca da Cunha Sousa L, Silva de Miranda A, Gasparini MR, Brant F, et al. Role of the suppressor of cytokine signaling 2 (SOCS2) during meningoencephalitis caused by Bovine herpesvirus 5 (BoHV-5). *Comp Immunol Microbiol Infect Dis.* (2016) 47:26–31. doi: 10.1016/j.cimid.2016.05.003
174. Fogh J, Lund RO. Continuous cultivation of epithelial cell strain (FL) from human amniotic membrane. *Proc Soc Exp Biol Med.* (1957) 94:532–7. doi: 10.3181/00379727-94-23003
175. Li J, Hu S, Zhou L, Ye L, Wang X, Ho J, et al. Interferon lambda inhibits herpes simplex virus type I infection of human astrocytes and neurons. *Glia.* (2011) 59:58–67. doi: 10.1002/glia.21076
176. Steiner I, Kennedy PGE, Pachner AR. The neurotropic herpes viruses: herpes simplex and varicella-zoster. *Lancet Neurol.* (2007) 6:1015–28. doi: 10.1016/S1474-4422(07)70267-3
177. Verweij MC, Wellish M, Whitmer T, Malouli D, Lapel M, Jonjić S, et al. Varicella viruses inhibit interferon-stimulated JAK-STAT signaling through multiple mechanisms. *PLoS Pathog.* (2015) 11:e1004901. doi: 10.1371/journal.ppat.1004901
178. Arvin AM. Humoral and cellular immunity to varicella-zoster virus: an overview. *J Infect Dis.* (2008) 197(Supplement_2):S58–60. doi: 10.1086/522123
179. Gandhi MK, Khanna R. Human cytomegalovirus: clinical aspects, immune regulation, and emerging treatments. *Lancet Infect Dis.* (2004) 4:725–38. doi: 10.1016/S1473-3099(04)01202-2
180. Heiden D, Ford N, Wilson D, Rodriguez WR, Margolis T, Janssens B, et al. Cytomegalovirus retinitis: the neglected disease of the AIDS pandemic. *PLoS Med.* (2007) 4:e334. doi: 10.1371/journal.pmed.0040334
181. Stewart MW. Optimal management of cytomegalovirus retinitis in patients with AIDS. *Clin Ophthalmol.* (2010) 4:285–99. doi: 10.2147/OPTH.S6700
182. Lancini D, Faddy HM, Flower R, Hogan C. Cytomegalovirus disease in immunocompetent adults. *Med J Aust.* (2014) 201:578–80. doi: 10.5694/mja14.00183
183. Holland GN, Tufail A, Jordan MC. Cytomegalovirus diseases. In: J Pepose, G Holland, K Wilhelmus, editors. *Ocular Infection and Immunity.* St. Louis, MO: Mosby Year Book (1996). p. 1088–128.
184. Lares AP, Tu CC, Spencer JV. The human cytomegalovirus US27 gene product enhances cell proliferation and alters cellular gene expression. *Virus Res.* (2013) 176:312–20. doi: 10.1016/j.virusres.2013.07.002
185. Holzerlandt R, Orengo C, Kellam P, Alba MM. Identification of new herpesvirus gene homologs in the human genome. *Genome Res.* (2002) 12:1739–48. doi: 10.1101/gr.334302
186. Kotenko SV, Saccani S, Izotova LS, Mirochnitchenko OV, Pestka S. Human cytomegalovirus harbors its own unique IL-10 homolog (cmvIL-10). *Proc Natl Acad Sci USA.* (2000) 97:1695–700. doi: 10.1073/pnas.97.4.1695
187. Gruber SG, Gloria Luciani M, Grundtner P, Zdanov A, Gasche C. Differential signaling of cmvIL-10 through common variants of the IL-10 receptor 1. *Eur J Immunol.* (2008) 38:3365–75. doi: 10.1002/eji.200837718
188. Nachtwey J, Spencer JV. HCMV IL-10 suppresses cytokine expression in monocytes through inhibition of nuclear factor-kappaB. *Viral Immunol.* (2008) 21:477–82. doi: 10.1089/vim.2008.0048
189. Crawford DH. Biology and disease associations of Epstein-Barr virus. *Philos Trans R Soc Lond B Biol Sci.* (2001) 356:461–73. doi: 10.1098/rstb.2000.0783
190. Young LS, Yap LF, Murray PG. Epstein–Barr virus: more than 50 years old and still providing surprises. *Nat Rev Cancer.* (2016) 16:789. doi: 10.1038/nrc.2016.92

191. Li QX, Young LS, Niedobitek G, Dawson CW, Birkenbach M, Wang F, et al. Epstein-Barr virus infection and replication in a human epithelial cell system. *Nature*. (1992) 356:347–50. doi: 10.1038/356347a0
192. Imai S, Nishikawa J, Takada K. Cell-to-cell contact as an efficient mode of epstein-barr virus infection of diverse human epithelial cells. *J Virol*. (1998) 72:4371–8.
193. Ni C, Chen Y, Zeng M, Pei R, Du Y, Tang L, et al. In-cell infection: a novel pathway for Epstein-Barr virus infection mediated by cell-in-cell structures. *Cell Res*. (2015) 25:785–800. doi: 10.1038/cr.2015.50
194. Chang Y, Cesarman E, Pessin M, Lee F, Culpepper J, Knowles D, et al. Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. *Science*. (1994) 266:1865–69. doi: 10.1126/science.7997879
195. Mesri EA, Cesarman E, Boshoff C. Kaposi's sarcoma and its associated herpesvirus. *Nat Rev Cancer*. (2010) 10:707. doi: 10.1038/nrc2888
196. Cai QL, Knight JS, Verma SC, Zald P, Robertson ES. EC5S ubiquitin complex is recruited by KSHV latent antigen LANA for degradation of the VHL and p53 tumor suppressors. *PLoS Pathog*. (2006) 2:e116. doi: 10.1371/journal.ppat.0020116
197. Carroll PA, Brazeau E, Lagunoff M. Kaposi's sarcoma-associated herpesvirus infection of blood endothelial cells induces lymphatic differentiation. *Virology*. (2004) 328:7–18. doi: 10.1016/j.virol.2004.07.008
198. Qin Z, Kearney P, Plaisance K, Parsons CH. Pivotal Advance: Kaposi's sarcoma-associated herpesvirus (KSHV)-encoded microRNA specifically induce IL-6 and IL-10 secretion by macrophages and monocytes. *J Leukoc Biol*. (2010) 87:25–34. doi: 10.1189/jlb.0409251
199. Nazerian K, Witter RL. Cell-free transmission and *in vivo* replication of Marek's disease virus. *J Virol*. (1970) 5:388–97.
200. Pomeranz LE, Reynolds AE, Hengartner CJ. Molecular biology of pseudorabies virus: impact on neurovirology and veterinary medicine. *Microbiol Mol Biol Rev*. (2005) 69:462–500. doi: 10.1128/MMBR.69.3.462-500.2005
201. McCordock HA, Smith MG. The visceral lesions produced in mice by the salivary gland virus of mice. *J Exp Med*. (1936) 63:303–10. doi: 10.1084/jem.63.3.303
202. Smith MG. Propagation of salivary gland virus of the mouse in tissue cultures. *Proc Soc Exp Biol Med*. (1954) 86:435–40. doi: 10.3181/00379727-86-21123
203. Hudson JB. The murine cytomegalovirus as a model for the study of viral pathogenesis and persistent infections. *Arch Virol*. (1979) 62:1–29. doi: 10.1007/BF01314900
204. Blaskovic D. Isolation of five strains of herpesviruses from two species of free living small rodents. *Acta Virol*. (1980) 24:468.
205. Nash AA, Dutia BM, Stewart JP, Davison AJ. Natural history of murine gamma-herpesvirus infection. *Philos Trans R Soc Lond B Biol Sci*. (2001) 356:569–79. doi: 10.1098/rstb.2000.0779
206. Speck SH, Virgin HW. Host and viral genetics of chronic infection: a mouse model of gamma-herpesvirus pathogenesis. *Curr Opin Microbiol*. (1999) 2:403–9. doi: 10.1016/S1369-5274(99)80071-X
207. Cieniewicz B, Santana AL, Minkah N, Krug LT. Interplay of murine gammaherpesvirus 68 with NF-kappaB signaling of the host. *Front Microbiol*. (2016) 7:1202. doi: 10.3389/fmicb.2016.01202
208. Flano E, Husain SM, Sample JT, Woodland DL, Blackman MA. Latent murine gamma-herpesvirus infection is established in activated B cells, dendritic cells, and macrophages. *J Immunol*. (2000) 165:1074–81. doi: 10.4049/jimmunol.165.2.1074
209. Li S, Wen X. Seropositivity to herpes simplex virus type 2, but not type 1 is associated with cervical cancer: NHANES (1999–2014). *BMC Cancer*. (2017) 17:726. doi: 10.1186/s12885-017-3734-2
210. Grossniklaus HE, Geisert EE, Nickerson JM. Chapter twenty-two - introduction to the retina. In: Hejtmancik JF and John MN, editors. *Progress in Molecular Biology and Translational Science*. Waltham, MA: Academic Press (2015). p. 383–96.
211. Medawar PB. Immunity to homologous grafted skin; the fate of skin homografts transplanted to the brain, to subcutaneous tissue, and to the anterior chamber of the eye. *Br J Exp Pathol*. (1948) 29:58–69.
212. Streilein JW. Ocular immune privilege: therapeutic opportunities from an experiment of nature. *Nat Rev Immunol*. (2003) 3:879–89. doi: 10.1038/nri1224
213. Taylor AW. Ocular immune privilege and transplantation. *Front Immunol*. (2016) 7:37. doi: 10.3389/fimmu.2016.00037
214. Hoover DR, Peng Y, Saah A, Semba R, Detels RR, Rinaldo CR, et al. Occurrence of cytomegalovirus retinitis after human immunodeficiency virus immunosuppression. *Arch Ophthalmol*. (1996) 114:821–7. doi: 10.1001/archopht.1996.01100140035004
215. Sugar EA, Jabs DA, Ahuja A, Thorne JE, Danis RP, Meinert CL, et al. Incidence of cytomegalovirus retinitis in the era of highly active antiretroviral therapy. *Am J Ophthalmol*. (2012) 153:1016–24 e1015. doi: 10.1016/j.ajo.2011.11.014
216. Jabs DA, Ahuja A, Van Natta ML, Lyon AT, Yeh S, Danis R. Long-term outcomes of cytomegalovirus retinitis in the era of modern antiretroviral therapy: results from a United States cohort. *Ophthalmology*. (2015) 122:1452–63. doi: 10.1016/j.ophtha.2015.02.033
217. Lurain NS, Chou S. Antiviral drug resistance of human cytomegalovirus. *Clin Microbiol Rev*. (2010) 23:689–712. doi: 10.1128/CMR.00009-10
218. Ahmed A. Antiviral treatment of cytomegalovirus infection. *Infect Disord Drug Targets*. (2011) 11:475–503. doi: 10.2174/187152611797636640
219. Prichard MN, Kern ER. The search for new therapies for human cytomegalovirus infections. *Virus Res*. (2011) 157:212–21. doi: 10.1016/j.virusres.2010.11.004
220. Harter G, Michel D. Antiviral treatment of cytomegalovirus infection: an update. *Expert Opin Pharmacother*. (2012) 13:623–7. doi: 10.1517/14656566.2012.658775
221. Vadlapudi AD, Vadlapatla RK, Mitra AK. Current and emerging antivirals for the treatment of cytomegalovirus (CMV) retinitis: an update on recent patents. *Recent Pat Antiinfect Drug Discov*. (2012) 7:8–18. doi: 10.2174/157489112799829765
222. Baldanti F, Underwood MR, Stanat SC, Biron KK, Chou S, Sarasini A, et al. Single amino acid changes in the DNA polymerase confer foscarnet resistance and slow-growth phenotype, while mutations in the UL97-encoded phosphotransferase confer ganciclovir resistance in three double-resistant human cytomegalovirus strains recovered from patients with AIDS. *J Virol*. (1996) 70:1390–5.
223. Schleiss MR. Cytomegalovirus vaccine development. *Curr Top Microbiol Immunol*. (2008) 325:361–82. doi: 10.1007/978-3-540-77349-8_20
224. Anderholm KM, Bierle CJ, Schleiss MR. Cytomegalovirus vaccines: current status and future prospects. *Drugs*. (2016) 76:1625–45. doi: 10.1007/s40265-016-0653-5
225. Dudgeon JA. Cytomegalovirus infection. *Arch Dis Child*. (1971) 46:581–3. doi: 10.1136/adc.46.249.581
226. Dix RD, Cousins SW. Murine cytomegalovirus retinitis during MAIDS: susceptibility correlates with elevated intraocular levels of interleukin-4 mRNA. *Curr Eye Res*. (2003) 26:211–7. doi: 10.1076/ceyr.26.3.211.14902
227. Dix RD, Cousins SW. Susceptibility to murine cytomegalovirus retinitis during progression of MAIDS: correlation with intraocular levels of tumor necrosis factor-alpha and interferon-gamma. *Curr Eye Res*. (2004) 29:173–80. doi: 10.1080/02713680490504876
228. Chalmer JE, Mackenzie JS, Stanley NF. Resistance to murine cytomegalovirus linked to the major histocompatibility complex of the mouse. *J Gen Virol*. (1977) 37:107–14. doi: 10.1099/0022-1317-37-1-107
229. Selgrade MK, Nedrud JG, Collier AM, Gardner DE. Effects of cell source, mouse strain, and immunosuppressive treatment on production of virulent and attenuated murine cytomegalovirus. *Infect Immun*. (1981) 33:840–7.
230. Atherton SS, Newell CK, Kanter MY, Cousins SW. Retinitis in euthymic mice following inoculation of murine cytomegalovirus (MCMV) via the supraciliary route. *Curr Eye Res*. (1991) 10:667–77. doi: 10.3109/02713689109013858
231. Bigger JE, Thomas CA III, Atherton SS. NK cell modulation of murine cytomegalovirus retinitis. *J Immunol*. (1998) 160:5826–31.
232. Dix RD. Systemic murine cytomegalovirus infection of mice with retrovirus-induced immunodeficiency results in ocular infection but not retinitis. *Ophthalmic Res*. (1998) 30:295–301. doi: 10.1159/000055488
233. Zhang M, Zhou J, Marshall B, Xin H, Atherton SS. Lack of iNOS facilitates MCMV spread in the retina. *Invest Ophthalmol Vis Sci*. (2007) 48:285–92. doi: 10.1167/iovs.06-0792

234. Duan Y, Ji Z, Atherton SS. Dissemination and replication of MCMV after supraciliary inoculation in immunosuppressed BALB/c mice. *Invest Ophthalmol Vis Sci*. (1994) 35:1124–31.
235. Haas M, Meshorer A. Reticulum cell neoplasms induced in C57BL/6 mice by cultured virus grown in stromal hematopoietic cell lines. *J Natl Cancer Inst*. (1979) 63:427–39.
236. Mosier DE, Yetter RA, Morse HC III. Retroviral induction of acute lymphoproliferative disease and profound immunosuppression in adult C57BL/6 mice. *J Exp Med*. (1985) 161:766–84. doi: 10.1084/jem.161.4.766
237. Watson RR. Murine models for acquired immune deficiency syndrome. *Life Sci*. (1989) 44:iii–xv. doi: 10.1016/0024-3205(89)90592-4
238. Cunnigham RK, Thacore HR, Zhou P, Terzian R, Nakeeb S, Zaleski MB. Murine AIDS: A model for the human disease or a distinct entity? *Immunol Res*. (1994) 13:21–8. doi: 10.1007/BF02918221
239. Quinnan GV Jr, Manischewitz JF. Genetically determined resistance to lethal murine cytomegalovirus infection is mediated by interferon-dependent and -independent restriction of virus replication. *J Virol*. (1987) 61:1875–81.
240. Scalzo AA, Fitzgerald NA, Simmons A, La Vista AB, Shellam GR. Cmv-1, a genetic locus that controls murine cytomegalovirus replication in the spleen. *J Exp Med*. (1990) 171:1469–83. doi: 10.1084/jem.171.5.1469
241. Pomeroy C, Delong D, Clabots C, Riciputi P, Filice GA. Role of interferon-gamma in murine cytomegalovirus infection. *J Lab Clin Med*. (1998) 132:124–33. doi: 10.1016/S0022-2143(98)90007-5
242. Geist LJ, Hinde SL. Susceptibility to cytomegalovirus infection may be dependent on the cytokine response to the virus. *J Investig Med*. (2001) 49:434–41. doi: 10.2310/6650.2001.33788
243. Hamelin-Bourassa D, Skamene E, Gervais F. Susceptibility to a mouse acquired immunodeficiency syndrome is influenced by the H-2. *Immunogenetics*. (1989) 30:266–72. doi: 10.1007/BF02421330
244. Watson RR, Prabhala RH, Darban HR, Yahya MD, Smith TL. Changes in lymphocyte and macrophage subsets due to morphine and ethanol treatment during a retrovirus infection causing murine AIDS. *Life Sci*. (1988) 43:v–xi. doi: 10.1016/0024-3205(88)90145-2
245. Yetter RA, Buller RM, Lee JS, Elkins KL, Mosier DE, Fredrickson TN, et al. CD4+ T cells are required for development of a murine retrovirus-induced immunodeficiency syndrome (MAIDS). *J Exp Med*. (1988) 168:623–35. doi: 10.1084/jem.168.2.623
246. Cheung SC, Chattopadhyay SK, Hartley JW, Morse HC, III, Pitha PM. Aberrant expression of cytokine genes in peritoneal macrophages from mice infected with LP-BM5 MuLV, a murine model of AIDS. *J Immunol*. (1991) 146:121–7.
247. Kim WK, Tang Y, Kenny JJ, Longo DL, Morse HC III. In murine AIDS, B cells are early targets of defective virus and are required for efficient infection and expression of defective virus in T cells and macrophages. *J Virol*. (1994) 68:6767–9.
248. Zhang M, Covar J, Marshall B, Dong Z, Atherton SS. Lack of TNF-α promotes Caspase-3–independent apoptosis during murine cytomegalovirus retinitis. *Invest Ophthalmol Vis Sci*. (2011) 52:1800–8. doi: 10.1167/iovs.10-6904
249. Sloka JS, Stefanelli M. The mechanism of action of methylprednisolone in the treatment of multiple sclerosis. *Mult Scler*. (2005) 11:425–32. doi: 10.1191/1352458505ms1190oa
250. Klinman DM, Morse HC III. Characteristics of B cell proliferation and activation in murine AIDS. *J Immunol*. (1989) 142:1144–9.
251. Morse HC III, Yetter RA, Via CS, Hardy RR, Cerny A, Hayakawa K, et al. Functional and phenotypic alterations in T cell subsets during the course of MAIDS, a murine retrovirus-induced immunodeficiency syndrome. *J Immunol*. (1989) 143:844–50.
252. Mosier DE, Yetter RA, Morse HC III. Functional T lymphocytes are required for a murine retrovirus-induced immunodeficiency disease (MAIDS). *J Exp Med*. (1987) 165:1737–42. doi: 10.1084/jem.165.6.1737
253. Makino M, Winkler DF, Wunderlich J, Hartley JW, Morse HC, Holmes KL. High expression of NK-1.1 antigen is induced by infection with murine AIDS virus. *Immunology*. (1993) 80:319–25.
254. Chen Y, Mendoza S, Davis-Gorman G, Cohen Z, Gonzales R, Tuttle H, et al. Neutrophil activation by murine retroviral infection during chronic ethanol consumption. *Alcohol Alcohol*. (2003) 38:109–14. doi: 10.1093/alcalc/agg049
255. Gazzinelli RT, Makino M, Chattopadhyay SK, Snapper CM, Sher A, Hugin AW, et al. CD4+ subset regulation in viral infection. Preferential activation of Th2 cells during progression of retrovirus-induced immunodeficiency in mice. *J Immunol*. (1992) 148:182–8.
256. Hartley JW, Fredrickson TN, Yetter RA, Makino M, Morse HC III. Retrovirus-induced murine acquired immunodeficiency syndrome: natural history of infection and differing susceptibility of inbred mouse strains. *J Virol*. (1989) 63:1223–31.
257. Thacore HR, Cunningham RK, Zhou P, Nakeeb S, Terzian R, Zaleski MB. Acquired immunodeficiency in murine lymphoproliferative disease: considerations on pathogenesis. *Immunobiology*. (1994) 190:195–211. doi: 10.1016/S0171-2985(11)80269-X
258. Klinken SP, Fredrickson TN, Hartley JW, Yetter RA, Morse HC III. Evolution of B cell lineage lymphomas in mice with a retrovirus-induced immunodeficiency syndrome, MAIDS. *J Immunol*. (1988) 140:1123–31.
259. Kanagawa O, Gayama S, Vaupel B. Functional and phenotypic change of T cells in murine acquired immune deficiency. *J Immunol*. (1994) 152:4671–9.
260. Crough T, Khanna R. Immunobiology of human cytomegalovirus: from bench to bedside. *Clin Microbiol Rev*. (2009) 22:76–98. doi: 10.1128/CMR.00034-08
261. Chien H, Dix RD. Evidence for multiple cell death pathways during development of experimental cytomegalovirus retinitis in mice with retrovirus-induced immunosuppression: apoptosis, necroptosis, and pyroptosis. *J Virol*. (2012) 86:10961–78. doi: 10.1128/JVI.01275-12
262. Dix RD, Cray C, Cousins SW. Antibody alone does not prevent experimental cytomegalovirus retinitis in mice with retrovirus-induced immunodeficiency (MAIDS). *Ophthalmic Res*. (1997) 29:381–92. doi: 10.1159/000268039
263. Dix RD, Ekworomadu CO, Hernandez E, Cousins SW. Perforin knockout mice, but not mice with MAIDS, show protection against experimental cytomegalovirus retinitis after adoptive transfer of immune cells with a functional perforin cytotoxic pathway. *Arch Virol*. (2004) 149:2235–44. doi: 10.1007/s00705-004-0370-3
264. Dix RD, Cousins SW. Cell-mediated cytotoxicity of murine cytomegalovirus-infected target cells allows for release of residual infectious virus. *Arch Virol*. (2005) 150:797–803. doi: 10.1007/s00705-004-0459-8
265. Dix RD, Cousins SW. Interleukin-2 immunotherapy of murine cytomegalovirus retinitis during MAIDS correlates with increased intraocular CD8+ T-cell infiltration. *Ophthalmic Res*. (2003) 35:154–9. doi: 10.1159/000070051
266. Dix RD, Cousins SW. Interleukin-2 immunotherapy and AIDS-related cytomegalovirus retinitis. *Curr HIV Res*. (2004) 2:333–42. doi: 10.2174/1570162043351066
267. Blalock EL, Chien H, Dix RD. Systemic reduction of interleukin-4 or interleukin-10 fails to reduce the frequency or severity of experimental cytomegalovirus retinitis in mice with retrovirus-induced immunosuppression. *Ophthalmol Eye Dis*. (2012) 4:79–90. doi: 10.4137/OED.S10294
268. Flowers LO, Johnson HM, Mujtaba MG, Ellis MR, Haider SM, Subramaniam PS. Characterization of a peptide inhibitor of Janus kinase 2 that mimics suppressor of cytokine signaling 1 function. *J Immunol*. (2004) 172:7510–8. doi: 10.4049/jimmunol.172.12.7510
269. Ahmed CM, Dabelic R, Martin JP, Jager LD, Haider SM, Johnson HM. Enhancement of antiviral immunity by small molecule antagonist of suppressor of cytokine signaling. *J Immunol*. (2010) 185:1103–13. doi: 10.4049/jimmunol.0902895
270. Ahmed CM, Dabelic R, Bedoya SK, Larkin J, III, Johnson HM. A SOCS1/3 antagonist peptide protects mice against lethal infection with influenza A virus. *Front Immunol*. (2015) 6:574. doi: 10.3389/fimmu.2015.00574
271. Cull VS, Bartlett EJ, James CM. Type I interferon gene therapy protects against cytomegalovirus-induced myocarditis. *Immunology*. (2002) 106:428–37. doi: 10.1046/j.1365-2567.2002.01423.x
272. He C, Yu CR, Sun L, Mahdi RM, Larkin J III, Egwuagu CE. Topical administration of a suppressor of cytokine signaling-1 (SOCS1) mimetic peptide inhibits ocular inflammation and mitigates ocular pathology during mouse uveitis. *J Autoimmun*. (2015) 62:31–8. doi: 10.1016/j.jaut.2015.05.011

273. Ahmed CM, Massengill MT, Brown EE, Ildefonso CJ, Johnson HM, Lewin AS. A cell penetrating peptide from SOCS-1 prevents ocular damage in experimental autoimmune uveitis. *Exp Eye Res*. (2018) 177:12–22. doi: 10.1016/j.exer.2018.07.020
274. Yu CR, Hayashi K, Lee YS, Mahdi RM, Shen de F, Chan CC, et al. Suppressor of cytokine signaling 1 (SOCS1) mitigates anterior uveitis and confers protection against ocular HSV-1 infection. *Inflammation*. (2015) 38:555–65. doi: 10.1007/s10753-014-9962-6
275. Yu CR, Mahdi RR, Oh HM, Amadi-Obi A, Levy-Clarke G, Burton J, et al. Suppressor of cytokine signaling-1 (SOCS1) inhibits lymphocyte recruitment into the retina and protects SOCS1 transgenic rats and mice from ocular inflammation. *Invest Ophthalmol Vis Sci*. (2011) 52:6978–86. doi: 10.1167/iovs.11-7688
276. Yu CR, Kim SH, Mahdi RM, Egwuagu CE. SOCS3 deletion in T lymphocytes suppresses development of chronic ocular inflammation via upregulation of CTLA-4 and expansion of regulatory T cells. *J Immunol*. (2013) 191:5036–43. doi: 10.4049/jimmunol.1301132
277. Alston CI. *Suppressor of Cytokine Signaling (SOCS)1 and SOCS3 Stimulation During Experimental Cytomegalovirus Retinitis: Virologic, Immunologic, or Pathologic Mechanisms*. Doctor of Philosophy Ph.D. Dissertation, Georgia State University (2017).

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

MINI REVIEW
published: 07 May 2019
doi: 10.3389/fimmu.2019.01008

Bovine Herpesvirus 1 Counteracts Immune Responses and Immune-Surveillance to Enhance Pathogenesis and Virus Transmission

*Clinton Jones**

Department of Veterinary Pathobiology, Center for Veterinary Health Sciences, Oklahoma State University, Stillwater, OK, United States

Infection of cattle by bovine herpesvirus 1 (BoHV-1) can culminate in upper respiratory tract disorders, conjunctivitis, or genital disorders. Infection also consistently leads to transient immune-suppression. BoHV-1 is the number one infectious agent in cattle that is associated with abortions in cattle. BoHV-1, as other α-herpesvirinae subfamily members, establishes latency in sensory neurons. Stressful stimuli, mimicked by the synthetic corticosteroid dexamethasone, consistently induce reactivation from latency in latently infected calves and rabbits. Increased corticosteroid levels due to stress have a two-pronged effect on reactivation from latency by: (1) directly stimulating viral gene expression and replication, and (2) impairing antiviral immune responses, thus enhancing virus spread and transmission. BoHV-1 encodes several proteins, bICP0, bICP27, gG, UL49.5, and VP8, which interfere with key antiviral innate immune responses in the absence of other viral genes. Furthermore, the ability of BoHV-1 to infect lymphocytes and induce apoptosis, in particular CD4+ T cells, has negative impacts on immune responses during acute infection. BoHV-1 induced immune-suppression can initiate the poly-microbial disorder known as bovine respiratory disease complex, which costs the US cattle industry more than one billion dollars annually. Furthermore, interfering with antiviral responses may promote viral spread to ovaries and the developing fetus, thus enhancing reproductive issues associated with BoHV-1 infection of cows or pregnant cows. The focus of this review is to describe the known mechanisms, direct and indirect, by which BoHV-1 interferes with antiviral immune responses during the course of infection.

Keywords: bovine herpesvirus 1 (BoHV-1), immune evasion, VP8, infected cells protein 0 (bICP0), abortion, bovine respiratory disease complex

OPEN ACCESS

Edited by:
Susmit Suvas,
School of Medicine, Wayne State University, United States

Reviewed by:
Richard D. Dix,
Georgia State University, United States
James Craig Forrest,
University of Arkansas for Medical Sciences, United States
Homayon Ghiasi,
Cedars-Sinai Medical Center, United States

***Correspondence:**
Clinton Jones
clint.jones10@okstate.edu

Specialty section:
This article was submitted to Viral Immunology, a section of the journal Frontiers in Immunology

Received: *26 October 2018*
Accepted: *18 April 2019*
Published: *07 May 2019*

Citation:
Jones C (2019) Bovine Herpesvirus 1 Counteracts Immune Responses and Immune-Surveillance to Enhance Pathogenesis and Virus Transmission. Front. Immunol. 10:1008. doi: 10.3389/fimmu.2019.01008

BOHV-1 IS AN IMPORTANT VIRAL PATHOGEN

Bovine herpesvirus 1 (BoHV-1) is an α-herpesvirinae subfamily member that causes significant economical losses to the cattle industry (1). Three well-defined subtypes exist, BoHV-1.1, BoHV-1.2a, and BoHV-1.2b (2b) (2). Subtype 1 virus isolates are prevalent in Europe, North America, and South America: these subtypes are frequently detected in cattle suffering from infectious bovine rhinotracheitis (IBR) and the respiratory tract of aborted fetuses. Subtype 2a strains are prevalent in Brazil and are associated with respiratory and genital tract infections, including IBR, infectious pustular vulvovaginitis (IPV), balanopostitis (IPV), and abortions (3). Subtype 2b strains, which are

frequently isolated in Australia or Europe (4), are associated with respiratory disease and IPV/IPB, but not abortion (3, 5). The seroprevelance of BoHV-1 ranges from 14 to 90% depending on the age of cattle and geographical location (6, 7). Serological testing and removal of infected animals has eliminated BoHV-1 from Denmark, Switzerland, and Austria (8).

BoHV-1 is the most frequently diagnosed cause of viral abortion in North American cattle (9). Exposure of a susceptible herd to BoHV-1 can result in abortion storms ranging from 25 to 60% of cows undergoing abortion. Commercially available modified live vaccines also induce abortions in pregnant cows. Furthermore, several studies concluded that naïve heifers vaccinated with an inactivated BoHV-1 vaccine are more likely to have a normal estrous cycle and significantly higher pregnancy rates relative to heifers vaccinated with a modified live (MLV) vaccine (9–13).

The incubation period for the genital forms of BoHV-1 is 2–6 day and initial clinical signs are frequent urination and a mild vaginal infection (14). It is also common to observe swollen vulva or small papules followed by erosions and ulcers on the mucosal surface. In bulls, similar lesions occur on the penis and prepuce. If secondary bacterial infections occur, inflammation of the uterus and transient infertility with purulent vaginal discharge occurs for several weeks. BoHV-1 infection, virulent field strains or modified live vaccines, of sero-negative heifers can target the ovary and corpus luteum during estrus and early in gestation (9).

Bovine respiratory disease complex (BRDC), a poly-microbial disease initiated by stress and/or virus infection, is the most economically important disease that affects beef and dairy cattle. Annual BRDC losses in the U.S. are ∼$1 billion (15–18). A gram negative bacterium, *Mannheimia haemolytica (MH),* exists in the upper respiratory tract of healthy ruminants (19, 20). Following stressful stimuli or co-infections with other viruses (21), this commensal relationship is disrupted and *MH* becomes the predominant organism that causes life threatening bronchopneumonia in many BRDC cases (22–25). BoHV-1 infection frequently causes upper respiratory tract disease (26, 27), high fever, conjunctivitis, and erodes mucosal surfaces of the upper respiratory tract. Consequently, colonization of *MH* occurs in the lower respiratory tract (22, 23, 25), thus enhancing interactions between the *MH* leukotoxin, bovine peripheral blood mononuclear cells, and neutrophils (28, 29). Co-infection of calves with BoHV-1 and *MH* consistently leads to pneumonia (30). Finally, a BoHV-1 protein that is required for virus entry was identified as a significant BRDC susceptibility gene in Holsteins (31) confirming BoHV-1 is an important BRDC cofactor.

THE BOHV-1 LATENCY-REACTIVATION CYCLE IS IMPORTANT FOR VIRUS TRANSMISSION

Acute Infection Leads to High Levels of Virus Shedding

Acute BoHV-1 infection of cattle is initiated on mucosal surfaces and results in high levels of programmed cell death (32, 33). Acute infection leads to high levels of virus production and secretion in ocular, oral, nasal, or genital cavities for 7–10 days after infection. BoHV-1 gene expression during productive infection is operationally divided into three distinct phases: immediate early (IE), early (E), or late (L) (32, 33). IE gene expression is stimulated by VP16, a tegument protein (34, 35). Thus, IE mRNA expression does not require *de novo* protein synthesis. Two IE transcription units exist: IE transcription unit 1 (IEtu1) and IEtu2. IE transcription unit 1 (IEtu1) encodes two transcriptional regulatory proteins, bICP0 and bICP4, because a single IE transcript is differentially spliced and then translated into bICP0 or bICP4 (36–38). The bICP0 protein is also translated from an E mRNA (E2.6) because a separate E promoter drives expression of the bICP0 E transcript (36–39). The bICP0 protein has similar properties as HSV-1 encoded ICP0 (40), including a RING finger that is crucial for stimulating viral promoters and productive infection (41, 42). bICP4 is likely to possess similar functions as the HSV-1 encoded ICP4. bICP4 autoregulates the IEtu1 promoter, but activates the bICP0 E promoter.

E gene expression requires *de novo* protein expression, including bICP0 and bICP4, which transactivate E viral promoters. In general, the E proteins encode proteins that promote DNA synthesis. Example of early viral proteins include the DNA polymerase, thymidine kinase, small and large subunits of the ribonucleotide reductase, dUTPase, and origin binding protein. In general, the E proteins are non-structural.

The L genes are divided into two classes: Gamma-1 and Gamma-2 genes. Transcription of Gamma-1 genes requires *de novo* protein synthesis, including bICP0 and bICP4, but does not require viral DNA replication. Transcription of Gamma-2 genes requires *de novo* protein synthesis, including bICP0 and bICP4, and abundant expression requires viral DNA replication. In general, L proteins encode structural proteins and their synthesis culminates in virion assembly and release.

Summary of Latency-Reactivation Cycle

Viral particles enter the peripheral nervous system via cell-cell spread. If infection is initiated within the oral, nasal, or ocular cavity, the primary site for latency is sensory neurons in trigeminal ganglia (TG). Viral gene expression (43) and infectious virus (44) are detected in TG from 2 to 6 days after infection. Lytic gene expression is then extinguished, and surviving infected neurons harbor viral genomes (establishment of latency).

Abundant expression of the viral encoded latency related (LR) gene occurs in latently infected neurons, but infectious virus is not readily detected (maintenance of latency) (32, 33, 45–48). LR-RNA overlaps the bICP0 gene (49, 50), has two open reading frames (ORF1 and ORF2), two reading frames lacking an initiating ATG, and encodes two micro-RNAs. A LR mutant virus strain with three stop codons at the N-terminus of ORF2 has reduced virus shedding from the eye, TG, or tonsils of infected calves (44, 51, 52). LR-encoded proteins are expressed late during productive infection when infected with wild-type (wt) or LR-rescued virus, but have reduced or no expression after infection with the LR mutant virus (53, 54). Wt BoHV-1, but not the LR mutant virus, reactivates from latency (44).

The anti-apoptosis activity of ORF2 (41, 55–57) and the micro-RNAs, which interfere with bICP0 expression (58) regulate the latency-reactivation cycle.

The synthetic corticosteroid dexamethasone (DEX) initiates reactivation from latency in latently infected calves or rabbits 100% of the time (27, 32, 33, 44, 47, 59). Within 6 h after latently infected calves are treated with DEX, viral regulatory proteins (ICP0 and VP16) (60, 61) and lytic cycle viral RNA expression are detected in TG neurons (62, 63). Within 3 h after DEX treatment, 11 cellular genes are induced more than 10-fold in TG (64). Pentraxin 3, a regulator of innate immunity and neuro-degeneration, is stimulated more than 30-fold at 3 or 6 h after DEX treatment. Two transcription factors, promyelocytic leukemia zinc finger (PLZF) and Slug are induced more than 15-fold 3 h after DEX treatment, which can enhance productive infection. Additional DEX induced transcription factors, SPDEF (Sam-pointed domain containing Ets transcription factor), Krüppel-like transcription factor 15 (KLF15), KLF4, KLF6, and GATA6, stimulate productive infection and certain key viral promoters. The finding that four KLF family members are stimulated during DEX induced reactivation from latency is intriguing because KLF family members resemble the Sp1 transcription factor family and both family of transcription factors interact with GC rich motifs, reviewed in Bieker (65) and Kaczynski et al. (66). The BoHV-1 genome is GC rich and many viral promoters contain Sp1 consensus binding sites and other GC rich motifs suggesting specific KLF transcription factors bind to viral sequences and stimulate viral transcription during early stages of reactivation from latency.

The IEtu1 promoter that drives bICP0 and bICP4 expression is stimulated by DEX and contains two consensus GR binding sites that are bound by the activated GR (67, 68). The GR and KLF15 are frequently expressed in the same TG neuron during reactivation and cooperatively stimulate productive infection and IEtu1 promoter activity. A host cellular factor 1 (HCF-1), which forms a complex with VP16 and Oct1 to bind to the IE enhancer core via the TAATGARAT motif, is important for GR mediated activation of the IEtu1 promoter suggesting glucocorticoid induction of viral reactivation may proceed via an HCF-1-GR mechanism in the absence of the viral IE activator VP16 (69). Stress-mediated activation of key viral promoters is predicted to be a very early event during reactivation from latency; then viral transactivators activate all other viral genes and virus production occurs. Hence, stress has a two-pronged effect on reactivation from latency by directly activating viral gene expression and indirectly enhancing viral spread via immunosuppression (70–72).

IMMUNE RESPONSE TO BOHV-1 FOLLOWING ACUTE INFECTION

Cattle acutely infected with BoHV-1 develop an innate immune response (73–76); however, efficient virus replication and spread occurs. For example, virus neutralizing antibodies are detected after acute infection that recognize envelope glycoproteins, including gB, gC, gD, and gH (77, 78). Cytotoxic T cell responses to viral glycoproteins occur in cattle following infection (79–81). Infection of cultured cells also induces inflammasome formation (82), consistent with inflammation in the nasal cavity and upper respiratory tract during acute infection.

Although the host immune response clears virus after acute infection, viral infection impairs immune-recognition on several levels impairs: (1) cell-mediated immunity (83–86), (2) CD8+ T cell recognition of infected cells (68, 87–89), (3) CD4+ T cell functions because BoHV-1 infect these cells and rapidly inducing apoptosis after viral entry (90, 91), and (4) interferon responses (92–95). The known viral genes that antagonize immune responses are discussed below (see **Figure 1** for a schematic that summarizes how viral genes impair immune responses).

VIRAL PROTEINS INTERFERE WITH INNATE IMMUNE RESPONSES AND IMMUNE-SURVEILLANCE

The amino-terminus of the bICP0 protein contains transcriptional activation domains, a nuclear localization signal (NLS) necessary for efficient transcriptional activation (99), and a C_3HC_4 zinc RING finger that is conserved in all ICP0 proteins (100, 101). Point mutations within the C_3HC_4 zinc RING finger domain of bICP0 interfere with transactivation of a simple viral promoter (99), stimulation of productive infection (41, 102), and reduces IFN-β promoter activity (92–95). bICP0 co-localizes with and disrupts the anti-viral promyelocytic leukemia (PML) protein-containing nuclear domains (41, 101). PML bodies are comprised of numerous proteins, which regulate the cell cycle, apoptosis, senescence, stress, DNA damage, and innate immune responses (103). Many DNA viruses reorganize or dissolve PML bodies, thus increasing viral replication. Interferon treatment increases components of PML bodies, Sp100, and PML for example (104, 105) and PML bodies increase beta-interferon (IFN-β) expression (106).

bICP0 inhibits IFN-β promoter activity in transient transfection studies (92, 94) by reducing IRF3 (interferon regulatory factor 3) protein levels. The RING finger of bICP0 (107) is an E3 ubiquitin ligase suggesting it mediates IRF3 degradation in a proteasome dependent manner. bICP0 also interacts with IRF7 and impairs activation of IFN-β promoter activity, but does not reduce IRF7 protein levels (94). IRF3 and IRF7 are transcription factors that stimulate IFN-β promoter activity (96–98). IRF3 directly binds several consensus DNA binding sites, including an ISRE (IFN response elements), and can activate IFN-stimulated promoters in the absence of IFN (108, 109). A recent study concluded PML regulates intrinsic and innate immune responses to HSV-1 infection, which is ablated by ICP0 (110). The ability of bICP0 to reduce IFN-β promoter activity correlates with IRF3 degradation, IRF7 interactions, and dissolving PML bodies.

The BoHV-1 bICP27 protein is expressed from an early promoter and based on similarity with the HSV-1 ICP27 is expected to shuttle RNA from the nucleus to the cytoplasm and regulate transcription (111). HSV-1 encoded ICP27 regulates IFN expression (112) by interfering with activation of the stimulator

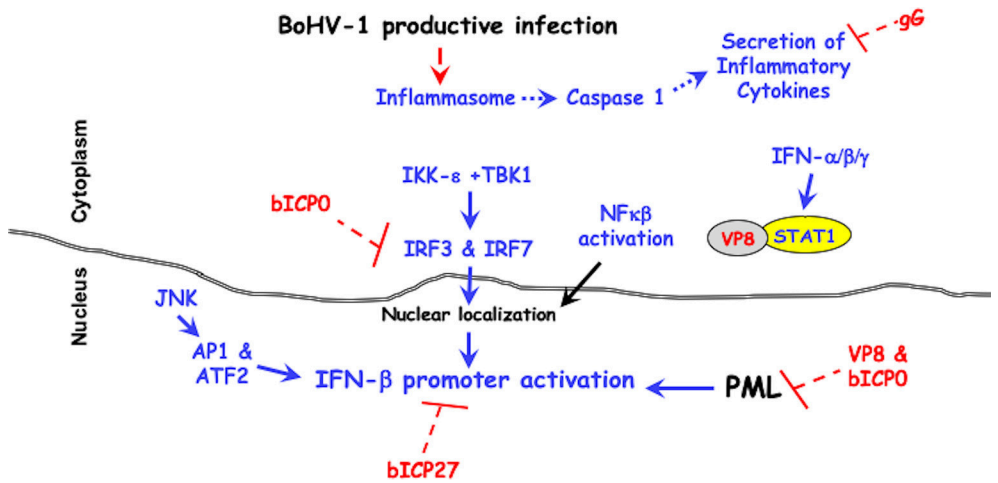


FIGURE 1 | BoHV-1 encoded immune-evasion genes that promote productive infection. Cellular mechanisms leading to innate immune antiviral signaling pathways are denoted in blue. Red lettering denotes viral genes that counteract antiviral signaling pathways. It is well-established that two protein kinases (IKK-ε +TBK1) activate the transcription factors (IRF3 and IRF7), which are required for activating the IFN-β promoter (96–98). The JNK protein kinase (c-Jun N-terminal kinases) activates the AP1 (activating protein 1) and ATF2 (activating transcription factor 2), which are also required for activating the IFN-β promoter (96–98). For further details, see the text.

of interferon genes (STING) by tank binding protein kinase 1 (TBK1) (113). Interestingly, bICP27 reduces bovine IFN-β1 and IFN-β3 promoter activity in transfected cells (114). *Bos Taurus* encodes three functional IFN-β genes; all have anti-viral activity but each gene contains a unique promoter (115, 116).

Glycoprotein G (gG) promotes cell to cell spread (117) and maintains adherence of infected cells (118). gG is a unique viral glycoprotein because it can exist in three isoforms: a full-length membrane-bound form, a smaller membrane-bound form, and a secreted form. gG interferes with chemokine binding to their specific receptors and glycosaminoglycans (119). Although it is not known what role gG plays during acute infection of calves, the ability of chemokines to control the migratory patterns and positioning of immune cells (120) would likely be altered by gG.

The BoHV-1 UL49.5 ORF, also known as glycoprotein N (gN), is a 96 amino acid protein (121). The BoHV-1 and pseudorabies virus UL49.5 proteins interfere with processing of the transporter-associated antigen processing (TAP)-mediated transport of cytosolic peptides into the endoplasmic reticulum because UL49.5 renders the TAP complex susceptible to proteolytic degradation (122, 123). Peptide transport by TAP is crucial for MHC class I antigen presentation and recognition of infected cells by $CD8^+$ T cells (122, 124–126). Infection of calves with a UL49.5 BoHV-1 mutant leads to increased levels of virus neutralizing antibody and cellular immune responses when compared to the parental wild-type virus (127).

VP8, the most abundant tegument protein in the virion, enhances growth in cultured cells and is required for pathogenesis in calves (128). VP8 interacts with DDB1 (DNA damaging-binding protein 1) that is associated with a E3 ubiquitin ligase complex (129), and remodels PML nuclear bodies (130). Recent studies demonstrated VP8 interacts with STAT1 (Signal transducer and activator of transcription 1) and prevents STAT1 from entering the nucleus (131). Stat1 is bound to the IFN-γ receptor and upon IFN-γ binding to its receptor (Jak1 and Jak2) phosphorylates specific tyrosine residues on STAT1. STAT1 subsequently enters the nucleus and stimulates GAS (IFN-γ activated sequences) setting off a second wave of IFN-γ (132). Following IFN-α or IFN-β stimulation, STAT1 forms a heterodimer with STAT2 and this heterodimer binds an ISRE element and activates transcription (133). VP8 also interferes with IFN-β signaling activity by reducing an interferon sensitive response element (ISRE) responsive promoter in transfected or infected cells. Thus, VP8 is a potent IFN antagonist that can interfere with host innate immune responses in the absence of *de novo* viral protein synthesis.

CONCLUSIONS/DISCUSSION

BoHV-1 is a very successful pathogen because it encodes several genes that impair intrinsic and innate immune responses throughout productive infection (see **Figure 1**). VP8 is likely the initial anti-viral protein that impairs antiviral IFN responses because high levels of VP8 are present in the tegument of incoming viral particles. bICP0, which is encoded by the IEtu1 promoter, would be an early interferon antagonist. bICP27 via unknown mechanisms interferes with IFN-β promoter activation. Three late proteins (gG, UL49.5, and VP8) would further antagonize immune-recognition. In summary, the presence of viral proteins in the virion and expression of viral proteins throughout productive infection allows for high levels of virus production during acute infection and reactivation from latency in cattle.

AUTHOR CONTRIBUTIONS

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

FUNDING

This research was supported by grants from the USDA-NIFA Competitive Grants Program (2018-06668 and 2016-09370), National Institute of Neurological Disorders and Stroke of the NIH (R21NS102290), support from the Oklahoma Center for Respiratory and Infectious Diseases (National Institutes of Health Centers for Biomedical Research Excellence Grant # P20GM103648), and funds derived from the Sitlington Endowment.

REFERENCES

1. Turin L, Russo S, Poli G. BHV-1: new molecular approaches to control a common and widespread infection. *Molec Med.* (1999) 5:261–84. doi: 10.1007/BF03402063
2. Metzler AE, Matile H, Gasman U, Engels M, Wyler R. European isolates of bovine herpesvirus 1: a comparison of restriction endonuclease sites, polypeptides and reactivity with monoclonal antibodies. *Arch Virol.* (1985) 85:57–69. doi: 10.1007/BF01317006
3. Oirschot JT. Bovine herpesvirus in semen of bulls and the risk of transmission: a brief overview. *Vet Quart.* (1995) 17:29–33. doi: 10.1080/01652176.1995.9694526
4. Edwards S, White H, Nixon P. A study of the predominant genotypes of bovine herpesvirus 1 isolated in the U.K. *Vet Microbiol.* (1990) 22:213–23. doi: 10.1016/0378-1135(90)90108-8
5. D'Arce RCF, Almedia RS, Silva TC, Spilki AC, Roehe PM, Ams CW. Restriction endonucleases and monoclonal antibody analysis of Brazilian isolates of bovine herpesvirus 1 and 5. *Vet Microbiol.* (2002) 88:315–34. doi: 10.1016/S0378-1135(02)00126-8
6. Straub OC. Infectious bovine rhinotracheitis virus. *In: Dinter Z, Morin B, editors. Virus Infections of Ruminants. Amsterdam: Elsevier.* (1990). *p.* 71–109. doi: 10.1016/B978-0-444-87312-5.50020-5
7. Wuyckhuise L, Van Bosch J, Franken P, Hage J, Verhoeff J, Zimmer G. The prevalence of infectious bovine rhinotracheitis (IBR) in the Netherlands. Paper presented at: *18th World Buiatrics Congress,* Bologna. (1994).
8. Ackermann M, Engals M. Pro and contra-IBR-eradication. *Vet Microbiol.* (2005) 113:293–302. doi: 10.1016/j.vetmic.2005.11.043
9. Chase C, Fulton RW, O'Toole D, Gillette B, Daly RF, Perry G, et al. Bovine herpesvirus 1 modified live vaccines for cattle reproduction: balancing protection with undesired effects. *Vet Microbiol.* (2017) 206:69–77. doi: 10.1016/j.vetmic.2017.03.016
10. Miller JM, Van der Matten MJ. Early embryonic death in heifers after inoculation with bovine herpesvirus-1 and reactivation of latency virus in reproductive tissues. *Am J Vet Res.* (1987) 48:1555–8.
11. O'Toole D, Miller MM, Cavender JL, Cornish TE. Pathology in practice. *Vet Med Today.* (2012) 241:189–91. doi: 10.2460/javma.241.2.189
12. O'Toole D, Corbett R. Letter to the editor, regarding Bovine herpesvirus 1 abortion and vaccination. *J Vet Diagn Invest.* (2013) 25:555. doi: 10.1177/1040638713496050
13. Perry G, Zimmerman AD, Daly RF, Butterbaugh RE, Rhoades J, Schultz D, et al. The effects of vaccination on serum hormone concentrations and conception rates in synchronized naive beef heifers. *Theriongenology.* (2013) 79:200–5. doi: 10.1016/j.theriogenology.2012.10.005
14. Yates WDG. A review of infectious bovine rhinotracheitis, shipping fever pneumonia, and viral-bacterial synergism in respiratory diesease of cattle. *Can J Comp Med.* (1982) 46:225–63.
15. National Agricultural Statistics Service (NASS). *Agricultural Statistics Board.* Washington, DC: U.S. Department of Agriculture (1996).
16. Edwards AJ. Respiratory diseases of feedlot cattle in teh central USA. *Bovine Pract.* (1996) 30:5–7.
17. Griffin D. Economic impact associated with respiratory disease in beef cattle. *Vet Clin North Am Food Anim Pract.* (1997) 13:367–77. doi: 10.1016/S0749-0720(15)30302-9
18. Kapil S, Basaraba RJ. Infectious bovine rhinotracheitis, parainfluenza-3, and respiratory coronavirus. *Vet Clin North Am Food Anim Pract.* (1997) 13:455–61. doi: 10.1016/S0749-0720(15)30308-X
19. Frank GH. (*ed.) Bacteria as Etiologic Agents in Bovine Respiratory Disease*. College Station, TX: Texas A&M University Press (1984).
20. Songer JG, Pos KW. (ed.) *The Genera Mannheimia and Pasteurella*. St. Louis, MO: Elsevier Saunders (2005).
21. Rice JA, Carrasco-Medina L, Hodgins DC, Shewen PE. Mannheimia haemolytica and bovine respiratory disease. *Anim Health Res Rev.* (2008) 8:117–28. doi: 10.1017/S1466252307001375
22. Highlander SK. Molecular genetic analysis of virulence in Mannheimia (Pasteurella) haemolytica. *Front Biosci. (2001) D1128–50.* doi: 10.2741/A574
23. Highlander SK, Fedorova ND, Dusek DM, Panciera R, Alvarez LE, Renehart C. Inactivation of pasteurella (Mannheimia) haemolytica leukotoxin causes partial attenuation of virulence in a calf challenge model. *Infect Immun.* (2000) 68:3916–22. doi: 10.1128/IAI.68.7.3916-3922.2000
24. Shewen PE, Hodkins DC. (*ed.) Pneumonic Pasteurellosis of Cattle*. Cape Town: Oxford University Press (2004).
25. Zecchinon L, Frett T, Desmecht D. How mannheimia haemolytica defeats host defense through a kiss of death mechanism. *Vet Res.* (2005) 36:133–56. doi: 10.1051/vetres:2004065
26. Hodgson PD, Aich P, Manuja A, Hokamp K, Roche FM, Brinkman FS, et al. Effect of stress on viral-bacterial synergy in bovine respiratoryt disease: novel mechanisms to regulate inflammation. *Comp Funct Genomics.* (2005) 6:244–50. doi: 10.1002/cfg.474
27. Jones C, Chowdhury S. Bovine herpesvirus type 1 (BHV-1) is an important cofactor in the bovine respiratory disease complex. In: Cooper VL, Broderson B, editors. *Veterinary Clinics of North America, Food Animal Practice, Bovine Respiratory Disease.* New York, NY: Elsevier, (2010). p. 303–21. doi: 10.1016/j.cvfa.2010.04.007
28. Leite F, Kuckleburg C, Atapattu D, Schulz R, Czuprynski CJ. BHV-1 infection and inflammatory cytokines amplify the interaction between mannheimia haemolytica lukotoxin with bovine peripheral blood mononuclear cells *in vitro*. *Vet Immunol Immunopathol.* (2004) 99:193–202. doi: 10.1016/j.vetimm.2004.02.004
29. Rivera-Rivas JJ, Kisiela D, Czuprynski CJ. Bovine herpesvirus type 1 infection of bovine bronchial epithelial cells increases neutrophil adhesion and activation. *Vet Immunol Immunopathol.* (2009) 131:167–76. doi: 10.1016/j.vetimm.2009.04.002
30. Yates WD, Babiuk LA, Jericho K W. Viral-bacterial pneumonia in calves: duration of the interaction between bovine herpesvirus 1 and pasteurella haemolytica. *Can J Comp Med.* (1983) 47:257–64.
31. Neibergs HL, Seabury CM, Wojtowicz AJ, Wang Z, Scraggs E, Kiser JN, et al. Susceptibility loci revealed for bovine respiratory disease complex in pre-weaned holstein calves. *BMC Genomics.* (2014) 15:1–19. doi: 10.1186/1471-2164-15-1164
32. Jones C. Alphaherpesvirus latency: its role in disease and survival of the virus in nature. *Adv Virus Res.* (1998) 51:81–133. doi: 10.1016/S0065-3527(08)60784-8
33. Jones C. Herpes simplex virus type 1 and bovine herpesvirus 1 latency. *Clin Micro Rev.* (2003) 16:79–95. doi: 10.1128/CMR.16.1.79-95.2003
34. Misra V, Bratanich AC, Carpenter D, O'Hare P. Protein and DNA elements involved in transactivation of the promoter of the bovine herpesvirus (BHV) 1 IE-1 transcription unit by the BHV alpha gene trans-inducing factor. *J Virol.* (1994) 68:4898–909.
35. Misra V, Walker S, Hayes S, O'Hare P. The bovine herpesvirus alpha gene trans-inducing factor activates transcription by mechanisms different from those of its herpes simplex virus type 1 counterpart VP16. *J Virol.* (1995) 69:5209–16.
36. Wirth UV, Fraefel C, Vogt B, Vlcek C, Paces V, Schwyzer M. Immediate-early RNA 2.9 and early RNA 2.6 of bovine herpesvirus 1 are 3' coterminal and encode a putative zinc finger transactivator protein. *J Virol.* (1992) 66:2763–72.

37. Wirth UV, Gunkel K, Engels M, Schwyzer M. Spatial and temporal distribution of bovine herpesvirus 1 transcripts. *J Virol.* (1989) 63:4882–9.
38. Wirth UV, Vogt B, Schwyzer M. The three major immediate-early transcripts of bovine herpesvirus 1 arise from two divergent and spliced transcription units. *J Virol.* (1991) 65:195–205.
39. Fraefel C, Zeng J, Choffat Y, Engels M, Schwyzer M, Ackermann M. Identification and zinc dependence of the bovine herpesvirus 1 transactivator protein BICP0. *J Virol.* (1994) 68:3154–62.
40. Boutell C, Everett RD. Regulation of alphaherpesvirus infections by the ICP0 family of proteins. *J Gen Virol.* (2013) 94:465–81. doi: 10.1099/vir.0.048900-0
41. Inman M, Zhang Y, Geiser V, Jones C. The zinc ring finger in the bICP0 protein encoded by bovine herpes virus-1 mediates toxicity and activates productive infection. *J Gen Virol.* (2001b) 82:483–92. doi: 10.1099/0022-1317-82-3-483
42. Saira S, Chowdhury S, Gaudreault N, Henderson G, Doster A, Jones C. The zinc RING finger of the bovine herpesvirus 1 encoded bICP0 protein is crucial for viral replication and virulence. *J Virol.* (2008) 82:12060–8. doi: 10.1128/JVI.01348-08
43. Schang L, Jones C. Analysis of bovine herpesvirus 1 transcripts during a primary infection of trigeminal ganglia of cattle. *J Virol.* (1997) 71:6786–95.
44. Inman M, Lovato L, Doster A, Jones C. A mutation in the latency related gene of bovine herpesvirus 1 interferes with the latency-reactivation cycle of latency in calves. *J Virol.* (2002) 76:6771–9. doi: 10.1128/JVI.76.13.6771-6779.2002
45. Jones C, Geiser V, Henderson G, Jiang Y, Meyer F, Perez S, et al. Functional analysis of bovine herpesvirus 1 (BHV-1) genes expressed during latency. *Vet Micro.* (2006) 113:199–210. doi: 10.1016/j.vetmic.2005.11.009
46. Kutish G, Mainprize T, Rock D. Characterization of the latency-related transcriptionally active region of the bovine herpesvirus 1 genome. *J Virol.* (1990) 64:5730–7.
47. Rock D, Lokensgard J, Lewis T, Kutish G. Characterization of dexamethasone-induced reactivation of latent bovine herpesvirus 1. *J Virol.* (1992) 66:2484–90.
48. Rock DL, Beam SL, Mayfield JE. Mapping bovine herpesvirus type 1 latency-related RNA in trigeminal ganglia of latently infected rabbits. *J Virol.* (1987) 61:3827–31.
49. Bratanich AC, Hanson ND, Jones C. The latency-related gene of bovine herpesvirus 1 inhibits the activity of immediate-early transcription unit 1. *Virology.* (1992) 191:988–91. doi: 10.1016/0042-6822(92)90278-W
50. Hossain A, Schang LM, Jones C. Identification of gene products encoded by the latency-related gene of bovine herpesvirus 1. *J Virol.* (1995) 69:5345–52.
51. Inman M, Lovato L, Doster A, Jones C. A mutation in the latency-related gene of bovine herpesvirus 1 leads to impaired ocular shedding in acutely infected calves. *J Virol.* (2001a) 75:8507–15. doi: 10.1128/JVI.75.18.8507-8515.2001
52. Perez S, Inman M, Doster A, Jones C. Latency-related gene encoded by bovine herpesvirus 1 promotes virus growth and reactivation from latency in tonsils of infected calves. *J Clin Micro.* (2005) 43:393–401. doi: 10.1128/JCM.43.1.393-401.2005
53. Jiang Y, Inman M, Zhang Y, Posadas NA, Jones C. A mutation in the latency related gene of bovine herpesvirus 1 (BHV-1) inhibits protein expression of a protein from open reading frame 2 (ORF-2) and an adjacent reading frame during productive infection. *J Virol.* (2004) 78:3184–9. doi: 10.1128/JVI.78.6.3184-3189.2004
54. Meyer F, Perez S, Jiang Y, Zhou Y, Henderson G, Jones C. Identification of a novel protein encoded by the latency-related gene of bovine herpesvirus 1. *J Neurovirology.* (2007) 13:569–78. doi: 10.1080/13550280701620754
55. Ciacci-Zanella J, Stone M, Henderson G, Jones C. The latency-related gene of bovine herpesvirus 1 inhibits programmed cell death. *J Virol.* (1999) 73:9734–40.
56. Henderson G, Perng C-G, Nesburn A, Wechsler S, Jones C. The latency related gene of bovine herpesvirus 1 can suppress caspase 3 and caspase 9 during productive infection. *J Neurovirol.* (2004) 10:64–70. doi: 10.1080/13550280490261716
57. Shen W, Jones C. Open reading frame 2 encoded by the latency related gene of bovine herpesvirus 1 has anti-apoptosis activity in transiently transfected neuroblastoma cells. *J Virol.* (2008) 82:10940–5. doi: 10.1128/JVI.01289-08
58. Jaber T, Workman A, Jones C. Small noncoding RNAs encoded within the bovine herpesvirus 1 latency-related gene can reduce steady-state levels of infected cell protein 0 (bICP0). *J Virol.* (2010) 84:6297–307. doi: 10.1128/JVI.02639-09
59. Jones C, Newby TJ, Holt T, Doster A, Stone M, Ciacci-Zanella J, et al. Analysis of latency in cattle after inoculation with a temperature sensitive mutant of bovine herpesvirus 1 (RLB106). *Vaccine.* (2000) 18:3185–95. doi: 10.1016/S0264-410X(00)00106-7
60. Frizzo da Silva Kook LI, Doster A, Jones C. Bovine herpesvirus 1 regulatory proteins, bICP0 and VP16, are readily detected in trigeminal ganglionic neurons expressing the glucocorticoid receptor during the early stages of reactivation from latency. *J Virol.* (2013) 87:11214–22. doi: 10.1128/JVI.01737-13
61. Kook I, Doster A, Jones C. Bovine herpesvirus 1 regulatory proteins are detected in trigeminal ganglionic neurons during the early stages of stress-induced escape from latency. *J Neurovirol.* (2015a) 21:585–91. doi: 10.1007/s13365-015-0339-x
62. Winkler MT, Doster A, Jones C. Persistence and reactivation of bovine herpesvirus 1 in the tonsil of latently infected calves. *J Virol.* (2000) 74:5337–46. doi: 10.1128/JVI.74.11.5337-5346.2000
63. Winkler MT, Doster A, Sur JH, Jones C. Analysis of bovine trigeminal ganglia following infection with bovine herpesvirus 1. *Vet Microbiol.* (2002) 86:139–55. doi: 10.1016/S0378-1135(01)00498-9
64. Workman A, Eudy J, Smith L, Frizzo da Silva L, Sinani D, Bricker H, et al. Cellular transcription factors induced in trigeminal ganglia during dexamethasone-induced reactivation from latency stimulate bovine herpesvirus 1 productive infection and certain viral promoters. *J Virol.* (2012) 86:2459–73. doi: 10.1128/JVI.06143-11
65. Bieker JJ. Kruppel-like factors: three fingers in many pies. *J Biol Chem.* (2001) 276:34355–8. doi: 10.1074/jbc.R100043200
66. Kaczynski J, Cook T, Urrutia R. Sp1- and Kruppel-like transcription factors. *Genome Biol.* (2003) 4:206.201. doi: 10.1186/gb-2003-4-2-206
67. El-Mayet FS, Sawant L, Thungunutla P, Jones C. Combinatorial effects of the glucocorticoid receptor and Krüppel-like transcription factor 15 on bovine herpesvirus 1 transcription and productive infection. *J Virol.* (2017) 91:e00904–17. doi: 10.1128/JVI.00904-17
68. Kook I, Henley C, Meyer F, Hoffmann F, Jones C. Bovine herpesvirus 1 productive infection and the immediate early transcription unit 1 are stimulated by the synthetic corticosteroid dexamethasone. *Virology.* (2015b) 484:377–85. doi: 10.1016/j.virol.2015.06.010
69. Sawant L, Kook I, Vogel JL, Kristie TM, Jones C. The cellular coactivator HCF-1 is required for glucocorticoid receptor-mediated transcription of bovine herpesvirus 1 immediate early genes. *J Virol.* (2018) 92:e00987–18. doi: 10.1128/JVI.00987-18
70. Barnes PJ. Anti-inflammatory actions of glucocorticoids: molecular mechanisms. *Clin Sci.* (1998) 94:557–72. doi: 10.1042/cs0940557
71. Funder JW. Glucocorticoids and mineralocorticoid receptors: biology and clinical relevance. *Annu Rev Med.* (1997) 48:231–40. doi: 10.1146/annurev.med.48.1.231
72. Schonevild OJLM, Gaemers IC, Lamers WH. Mechanisms of glucocorticoid signalling. *Biochem Biophys Acta.* (2004) 1680:114–28. doi: 10.1016/j.bbaexp.2004.09.004
73. Babiuk LA, Bielefeldt Ohamnn H, Gifford H, Czarniecki CW, Scialli VT, Hamilton EB. Effect of bovine alpha 1 interferon on bovine herpesvirus type 1 induced respiratory disease. *J Gen Virol.* (1985) 66:2383–94. doi: 10.1099/0022-1317-66-11-2383
74. Jensen J, Schulz RD. Bovine natuaral cell mediated cytotoxicity (NCMC): activation by cytokines. *Vet Immunol Immunopathol.* (1990) 24:113–29. doi: 10.1016/0165-2427(90)90014-J
75. Lawman MJP, Gifford G, Gyongyossy-Issa M, Dragan R, Heise J, Babiuk LA. Activity of polymorphonuclear (PMN) leukocytes diseases bovine herpesvirus-1 induced repiratory disease: Effect of recombinant bovine interferon alpha I. *Antiviral Res.* (1987) 8:225–38. doi: 10.1016/S0166-3542(87)80001-3
76. Straub OC, Ahl R. Lokale interferonbildung beim rind nach intranasaler infektion mit avirulentm IBR-IPV-virus und deren wirkung auf eine anschliebende inkection mit maul und klauenseuche-virus. *Zbl Vet Med B.* (1976) 23:470–82. doi: 10.1111/j.1439-0450.1976.tb01627.x

77. Marshall RL, Israel BA, Letchworth GJ. Monoclonal antibody analysis of bovine herpesvirus-1 glycoprotein antigenic areas relevant to natural infection. *Virology.* (1988) 165:338–47. doi: 10.1016/0042-6822(88)90578-8
78. Van Drunen Littel-van den Hurk S, Babiuk LA. Polypeptide specificity of the antibody reponse after primary and recurrent infection with bovine herpesvirus 1. *J Clin Microbiol.* (1986) 23:274–82.
79. Denis M, Slaoui M, Keil GM, Babiuk LA, Ernst E, Pastorer PP, et al. Identification of different target glycoproteins for bovine herpesvirus-1-specific cytotoxic T lymphocytes depending on the method of *in vitro* stimulation. *Immunology.* (1993) 78:7–13.
80. Huang Y, Babiuk LA, van Drunen Littel-van den Hurk S. Immunization with a bovine heprsvirus-1 glycoprotein B DNA vaccine induces cytotoxic T lymphocyte responses in mice and cattle. *J Gen Virol.* (2005) 88:887–98. doi: 10.1099/vir.0.80533-0
81. Van Drunen Littel-van den Hurk S. Cell-mediated immune responses induced by BHV-1: rational vaccine design. *Expert Rev Vaccines.* (2007) 6:369–80. doi: 10.1586/14760584.6.3.369
82. Wang J, Alexander J, Wiebe M, Jones C. Bovine herpesvirus 1 productive infection stimulates inflammasome formation and caspase 1 activity. *Virus Res.* (2014) 185:72–6. doi: 10.1016/j.virusres.2014.03.006
83. Carter JJ, Weinberg AD, Pollard A, Reeves R, Magnuson JA, Magnuson NS. Inhibition of T-lymphocyte mitogenic responses and effects on cell functions by bovine herpesvirus 1. *J Virol.* (1989) 63:1525–30.
84. Griebel P, Ohmann HB, Lawman MJ, Babiuk LA. The interaction between bovine herpesvirus type 1 and activated bovine T lymphocytes. *J Gen Virol.* (1990) 71:369–77. doi: 10.1099/0022-1317-71-2-369
85. Griebel P, Qualtiere L, Davis WC, Gee A, Bielefeldt Ohmann H, Lawman MJ, et al. T lymphocyte population dynamics and function following a primary bovine herpesvirus type-1 infection. *Viral Immunol.* (1987) 1:287–304. doi: 10.1089/vim.1987.1.287
86. Griebel PJ, Qualtiere L, Davis WC, Lawman MJ, Babiuk LA. Bovine peripheral blood leukocyte subpopulation dynamics following a primary bovine herpesvirus-1 infection. *Viral Immunol.* (1987) 1:267–86. doi: 10.1089/vim.1987.1.267
87. Hariharan MJ, Nataraj C, Srikumaran S. Down regulation of murine MHC class I expression by bovine herpesvirus 1. *Viral Immunol.* (1993) 6:273–84. doi: 10.1089/vim.1993.6.273
88. Hinkley S, Hill AB, Srikumaran S. Bovine herpesvirus-1 infection affects the peptide transport activity in bovine cells. *Virus Res.* (1998) 53:91–6. doi: 10.1016/S0168-1702(97)00128-7
89. Nataraj C, Eidmann S, Hariharan MJ, Sur JH, Perry GA, Srikumaran S. Bovine herpesvirus 1 downregulates the expression of bovine MHC class I molecules. *Viral Immunol.* (1997) 10:21–34. doi: 10.1089/vim.1997.10.21
90. Eskra L, Splitter GA. Bovine herpesvirus-1 infects activated CD4+ lymphocytes. *J Gen Virol.* (1997) 78:2159–66. doi: 10.1099/0022-1317-78-9-2159
91. Winkler MT, Doster A, Jones C. Bovine herpesvirus 1 can infect CD4(+) T lymphocytes and induce programmed cell death during acute infection of cattle. *J Virol.* (1999) 73:8657–68.
92. Henderson G, Zhang Y, Jones C. The bovine herpesvirus 1 gene encoding infected cell protein 0 (bICP0) can inhibit interferon-dependent transcription in the absence of other viral genes. *J Gen Virol.* (2005) 86:2697–702. doi: 10.1099/vir.0.81109-0
93. Jones C. Regulation of innate immune responses by bovine herpesvirus 1 and infected cell protein 0. *Viruses.* (2009) 1:255–75. doi: 10.3390/v1020255
94. Saira K, Zhou Y, Jones C. The infected cell protein 0 encoded by bovine herpesvirus 1 (bICP0) induces degradation of interferon response factor 3 (IRF3), and consequently inhibits beta interferon promoter activity. *J Virol.* (2007) 81:3077–86. doi: 10.1128/JVI.02064-06
95. Saira K, Jones C. The infected cell protein 0 encoded by bovine herpesvirus 1 (bICP0) associates with interferon regulatory factor 7 (IRF7), and consequently inhibits beta interferon promoter activity. *J Virol.* (2009) 83:3977–81. doi: 10.1128/JVI.02400-08
96. Goodbourn S, Zinn K, Maniatis T. Human beta-interferon gene expression is regulated by an inducible enhancer element. *Cell.* (1985) 41:509–20. doi: 10.1016/S0092-8674(85)80024-6
97. Honda K, Yanai H, Negishi H, Asagiri M, Saton M, Mizutani T, et al. IRF-7 is the master regulator of type-I interferon-dependent immune responses. *Nature.* (2005) 434:772–7. doi: 10.1038/nature03464
98. Wathelet MG, Lin CH, Parekh BS, Ronco LV, Howley PM, Maniatis T. Virus infection induces the assembly of coordinately activated transcription factors on the IFN-b enhancer *in vivo*. *Mol Cell.* (1998) 1:507–18. doi: 10.1016/S1097-2765(00)80051-9
99. Zhang Y, Jones C. Identification of functional domains within the bICP0 protein encoded by BHV-1. *J Gen Virol.* (2005) 86:879–86. doi: 10.1099/vir.0.80698-0
100. Everett RD. ICP0, a regulator of herpes simplex virus during lytic and latent infection. *Bioessays.* (2000) 22:761–70. doi: 10.1002/1521-1878(200008)22:8<761::AID-BIES10>3.0.CO;2-A
101. Parkinson J, Everett RD. Alphaherpesvirus proteins related to herpes simplex virus type 1 ICP0 affect cellular structures and proteins. *J Virol.* (2000) 74:10006–17. doi: 10.1128/JVI.74.21.10006-10017.2000
102. Geiser V, Jones C. Stimulation of bovine herpesvirus 1 productive infection by the adneovirus E1A gene and a cell cycle regulatory gene, E2F-4. *J Gen Virol.* (2003) 84:929–38. doi: 10.1099/vir.0.18915-0
103. Scherer M, Stamminger T. Emerging role of PML nuclear bodies in innate immune signaling. *J Virol.* (2016) 90:5850–4. doi: 10.1128/JVI.01979-15
104. Chee AV, Pandolfi LP, Roizman B. Promyelocytic leukemia protein mediates interferon-based anti-herpes simplex virus 1 effects. *J Virol.* (2003) 77:7101–5. doi: 10.1128/JVI.77.12.7101-7105.2003
105. Regad T, Saib A, Lallemand-Breitenbach V, Pandolfi PP, de The H, Chelbi-Alix MK. PML mediates the interferon-induced antiviral state against a complex retrovirus via its association with the viral transactivator. *Embo J.* (2001) 20:3495–505. doi: 10.1093/emboj/20.13.3495
106. Chen Y, Wright J, Meng X, Leppard KN. Promyelocytic leukemia protein isoform II promotes transcription factor recruitment to activate interferon beta and interferon-responsive gene expression. *Molec Cell Biol.* (2015) 35:1660–72. doi: 10.1128/MCB.01478-14
107. Dia L, Zhang B, Fan J, Gao X, Sun S, Yang K, et al. Herpes virus proteins ICP0 and BICP0 can activate NF-kB by catalyzing IkBa ubiquitination. *Cell Signal.* (2005) 17:217–29. doi: 10.1016/j.cellsig.2004.07.003
108. Guo J, Peters KL, Sen GC. Induction of the human protein P56 by interferon, double stranded RNA, or virus infection. *Virology.* (2000) 267:209–19. doi: 10.1006/viro.1999.0135
109. Mossman KL, Macgregor PF, Rozmus JJ, Goryachev AB, Edwards AM, Smiley JR. Herpes simplex virus triggers and then disarms a host antiviral response. *J Virol.* (2001) 75:750–8. doi: 10.1128/JVI.75.2.750-758.2001
110. Alandijany T, Roberts APE, Con KL, Loney C, McFarlane S, Boutell C. Distinct temporal roles for the promyelocytic leukaemia (PML) protein in the sequential regulation of intracellular host immunity to HSV-1 infection. *PloS Pathog.* (2018) 14:e1006769. doi: 10.1371/journal.ppat.1006769
111. Sandri-Goldin RM. The many roles of the highly interactive HSV protein ICP27, a key regulator of infection. *Future Microbiol.* (2011) 6:1261–77. doi: 10.2217/fmb.11.119
112. Johnson KE, Song B, Knipe DM. Role for herpes simplex virus 1 ICP27 in the inhibition of type I interferon signaling. *Virology.* (2008) 374:487–94. doi: 10.1016/j.virol.2008.01.001
113. Christensen M, Jensen SB, Miettinen JJ, Luecke S, Prabakaran T, Reinert LS, et al. HSV-1 ICP27 targets the TBK1-activated STING signalsome to inhibit virus-induced type *I* IFN expression. *Embo J.* (2016) 35:1385–99.doi: 10.15252/embj.201593458
114. da Silva LF, Jones C. The ICP27 protein encoded by bovine herpesvirus type 1 (bICP27) interferes with promoter activity of the bovine genes encoding beta interferon 1 (IFN-β1) and IFN-β3. *Virus Res.* (2012) 162–8. doi: 10.1016/j.virusres.2012.07.023
115. Valarcher, J-F, Furze J, Wyld S, Cook R, Conzelman K-K, Taylor G. Role of alpha/beta interferons in the attenuation and immunogenecity of recombinant bovine respiratory syncitial viruses lacking NS proteins. *J Virol.* (2003) 77:8426–39. doi: 10.1128/JVI.77.15.8426-8439.2003
116. Wilson V, Jeffreys AJ, Barrie PA. A comparison of vertebrate interferon gene families deteced by hybridization with human interferon DNA. *J Mol Biol.* (1983) 166:457–75. doi: 10.1016/S0022-2836(83)80281-2

117. Nakamichi K, Ohara N, Kuroki D, Otsuka H. Bovine herpesvirus 1 glycoprotein G is required for viral growth by cell-to-cell infection. *Virus Res.* (2000) 68:175–81. doi: 10.1016/S0168-1702(00)00171-4
118. Nakanishi K, Maruyama M, Shibata T, Morishima N. Identification of a caspase-9 substrate and detection of its cleavage in programmed cell death during mouse development. *J Biol Chem.* (2001) 276:41237–44. doi: 10.1074/jbc.M105648200
119. Bryant NA, Davis-Poynter N, Vanderplasschen A, Alcami A. Glycoprotein G isoforms from some alphaherpesvirus function as broad-spectrum chemokine binding proteins. *EMBO J.* (2003) 22:833–46. doi: 10.1093/emboj/cdg092
120. Griffith J, Sokol CL, Luster AD. Chemokines and chemokine receptors: position cells for host defense and immunity. *Ann Rev Immunol.* (2014) 32:659–702. doi: 10.1146/annurev-immunol-032713-120145
121. Liang X, Tang M, Manns B, Babiuk LA, Zamb TJ. Identification and deletion mutagenesis of the bovine herpesvirus 1 dUTPase gene and a gene homologous to herpes simplex virus UL49.5. *Virology.* (1993) 195:42–50. doi: 10.1006/viro.1993.1344
122. Koppers-Lalic DE, Reits AJ, Ressing ME, Lipinska AD, Abele R, Koch J, et al. Varicelloviruses avoid T cell recognition by UL49.5-mediated inactivation of the transporter associated with antigen processing. *Proc Nat Acad Sci.* (2005) 102:5144–9. doi: 10.1073/pnas.0501463102
123. Lipinska AD, Koppers-Lalic D, Rychlowski M, Admiraal P, Rijsewijk FAM, Bienkowska-Szewczyk K, et al. Bovine herpesvirus 1 UL49.5 protein inhibits the transporter associated with antigen processing despite complex formation with glycoprotein M. *J Virol.* (2006) 81:5822–32. doi: 10.1128/JVI.02707-05
124. Ahn K, Meyer TH, Uebel S, Sempe P, Djaballah H, Yang Y, et al. Molecular mechanism and species specificity of TAP inhibition by herpes simplex virus ICP47. *EMBO J.* (1996) 15:3247–55. doi: 10.1002/j.1460-2075.1996.tb00689.x
125. Ambagala AP, Gopinath RS, Srikumaran S. Peptide transport activity of the transporter associated with antigen processing (TAP) is inhibited by an early protein of equine herpesvirus-1. *J Gen Virol.* (2004) 66:2383–94. doi: 10.1099/vir.0.19563-0
126. Hughes EA, Hammond C, Cresswell P. Misfolded major histocompatibility complex class I heavy chains are translocated into the cytoplasm and degraded by the proteosome. *Proc Nat Acad Sci.* (1997) 94:1896–901. doi: 10.1073/pnas.94.5.1896
127. Wei H, He J, Paulsen DB, Chowdhury SI. Bovine herpesvirus type 1 (BHV-1) mutant lacking UL49.5 luminal domain residues 30-32 and cytoplasmic tail residues 80-96 induces more rapid onset of virus neutralizing antibody and cellular immune responses in calves than the wild-type strain Cooper. *Vet Immunol Immunopathol.* (2012) 147:223–9. doi: 10.1016/j.vetimm.2012.04.015
128. Lobanov VA, Maher-Sturgess SL, Snider MG, Lawman Z, Babiuk LA, van Drunen Littel-van den Hurk S. A UL47 gene deletion mutant of bovine herpesvirus type 1 exhibits impaired growth in cell culture and lack of virulence in cattle. *J Virol.* (2010) 84:445–58. doi: 10.1128/JVI.01544-09
129. Fruh M, Fruh K. Viral modulators of cullin RING ubiquitin ligases: culling the host defense. *Sci STKE.* (2006) 335:pe21. doi: 10.1126/stke.3352006pe21
130. Zhang K, Afroz S, Brownlie R, Snider M, van Drunen Littel-van den Hurk S. Regulation and function of phosphorylation on VP8, the major tegument protein of bovine herpesvirus 1. *J Virol.* (2015) 89:4598–611. doi: 10.1128/JVI.03180-14
131. Afroz S, Brownlie R, Fodje M, van Drunen Littel-van den Hurk S. VP8, the major tegument protein of bovine herpesvirus 1, interacts with cellular STAT1 and inhibits interferon beta signaling. *J Virol.* (2016) 90:4889–904. doi: 10.1128/JVI.00017-16
132. Sikorski K, Chmielewski S, Olejnik A, Wesoly JZ, Heemann U, Baumann M, et al. STAT1 as a central mediator of IFN-gamma and TLR4 signal integration in vascular dysfunction. *Landes Biosci.* (2012) 1:241–9. doi: 10.4161/jkst.22469
133. Katze M, He Y, Gale M Jr. Viruses and interferon: a fight for supremacy. *Nat Rev Imunology.* (2002) 2:675–87. doi: 10.1038/nri888

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

MINI REVIEW
published: 10 June 2019
doi: 10.3389/fimmu.2019.01155

Subversion of Immune Response by Human Cytomegalovirus

*A. Raj Kumar Patro**

Infectious Disease Biology Group, Institute of Life Sciences (ILS), Bhubaneswar, India

Human cytomegalovirus (HCMV) is the most common cause of congenital infections and is an important pathogen in immunocompromised individuals. Despite a robust host immune system, HCMV able to replicate, evade host defenses, establish latency for life. A significant portion of HCMV genome dedicated to encode gene products for modulation of host immune response. Growing number of HCMV gene products are being recognized to play role in immune evasion. Information on viral immune evasion mechanisms by which HCMV persists in host will be useful in devising antiviral intervention strategies and development of new vaccines. This minireview provides a brief overview of immune evasion strategy adapted by HCMV by utilizing its gene products in modulation of host immune response.

Keywords: HCMV (human cytomegalovirus), immune evasion, pathogenesis, superinfection, vaccine

OPEN ACCESS

Edited by:
Richard D. Dix,
Georgia State University,
United States

Reviewed by:
Edward Mocarski,
Emory University, United States
Sarah Rowland-Jones,
University of Oxford, United Kingdom

***Correspondence:**
A. Raj Kumar Patro
rajkumarpatro@yahoo.com

Specialty section:
This article was submitted to Viral Immunology, a section of the journal Frontiers in Immunology

Received: *28 November 2018*
Accepted: *07 May 2019*
Published: *10 June 2019*

Citation:
Patro ARK (2019) Subversion of Immune Response by Human Cytomegalovirus. Front. Immunol. 10:1155. doi: 10.3389/fimmu.2019.01155

INTRODUCTION

The human cytomegalovirus (HCMV) is a ubiquitous β-herpesvirus that establishes lifelong persistent infection following introduction to an immunocompetent host. Primary infection in a healthy individual leads to mild febrile illness, whereas HCMV causes serious complications in immunosuppressed subjects, especially in transplant recipients and in immunocompromised patients (1, 2). Human cytomegalovirus is the most common cause of congenital infections leading to neurodevelopmental sequelae. Each year, 20,000–40,000 children are born with congenital human CMV infection in the US, of which 10–15% develops permanent sequelae including sensorineural hearing loss (3–5). Furthermore, substantial fraction of the asymptomatic children develops late onset hearing loss. In an attempt to reduce these disabilities and loss of life, as well as the associated economic cost, the Institute of Medicine of National Academy of Sciences, USA have ranked the development of HCMV vaccine as a highest priority (6, 7).

Decades of research on cytomegalovirus has provided novel insight in understanding the host immune response and evasion strategies adapted by the virus. HCMV has dedicated more than half of its genome encoding for modulation of host response to infection (8, 9). This mini-review article discusses on current understanding of HCMV gene products in modulation of host immune response with an emphases on the immune evasion by interference in antigen presentation and activation of NK cells, viral strain diversity and superinfection in immune subject.

MODULATION OF IMMUNE RESPONSES BY HCMV GENE PRODUCTS

The virus has co-evolved with its host organism for 200 million years (9, 10). HCMV has a large genome size of 236 kb with unique long (UL) and unique short (US) regions flanked by terminal repeats and internal repeats. The genome has been annotated and encodes 167 gene products, as

well as non-coding RNAs, microRNAs, and with an extensive alternate mRNA splicing. However, recent report suggested that HCMV encode to have more than 750 translated ORFs (11). More than 40 HCMV gene products are recognized to have a role in modulating the host immune response following infection (12, 13). Both the innate and adaptive arms of the immune system play a crucial role in controlling HCMV infection (12, 14). Despite a robust host immune system, HCMV is able to establish latency and once infected the HCMV remains in the host for life. The virus remains latent in the myeloid progenitor cells during its dormant phase; however on stimulation, or when the immune system is suppressed, the virus can once again become active (15). The battle between the host immune system and the virus continues throughout life, with HCMV having evolved multiple mechanisms to evade the host immune response. The divergence of the immune response and incomplete viral control may be attributed to the diversity of immune modulators encoded by HCMV gene products [**Figure 1**, **Table 1**]. Many of these gene products are homologs of host genes involved in the immune response.

To eliminate the virus, the host needs to have an effective immune system. After viral infection, host antigen presenting cells must present viral antigen to the immune cells in order to stimulate effector cells to eliminate the virus. However, HCMV has devised strategies to limit this presentation. NK cells are normally responsible for immediate control of viral infections; however, there are number of HCMV gene products that block NK cell mediated recognition. Approximately, there are 12 HCMV gene products, US20, UL16, UL17, UL18, UL40, UL43, UL140, UL83, UL141-UL144, and UL148, known to control NK cell modulation (**Table 1**). HCMV UL16, UL17, UL40, UL140, and UL142 genes all encode products that down-regulate NK cell activity by imitating the host HLA class I. For example, UL40 encodes a canonical ligand for HLA-E and negatively regulates NK cells, which results in down-regulation of activating ligand CD155 (20). Individuals with impaired NK cell function, succumbs to severe herpesvirus infections (31). In addition, HCMV gene products UL18 and UL83 (pp65) encode for an MHC-I homolog, modulate expression of other HCMV genes and inhibit NK cell lysis (12, 20). Furthermore, the HCMV microRNA miR-UL122 acts to suppress host MICB surface expression (13, 20, 29).

As is a common characteristic of herpes viruses, HCMV is able to interfere with the class I MHC molecule involved in antigen presentation to CD8+ T cells. HCMV establishes persistent infection by producing host homologous molecules that prevent recognition and interfere with antigen presentation, subverting the cytotoxic T lymphocytes (CTLs). Viral antigens are normally presented by the MHC class I proteins on the infected cell surface. HCMV gene products obstruct peptide translocation to the ER lumen and stimulate degradation of the MHC class I proteins before they can reach the cell surface. For example, the HCMV US3 gene product degrades the MHC class I heavy chain by interacting with Tapasin and retaining the class I molecule at the site of synthesis, in the ER. In addition, the US2 and US11 gene products relocate the heavy chain of MHC class I into the ER for proteosomal degradation. Similarly, another gene product of HCMV, US6, prevents peptide loading by inhibiting the binding of ATP to TAP, thereby preventing the transport of peptides through the TAP pore. The combined functions of the HCMV gene products US2, US3, US6, and US11, therefore, lead to peptide transport blockade, retention of MHC class I in the ER and ultimately proteasomal degradation. In addition, the gene product US2 interferes with MHC class II signal transduction by degradation of MHC class II proteins. US2 targets the class II DR and DMα chains for degradation in the cytosol, thereby preventing antigen presentation to CD4+ T lymphocytes (12, 14, 16, 32).

In addition to the above, the HCMV UL83 gene product, pp65 blocks the processing of immediate early-1 in the proteasome by phosphorylation. Besides, the tegument Protein UL82 evades antiviral immunity by inhibiting stimulator of interferon (STING) signaling (21) and may be responsible for induction of latency (15, 22). Recently, Nightingale et al. reported that the HCMV gene product UL145 facilitates degradation of the antiviral factor helicase like transcription factor (HLTF) by recruiting the host Cullin4 E3 ligase complex, and captures Cullin3 to invoke the strategy of immune evasion (27). Additionally, the HCMV late gene product UL111A encodes cmvIL-10, a homolog of human IL-10, which is expressed during viral latency, and causes a state of immune suppression (23). The cytokine Interleukin-10 has an immunosuppressive role on several effector cells of the immune system. The HCMV gene product cmvIL-10 exerts an immunosuppressive effect on the host by modulating the expression of the MHC class I and II molecules and interfering with dendritic cell (DC) function (24). In a murine model of CMV, following productive infection with CMV both *in vitro* and *ex vivo*, the virus reduced the expression of MHC as well as co-stimulation of DC. This eventually led to loss of expression of IL-2 and IL-12 and hindrance of DC differentiation (33–35). A recent report by Wang et al also demonstrated that the HCMV UL148 gene product suppresses co-stimulation and expression of the cell adhesion molecule CD58, endorsing cellular immune defense evasion by impairing NK and T cell activation (28). This work was further supported by HCMV UL148 mediated tropism and immune evasion by unfolded protein response (36). In Rhesus model, Rh159, a homolog of HCMV UL148 involved in retention of distinct set of costimulatory molecules and involved in NK cell evasion (37). HCMV UL148 gene products encode for avoidance of killing of HCMV infected cells from NK cells by down regulating MICA (38).

HCMV possesses a unique challenge, as it is able to super-infect in a subject already infected with the virus, even in the presence of a strong specific immune response. Several studies have demonstrated congenital HCMV infection in offspring of immune mothers because of reinfection with a different strain of virus (39–43). Further, congenital infected infant born to immune mother may develop sequelae similar to infants born to mother with primary infection during pregnancy. It has also been observed that infection with more than one strain of HCMV is common in nature (39, 40, 44). HCMV strain polymorphism could contribute to immune evasion. Since HCMV glycoproteins are highly polymorphic, antibody response to one strain may

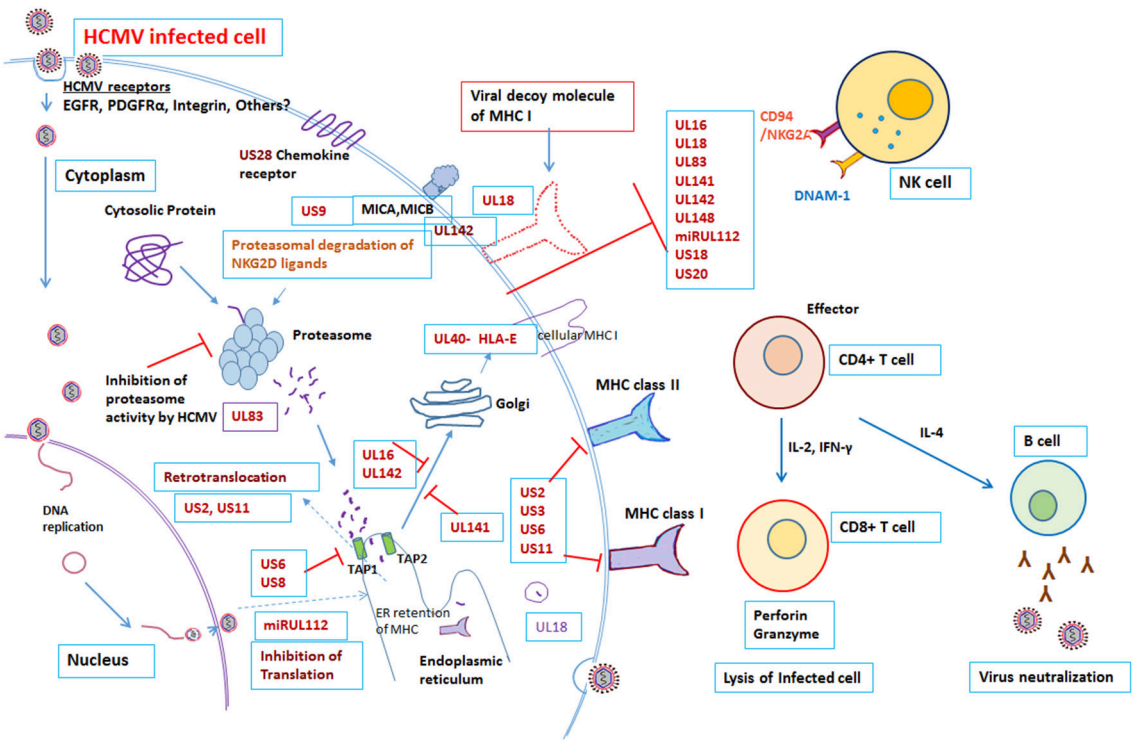


FIGURE 1 | Modulation of Immune response by human cytomegalovirus. Overview of the interactions between HCMV and the immune system. Red "T" bars indicate inhibition. Blue arrows indicate activation. Detail mechanisms explained in the text.

not efficiently neutralize infection with a different strain and this could enable to superinfection (45–47). In addition to interference in antigen presentation, the CMV gene products US2, US3, US6, and US11 encode for human homologs that interfere with the function of CD8+ T cells. This allowed viral replication and super-infection with a different strain of virus in a rhesus macaque model. This was confirmed, by the observation that US2-11 mutant virus, although able to produce infection, was unable to super-infect (17). However, further studies are needed to decipher the detailed mechanisms of the CTL response in contending with the combined action of these HCMV gene products. The large genome size of HCMV enables it to utilize an array of genes for host immune evasion, which allows long-term association and adaption of the virus in the host. In an immunocompetent host, viral latency is critical for its survival. After primary infection, the virus persists for a lifetime regardless of pre-existing immunity. During latency, the viral genome is maintained in the host without active replication and retains the capacity to reactivate in response to activation signals (48). Studies have linked various latency-associated determinants to HCMV latency (15), however, the detailed mechanisms of immune evasion during latency and how the virus persists in the host for life remains elusive. Deciphering these mechanisms could provide clues to allow us to prevent reactivation of this latent virus in congenital and transplant setup. Further, a note of caution is required; HCMV is strictly species specific. Since much of our understanding on cytomegalovirus biology is derived from *in vitro* cell culture studies and animal models, it is necessary to test these immune evasion functions in the appropriate setting. For instance, the UL18 gene product of HCMV encoding an MHC class I homolog was proposed to block NK cell activity by binding with KIR receptors; however, later studies have found it to enhance killing of infected fibroblasts by NK cells (12).

Further, extensive genetic variability has been observed in clinical isolate of HCMV (4, 40, 49–51), and even within a single host (4, 44, 52–54). High throughput sequencing of HCMV clinical isolates reveals that intrahost HCMV populations were as variable as seen in RNA virus quasispecies (52, 53). Viral strain diversity, differences in culture systems and population heterogeneity, make the generalization of genetic information difficult. In addition, a recent report showed that HCMV seroprevalence is related to a shift in immune phenotype along an age axis (55). This immunotypes varies in younger vs. elder individuals (55–57). In due course of evolution with the host, HCMV has been significant in shaping host immune system (57). HCMV also affects the host in response to infection with other pathogen. In HCMV seropositive children and in aging individuals have negative impact to Influenza; however, in younger individuals HCMV infection enhance immune response to influenza (58). Viral strain diversity could limit effective antiviral function, and the evasion strategy adapted by HCMV further complicates the development of an effective vaccine (45, 59). This underscores the need for large-scale genetic and immunological profiling studies, which could provide

TABLE 1 | HCMV gene products involved in modulation of host immune response.

HCMV Gene Product	Effect on host immune system & mechanism of evasion	Reference(s)
US2, US3, US6, US11	MHC class –I down regulation and impairment of expression; Further reduction in HCMV antigen presentation to CD8+ cells; Evasion of CD8+ T cell Immunity; Superinfection	(16, 17)
US2, HCMV Immediate Early/ Early	MHC class –II down regulation; Further reduction in HCMV antigen presentation to CD4+ cells	(12, 18)
US18 and US20	Interfere with B7-H6 surface expression involving endosomal degradation; escapes immune recognition by NK cells	(19)
UL18	Expression of human MHC class –I homolog; downregulate CTLs; Ligand decoy for NK receptors	(14, 16)
UL16	Regulation of NK cell ligand NKG2D; NK cells function impairment	(18)
UL40	NK cell evasion; HLA-E Over expression	(20)
UL83 (pp65)	IE-I sequestration; inhibit proteasome processing; Reduce action of NKp30; hinders antiviral gene expression	(21)
IE2 (immediate early) gene product	Overexpression of anti-apoptotic FLIP protein	(16, 18)
US28 (viral GPCR)	Targeting chemokine receptor; reduced inflammatory response	(12)
UL82 (pp71)	The tegument protein binds with stimulator of interferon genes to inhibit antiviral response.	(21, 22)
UL111A	HCMV encodes cmv IL-10, an homolog of human IL-10, thereby modulate immune system results in immune suppression	(23, 24)
UL141	CD155 down regulation	(14)
UL142	Inhibition of MICA	(12, 18)
UL36	Inhibition of pro-apoptotic recruitment of pro-caspase 8 to the DISC Decline in phagocytic activity (infected APCs)	(12)
UL37	Inhibition of pro-apoptotic Bcl-2 family Bak and Bax protein Apoptosis inhibition	(18)
UL97	Along with HCMV pp65 mediated immune evasion; Protein Kinase UL97 Forms a Complex with the Tegument Phosphoprotein pp65	(14, 25)
IE gene products	Induction of TGF-β: HCMV induce transcription & release of TGF-β	(26)
UL138	Latency associated; Sensitizes cells to TNF-α signaling	(15)
UL141- UL144	Encodes for homolog of TNFR; This HCMV encoded gene product inhibits cell surface expression of CD155 and CD112 (NK cell activating ligands) and the death receptor for the TNF family ligand TRAIL	(8, 14)
UL145	degradation of helicase like transcription factor- (HLTF) by recruitment of Cullin4/DDB ligase complex	(27)
UL146	Chemokine; role in inflammatory response	(14)
UL148	Suppression of CD58; Potent Modulator of CTL Function	(28)
miR-UL112	Escape from NK cell by down regulation of MICB; recognition from T cells by NKG2D decreased	(29, 30)

List of HCMV gene products involved in immune evasion.
[US, Unique short; UL, Unique long; miR, Micro RNA; MHC, major histocompatibility complex, TAP, Transporter associated with antigen processing; NK cells, natural killer cells; CTL, cytotoxic T cell l; LIR-1, Leukocyte Immunoglobulin-like receptor 1; HLA, human leukocyte antigen; IE, Immediate early; FLIP, FLICE-inhibitory protein; FLICE, cysteine proteases (caspase-8/MACH/Mch5), CRP- C-reactive protein, MICA, MHC class I polypeptide-related sequence A; un, unknown; DISC, death-inducing signaling complex; APC, Antigen presenting cells; Bak- BCL2 Antagonist/Killer, Bax- BCL2 Associated X, Bcl-2- B-cell lymphoma 2; pp65, phospho protein 65; TGF-β, Transforming growth factor –β; TNFR, tumor necrosis factor receptor; Cullin4/DDB, Cullin-4A·DNA Damage-binding Protein; CD, cluster of differentiation].

a decisive correlation on the nature of protective immune responses (56, 59–61).

HCMV has devised multiple strategies to interfere with antigen presentations and escape from CTL response, but this does not abrogate with the development of CTL response by host. This underscores the critical role of CD8+ T cells in HCMV infected cells as targets for immune clearance. Studies from adaptive transfer of HCMV specific CTL, in bone marrow transplant subjects, provide protection from HCMV disease (62). The complex interaction between the HCMV immune-evasins and host factors contributes to the levels of viral persistence in host (63). Information on viral immune evasion mechanisms by which HCMV persists in host will be useful in devising antiviral intervention strategies and development of new vaccines. Deletion of immune evasions could be a novel strategy for virus attenuation for vaccine candidate without compromising CD8 T cell response (64). Hansen et al reported that Simian immunodeficiency virus (SIV) protein expressing rhesus cytomegalovirus vector elicits SIV specific CD8+ T cells which recognizes unusual, diverse epitopes and results in immune clearance (65, 66). Thus, CMV vectors, genetically altered for diverse CD8+ T cell response could be useful for effective prophylactic and therapeutic vaccination (9, 65–68). Further, this could be useful in ultimately

designing an effective vaccine that could protect primary as well as reinfections.

CONCLUSIONS

In conclusion, human cytomegalovirus is a master of disguise. HCMV has evolved mechanisms to replicate and evade the host immune system by targeting the host cell machinery. Information on the host cell receptor targeted by this virus and the mechanisms utilized to operate cellular processes and evade the host immune system will provide clues to viral pathogenesis. An increasing number of HCMV gene products have been reported to play roles in immune evasion. These gene products sophistically orchestrate to modulate the host immune system, thereby allowing persistent and latent infection and life-long existence in the host. Information on viral escape mechanisms will be useful in rational design of antiviral drugs and should bring us one step closer to development of an effective vaccine.

AUTHOR CONTRIBUTIONS

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

FUNDING

AP was supported by grant from Department of Science and Technology, Science Engineering Research Board (Grant no. YSS/2015/00389), Govt. of India. The Institute of Life Sciences, Bhubaneswar is supported by core funds made available by Department of Biotechnology, Government of India.

ACKNOWLEDGMENTS

I would like to apologize to those authors whose work could not be cited due to space limitations.

REFERENCES

1. Manicklal S, Emery VC, Lazzarotto T, Boppana SB, Gupta RK. The "silent" global burden of congenital cytomegalovirus. *Clin Microbiol Rev*. (2013) 26:86–102. doi: 10.1128/CMR.00062-12
2. Griffiths P, Baraniak I, Reeves M. The pathogenesis of human cytomegalovirus. *J Pathol*. (2015) 235:288–97. doi: 10.1002/path.4437
3. Schleiss MR, Permar SR, Plotkin SA. Progress toward development of a vaccine against congenital cytomegalovirus infection. *Clin Vaccine Immunol*. (2017) 24:e00268-17. doi: 10.1128/CVI.00268-17.
4. Ross SA, Novak Z, Pati SK, Patro RK, Blumenthal J, Danthuluri VR, et al. Mixed infection and strain diversity in congenital cytomegalovirus infection. *J Infect Dis*. (2011) 204:1003–7. doi: 10.1093/infdis/jir457
5. Fowler KB, Boppana SB. Congenital cytomegalovirus infection. *Semin Perinatol*. (2018) 42:149–154. doi: 10.1053/j.semperi.2018.02.002
6. Britt WJ. Congenital human cytomegalovirus infection and the enigma of maternal immunity. *J Virol*. (2017) 91:e02392-16. doi: 10.1128/JVI.02392-16
7. Institute of Medicine Disivion of Health Promotion and Disease Prevention. *Vaccines for the 21st Century: a Tool for Decision Making. IOM Committee to Study National Priorities for Vaccine Development*. Washington, DC: National Academies Press (2000).
8. Benedict CA. A CMV vaccine: TREATing despite the TRICKs. *Exp Rev Vacc*. (2013) 12: 1235-1237. doi: 10.1586/14760584.2013.844653
9. Barry PA. Exploiting viral natural history for vaccine development. *Med Microbiol Immunol*. (2015) 204:255–62. doi: 10.1007/s00430-015-0406-1
10. McGeoch DJ, Cook S, Dolan A, Jamieson FE, Telford EA. Molecular phylogeny and evolutionary timescale for the family of mammalian herpesviruses. *J Mol Biol*. (1995) 247:443-58. doi: 10.1006/jmbi.1995.0152
11. Stern-Ginossar N, Weisburd B, Michalski A, Le VT, Hein MY, Huang SX, et al. Decoding human cytomegalovirus. *Science*. (2012) 338:1088–93. doi: 10.1126/science.1227919
12. Mocarski ES Jr, Shenk T, Griffiths PD, Pass RF. Cytomegalovirus. In: Knipe DM and Howley PM, editors. *Fields Virology, 6th ed*. Philadelphia, PA: Wolters Kluwer, Lippincott Williams & Wilkins (2013) pp. 1960–2014.
13. Stern-Ginossar N, Saleh N, Goldberg MD, Prichard M, Wolf DG, Mandelboim O. Analysis of human cytomegalovirus-encoded microRNA activity during infection. *J Virol*. (2009) 83:10684–93. doi: 10.1128/JVI.01292-09
14. Jackson SE, Mason GM, Wills MR. Human cytomegalovirus immunity and immune evasion. *Virus Res*. (2011) 157:151–60. doi: 10.1016/j.virusres.2010.10.031
15. Goodrum F. Human cytomegalovirus latency: approaching the gordian knot. *Annu Rev Virol*. (2016) 3:333-357. doi: 10.1146/annurev-virology-110615-042422
16. Gilbert MJ, Riddell SR, Plachter B, Greenberg PD. Cytomegalovirus selectively blocks antigen processing and presentation of its immediate-early gene product. *Nature*. (1996) 383:720–2. doi: 10.1038/383720a0
17. Hansen SG, Powers CJ, Richards R, Ventura AB, Ford JC, Siess D, et al. HCMV Evasion of CD8+ T Cells Is Critical for Superinfection by Cytomegalovirus. *Science*. (2010) 328:102–6 doi: 10.1126/science.1185350
18. Tortorella D, Gewurz BE, Furman MH, Schust DJ, Ploegh HL. Viral subversion of the immune system. *Annu Rev Immunol*. (2000) 18:861–926. doi: 10.1146/annurev.immunol.18.1.861
19. Charpak-Amikam Y, Kubsch T, Seidel E, Oiknine-Djian E, Cavaletto N, Yamin R, et al. Human cytomegalovirus escapes immune recognition by NK cells through the downregulation of B7-H6 by the viral genes US18 and US20. *Sci Rep*. (2017) 7:8661. doi: 10.1038/s41598-017-08866-2
20. Patel M, Vlahava VM, Forbes SK, Fielding CA, Stanton RJ, Wang ECY. HCMV-encoded NK modulators: lessons from *in vitro* and *in vivo* genetic variation. *Front Immunol*. (2018) 9:2214. doi: 10.3389/fimmu.2018.02214
21. Fu YZ, Su S, Gao YQ, Wang PP, Huang ZF, Hu MM, et al. Human cytomegalovirus tegument protein UL82 inhibits STING-mediated signaling to evade antiviral immunity. *Cell Host Microbe*. (2017) 21:231–243. doi: 10.1016/j.chom.2017.01.001
22. Kalejta RF, Shenk T. The Human Cytomegalovirus UL82 Gene Product (pp71) Accelerates Progression through the G1 Phase of the Cell Cycle. *J Virol*. (2003) 77:3451–9. doi: 10.1128/JVI.77.6.3451-3459.2003
23. Kotenko SV, Saccani S, Izotova LS, Mirochnitchenko OV, Pestka S. Human cytomegalovirus harbors its own unique IL-10 homolog (cmvIL-10). *Proc Natl Acad Sci USA*. (2000) 97:1695–700. doi: 10.1073/pnas.97.4.1695
24. Chang WL, Baumgarth N, Yu D, Barry PA. Human cytomegalovirus-encoded interleukin-10 homolog inhibits maturation of dendritic cells and alters their functionality. *J Virol*. (2004) 78:8720–31. doi: 10.1128/JVI.78.16.8720-8731.2004
25. Prichard MN, Britt WJ, Daily SL, Hartline CB, Kern ER. Human cytomegalovirus UL97 Kinase is required for the normal intranuclear distribution of pp65 and virion morphogenesis. *J Virol*. (2005) 79:15494–502. doi: 10.1128/JVI.79.24.15494-15502.2005

26. Yoo YD, Chiou CJ, Choi KS, Yi Y, Michelson S, Kim S, et al. The IE2 refulatory protein of human cytomegalovirus induces expression of the human transforming growth factor beta 1 gene through an Egr-1 binding site. *J Virol*. (1996) 70:7062–70.
27. Nightingale K, Lin KM, Ravenhill BJ, Davies C, Nobre L, Fielding CA, et al. High-definition analysis of host protein stability during human cytomegalovirus infection reveals antiviral factors and viral evasion mechanisms. *Cell Host Microbe*. (2018) 24:447–460.e11. doi: 10.1016/j.chom.2018.07.011
28. Wang ECY, Pjechova M, Nightingale K, Vlahava VM, Patel M, Ruckova E, et al. Suppression of costimulation by human cytomegalovirus promotes evasion of cellular immune defenses. *Proc Natl Acad Sci USA*. (2018) 115:4998–5003. doi: 10.1073/pnas.1720950115
29. Nachmani D, Lankry D, Wolf DG, Mandelboim O. The human cytomegalovirus microRNA miRUL112 acts synergistically with a cellular microRNA to escape immune elimination. *Nat Immunol*. (2010) 11:806–813. doi: 10.1038/ni.1916
30. De Pelsmaeker S, Romero N, Vitale M, Favoreel HW. Herpesvirus evasion of natural killer cells. *J Virol*. (2018) 92:e02105-17. doi: 10.1128/JVI.02105-17
31. Biron CA, Byron KS, Sullivan JL. Severe herpesvirus infections in an adolescent without natural killer cells. *N Engl J Med*. (1989) 320:1731–5. doi: 10.1056/NEJM198906293202605
32. Ploegh HL. Viral strategies of immune evasion. *Science*. (1998) 10:248–53. doi: 10.1126/science.280.5361.248
33. Andrews DM, Andoniou CE, Granucci F, Ricciardi-Castagnoli P, Degli-Esposti MA. Infection of dendritic cells by murine cytomegalovirus induces functional paralysis. *Nat Immunol*. (2001) 2:1077–84. doi: 10.1038/ni724
34. Moutaftsi M1, Mehl AM, Borysiewicz LK, Tabi Z. Human cytomegalovirus inhibits maturation and impairs function of monocyte-derived dendritic cells. *Blood*. (2002) 99:2913–21. doi: 10.1182/blood.V99.8.2913
35. Gredmark-Russ S, Söderberg-Nauclér C. Dendritic cell biology in human cytomegalovirus infection and the clinical consequences for host immunity and pathology. *Virulence*. (2012) 3:621–34. doi: 10.4161/viru.22239
36. Siddiquey MNA, Zhang H, Nguyen CC, Domma AJ, Kamil JP. The human cytomegalovirus endoplasmic reticulum-resident glycoprotein UL148 activates the unfolded protein response. *J Virol*. (2018) 92:e00896-18. doi: 10.1128/JVI.00896-18
37. Sturgill ER, Malouli D, Hansen SG, Burwitz BJ, Seo S, Schneider CL, et al. Natural Killer Cell Evasion Is Essential for Infection by Rhesus Cytomegalovirus. *PLoS Pathog*. (2016) 12:e1005868. doi: 10.1371/journal.ppat.1005868
38. Dassa L, Seidel E, Oiknine-Djian E, Yamin R, Wolf DG, Le-Trilling VTK, et al. The human cytomegalovirus protein UL148A downregulates the NK cell-activating ligand MICA to avoid NK cell attack. *J Virol*. (2018) 92:e00162-18. doi: 10.1128/JVI.00162-18
39. Boppana SB, Rivera LB, Fowler KB, Mach M, Britt WJ. Intrauterine transmission of cytomegalovirus to infants of women with preconceptional immunity. *N Engl J Med*. (2001) 344:1366-71. doi: 10.1056/NEJM200105033441804
40. Novak Z, Ross SA, Patro RK, Pati SK, Kumbla RA, Brice S, et al. Cytomegalovirus strain diversity in seropositive women. *J Clin Microbiol*. (2008) 46:882–6. doi: 10.1128/JCM.01079-07
41. Yamamoto AY, Mussi-Pinhata MM, Boppana SB, Novak Z, Wagatsuma VM, Oliveira Pde F, et al. Human cytomegalovirus reinfection is associated with intrauterine transmission in a highly cytomegalovirus-immune maternal population. *Am J Obstet Gynecol*. (2010) 202:297.e1-8 doi: 10.1016/j.ajog.2009.11.018
42. Gaytant MA, Rours GI, Steegers EA, Galama JM, Semmekrot BA. Congenital cytomegalovirus infection after recurrent infection: case reports and review of the literature. *Eur J Pediatr*. (2003) 162:248–53.
43. Ikuta K, Minematsu T, Inoue N, Kubo T, Asano K, Ishibashi K, Imamura T, Nakai H, Yoshikawa T, Moriuchi H, Fujiwara S, Koyano S, Suzutani T. Cytomegalovirus (CMV) glycoprotein H-based serological analysis in Japanese healthy pregnant women, and in neonates with congenital CMV infection and their mothers. *J Clin Virol*. (2013) 58:474–8. doi: 10.1016/j.jcv.2013.07.004
44. Pati SK, Pinninti S, Novak Z, Chowdhury N, Patro RK, Fowler K, et al. NIDCD CHIMES Study Investigators. Genotypic diversity and mixed infection in newborn disease and hearing loss in congenital cytomegalovirus infection. *Pediatr Infect Dis J*. (2013) 32:1050–4. doi: 10.1097/INF.0b013e31829bb0b9
45. Gardner TJ, Tortorella D. Virion glycoprotein-mediated immune evasion by human cytomegalovirus: a sticky virus makes a slick getaway. *Microbiol Mol Biol Rev*. (2016) 80:663–77. doi: 10.1128/MMBR.00018-16
46. Burkhardt C, Himmelein S, Britt W, Winkler T, Mach M. Glycoprotein N subtypes of human cytomegalovirus induce a strain-specific antibody response during natural infection. *J Gen Virol*. (2009) 90(Pt 8):1951–61. doi: 10.1099/vir.0.010967-0
47. Pati SK, Novak Z, Purser M, Arora N, Mach M, Britt WJ, Boppana SB. Strain-specific neutralizing antibody responses against human cytomegalovirus envelope glycoprotein N. *Clin Vaccine Immunol*. (2012) 19:909-13. doi: 10.1128/CVI.00092-12
48. Söderberg-Nauclér C, Fish KN, Nelson JA. Reactivation of latent human cytomegalovirus by allogeneic stimulation of blood cells from healthy donors. *Cell*. (1997)91:119–26. doi: 10.1016/S0092-8674(01)80014-3
49. Prichard MN, Penfold ME, Duke GM, Spaete RR, Kemble GW. A review of genetic differences between limited and extensively passaged human cytomegalovirus strains. *Rev Med Virol*. (2001) 11:191–200. doi: 10.1002/rmv.315
50. Patro AR, Dar L, Pati SK, Agarwal SK, Guleria S, Broor S, et al. Human cytomegalovirus UL73 polymorphisms among renal transplant recipients in India. *BMC Infect Dis*. (2014)14:66. doi: 10.1186/1471-2334-14-S3-P66
51. Arav-Boger R. Strain variation and disease severity in congenital cytomegalovirus infection: In search of a viral marker. *Infect Dis Clin North Am*. (2015) 29:401–14. doi: 10.1016/j.idc.2015.05.009
52. Renzette N, Pokalyuk C, Gibson L, Bhattacharjee B, Schleiss MR, Hamprecht K, et al. Limits and patterns of cytomegalovirus genomic diversity in humans. *Proc Natl Acad Sci USA*. (2015) 112:E4120–8. doi: 10.1073/pnas.1501880112
53. Sackman AM, Pfeifer SP, Kowalik TF, Jensen JD. On the demographic and selective forces shaping patterns of human cytomegalovirus variation within hosts. *Pathogens*. (2018) 7:E16. doi: 10.3390/pathogens7010016
54. Cudini J, Roy S, Houldcroft CJ, Bryant JM, Depledge DP, Tutill H, et al. Human cytomegalovirus haplotype reconstruction reveals high diversity due to superinfection and evidence of within-host recombination. *Proc Natl Acad Sci USA*. (2019) 116:5693–8. doi: 10.1073/pnas.1818130116
55. Kaczorowski KJ, Shekhar K, Nkulikiyimfura D, Dekker CL, Maecker H, Davis MM, et al. Continuous immunotypes describe human immune variation and predict diverse responses. *Proc Natl Acad Sci USA*. (2017) 114:E6097–106. doi: 10.1073/pnas.1705065114
56. Davis MM, Brodin P. Rebooting human immunology. *Annu Rev Immunol*. (2018) 36:843–64. doi: 10.1146/annurev-immunol-042617-053206
57. Picarda G, Benedict CA. Cytomegalovirus: shape-shifting the immune system. *J Immunol*. (2018) 200:3881–9. doi: 10.4049/jimmunol.1800171
58. Furman D, Jojic V, Sharma S, Shen-Orr SS, Angel CJ, Onengut-Gumuscu S, et al. Cytomegalovirus infection enhances the immune response to influenza. *Sci Transl Med*. (2015) 7:28. doi: 10.1126/scitranslmed.aaa2293
59. Britt WJ. Maternal immunity and the natural history of congenital human cytomegalovirus infection. *Viruses*. (2018) 10:E405. doi: 10.3390/v10080405
60. Prober CG, Enright AM. Congenital cytomegalovirus (CMV) infections: hats off to Alabama. *J Pediatr*. (2003)143:4–6. doi: 10.1016/S0022-3476(03)00290-7
61. Liston A, Goris A. The origins of diversity in human immunity. *Nat Immunol*. (2018) 19:209–210. doi: 10.1038/s41590-018-0047-9
62. Cobbold M, Khan N, Pourgheysari B, Tauro S, McDonald D, Osman H, et al. Adoptive transfer of cytomegalovirus-specific CTL to stem cell transplant patients after selection by HLA-peptide tetramers. *J Exp Med*. (2005) 202:379–86. doi: 10.1084/jem.20040613
63. Ameres S, Besold K, Plachter B, Moosmann A. CD8 T cell-evasive functions of human cytomegalovirus display pervasive MHC allele specificity, complementarity, and cooperativity. *J Immunol*. (2014) 192:5894–905. doi: 10.4049/jimmunol.1302281
64. Goodier MR, Jonjić S, Riley EM, Juranić Lisnić V. CMV and natural killer cells: shaping the response to vaccination. *Eur J Immunol*. (2018) 48:50–65. doi: 10.1002/eji.201646762
65. Hansen SG, Sacha JB, Hughes CM, Ford JC, Burwitz BJ, Scholz I, et al. Cytomegalovirus vectors violate CD8+ T cell epitope recognition paradigms. *Science*. (2013) 340:1237874. doi: 10.1126/science.1237874

66. Früh K, Picker L. CD8+ T cell programming by cytomegalovirus vectors: applications in prophylactic and therapeutic vaccination. *Curr Opin Immunol.* (2017) 47:52–6. doi: 10.1016/j.coi.2017.06.010
67. Hill AB. The immune response to CMV infection and vaccination in mice, monkeys and humans: recent developments. *Curr Opin Virol.* (2018) 28:161–66. doi: 10.1016/j.coviro.2018.01.006
68. Gerna G, Lilleri D. Human cytomegalovirus (HCMV) infection/re-infection: development of a protective HCMV vaccine. *New Microbiol.* (2019) 42:1–20.

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: info@frontiersin.org | +41 21 510 17 00

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership