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Herpes simplex viruses lacking the virion host shutoff function (Δvhs) are avirulent and hypersensitive to type I and type II interferon (IFN). In this study, we demonstrate that even in the absence of IFN responses in AG129 (IFN-αβγR$^{-/-}$) mice, Δvhs remains highly attenuated via corneal infection but is fully virulent via intracranial infection. The data demonstrate that the interferon-independent inherent replication defect of Δvhs has a significant impact upon peripheral replication and neuroinvasion.

Herpes simplex virus (HSV) is a common human pathogen and a significant source of morbidity (33). Severe disease and mortality are rare in most populations, occurring most frequently in immunocompromised individuals (24, 26). Those lacking Stat1 or having interferon (IFN) signaling defects are among the most susceptible, especially to central nervous system (CNS) infection (3, 7). IFN responses represent a critical early barrier to infection, and in common with all viruses, HSV encodes a variety of functions that counter the antiviral effects of IFN (18). One such function pertinent to this study is virion host shutoff (vhs), encoded by the UL41 gene of HSV (25). vhs is a tegument-derived endoribonuclease (13, 31) that is conserved among neurotropic herpesviruses (2). Consistent with the ability of vhs to destabilize a broad spectrum of mRNAs (30), vhs modulates various aspects of the host immune response, including IFN responses, dendritic cell maturation, antigen presentation, and cytokine/chemokine elicitation (4, 6, 19, 32). Correspondingly, the replication and virulence of HSV strains lacking vhs is significantly reduced in wild-type mice but significantly increased in mice lacking IFN responses (1, 20, 23, 28). While vhs is critical for HSV replication *in vivo*, its impact in cell culture varies greatly by cell type (5). The growth deficits observed in certain cells were correlative with induction of stress granules (indicating stalled translation) and decreased accumulation of viral late genes. These inherent *in vitro* growth defects were shown to be independent of IFN responses and consistent with a cell type-specific growth deficit (5). In this study, we sought to examine the role of vhs in promoting viral replication and virulence *in vivo* in the absence of type 1 and type II IFN responses. Previous studies of a vhs-deleted HSV-1 strain (UL41NHB [28], hereinafter referred to as Δvhs) in Stat1$^{-/-}$ mice showed incomplete restoration of growth and virulence relative to those of a wild-type (KOS) virus (23). While these Stat1$^{-/-}$ mice were highly susceptible to HSV infection, they maintain some persistent IFN-dependent responses that affect viral growth and pathogenesis (21), possibly explaining the incomplete restoration of growth and virulence to Δvhs. Testing Δvhs in type I/II IFN receptor knockout mice (AG129) therefore affords direct analysis of the role of IFN responses in controlling Δvhs replication and virulence and allows the examination of IFN-independent defects of Δvhs replication *in vivo*.

To assess the extent to which the replication of Δvhs might be inherently limited in an IFN-independent fashion in cells derived from AG129 mice, we compared the replication of Δvhs to that of wild-type KOS using multistep growth analyses at a multiplicity of infection (MOI) of 0.01 in bone marrow-derived dendritic cells (BMDCs) from wild-type (strain 129) and AG129 mice as previously described (17). BMDCs are a challenging environment for HSV replication, especially for viruses with engineered deletions in virulence genes (8, 9). Consistent with this, the replication of Δvhs was significantly reduced ($P < 0.0005$ at 48 h) in wild-type (strain 129/SvEv mice, Taconic Farms) BMDCs relative to the replication of KOS in these cells (Fig. 1A and B). In AG129 BMDCs, both Δvhs and KOS virus showed significantly more replication ($P < 0.005$ at 48 h) than in wild-type BMDCs, but the replication of Δvhs was not restored to the levels observed for KOS ($P < 0.002$ at 48 h). This is in contrast to the restoration of Δvhs replication observed in mouse embryonic fibroblasts (MEFs) derived from AG129 mice (22).

Previous *in vivo* work showed that vhs-deficient virus replication was significantly reduced in the wild-type mouse cornea (27, 28), and replication was only partially restored in mice lacking Stat1 (23). We therefore wished to examine the replication of Δvhs in the corneas of AG129 mice using methods previously described (28). Mice were infected with 2×10^6 PFU per scarified eye of KOS or Δvhs, and replication measured by eye swab assays from day 1 through 7 postinfection (Fig. 1C and D). The replication of both KOS and Δvhs was significantly enhanced ($P < 0.02$ at 3 days) in AG129 mice relative to that in wild-type mice, although the increased replication of Δvhs in AG129 corneas was not restored to the levels observed for KOS in these IFN-deficient mice. These data suggested that while IFN was limiting to the replication of both viruses, the replication defect of Δvhs observed in both BMDCs and corneas was inherent to vhs deficiency and yet independent of IFN.

To further assess the impact of vhs on the tropism and pathogen-

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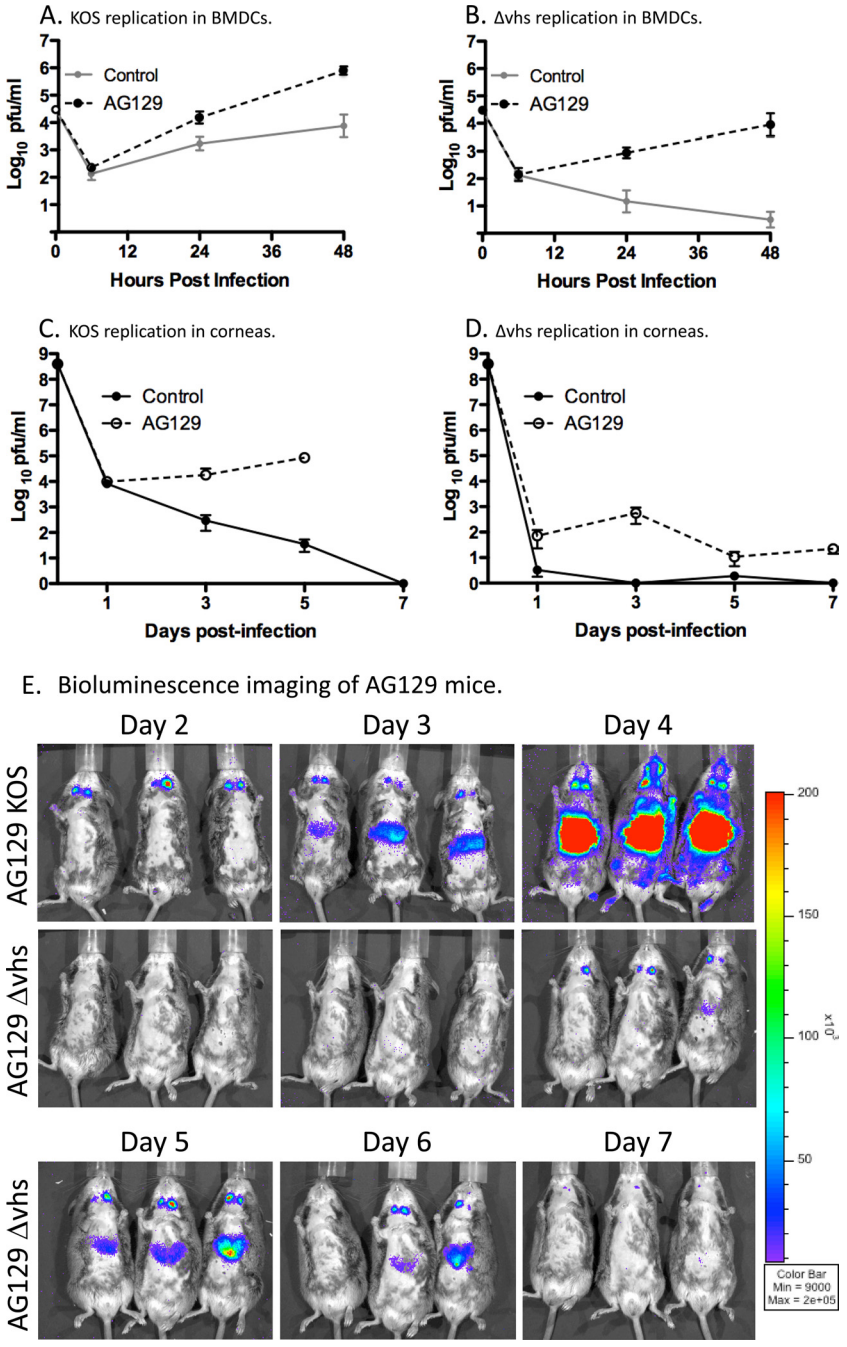

FIG 1 Replication of KOS and Δvhs in bone marrow-derived dendritic cells (BMDCs) and corneas of wild-type and IFN-deficient mice, and their virulence and spread following corneal infection. (A) Multiple-step growth curve of KOS in BMDCs derived from either wild-type or AG129 mice. (B) Multiple-step growth curve of Δvhs in BMDCs derived from either wild-type or AG129 mice. (C) Replication of KOS in scarified corneas of wild-type or AG129 mice following inoculation of 2×10^6 PFU per eye. (D) Replication of Δvhs in scarified corneas of wild-type or AG129 mice following inoculation of 2×10^6 PFU per eye. No corneal titers are shown for KOS in AG129 mice beyond day 5 due to mortality. (E) Tropism and spread of KOSdlux and Δvhsdlux shown by bioluminescence imaging following corneal infection with 2×10^6 PFU per eye. Living mice were imaged daily by IVIS (Caliper Lifesciences) following anesthesia and injection of 150 μg/g D-luciferin. Images were formatted on an identical photon flux scale as 10^3 photons/s/sr/cm^2. No images are shown for KOSdlux in AG129 mice beyond day 4 due to mortality. Data shown are derived from or representative of at least 2 experiments.

esis of HSV-1 in AG129 mice, we performed bioluminescence imaging as previously described (14–16, 21). The viruses used were KOS/dlux/ori$_L$ (29) (hereinafter referred to KOSdlux) and a new virus, Δvhsdlux, in which the firefly luciferase-expressing cassette described previously for making KOSdlux was inserted into the UL49.5 locus (29) of the vhs-deficient mutant UL41NHB using homologous recombination (28). Following corneal infection with KOSdlux, as expected, the previously published pattern of bioluminescence was observed (15), with the appearance of light signals in cervical lymph nodes by day 2 and increasingly strong signals in nodes and livers

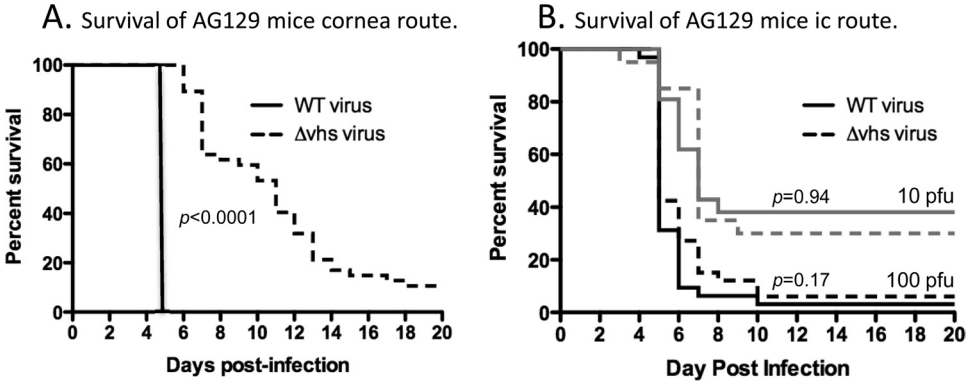

FIG 2 Survival of wild-type and AG129 mice following corneal or intracerebral (ic) challenge with KOS and Δvhs viruses. (A) Survival of AG129 mice following corneal challenge with 2×10^6 PFU of either KOS or Δvhs. (B) Survival of AG129 mice following intracerebral challenge with 1×10^1 PFU or 1×10^2 PFU of either KOS or Δvhs. Results are derived from at least 2 independent experiments for each panel.

from day 3 to day 4 (Fig. 1E). Mortality precluded imaging of the KOSdlux-infected mice on and beyond day 5. Overall, Δvhsdlux showed a tropism similar to that of KOSdlux in AG129 mice, but peak signals in all tissues were delayed by 1 day and attenuated approximately 200-fold. Interestingly, the AG129 mice cleared the Δvhs visceral infection by day 7 (Fig. 1E and data not shown) and yet began to show mortality at and beyond this time point (Fig. 2A). Taken together, these data demonstrate, both *in vitro* and *in vivo*, the strong impact of IFN-independent defects on the replication of a *vhs*-deficient HSV-1 strain in BMDCs and peripheral tissues.

One possible explanation for these data is that the cornea serves as an IFN-independent bottleneck to Δvhs infection and that, once Δvhs gains access to the CNS, it is able to replicate robustly and cause mortality. To test this possibility further, we examined the virulence and replication of KOS and Δvhs in the brain following corneal and intracerebral infection, as previously described (28). Observation of wild-type and AG129 mice infected via the cornea revealed a striking difference in survival following infection with KOS or Δvhs. All wild-type mice survived infection with either virus (data not shown). KOS-infected AG129 mice (13/13) all died synchronously on day 5, while the Δvhs-infected mice had a median survival of 11 days, with a more gradual mortality (42/47) out to day 21 (Fig. 2A). Despite the significantly ($P < 0.0001$) longer survival time for Δvhs-infected mice, this high degree of lethality was unexpected given the significant attenuation of Δvhs for corneal replication in these mice relative to the level of replication of KOS (Fig. 1C and D). This also contrasted strongly with Δvhs infection in corneas of Stat1$^{-/-}$ mice in which ~90% of mice survived to day 21 (20, 23). When wild-type mice were intracerebrally infected (28) with 2×10^6 PFU, KOS was significantly more virulent (6% survival) than Δvhs (79% survival; data not shown) in wild-type mice. In contrast, in AG129 mice, the virulence of Δvhs was indistinguishable from that of KOS ($P > 0.17$), even following infection with only 1×10^1 or 1×10^2 PFU (Fig. 2B). To assess this further, we examined virus replication in brain stems in wild-type and AG129 mice following corneal (2×10^6 PFU) or intracerebral (1×10^2 PFU) infection with either KOS or Δvhs (Fig. 3). Both corneal and intracerebral infection of wild-type mice resulted in detectable titers ($\leq 8 \times 10^3$ PFU) of KOS per brain stem, while Δvhs remained mostly undetectable (Fig. 3A and B). In AG129 mice following corneal infection, KOS was readily detectable by day 3 in the brain stem and reached $>1 \times 10^5$ PFU per brain on day 5, when 100% mortality was seen (Fig. 3C). Following corneal infection with Δvhs, increasing brain stem titers were observed on days 3 to 5, with titers of $>1 \times 10^4$ on day 7, at which time the first mortality was observed (Fig. 2A). For all data shown in Fig. 3B and C, the replication differences between KOS and Δvhs were statistically significant (P values ranging from 0.017 to 7×10^{-7}). Intracerebral infection of AG129 mice with 100 PFU of KOS or Δvhs resulted in high (10^5 to 10^6 PFU/brain stem) titers on days 3 and 5 (Fig. 3D). These titers were statistically indistinguishable between KOS and Δvhs ($P = 0.28$) on day 3. Although significant differences between these viruses were seen on day 5 ($P = 0.002$), the 10-fold difference in KOS and Δvhs titers in this tissue is in stark

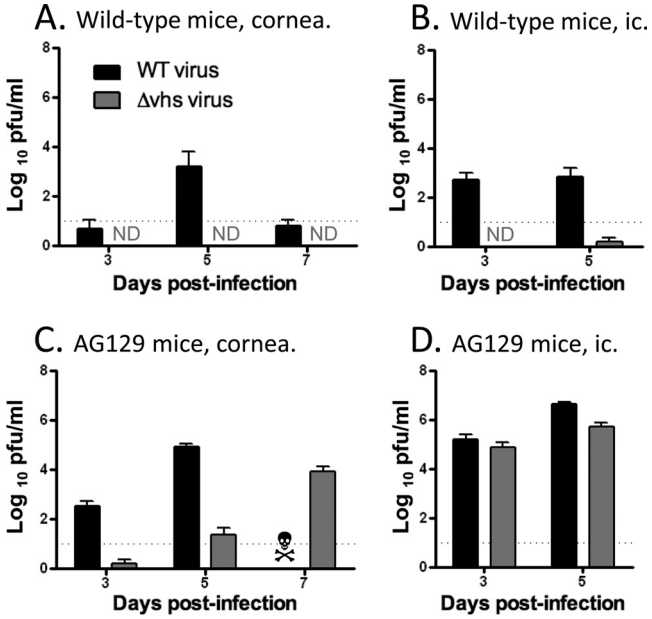

FIG 3 Virus replication in brain stems in wild-type and AG129 mice following corneal or intracerebral (ic) infection with either KOS or Δvhs viruses. "ND" indicates virus not detected. (A) Brain stem titers following corneal infection of wild-type mice with 2×10^6 PFU KOS or Δvhs per eye. (B) Brain stem titers following intracerebral infection of wild-type mice with 2×10^2 PFU KOS or Δvhs. (C) Brain stem titers following corneal infection of AG129 mice with 2×10^6 PFU KOS or Δvhs. KOS-infected AG129 mice died on day 5, precluding corneal swab sampling on day 7. (D) Brain stem titers following intracerebral infection of AG129 mice with 1×10^2 PFU KOS or Δvhs. Results are derived from at least 2 independent experiments for each panel.

contrast with the almost-100,000-fold difference observed in the AG129 corneas at this time point. These data therefore show that in AG129 mice, the virulence of Δvhs is attenuated by the corneal route but largely restored by the intracerebral route.

Humans and experimental animal models show significant resistance to corneal infection by ocular pathogens, unless the cornea is physically damaged or compromised through IFN deficiency (12). This is also consistent with the observation that significant bottlenecks for the transition of poliovirus from the periphery to the nervous system include both physical and IFN-responsive barriers (11). The attenuation of Δvhs in AG129 mice by the corneal route contrasts with significantly higher virulence by the intracerebral and intraperitoneal routes (data not shown), and these data together support the hypothesis that the cornea is a potent replication bottleneck to Δvhs replication, even in the absence of IFN responses. Although other tissues probably also limit the replication of vhs-deficient viruses (as evidenced by the lack of spread from the viscera by Δvhs), the limitation in the cornea is especially pertinent since it is both a common natural and experimental site of infection. This study also provides an *in vivo* parallel to previous work (5) that showed that the ability of vhs to augment HSV replication was highly cell-type dependent *in vitro*. Previous observations of the conservation of *vhs* among neurotropic herpesviruses, the attenuation of vhs-deficient viruses in the nervous system, and the role of vhs in evading the IFN response have collectively suggested that *vhs* must be critical for the neuropathogenesis of HSV (2, 10, 20, 28). This study, however, suggests that the inherent growth defect of vhs-deficient HSV-1 strains in the periphery is a critical determinant of their attenuation.

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