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PATHOGENESIS AND IMMUNITY

Herpes Simplex Virus 1 Specifically Targets Human CD1d Antigen Presentation To Enhance Its Pathogenicity

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ABSTRACT Herpes simplex virus 1 (HSV-1) is one of the most prevalent herpesviruses in humans and represents a constant health threat to aged and immunocompromised populations. How HSV-1 interacts with the host immune system to efficiently establish infection and latency is only partially known. CD1d-restricted NKT cells are a critical arm of the host innate immune system and play potent roles in anti-infection and antitumor immune responses. We discovered previously that upon infection, HSV-1 rapidly and efficiently downregulates CD1d expression on the cell surface and suppresses the function of NKT cells. Furthermore, we identified the viral serine/threonine protein kinase US3 as a major viral factor downregulating CD1d during infection. Interestingly, neither HSV-1 nor its US3 protein efficiently inhibits mouse CD1d expression, suggesting that HSV-1 has coevolved with the human immune system to specifically suppress human CD1d (hCD1d) and NKT cell function for its pathogenesis. This is consistent with the fact that wild-type mice are mostly resistant to HSV-1 infection. On the other hand, *in vivo* infection of CD1d-humanized mice (hCD1d knock-in mice) showed that HSV-1 can indeed evade hCD1d function and establish infection in these mice. We also report here that US3-deficient viruses cannot efficiently infect hCD1d knock-in mice but infect mice lacking all NKT cells at a higher efficiency. Together, these studies supported HSV-1 evasion of human CD1d and NKT cell function as an important pathogenic factor for the virus. Our results also validated the potent roles of NKT cells in antiherpesvirus immune responses and pointed to the potential of NKT cell ligands as adjuvants for future vaccine development.

IMPORTANCE Herpes simplex virus 1 (HSV-1) is among the most common human pathogens. Little is known regarding the exact mechanism by which this virus evades the human immune system, particularly the innate immune system. We reported previously that HSV-1 employs its protein kinase US3 to modulate the expression of the key antigen-presenting molecule, CD1d, so as to evade the antiviral function of NKT cells. Here we demonstrated that the virus has coevolved with the human CD1d and NKT cell system and that NKT cells indeed play potent roles in anti-HSV immune responses. These studies point to the great potential of exploring NKT cell ligands as adjuvants for HSV vaccines.

KEYWORDS CD1d, HSV-1, NKT cell, US3, immune evasion, mouse model, pathogenicity

Herpesviruses are categorized into three major groups: alpha-, beta-, and gammaherpesviruses. Herpes simplex virus 1 (HSV-1) is a member of the alphaherpesvirus family and can cause acute and latent oral, ocular, and genital infections. In addition to causing frequent oral blister infections in the general population and severe encephalitis in elderly and immunocompromised patients, HSV-1 is the leading etiological pathogen for infection-caused blindness. It is estimated that globally there are

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1,000,000 new cases and 9,000,000 recurrent episodes of ocular HSV infection each year. HSV is also responsible for 1,000,000 cases of visual disability worldwide every year (1, 2). In the United States, patients make four doctor's visits for the first ocular infection episode and at least six visits for recurring episodes. It is estimated that 444,000 days of work are lost per year due to ocular HSV infections (2, 3). Therefore, in addition to causing high morbidity in millions of patients, HSV imposes a severe social and economic burden. Despite intensive studies, our understanding of HSV-1 pathogenesis is still limited. As a consequence, no vaccine for these highly prevalent viruses is currently available. Understanding the interaction of the virus with the immune system, particularly the innate immune system, will provide clues for novel antivirals and valuable information for the generation of successful vaccines.

As a key component of innate immunity, NKT cells are an unconventional subset of T cells coexpressing the T-cell receptor (TCR) and typical surface receptors for NK cells. Over the past 2 decades, studies have shown that NKT cells function potently in diverse immune responses, including immunity to tumors and infectious diseases, autoimmune diseases, and allergies (4). The major population of NKT cells expresses a single TCRα chain—Vα24Jα18 in humans and Vα14Jα18 in mice—and is often called invariant NKT (iNKT) cells. As part of the innate immune system, NKT cells are among the first responders in the periphery during immune responses and are typically activated within hours. Upon activation, they rapidly produce copious amounts of cytokines, both the Th1 and Th2 types, and play a critical immunomodulatory role in the ensuing adaptive immune responses (5). While conventional $CD8^+$ and $CD4^+$ T cells are restricted by major histocompatibility complex (MHC) class I and II molecules, respectively, NKT cells are restricted by the nonpolymorphic MHC class I-like antigen-presenting molecule CD1d (6). In contrast to MHC class I and II molecules, which present peptide ligands to conventional T cells, CD1d molecules present antigenic phospholipids or glycolipids to NKT cells (7, 8).

A common tactic adopted by viruses to evade T cell function is to downregulate the antigen-presenting molecule (9, 10). Viral evasion of antigen presentation by MHC class I and II molecules has been well documented (9, 10). Much less is known about how viruses overcome CD1d antigen presentation. Many viruses, including HIV (11–15), Kaposi's sarcoma-associated herpesvirus (KSHV) (16), HSV-1 (17–19), lymphocytic choriomeningitis virus (LCMV) (20–23), vesicular stomatitis virus (24, 25), and vaccinia virus (25, 26), use different mechanisms to either inhibit NKT cell function directly or inhibit CD1d expression and thus suppress the function of NKT cells. HIV Nef protein interacts with the cytoplasmic tail of CD1d, accelerates its endocytosis, and relocalizes it to the *trans*-Golgi network (TGN) (11). KSHV uses its MIR-2 (modulator of immune recognition 2) protein to ubiquitinate human CD1d (hCD1d) at its cytoplasmic tail and enhance CD1d endocytosis (16). Other viruses, including vaccinia virus and vesicular stomatitis virus, inhibit NKT function not by inhibiting CD1d surface expression but by modulating the signaling pathways and the quality of CD1d lipid presentation (24–26). Little is known about how CD1d biogenesis and trafficking are affected in these infections. For HSV-1, we reported previously that the major cellular target of the evasion gene US3 is the motor protein KIF3A (27, 28). However, whether and how US3 suppression of NKT cell function enhances viral pathogenicity are currently unknown.

Accumulating studies show that CD1d-restricted NKT cells play critical roles in immunity to different groups of viruses. Patients with different deficiencies in NKT cell development are hypersensitive to herpesvirus infection (29). There is a selective loss of circulating NKT cells during HIV-1 infection (30). Interestingly, current treatment of HIV-1 patients with either interleukin 2 (IL-2) or highly active antiretroviral therapy (HAART) rapidly restores iNKT cell populations (31, 32). With regard to animal models, NKT cell-deficient mice are much more susceptible than wild-type mice to infection by herpesviruses (33), influenza viruses (34), respiratory syncytial virus (35), and encephalomyocarditis virus (EMCV) (36). On the other hand, activation of NKT cells, mostly by a prototype NKT cell ligand, α-galactosylceramide (α-GalCer), greatly reduces the replication of mouse cytomegalovirus (37), hepatitis B virus (38), influenza virus (39),

respiratory syncytial virus (35), LCMV (40), and EMCV (36, 41). Nevertheless, it is largely unknown exactly how NKT cells function in antiviral immunity, and particularly what kind of effector molecules and cells are downstream of NKT cell activation. Understanding the antiviral mechanism of NKT cells is critical for the future development and optimization of NKT cell-based antiviral immunotherapies as well as for vaccine development.

We have demonstrated previously that HSV-1 rapidly and efficiently downregulates CD1d from infected human primary dendritic cells and suppressed NKT cell activation. In this report, we endeavored to understand how this evasion of CD1d and NKT cell function plays a role in HSV-1 infection and pathogenesis *in vivo*.

RESULTS

1. NKT cells play potent roles in antiviral immune responses during ocular HSV-1 infection. Despite many studies by several groups, there have been debates on whether NKT cells play a critical role in anti-HSV-1 immunity (17–19, 33, 42–44). The different results most likely arose from differences in infection routes, virus and mouse strains used, and experimental designs, including the virus dosage, mouse gender, and immune status. An additional factor is that many of these studies used a flank infection model, in which the infection site and route may not be physiologically or clinically relevant. In an effort to understand the exact antiviral role(s) of NKT cells in a clinically relevant model, we employed an ocular infection model for HSV-1 (2, 45–47).

Previous ocular HSV-1 infection experiments were performed mostly in BALB/c strains, presumably for better illustration of virus-induced epithelial and stromal keratitis (44, 47). We first performed ocular HSV-1 infections in BALB/c mice. Five million HSV-1 strain F viruses were inoculated into the corneas of 10-week-old wild-type or CD1d$^{-/-}$ mice. Mouse eyes were swabbed in order to determine the virus titers in the eyes. Mouse periocular disease was scored using an established disease-scoring system (48–51) described in Materials and Methods. Our results showed clearly that eye disease and inflammation were much more severe in CD1d$^{-/-}$ mice (lacking all NKT cells) than in wild-type mice (Fig. 1A). As early as 1 day postinfection, CD1d$^{-/-}$ mice started to present periocular disease, while wild-type BALB/c did not show even a low level of disease until 1 day later. Over the course of 11 days postinfection, the pathology scores in CD1d$^{-/-}$ mice were constantly higher than those in wild-type mice (Fig. 1A and B). Similarly, viruses were cleared much faster in wild-type mice than in CD1d$^{-/-}$ mice (Fig. 1C). While the viruses could be detected up to at least 7 days postinoculation in CD1d$^{-/-}$ mice, all the viruses were cleared by the third day after inoculation in wild-type BALB/c mice. These results suggested that CD1d-restricted NKT cells exert a potent antiviral function in wild-type mice.

To examine whether the anti-HSV-1 function of NKT cells is mouse strain dependent, we performed the experiment using C57BL/6 mice. Wild-type or CD1d$^{-/-}$ C57BL/6 mice were infected with 50 million HSV-1 strain F viruses through their corneas. C57BL/6 mice are more resistant to HSV-1 infection than BALB/c mice, and a higher dosage of HSV-1 inoculum is required to cause pathogenesis (33, 44). Overall, the disease scores in C57BL/6 mice were lower than those in BALB/c mice, even with a 10-times-higher HSV-1 inoculum. Importantly, periocular disease scores were significantly higher in CD1d$^{-/-}$ mice. While scores of 2 to 3 could be detected in CD1d$^{-/-}$ mice at day 10 postinfection, no obvious disease could be detected in wild-type mice (Fig. 1D and E). Also, the inoculated viruses were cleared from the eyes much faster in wild-type mice than in CD1d$^{-/-}$ mice, in agreement with the previous report that C57BL/6 mice are largely resistant to HSV-1 infection (44). At 1 day postinfection, significantly lower virus titers were detected in the eyes of wild-type mice than in those of CD1d$^{-/-}$ mice (Fig. 1F). All these results suggest that NKT cells can participate in early anti-HSV-1 immune responses. To our knowledge, our results are the first demonstration that NKT cells can play a critical role in anti-HSV-1 immune responses in *in vivo* ocular infection.

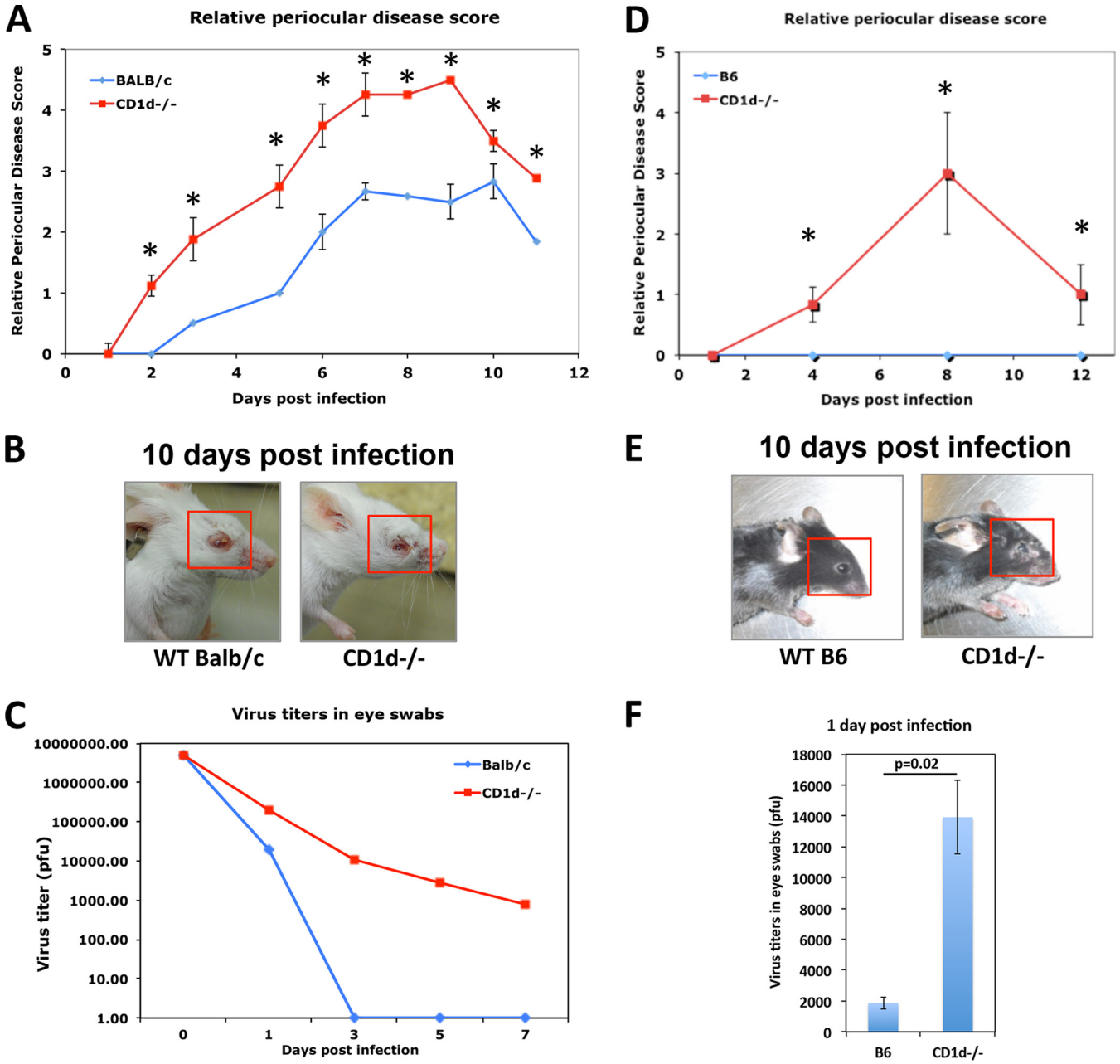

FIG 1 NKT cells can play potent antiviral roles in anti-HSV-1 immunity. (A, B, and C) Eight- to 10 week-old age- and gender-matched wild-type or CD1d$^{-/-}$ BALB/c mice (*n*, 5 each) were anesthetized with ketamine and xylazine and were infected via their corneas with 5 million HSV-1 strain F viruses. (A) The severity of periocular disease was scored in each group of mice at the indicated time points postinfection. Asterisks indicate significant differences ($P < 0.05$) between the two mouse groups at the indicated time points. (B) Representative eye images for BALB/c mice at 10 days postinfection. Areas boxed in red were examined for scoring. (C) Mouse eyes were swabbed at the indicated time points postinfection, and viral titers in swabs from a representative mouse in each group were determined by plaque assays in Vero cells. (D, E, and F) Eight- to 10-week-old wild-type or CD1d$^{-/-}$ C57BL/6 (B6) mice were infected with 50 million HSV-1 strain F viruses. (D) The severity of periocular disease was scored at the indicated time points postinfection. (E) Representative eye images for C57BL/6 mice at 10 days postinfection. (F) Viral titers in eye swabs at 1 day postinfection.

HSV-1 specifically inhibits the human CD1d/NKT cell antigen presentation pathway. Previously, we and others have demonstrated that upon infection, HSV-1 rapidly and efficiently downregulates CD1d in humans and inhibits the activation of NKT cells (17, 18, 27, 28). To investigate, using mouse models, how this immune evasion mechanism enhances HSV-1 pathogenesis, we first examined whether HSV-1 downregulates mouse CD1d (mCD1d) as it downregulates human CD1d in the human system. The mouse NIH 3T3 cell, line stably expressing mouse CD1d, was infected with

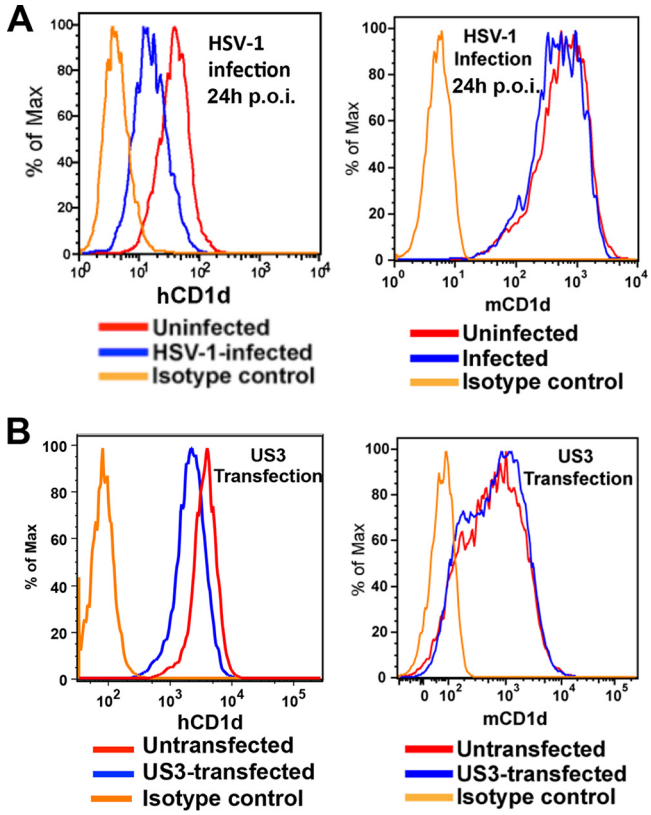

FIG 2 HSV-1 specifically downregulates human CD1d antigen presentation. (A) HeLa.CD1d and NIH 3T3.mCD1d cells were infected by HSV-1 strain F at an MOI of 5. Twenty-four hours postinfection (p.o.i.), human and mouse CD1d expression levels on cell surfaces were analyzed by flow cytometry using anti-hCD1d MAb 51.1.3 and anti-mCD1d MAb 1B1. Uninfected HeLa.CD1d or NIH 3T3.mCD1d cells were used as controls. Max, maximum. (B) HeLa.CD1d and 293T.mCD1d cells were transfected with US3-expressing plasmids coexpressing GFP and cell surface human or mouse CD1d, respectively, and CD1d expression levels were measured by flow cytometry. Transfected cells were gated into US3-expressing (GFP-positive) and non-US3-expressing (GFP-negative) populations, and the CD1d expression levels of the two populations were compared.

HSV-1 strain F at a multiplicity of infection (MOI) of 5 for 24 h, and the expression of mouse CD1d on the cell surface was examined by flow cytometry. Interestingly, HSV-1 infection minimally downregulated mouse CD1d on the cell surface (Fig. 2A). This result suggests that during HSV-1 infection of wild-type mice (Fig. 1B and E), mCD1d expression in antigen-presenting cells was not downregulated. Therefore, the antiviral function of NKT cells, which contributes to the overall anti-HSV-1 immune response, was not impeded. This is consistent with the HSV-1 resistance phenotype of wild-type mice.

We have previously identified the US3 protein kinase of HSV-1 as the major viral protein downregulating human CD1d (27, 28). To examine whether the US3 protein specifically downregulates human CD1d, we expressed US3 in HeLa.CD1d or 293T.mCD1d cells. The cell surface expression levels of CD1d in transfected (green fluorescent protein [GFP]-positive) and untransfected (GFP-negative) cells were compared. US3 efficiently downregulated hCD1d expression (Fig. 2B, left), as reported previously (27, 28), while it barely affected mCD1d expression levels (Fig. 2B, right). This result, together with the whole-virus infection results (Fig. 2A), strongly suggests that HSV-1 has coevolved with the human host and specifically downregulates human CD1d expression to evade the function of human NKT cells. It is noteworthy that another human-tropic virus, Kaposi's sarcoma-associated herpesvirus (KSHV), and the evasin gene MIR-2 have evolved to specifically suppress human CD1d and NKT cell function (16).

HSV-1 evasion of human CD1d function is a pathogenicity factor during viral infection. The inability of HSV-1 to downregulate the expression of mouse CD1d

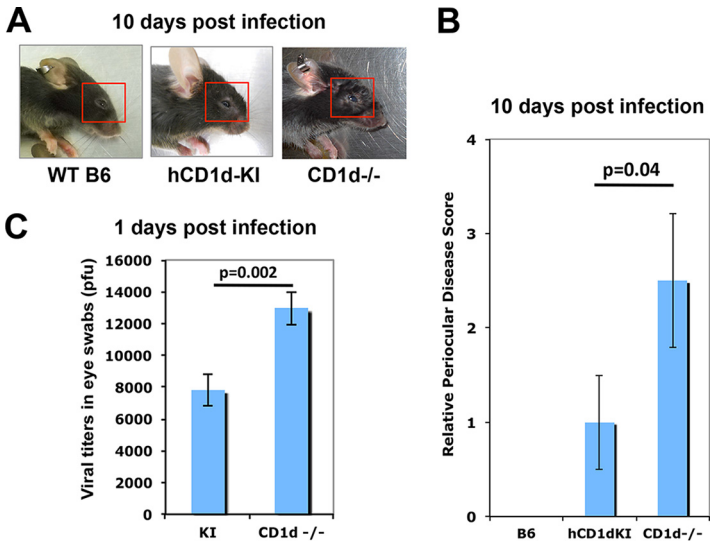

FIG 3 Evasion of human CD1d antigen presentation is a pathogenicity factor for HSV-1. Eight- to 10-week-old age- and gender-matched C57BL/6 (B6), hCD1d-KI, and CD1d$^{-/-}$ mice (*n*, 5 each) were anesthetized with ketamine-xylazine and were ocularly infected with 50 million HSV-1 strain F viruses. (A) Representative mouse eye images at 10 days postinfection. Areas boxed in red were examined for scoring. (B) The severity of periocular disease at 10 days postinfection was scored. (C) Virus titers in mouse eye swabs at 1 day postinfection were determined by plaque assays in Vero cells.

suggests that the function of mouse NKT cells is not suppressed during HSV-1 infection of wild-type mice. The species-specific nature of the evasion of human CD1d and NKT cell function undermines the usefulness of conventional mouse models for investigating the interaction of HSV-1 and the CD1d/NKT antigen presentation system. To build a more relevant mouse model for these studies, our laboratory has generated a novel human CD1d knock-in (hCD1d-KI) mouse model (52). The human CD1d supports the development of NKT cells in the resultant mice. More importantly, the developed NKT cells showed striking similarities to those in humans in the abundance, composition, and pattern of coreceptor expression (52). Considering the significant differences in NKT cell populations between humans and mice (52–55), our new hCD1d-KI mouse represents a more relevant model with which to study the interaction of HSV-1 with the CD1d/NKT cell system *in vivo*. From here on, we performed all our experiments with hCD1d-KI mice.

To investigate the role of HSV-1 downregulation of CD1d expression and NKT cell function during *in vivo* infection, we infected hCD1d-KI mice with HSV-1 strain F. Interestingly, the infected mice showed clear eyelid swelling with some hair loss, an eye pathology more severe than that in wild-type C57BL/6 mice (Fig. 3A and B). On average, we could observe relative periocular disease scores of 1, suggesting that HSV-1 evasion of human CD1d function enhances HSV-1 pathogenicity. Nevertheless, the average disease scores for hCD1d-KI mice were clearly lower than those for CD1d$^{-/-}$ mice (Fig. 3A and B). Titers in mouse eye swabs were determined, and we could also detect a significant difference in virus titers between hCD1d-KI and CD1d$^{-/-}$ mice (Fig. 3C), suggesting that virus clearance is more active in hCD1d-KI mice. The greater severity of viral pathology in hCD1d-KI mice than in wild-type mice suggests that the evasion of human CD1d and NKT cell function in hCD1d-KI mice enhances viral pathogenicity. On the other hand, the lesser severity of eye disease in infected hCD1d-KI mice than in CD1d$^{-/-}$ mice suggested that viral inhibition of human CD1d expression did not lead to complete suppression of NKT cell function, a notion consistent with our previous *in vitro* study showing that HSV-1 infection leads to 50%-to-70% lower CD1d expression and a similar level of inhibition of NKT cell function (17, 27). The residual NKT cell function may still exhibit some anti-HSV-1 activity *in vivo*. This partial suppression of human CD1d and NKT cell function may result from the coevolution of HSV-1 and the

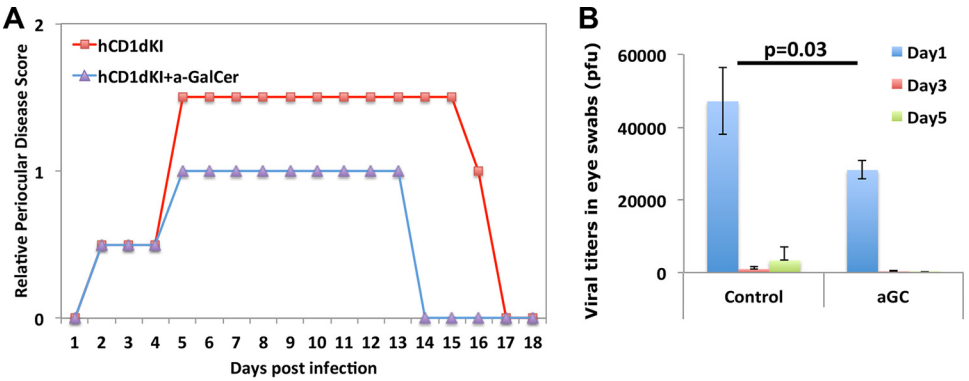

FIG 4 NKT cells possess potent anti-HSV-1 function in hCD1d-KI mice. Eight- to 10-week-old hCD1d-KI mice were injected intravenously with a vehicle control or with 2 μg of α-GalCer in PBS. Two days posttreatment, mice were ocularly infected with 50 million HSV-1 strain F viruses. (A) The severity of periocular disease was scored at the indicated time points postinfection. (B) Virus titers in mouse eye swabs at the indicated time points postinfection were determined by plaque assays in Vero cells. aGC, α-GalCer.

human host. To wit, while partial suppression of NKT cells allows HSV-1 to establish infection, the incomplete suppression of NKT cell function can ensure that the viral infection does not lead to too-severe damage to the host.

Our conclusion that HSV-1 evasion of human CD1d function is an important pathogenic factor is based on the premise that the NKT cells in hCD1d-KI mice indeed play critical antiviral roles. To investigate whether the NKT cells in hCD1d-KI mice, at a human-like abundance (52, 54), can play efficient antiviral roles, we preactivated the major group of NKT cells, invariant NKT (iNKT) cells, by administering α-galactosylceramide (α-GalCer), the prototype ligand of iNKT cells (56). Two micrograms of α-GalCer was first injected into the mice, and 2 days later, they were inoculated with HSV-1 strain F. The resulting eye disease was clearly milder in α-GalCer-treated mice than in untreated mice (Fig. 4A). Furthermore, virus titers were significantly lower in α-GalCer-treated hCD1d-KI mice than in untreated mice (Fig. 4B), suggesting that iNKT cells, though present at a much lower abundance than in wild-type mice (52), can play an important antiviral role in hCD1d-KI mice.

The HSV-1 evasin gene US3 is a pathogenesis factor targeting NKT cells. We have demonstrated previously that the protein kinase US3 potently downregulates CD1d expression in antigen-presenting cells and leads to the suppression of NKT cell function (27, 28) (Fig. 2). By use of US3-deficient viruses, it has been demonstrated that this viral protein kinase plays a critical role in viral pathogenesis (57–59). Nevertheless, it was not known how US3 enhances viral pathogenesis during HSV-1 ocular infection. We hypothesized that US3, as an important immune evasin, suppresses the functions of CD1d and NKT cells to enhance pathogenicity and allow the virus to establish infection. To test this hypothesis, we first infected hCD1d-KI mice with either strain F or the cognate US3-deficient (US3Δ) virus. Remarkably, without US3, HSV-1 completely lost its pathogenicity in hCD1d-KI mice (periocular disease score, 0) (Fig. 5A). In contrast, at 10 days postinfection, HSV-1 strain F caused overt ocular inflammation (Fig. 3A and B and Fig. 5A). In addition, wild-type strain F virus replicated much more efficiently than the US3Δ virus did in hCD1d-KI mice, retaining $>2 \times 10^4$ viable virions at day 1 postinfection, while the US3Δ virus was barely detectable at this time point ($<$1,000 virions) (Fig. 5B). To investigate whether the suppressed replication of the US3Δ virus is due to the antiviral function of NKT cells, we infected CD1d$^{-/-}$ mice with the US3Δ virus. Remarkably, significantly more viable US3Δ virus ($>$4,000 viable virions) was detected in CD1d$^{-/-}$ mice than in hCD1d-KI mice at day 1 postinfection (Fig. 5C), suggesting that at least one major pathogenic function of US3 protein in wild-type strain F is to suppress NKT cell function.

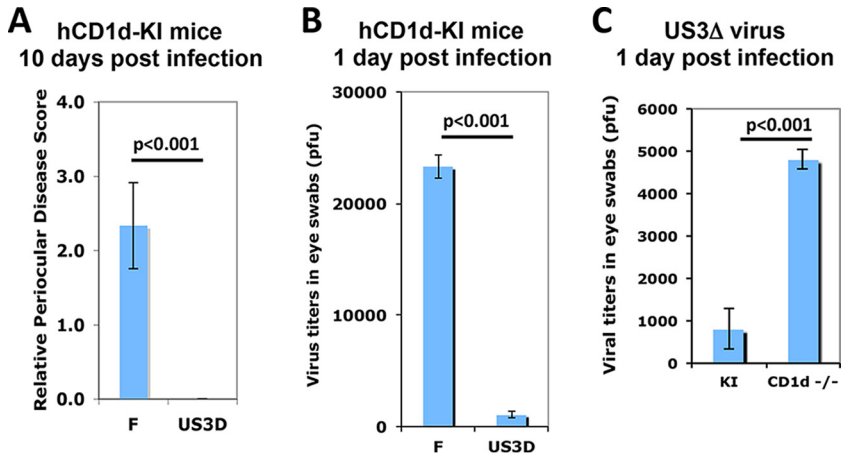

FIG 5 The US3 gene of HSV-1 is a critical pathogenicity factor targeting NKT cells. (A and B) Eight- to 10-week-old hCD1d-KI mice were infected with 50 million HSV-1 strain F or US3Δ (US3D) viruses. (A) The severity of periocular disease was scored at 10 days postinfection. (B) Virus titers in mouse eye swabs at 1 day postinfection were determined by plaque assays in Vero cells. (C) Eight- to 10-week-old hCD1d-KI or CD1d$^{-/-}$ mice were infected with 50 million HSV-1 US3Δ viruses. Virus titers in eye swabs at 1 day postinfection were determined by plaque assays in Vero cells.

DISCUSSION

A proper mouse model for studying the antiviral roles of NKT cells in antimicrobial immunity. Based on the overall conservation of immune systems in mammals, mouse models have been extensively used for human pathogen studies. They have provided a tremendous amount of valuable information in pathogenesis studies and have helped to establish the framework of pathogenesis for many human pathogens. However, human-specific pathogens have coevolved with humans for millions of years and have specifically overcome human immune system to establish infection. The profound differences between human and mouse immune systems (54, 60, 61) have therefore undermined the usefulness of conventional mice in modeling the pathogenesis of human-tropic pathogens. This limitation becomes more critical during *in vivo* pathogenesis investigations, as well as during antimicrobial-drug development, due to the differences between the molecular interactions of the drugs with target proteins from humans and their interactions with target proteins from mice (62, 63). One recent development in studies of human pathogens *in vivo* has been the use of humanized mice, some of which have repopulated human hematopoietic cells in an immune-deficient mouse background (64, 65). However, these newly developed mouse models have been used mostly to study the roles of major immune cell populations with high abundance (64, 65). NKT cells are a group of unconventional immunoregulatory cells that have a particularly low abundance in human populations. Yet they have critical immune-regulatory and antimicrobial functions (66, 67). NKT cells are barely detectable in humanized SCID/NOD mice (68), the commonly used humanized mice, most likely because these humanized mice still lack the thymic development and peripheral homeostasis support for human NKT cell survival (68). In this respect, our hCD1d-KI mice represent a genetically stable mouse model with which to more reliably study the antimicrobial roles of human-CD1d-restricted NKT cells (52). This model enables us, for the first time, to specifically investigate the interaction of human pathogens with human CD1d molecules. In this study, we indeed demonstrated that HSV-1 evasion of human CD1d function is a pathogenic factor. The ability to recapitulate immune evasion in our humanized mouse model may help to resolve the debate on the importance of NKT cells in HSV-1 infection that has arisen with other infection routes and models (42, 44). Moreover, given the striking resemblance of NKT cells in hCD1d-KI mice to those in the human population with regard to abundance and composition (52), our hCD1d-KI mice can be instrumental in the development of novel NKT cell ligands for anti-HSV-1 immunotherapy and vaccine adjuvants.

How do NKT cells exert their anti-HSV-1 function? Our results from plaque assays and periocular disease scores suggested that NKT cells play an anti-HSV-1 role very early, within the first couple of days postinfection (Fig. 1, 3, and 5). Previously, a large amount of data had demonstrated that during infection and other immune responses, NKT cells are activated within a few hours, either via the CD1d presentation of pathogen-derived NKT cell ligands (69) or via the inflammatory milieu (70, 71). The factor(s) that leads to NKT cell activation during HSV-1 infection is currently unknown. In general, viruses do not carry foreign lipid antigens as bacterial pathogens (66). Therefore, either CD1d presentation of an altered lipid ligand or an inflammatory milieu is more likely the trigger for NKT cell activation during HSV-1 infection. It will be very interesting to pursue the exact molecular factors that mediate this activation.

Very little is known about the exact mechanism by which NKT cells play critical roles in anti-HSV-1 immunity. Our results for HSV-1 ocular infection are consistent with other reports that NKT cells play critical roles in the early clearance of HSV (33, 43, 44, 72). In those studies, HSV-1 or HSV-2 was inoculated into the skin (33, 72) or by the oral (44) or vaginal (43) route. A large amount of data has demonstrated that neutrophils play essential roles in the early clearance of herpes simplex viruses (73, 74). It is therefore tempting to hypothesize that one of the major antiviral roles of NKT cells is to rapidly recruit and activate the neutrophils. Activated NKT cells secret large amounts of chemokines, including macrophage inflammatory protein 1α (MIP-1α), MIP-1β, RANTES, and leukotriene, as well as the cytokines tumor necrosis factor alpha (TNF-α) and IL-6 (75), all of which have been shown to play key roles in neutrophil recruitment and activation (76). In hepatitis and renal ischemia-reperfusion injuries, NKT cells play critical roles in neutrophil recruitment and function (77, 78). Moreover, NKT cells also contribute to neutrophil recruitment in the ocular mucosal environment (79). It will be particularly exciting to investigate further whether activation of NKT cells during HSV-1 infection also leads to rapid neutrophil influx and ultimately to clearance of the viruses and virus-infected cells.

MATERIALS AND METHODS

Viruses, cells, antibodies, and DNA constructs. Wild-type HSV-1 F and HSV-1 US3Δ (R7041) viruses were generous gifts from Bernard Roizman (University of Chicago, Chicago, IL). The HeLa.CD1d cell line was generated by transducing HeLa cells human CD1d gene-expressing construct based on the retroviral vector pLPCX (Clontech) and has been described previously (27, 80). For the generation of mouse cell lines stably expressing mouse CD1d, mouse CD1d1 cDNA was cloned into pLPCX. Retroviruses were generated and were transduced into NIH 3T3 or 293T cells. Puromycin was used to select cells stably expressing mouse CD1d as described previously (27). Expression of human or mouse CD1d was detected by monoclonal antibodies (MAbs) 51.1.3 (kindly provided by Steven Porcelli of the Albert Einstein College of Medicine, Bronx, NY) and 1B1 (Invitrogen), respectively. The HSV-1 US3 gene (strain 17) was subcloned from pLPCX.US3 (27) into the EcoRI/NotI sites of the pTracer-EF-V5-HisA vector (Invitrogen) to generate plasmid pTracer.US3.

Mice. C57BL/6 background mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were bred locally. C57BL/6 background CD1d$^{-/-}$ mice, with both the CD1d1 and CD1d2 genes knocked out, were generously provided by Chyung-Ru Wang of Northwestern University. The generation of hCD1d-KI mice in the C57BL/6 background has been reported previously (52). BALB/c background CD1d$^{-/-}$ mice were obtained from Michael J. Grusby, Harvard Medical School, and were bred into the BALB/c strain for $>$10 generations (81). All mice were bred into homozygotes. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Southern California.

Ocular infection of mice with HSV-1. Eight- to 10-week-old age- and gender-matched wild-type, hCD1d-KI, or CD1d$^{-/-}$ mice (*n*, 5 each) were anesthetized with intraperitoneally injected ketamine (120 mg/kg) and xylazine (10 mg/Kg), and their corneas infected as described previously with the amounts of viruses indicated in the figure legends (48–50). Briefly, 8- to 10-week-old mice were anesthetized, and their corneas were scarified by gentle scratching with syringe needles. A 5-μl volume of HSV-1 inoculum was then added on top of the cornea, and the eyelid was then rubbed to allow virus absorption. From day 1 to day 7, infected mice were anesthetized, and their eyes were swabbed with Q-tips. The absorbed viruses were then diluted and titers were determined by plaque assays on Vero cells. For infections with HSV-1 US3Δ, the eyes of infected mice were swabbed up to 3 days postinfection for the determination of virus titers. For α-galactosylceramide (α-GalCer) treatment of hCD1d-KI mice, 2 μg of α-GalCer (Funakoshi) in phosphate-buffered saline (PBS) per mouse was intravenously injected into the tail veins of 8- to 10-week-old mice. Forty-eight hours later, the mice were inoculated with HSV-1 and subsequently examined for eye diseases and virus titers.

Periocular skin disease scoring. After HSV-1 infection, the severity of mouse periocular skin disease was scored according to a method reported previously (48–50). In this scoring system, normal or disease-free status was scored as 0. When only the eyelid was affected with swelling and inflammation, the disease severity was scored as 1. Hair loss $<$3 mm from mouse eyes was scored as 2. Hair loss of $\leq$50%, 50 to 75%, or $>$75% on the infected side of the mouse face was scored as 3, 4, or 5, respectively. In addition, skin breakdown, as indicated by bleeding and/or scabbing/swelling of the eyelid to the point of eye closure, added 0.5 to the score.

DNA transfection and examination of CD1d downregulation in transfected cells. HeLa.CD1d or 293T.mCD1d cells were transiently transfected with the pTracer.US3 plasmid by use of the polyethylenimine (PEI) method. Forty-eight hours posttransfection, cells were stained with MAb 51.1.3 or 1B1 against hCD1d or mCD1d, respectively. Cells were gated at the GFP channel in flow cytometry, and the expression levels of hCD1d or mCD1d in GFP-positive (US3-expressing) cells and GFP-negative (control, non-US3-expressing) cells were compared.

Plaque assays, flow cytometry, and statistical tests. Plaque assays, flow cytometry, and statistical tests were performed as described previously (17, 27). For statistical analyses, we used Student's *t* test and analysis of variance (ANOVA) with pairwise multiple comparisons using Bonferroni's test. A *P* value of $<$0.05 was considered significant. All data are presented as means $\pm$ standard deviations.

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