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Invariant CD1d-restricted NKT (iNKT) cells play important roles in generating protective immune responses against infections. In this study, we have investigated the role of human iNKT cells in HSV-1 infection and their interaction with epidermal keratinocytes. These cells express CD1d and are the primary target of the virus. Keratinocytes loaded with α-galactosyl ceramide (α-GalCer) could stimulate IFN-γ production and CD25 upregulation by iNKT cells. However, both α-GalCer-dependent and cytokine-dependent activation of iNKT cells was impaired after coculture with HSV-1-infected cells. Notably, CD1d downregulation was not observed on infected keratinocytes, which were also found to inhibit TCR-independent iNKT cell activation. Further examination of the cytokine profile of iNKT–keratinocyte cocultures showed inhibition of IFN-γ, IL-5, IL-10, IL-13, and IL-17 secretion but upregulation of IL-4 and TNF-α after the infection. Moreover, cell-to-cell contact between infected keratinocytes and iNKT cells was required for the inhibition of activation, as the cell-free supernatants containing virus did not affect activation. Productive infection of iNKT cells was however not required for the inhibitory effect. After coculture with infected cells, iNKT cells were no longer responsive to further stimulation with α-GalCer–loaded CD1d-expressing cells. We found that exposure to HSV-1–infected cells resulted in impaired TCR signaling downstream of ZAP70. Additionally, infected cells upregulated the expression of the negative T cell regulator, galectin-9; however, blocking experiments indicated that the impairment of iNKT cell responses was independent of galectin-9. Thus, interference with activation of human iNKT cells by HSV-1 may represent a novel immunoevasive strategy used by the virus to avoid immune clearance. The Journal of Immunology, 2012, 188: 6216–6224.

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Abbreviations used in this article: α-GalCer, α-galactosyl ceramide; iNKT, invariant CD1d-restricted NKT; MOI, multiplicity of infection; PAA, phosphonoacetic acid; TIM-3, T cell Ig mucin-3.

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of HSV (28–30). Increased disease severity and impaired clearance of HSV has been observed in iNKT cell-deficient mice (31, 32), although the contribution of iNKT cells to disease resolution may depend on the virus strain as shown by Cornish et al. (33). More recently, however, two human in vitro studies have provided indirect evidence for the role of iNKT cells in anti-HSV immunity. HSV-1 was shown to downregulate CD1d expression on the surface of infected dendritic cells and monocytes (34, 35). This is, at least in part, due to two viral proteins, Us3 and gb, which act to inhibit CD1d recycling (36). Indeed, other viral pathogens including HIV-1, vaccinia virus, and Kaposi’s sarcoma-associated herpesvirus also have the capacity to interfere with CD1d Ag presentation (37–40). However, whether HSV-1 can also inhibit iNKT cell activation directly has not been addressed to date. As HSV infection is primarily restricted to the epidermal layers, we were interested in examining the effects of HSV on skin-resident CD1d-expressing keratinocytes and how this infection might modulate the ability of iNKT cells to generate an appropriate antiviral immune response.

Materials and Methods

Culture of iNKT cells

PBMCs of healthy donors were cultured in RPMI 1640 (Invitrogen, Paisley, U.K.) supplemented with 10% FCS (Invitrogen), 2.05 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES (HyClone, Logan, UT), and 100 ng/ml α-GalCer (Enzo Life Sciences, Plymouth Meeting, PA) to stimulate proliferation of donor iNKT cells. After initial 24 h of culture, the medium was supplemented with 10 ng/ml recombinant human IL-2 (Peprotech, London, U.K.). Ten to fourteen days later, iNKT cells were purified by immunomagnetic cell sorting. Briefly, cells were incubated with 100 ng/ml α-GalCer 1 h prior to HSV-1 infection. For cytokine-mediated stimulation of iNKT cells, exogenous IL-12 (100 ng/ml; Peprotech) and IL-18 (200 ng/ml; R&D Systems) were added for the duration of the coculture. In blocking experiments, iNKT cells or keratinocytes were incubated with 10 μg/ml T cell Ig mucin-3 (TIM-3) Ab 2E2 (R&D Systems) 1 h prior to coculture with infected cells.

Cytokine analysis from cell culture supernatants

iNKT cells were cultured alone or in contact with uninfected or HSV-1–infected keratinocytes with or without α-GalCer as described above. Cell-free supernatants were collected in duplicate at 24 and 48 h after coculture and used in a multiplex cytokine assay (Invitrogen). Supernatants of iNKT cells stimulated with immobilized CD3 mAb and soluble CD28 were used as a positive control. Supernatants from cultures containing keratinocytes alone were also included in the assay.

Phospho-specific flow cytometry

TCR-induced signaling events were examined by flow cytometry according to the manufacturer’s protocol (BD Phosflow). Briefly, iNKT cell activation was triggered by TCR cross-linking with a purified mouse anti-human CD3 mAb (UCHT1, 10 μg/ml; BD Pharmingen) and goat anti-mouse Ig (5 μg/ml; BD Pharmingen). The activation was terminated by fixation in BD Cytofix so- lution (BD Biosciences) followed by analysis using FlowJo software (Tree Star, Ashland, OR).

Results

HSV-1 inhibits activation of iNKT cells by epithelial keratinocytes

Keratinocytes are highly susceptible to HSV-1 infection and are the major virus target during primary and recurrent herpes infection. Because keratinocytes express CD1d on their surface (42), we set out to determine the effects of HSV-1 infection on CD1d expression on human epithelial keratinocytes and their ability to activate iNKT cells. As expected, keratinocytes induced iNKT cell activation by α-GalCer and maintained in culture medium supplemented with IL-2 (8). The expression of α-GalCer was confirmed by flow cytometry. iNKT cells were incubated with 100 ng/ml α-GalCer 1 h prior to HSV-1 infection. For cytokine-mediated stimulation of iNKT cells, exogenous IL-12 (100 ng/ml; Peprotech) and IL-18 (200 ng/ml; R&D Systems) were added for the duration of the coculture. In blocking experiments, iNKT cells or keratinocytes were incubated with 10 μg/ml T cell Ig mucin-3 (TIM-3) Ab 2E2 (R&D Systems) 1 h prior to coculture with infected cells.

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Several studies have demonstrated that the requirement for exogenous glycolipid Ags such as α-GalCer for the activation of iNKT cells can be overcome in the presence of proinflammatory cytokines such as IL-12 and/or IL-18 (22–26). In this scenario, iNKT cells recognize endogenous glycolipids bound to CD1d. Thus, we examined if HSV-1 infection also affected cytokine-driven iNKT cell activation. Indeed, whereas the cytokines IL-12 and IL-18 induced iNKT cell activation even in the absence of α-GalCer, iNKT cells cocultured with HSV-1–infected keratinocytes by flow cytometry in the absence or presence of PMA–ionomycin or the combination of exogenous IL-12 and IL-18 (100 ng/ml and 200 ng/ml, respectively). (C) Expression of activation marker CD25 on purified iNKT cells that were activated via CD3/CD28 mAb stimulation for 4 h or 6 h prior to coculture with uninfected or HSV-1–infected keratinocytes for further 24 h. (D) Viability of iNKT cells after 24 h incubation with 10 MOI of cell-free HSV-1 or HSV-1–infected keratinocytes. Viability was assessed by flow cytometry using staining with Pacific blue-conjugated annexin V and Live/Dead near-IR fixable stain. Events shown in the above plots were from the CD3+ gate. (E) Expression of CD1d by keratinocytes was analyzed by flow cytometry using surface staining and compared with the expression found on uninfected and HSV-1–infected (5 PFU/cell) monocyte-derived dendritic cells. The results are representative of three independent experiments.

HSV-1 interferes with NKT cell activation

HSV-1 has previously been shown to interfere with CD1d recycling and thereby its expression on the surface of APCs (34, 36). Thus, it was possible that the observed impairment of iNKT cell activation was due to the same mechanism. Indeed, we were able to confirm the previous findings on HSV-1–infected dendritic cells (Fig. 1E). In contrast, HSV-1 infection of keratinocytes did not result in the inhibition of CD1d expression (Fig. 1E), suggesting that this effect may be cell type dependent (Fig. 1E). Collectively, these data suggest a novel strategy used by HSV-1 to interfere with iNKT cell activation.

HSV-1 alters the cytokine pattern of iNKT cells after stimulation with α-GalCer

The above results demonstrate that IFN-γ production was inhibited by HSV-1. Thus, it was of interest to determine if the virus

FIGURE 1. HSV-1 inhibits CD1d-mediated iNKT cell activation by keratinocytes. Keratinocytes were either left uninfected or infected with HSV-1 at MOI of 1 in the presence or absence of α-GalCer. After 18 h, the cells were harvested, washed, and resuspended in culture medium. (A) IFN-γ production by iNKT cells after coculture with keratinocytes. Uninfected or HSV-1–infected keratinocytes were cocultured for 6 h at 1:1 ratio with purified iNKT cells in the presence of brefeldin A. (B) Expression of activation marker CD25 on purified iNKT cells after 24-h culture with unpulsed or α-GalCer–pulsed keratinocytes by flow cytometry in the absence or presence of PMA–ionomycin or the combination of exogenous IL-12 and IL-18 (100 ng/ml and 200 ng/ml, respectively). (C) Expression of activation marker CD25 on purified iNKT cells that were activated via CD3/CD28 mAb stimulation for 4 h or 6 h prior to coculture with uninfected or HSV-1–infected keratinocytes for further 24 h. (D) Viability of iNKT cells after 24 h incubation with 10 MOI of cell-free HSV-1 or HSV-1–infected keratinocytes. Viability was assessed by flow cytometry using staining with Pacific blue-conjugated annexin V and Live/Dead near-IR fixable stain. Events shown in the above plots were from the CD3+ gate. (E) Expression of CD1d by keratinocytes was analyzed by flow cytometry using surface staining and compared with the expression found on uninfected and HSV-1–infected (5 PFU/cell) monocyte-derived dendritic cells. The results are representative of three independent experiments.
caused a general suppression of cytokine secretion or a change in the pattern of cytokine release. To this end, we tested cell-free supernatants from iNKT cell–keratinocyte cocultures in the presence or absence of α-GalCer as well as supernatants from iNKT cells stimulated with CD3 Ab using the Luminex technology. The multiplex cytokine assay confirmed the initial data that IFN-γ production was suppressed by HSV-1 (Fig. 2). In contrast, production of IL-4 and TNF-α was increased in HSV-1–infected cocultures, which suggested a reversal of the pattern of cytokine release (Fig. 2). Notably, IL-10 production was also inhibited by the virus as was the release of several other Th1- and Th2-type cytokines including IL-5, IL-13, and IL-17 (Fig. 2). These results show that HSV-1 alters the cytokine pattern of iNKT cells and inhibits the secretion of soluble factors that are critical for the adequate protection against herpes infection.

**NKT cells are resistant to HSV-1 infection**

Because HSV-1–infected keratinocytes did not display reduced levels of CD1d on their surface, a possible explanation for the failure of iNKT cells to respond to α-GalCer stimulation could be their direct infection by HSV-1. Activated T lymphocytes have previously been shown to be susceptible to HSV-1 infection (45). Therefore, we examined if human iNKT cells could support HSV-1 infection. Infection was detected using an mAb against one of the structural proteins of HSV-1, which is typically expressed on the surface of infected cells after viral DNA replication. No expression of viral Ags was observed by flow cytometry when iNKT cells were infected with cell-free HSV-1 (Fig. 3A). The resistance of iNKT cells to HSV infection was further demonstrated by the lack of viral protein expression by iNKT cells even in the presence of high titers of cell-free virus, compared with keratinocytes, which were highly susceptible (Fig. 3A).

However, when iNKT cells were cocultured in direct contact with HSV-1–infected keratinocytes, >50% of iNKT cells were viral Ag positive as detected by flow cytometry (Fig. 3B). In contrast, there was little or no expression of the viral Ag by iNKT cells if they were separated from keratinocytes by a Transwell membrane (Fig. 3B). These differences could be explained...
by the ability of HSV-1 to infect more efficiently via cell-to-cell contact rather than as cell-free virions. However, there was still a possibility that the viral Ag detected on the surface of iNKT cells was not due to de novo production in iNKT cells but rather due to transfer of viral proteins from the membrane of keratinocytes. Expression of the majority of viral glycoproteins and other structural proteins is dependent on viral DNA replication. Viral DNA polymerase inhibitors can effectively block this step in virus replication. Therefore, to examine if the expression of viral glycoprotein on the surface of iNKT cells was due to direct infection, iNKT cells were incubated with infected keratinocytes in the presence of a viral DNA polymerase inhibitor, phosphonoacetic acid (PAA). Viral Ag expression on iNKT cells was unaltered by the presence of PAA, indicating that the expression was not due to productive infection (Fig. 3B). Together, the above results show the resistance of human iNKT cells to HSV-1 infection and suggest that the inhibition of iNKT cell activation is a distinct mechanism mediated by permissive cells such as keratinocytes.

**HSV-1–infected keratinocytes induce a sustained inhibition of iNKT cell activation**

Next, we were interested if iNKT cell inactivation was sustained beyond contact with HSV-1–infected cells. Thus, we transferred iNKT cells after 24-h incubation with uninfected or infected keratinocytes to cultures containing α-GalCer–pulsed CD1d-expressing 293T cells. iNKT cell activation was inhibited only in cocultures where direct contact between the infected keratinocytes and iNKT cells was allowed (Fig. 4A). In contrast, when iNKT cells were separated from the keratinocytes by a membrane, only allowing contact with the cell-conditioned medium and cell-free virus, no inhibition of IFN-γ production was seen (Fig. 4B). These results collectively indicate that inhibition of iNKT cell activation was a specific event dependent on close cell-to-cell contact and that this inhibition was sustained after the removal of HSV-1–infected cells.

**HSV-1 infection of keratinocytes induces galectin-9 expression**

Because inhibition of iNKT cell activation was mediated via close contact with HSV-1–infected cells in the absence of direct virus infection of iNKT cells (Fig. 4), we were interested in possible factors expressed by infected keratinocytes that could affect iNKT cell activation. A number of recent studies have revealed several members of the galectin family as important regulators of cellular immunity (46–48). Two of the members, namely galectin-1 and galectin-9, have been shown to suppress Th1 responses through inhibition of IFN-γ secretion in activated Th1-type T lymphocytes (46–48). Thus, we examined the expression patterns of the two galectins by keratinocytes. We found that whereas

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**FIGURE 3.** HSV-1 transfer from keratinocytes to iNKT cells occurs via cell-to-cell contact. (A) The proportion of HSV-1–infected iNKT cells and keratinocytes after 18-h infection with cell-free HSV-1. (B) Expression of viral Ags by iNKT cocultured with keratinocytes. Keratinocytes were either left uninfected or infected for 18 h with HSV-1 at MOI of 1. The cells were then harvested, washed, and cocultured for 24 h at 1:1 ratio with purified iNKT cells. Some keratinocytes were separated from iNKT cells by a 3.0-μm Transwell membrane to prevent cell-to-cell contact or cultured in the presence of PAA to inhibit virus replication. Infection of iNKT cells was analyzed by flow cytometry. Gray-tinted histograms, iNKT cells exposed to uninfected keratinocytes; clear histograms, iNKT cells exposed to HSV-1–infected keratinocytes. The results are representative of three independent experiments.

**FIGURE 4.** Direct contact to HSV-1–infected keratinocytes renders human iNKT cells unresponsive. Activation of iNKT cells after coculture with uninfected or HSV-1–infected keratinocytes for 24 h either in direct contact (A) or separated by a 3-μm Transwell membrane (B). iNKT cells were then mixed with untreated or α-GalCer–pulsed CD1d-transfected 293T cells at 1:1 ratio in the presence of brefeldin A for 6 h. iNKT cell activation was assessed by flow cytometry. The results are representative of three independent experiments.
there was no significant expression of either galectin on the surface of uninfected keratinocytes, there was a significant upregulation of galectin-9 but not galectin-1 after HSV-1 infection (Fig. 5A). We confirmed the flow cytometry findings with fluorescence confocal microscopy, which revealed that galectin-9 was specifically expressed by HSV-1-infected keratinocytes with perinuclear, cytosolic, and membrane staining (Fig. 5B). Inhibition of IFN-γ secretion by galectin-9 is believed to be mediated through its binding to TIM-3, which is expressed on activated but not naive T lymphocytes (46, 47). Therefore, we examined the relative expression of TIM-3 on purified iNKT cells. Resting iNKT cells already had a detectable expression of TIM-3 on the surface, consistent with their constitutive “memory” phenotype. As has been shown previously for conventional T cells (46), iNKT cell activation led to upregulation of TIM-3 expression (Fig. 5C). However, TIM-3 blockade using the mAb 2E2 (49, 50) and galectin-9 blockade using the mAb 9MI-3 (50) failed to rescue the production of IFN-γ (Fig. 5, Supplemental Fig. 1), suggesting that other inhibitory signals were responsible for the inhibition of iNKT cell activation.

**HSV-1 inhibits ERK activation in iNKT cells**

Considering that inhibition of IFN-γ response occurred after contact with infected cells, we wanted to examine if there was interference with the TCR signal transduction in iNKT cells. Using phospho-specific flow cytometry, we examined activation of TCR proximal factor ZAP70 and its substrate SLP76 after the engagement of TCR receptor and subsequent ERK phosphorylation, which was shown to be required for TCR-mediated IFN-γ secretion (51–53). Our results indicate that the levels of ZAP70 and SLP76 activation were unchanged by the virus, whereas ERK1/2 phosphorylation was reduced (Fig. 6). This was also reflected in the reduced CD25 upregulation 24 h later (data not shown). These results indicate that the contact with HSV-1–infected cells directly suppresses iNKT cell activation via the TCR downstream of ZAP70.

**Discussion**

HSV has developed multiple strategies to avoid immune recognition, from inhibition of MHC class I and II presentation to interference with dendritic cell maturation (54). In this study, we show yet another mechanism of immune evasion that directly targets iNKT cells. Coculture with HSV-infected keratinocytes rapidly led to inhibition of iNKT cell activation and IFN-γ secretion. Considering the importance of IFN-γ in the control of HSV infection, interference with its production, especially in the early stages of the response, represents a powerful immunoevasive strategy. Inhibition of IFN-γ secretion by HSV-1 in conventional T lymphocytes has been attributed to selective production of IL-10 (55). However, in our experiments IL-10 production, which was induced in response to α-GalCer, was inhibited by the virus suggesting a distinct interference mechanism. Indeed, production of other cytokines was inhibited in HSV-1–infected cocultures including IL-5, IL-13, and IL-17. In contrast, the levels of TNF-α and IL-4 were raised in HSV--
FIGURE 6. Contact with HSV-1–infected cells inhibits TCR signaling. Uninfected or HSV-1–infected cells were cocultured with iNKT cells for 2 h followed by TCR cross-linking with a UCHT-1 mAb. Activation of ZAP70, SLP76, and ERK1/2 were assessed using phospho-specific mAbs at 1 and 10 min after cross-linking, respectively. TCR cross-linking and PMA–ionomycin served as a positive control for ZAP70, SLP76, and ERK1/2, respectively. The results are representative of three independent experiments.

Infected cocultures. This increase in the two cytokines could be speculated to be of some benefit to the host at the site of herpetic lesion by acting on infiltrating macrophages and/or resident dendritic cells and promoting their activation (56). These APCs may subsequently enhance activation of lymphocytes, including iNKT cells, through secretion of proinflammatory cytokines such as IL-12 and IL-18 (57–59). However, our data demonstrated that HSV-1 is also able to prevent this cytokine-driven iNKT cell activation. Although HSV can inhibit CD1d expression on APCs (34, 35), we found that inhibition of iNKT cell activation was a distinct phenomenon, as iNKT cells exposed to HSV-infected cells did not respond to further stimulation. Notably, this occurred in the absence of productive infection, as we found that iNKT cells did not support virus replication even at high MOI. The fact that HSV–mediated inhibition of iNKT cell activation is independent of CD1d downregulation was further supported by the observation that activation induced by PMA–ionomycin was inhibited by HSV-1 and that the activation of ZAP70 and SLP76, which are proximal to the TCR, was unaffected by the virus. In contrast, ERK1/2 activation was inhibited after coincubation with the infected cells. This is in agreement with the observed reduction in IFN-γ production by iNKT cells, as its synthesis in response to TCR engagement has been shown to be dependent on signal transduction through the MEK–ERK pathway (51–53). In conventional T lymphocytes, HSV-1 was shown to inhibit LAT phosphorylation, which also leads to inhibition of signaling through ERK (60); however, we have not been able to obtain reliable detection of LAT phosphorylation to confirm that the same is true for iNKT cells. TCR activation can lead to LAT-independent ERK activation (61, 62), suggesting that HSV-1 may target other parts of the TCR signaling cascade to inhibit iNKT cell activation. Our results also indicate that the virus is able to override prior TCR-mediated activation of iNKT cells, which may have implications for the possible antiherpes therapies utilizing glycolipids.

Considering that close cellular contact was required for the inhibition of IFN-γ secretion, we focused our attention on putative inhibitory receptors that could be expressed on infected cells and suppress iNKT cell activation. We discovered that HSV-1–infected cells upregulate expression of galectin-9, a β-galactosidase–binding protein, which negatively regulates cellular immunity. Galectin-9 binding to its receptor TIM-3 on the surface of activated conventional T lymphocytes leads to multiple inhibitory effects including suppression of IFN-γ secretion and apoptosis (48). In addition, in chronic HIV-1 and HCV infections, exhausted CD8+ T cells express elevated levels of TIM-3 (49, 63). More recently, induction of galectin-9 was also seen in HSV infection in mice, and animals deficient in galectin-9 had improved antiviral CD8+ T cell responses (64). Although we found that human iNKT cells expressed TIM-3, our blocking experiments failed to prove that this axis was responsible for the inhibition of iNKT cell responses. Although the reasons for this are unclear at present, it is possible that the signaling events that lead to suppression of activation of iNKT cells vary from those of conventional T lymphocytes. Support for this type of scenario comes from studies in chronic HIV-1 infection in which elevated expression of inhibitory receptor PD-1 is observed in functionally impaired CD8+ T lymphocytes as well as iNKT cells (65–67). In these studies, blockade of PD-1 and its ligands, PD-L1 and PD-L2, led to improved CD8+ T cell responses but failed to restore iNKT cell responses. Notably, we have also examined the contribution of PD-1 in the suppression of iNKT cell activation by HSV-infected cells but did not see any effect of receptor blockade (alone or in combination with TIM-3 blocking) on restoration of IFN-γ (Supplemental Fig. 1). However, another explanation for the inability of TIM-3 and/or PD-1 blocking to rescue the IFN-γ production by iNKT cells may be that inhibition mediated by HSV may be due to another more dominant inhibitory pathway. Optimal activation of Th1 responses by iNKT cells appears to be dependent on the localization of CD1d–glycolipids complexes to lipid rafts (68). Therefore, it can be speculated that HSV-1 infection of keratinocytes may lead to redistribution of CD1d away from such structures and thus compromise the adjuvant iNKT activation. Although iNKT cells were resistant to productive infection by cell-free HSV-1, we cannot exclude the possibility that the virus entered the cells. This is a likely scenario, as we observed transfer of viral glycoproteins from infected cells to iNKT cells even in the presence of a viral DNA replication inhibitor. It is thus possible that the viral structural proteins may be involved in the inhibition of iNKT cell activation. This has been shown for conventional T lymphocytes where transfer of virus from infected fibroblasts led to inhibition of T cell activation (60).

The presence of multiple pathways of iNKT cell interference such as inhibition of activation and CD1d downregulation suggests an important role for these cells in the defense against HSV infection. As evidence for the role of iNKT cells in human HSV infection is lacking to date, data from murine models are our only reference. In this system, contribution of iNKT cells in the control of HSV is still open to debate, as conflicting data exist (31–33). There is no information available on the effects of HSV infection on CD1d expression in the mouse or the effects of such infection on murine iNKT cells. However, assuming such immunovesicative mechanism exists in the murine system, the general contribution of iNKT cells to the resolution of herpes infection may be underrated. Good evidence for the possible adjuvant role of iNKT cells in HSV infection comes from a recent study showing that α-GalCer, when used as adjuvant, induces highly protective immune responses to genital HSV challenge (69). Together, these findings underscore the importance of iNKT cells in the generation of effective anti-HSV immune responses and further highlight the complex interactions between a highly host-adapted pathogen, such as HSV-1, and the immune system.

Disclosures
The authors have no financial conflicts of interest.

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