

VACCINES

HVEM signaling promotes protective antibody-dependent cellular cytotoxicity (ADCC) vaccine responses to herpes simplex viruses

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Herpes simplex virus (HSV) glycoprotein D (gD) not only is required for virus entry and cell-to-cell spread but also binds the host immunomodulatory molecule, HVEM, blocking interactions with its ligands. Natural infection primarily elicits neutralizing antibodies targeting gD, but subunit protein vaccines designed to induce this response have failed clinically. In contrast, preclinical studies demonstrate that an HSV-2 single-cycle strain deleted in gD, Δ gD-2, induces primarily non-neutralizing antibodies that activate Fc γ receptors (Fc γ R) to mediate antibody-dependent cellular cytotoxicity (ADCC). These studies were designed to test the hypothesis that gD interferes with ADCC through engagement of HVEM. Immunization of *Hvem*^{-/-} mice with Δ gD-2 resulted in significant reduction in HSV-specific IgG2 antibodies, the subclass associated with Fc γ R activation and ADCC, compared with wild-type controls. This translated into a parallel reduction in active and passive vaccine protection. A similar decrease in ADCC titers was observed in *Hvem*^{-/-} mice vaccinated with an alternative HSV vaccine candidate (dl5-29) or an unrelated vesicular stomatitis virus–vectored vaccine. Unexpectedly, not only did passive transfer of immune serum from Δ gD-2-vaccinated *Hvem*^{-/-} mice fail to protect wild-type mice but transfer of immune serum from Δ gD-2-vaccinated wild-type mice failed to protect *Hvem*^{-/-} mice. Immune cells isolated from *Hvem*^{-/-} mice were impaired in Fc γ R activation, and, conversely, addition of gD protein or anti-HVEM antibodies to in vitro murine or human Fc γ R activation assays inhibited the response. These findings uncover a previously unrecognized role for HVEM signaling in generating and mediating ADCC and an additional HSV immune evasion strategy.

INTRODUCTION

Herpes simplex virus types 1 and/or 2 (HSV-1 and HSV-2) infect most of the world's population and are responsible for recurrent mucocutaneous lesions, infectious encephalitis, corneal blindness, and neonatal disease (1, 2). Prophylactic vaccines evaluated in clinical trials have predominantly been adjuvanted subunit vaccines designed to generate neutralizing antibodies (nAbs) targeting the major envelope glycoprotein D (gD) (3–6). For example, a recombinant gD (rgD) vaccine adjuvanted with aluminum (alum) and monophosphoryl lipid A (MPL) (gD-2/AS04) protected mice and guinea pigs from disease after challenge with laboratory-adapted strains of HSV-2 but did not fully prevent latency (7–9). Human clinical trials, however, yielded disappointing results. In studies conducted among serodiscordant partners, gD-2/AS04 protected doubly (HSV-1 and HSV-2) seronegative women but failed to protect men or HSV-1–seropositive women (4). In a subsequent field trial that enrolled only doubly seronegative women, there was no protection against HSV-2, although partial protection against genital HSV-1 was observed (5).

These experiences highlight the need for alternative vaccine strategies. We conducted preclinical murine studies with a single-cycle HSV-2 virus deleted in gD, designated Δ gD-2. Two doses completely protected female or male mice from vaginal and/or skin challenge

with clinical isolates of either HSV serotype and prevented the establishment of latency (10–12). Unlike gD-2/AS04, Δ gD-2 induced antibodies (Abs) that were weakly neutralizing but potently activated Fc γ receptors (Fc γ R) to elicit antibody-dependent cellular cytotoxicity (ADCC). Passive transfer studies showed that these Abs were sufficient to protect naïve mice from lethal HSV vaginal or skin challenge (10–12).

The predominance of an ADCC response to Δ gD-2, but a neutralizing response to gD-2/AS04 and to natural infection, suggests that gD may play an immunomodulatory role and skew the immune response away from Fc γ R-mediated responses. This could provide a survival advantage because HSV can escape nAbs by spreading from infected to uninfected cells through cell junctions (13, 14). We hypothesized that this could reflect interactions between gD and herpes virus entry mediator (HVEM or TNFRSF14) (15–20).

HVEM is a bidirectional costimulatory and coinhibitory signaling molecule broadly expressed on most immune cells (17, 21–23). HVEM binds the tumor necrosis factor (TNF)–related activating ligands, LIGHT (TNFSF14) and lymphotoxin- α (LT α) (24); the immunoglobulin superfamily members B and T lymphocyte attenuator (BTLA) (25) and CD160 (26); and synaptic adhesion–like molecule 5 (27). HVEM activates BTLA inhibitory signaling and limits both innate and adaptive immune responses to some infections. LIGHT–HVEM signaling drives proinflammatory responses, but its role in immune responses to infections is unknown (20). gD competes with BTLA and LIGHT binding to HVEM and also downregulates HVEM expression (17, 28–30). The competition and mimicry between gD and its natural cellular ligands suggest that gD could interfere with HVEM signaling to modulate host immune responses,

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which could contribute to the different immune response after Δ gD-2 vaccination.

To test this hypothesis, we compared the immunogenicity and efficacy of Δ gD-2 and rgD protein vaccines in mice deficient in HVEM signaling. These mice are fully susceptible to HSV infections because the virus uses the dominant gD receptor, nectin-1, for entry and neuronal spread (31). The results of these studies demonstrate that Fc γ R-activating antibody responses provide a strong correlate of immune protection against HSV and that HVEM signaling is required for generating and mediating this protective immune response.

RESULTS

Δ gD-2 completely protects against HSV-2 after active or passive immunization of wild-type mice

Female C57BL/6 mice were prime-boost vaccinated subcutaneously 3 weeks apart with 5×10^5 plaque-forming units (PFU) per mouse of Δ gD-2, 5 μ g of rgD-2 adjuvanted with AS04 (GlaxoSmithKline) or an uninfected cell lysate. Mice were subsequently challenged on the skin with the HSV-2 clinical isolate, SD90, at 10 times the lethal dose for 90% of mice ($10 \times$ LD90) (Fig. 1A). Δ gD-2 protected 100% of mice, whereas gD-2/AS04 provided only 20% protection. Similar results were obtained when a different mouse strain, BALB/c, was challenged with HSV-2 MS-luciferase, and infection was monitored by imaging for luciferase expression (fig. S1). These findings confirm the previous studies in male mice comparing Δ gD-2 with rgD-2 protein combined with alum and MPL, a formulation similar to gD-2/AS04 (12).

Studies were conducted to determine whether the differences in vaccine efficacy between the single-cycle and sub-unit vaccines were associated with the quantity and/or functionality of Ab responses. Total HSV-2-specific or gD-2-specific immunoglobulin G (IgG) levels were quantified 1 week after the second vaccine dose. Δ gD-2 elicited a significantly greater total HSV-specific Ab response with little or no gD-specific Abs, whereas gD-2-AS04 induced a robust gD-specific response (Fig. 1, B and C). The functionality of the Abs also differed. rgD-2/AS04 induced the highest neutralizing titer ($P < 0.0001$ relative to control lysate) (Fig. 1D) but little or no Fc γ RIV activation (Fig. 1E). Conversely, Δ gD-2 induced significant Fc γ RIV responses but little neutralizing activity. These functional differences were reflected in the relative amounts of HSV-2-specific IgG1 and IgG2. In mice, IgG2 is the isotype most strongly associated with activation of Fc γ RIV, whereas IgG1 is associated with nAbs (32–35). Δ gD-2 induced a predominantly

IgG2 response, whereas gD-2/AS04 generated a predominantly IgG1 response (Fig. 1F).

HVEM plays a key role in the generation of ADCC responses

The functional differences in immune responses to the different vaccines may reflect the absence of the major neutralizing target and/or an immunomodulatory effect of gD, possibly through its interactions with HVEM on immune cells. To explore the latter hypothesis, the antibody responses in *Hvem*^{-/-} and WT mice were compared. rgD-2 combined with alum and MPL (rgD-2/Alum-MPL) was used in these studies because of limited gD-2/AS04 availability (12). The two formulations provide similar protection, although the former elicits lower nAb responses compared with gD-2/AS04 (Figs. 1 and 2). There was no difference in total HSV-specific (Fig. 2A) or nAb titers (Fig. 2B) after Δ gD-2 or rgD-2/Alum-MPL vaccination in *Hvem*^{-/-} compared with wild-type (WT) mice, but there was a significant decrease in Fc γ RIV activation in response to Δ gD-2 vaccination in *Hvem*^{-/-} mice ($P < 0.0001$) (Fig. 2C). This was associated with a parallel decrease in IgG2c responses ($P < 0.01$) (Fig. 2D). The loss of ADCC responses in *Hvem*^{-/-} mice translated into a loss in protection after skin ($P < 0.0001$) or vaginal ($P < 0.05$) challenge with a $10 \times$ LD90 dose of HSV-2

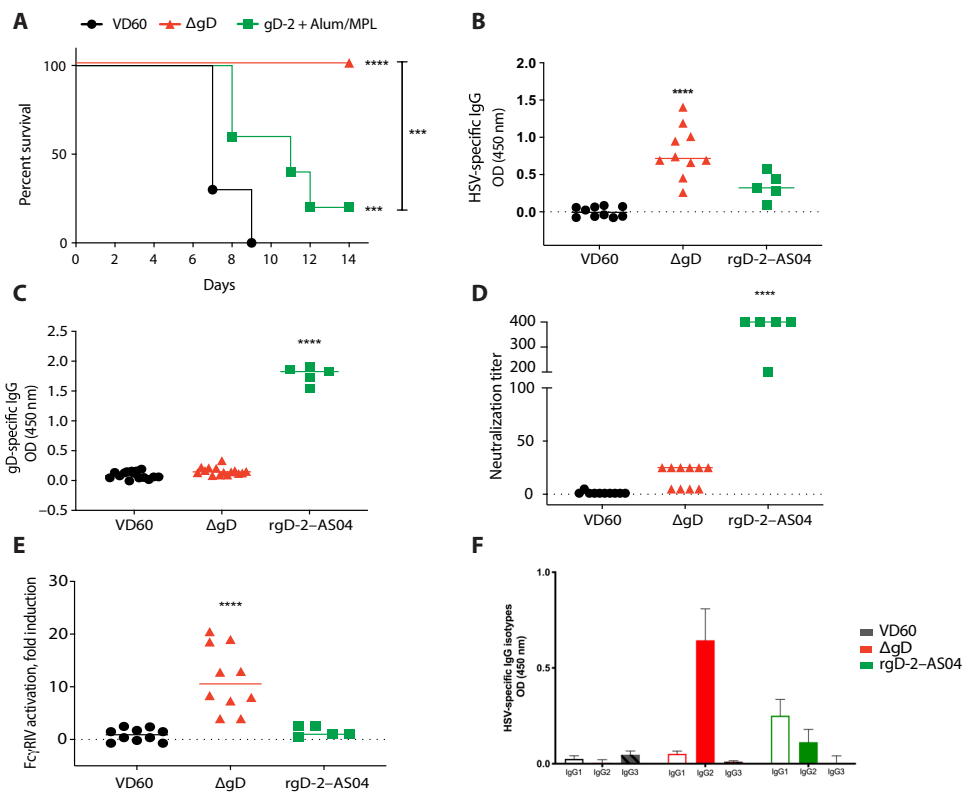
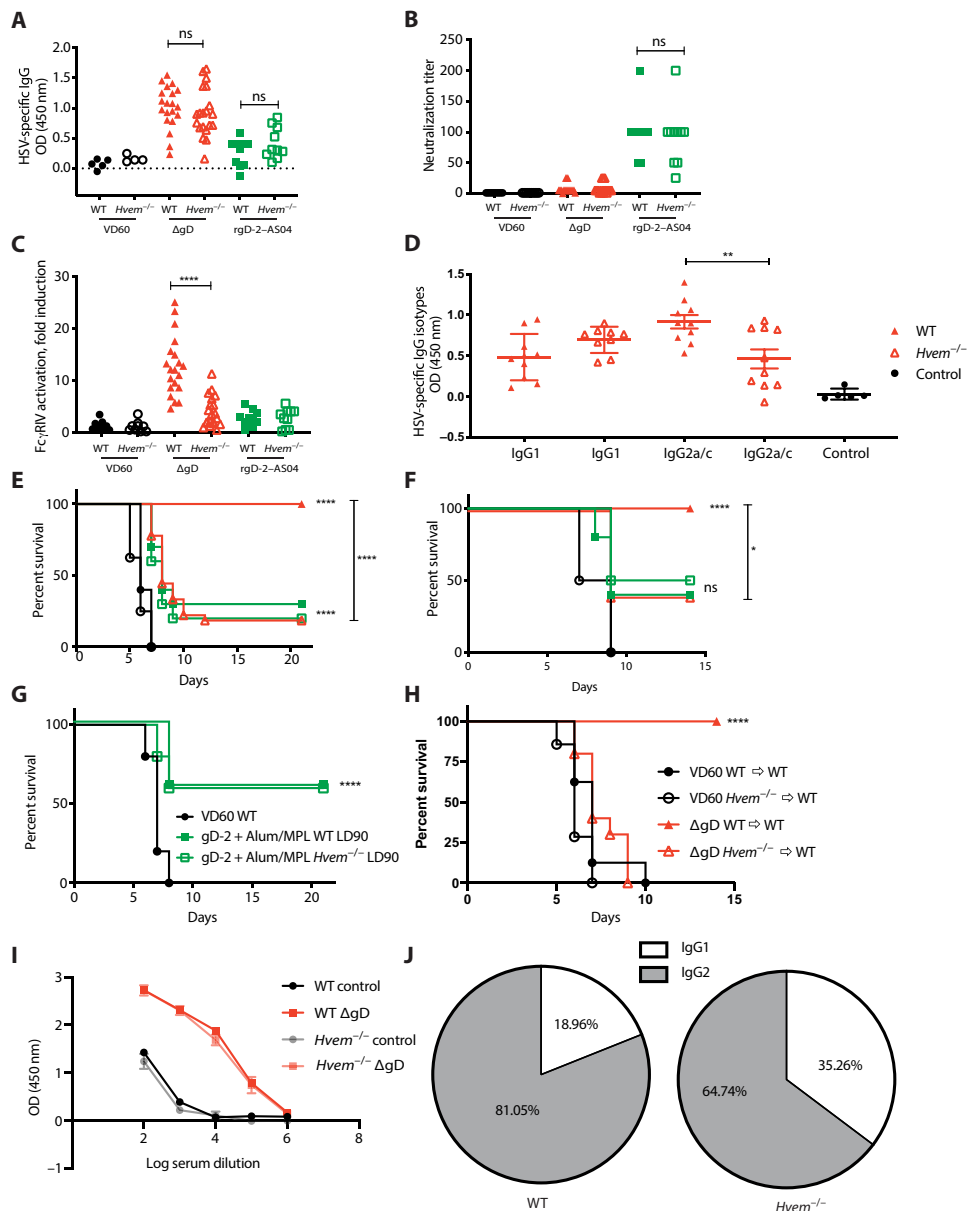


Fig. 1. Δ gD and rgD-2 differ in efficacy in mice challenged with HSV-2 (SD90). Female C57BL/6 mice were subcutaneously vaccinated with 5×10^5 PFU of Δ gD-2, 5 μ g of gD-2/AS04, or an uninfected lysate of VD60 cells (control). (A) Percentage survival after challenge on the skin with a $10 \times$ lethal dose (LD90) of HSV-2 (SD90). Asterisks indicate significant survival relative to VD60 control vaccine or comparing Δ gD-2 with gD-2/AS04 vaccine (Gehan-Breslow-Wilcoxon with Bonferroni correction; *** $P < 0.001$ and **** $P < 0.0001$). Serum samples were collected 1 week after the second vaccine dose and assayed for (B) HSV-2-specific IgG titer [1:90,000 dilution, at optical density (OD) 450 nm], (C) gD-2-specific IgG (1:10,000 dilution), (D) neutralization titer, (E) mFc γ RIV activation (1:5 dilution), or (F) HSV-2 isotype-specific Abs (1:1000 dilution). Responses were compared with VD60 control by one-way ANOVA (**** $P < 0.0001$). Two independent experiments were conducted with $n = 5$ mice per group in each experiment except for rgD-2-AS04 (one experiment with five mice).

Fig. 2. Vaccination of *Hvem*^{-/-} mice with Δ gD abrogates protection. WT or *Hvem*^{-/-} mice (male and female) were vaccinated with 5×10^5 PFU of Δ gD-2 or 5 μ g of rgD-2–Alum/MPL (two doses administered 3 weeks apart). One week after the second dose, serum was assayed for (A) HSV-2-specific IgG titer (1:90,000 dilution), (B) neutralizing titers, and (C) Fc γ RIV activation (1:5 dilution). (D) HSV-2 isotype-specific Abs (1:1000 dilution) were also determined. Percent survival is shown after (E) skin (male and female) and (F) intravaginal (female) challenge with a 10 \times LD90 dose of SD90. (G) Mice vaccinated with rgD-2–Alum/MPL were also challenged on the skin with a lower dose of SD90 (1 \times LD90). (H) WT C57BL/6 mice received immune serum containing 750 μ g of total IgG from VD60 (control) or Δ gD-2–vaccinated WT or *Hvem*^{-/-} mice 1 day before challenge on the skin with an LD90 dose of HSV-2 (4674). In (E), (F), and (H), each group is compared with its own WT control mice, and in (G), rgD-2–Alum/MPL–vaccinated mice are compared with VD60–vaccinated mice by Gehan-Breslow-Wilcoxon test. Responses in (A to D) were compared between WT and *Hvem*^{-/-} mice by ANOVA (**P* < 0.05, ***P* < 0.01, and *****P* < 0.0001); *n* = 10 to 20 animals per group combined from two independent experiments. (I and J) Serum collected 1 week after the second vaccine dose from mice immunized with 5×10^5 PFU per mouse of Δ gD-2 or VD60 control lysate was assayed for gB specificity by ELISA. Total (I) and isotype-specific (J) (1:1000) gB responses were quantified using subclass-specific anti-mouse secondary anti-IgG1, IgG2a/c, or IgG2b. For (I), *n* = 5 mice per group from two independent experiments; for (F and J), *n* = 5 mice per group from a single experiment. ns, not significant.



(SD90) (Fig. 2, E and F). The differences could not be attributed to increased susceptibility or generalized immune deficiency in *Hvem*^{-/-} mice because no differences in disease progression or lethality were observed in control-vaccinated *Hvem*^{-/-} versus WT mice. Moreover, when mice were challenged with a lower dose of SD90, rgD-2–Alum/MPL protected 60% of both WT and *Hvem*^{-/-} mice (Fig. 2G). Consistent with the reduction in ADCC responses in *Hvem*^{-/-} mice, passive transfer of immune serum from Δ gD-2–vaccinated *Hvem*^{-/-} into WT mice provided no protection, whereas all of the WT mice that received immune serum from Δ gD-2–vaccinated mice were completely protected (Fig. 2H).

Glycoprotein B (gB) is one of the targets of the Ab response elicited by Δ gD-2 (10). We confirmed this by enzyme-linked immunosorbent assay (ELISA) and compared the gB-specific response in WT and *Hvem*^{-/-} mice. There was no difference in the total gB-specific ELISA titer, but the proportion of IgG2, and specifically IgG2c, compared with IgG1 decreased substantially in *Hvem*^{-/-} compared with WT mice (Fig. 2, I and J).

A reduction in ADCC responses and protection was also observed when *Hvem*^{-/-} mice were vaccinated with the replication-

defective HSV-2 candidate vaccine, dl5-29 (Fig. 3, A to C). Prior studies demonstrated that dl5-29, which expresses gD at lower levels compared with replication-competent virus, elicits both neutralizing and ADCC responses, although the ADCC responses are lower than those after Δ gD-2 vaccination (36, 37). Depletion of gD-specific Ab from dl5-29 immune serum resulted in a significant reduction in total HSV-binding and nAb titers but had no effect on the ADCC levels (Fig. 3, D to F), indicating that gD is the primary target of nAbs but not ADCC Abs. Moreover, after controlling for the total IgG concentration, the gD-depleted immune serum provided greater protection than nondepleted serum in passive transfer studies (Fig. 3G).

To determine whether HVEM facilitates the generation of ADCC only for HSV or is more generalizable, WT and *Hvem*^{-/-} mice were vaccinated with a pseudotyped vesicular stomatitis virus expressing Ebola virus glycoprotein (rVSV-EBOV GP). There was a reduction

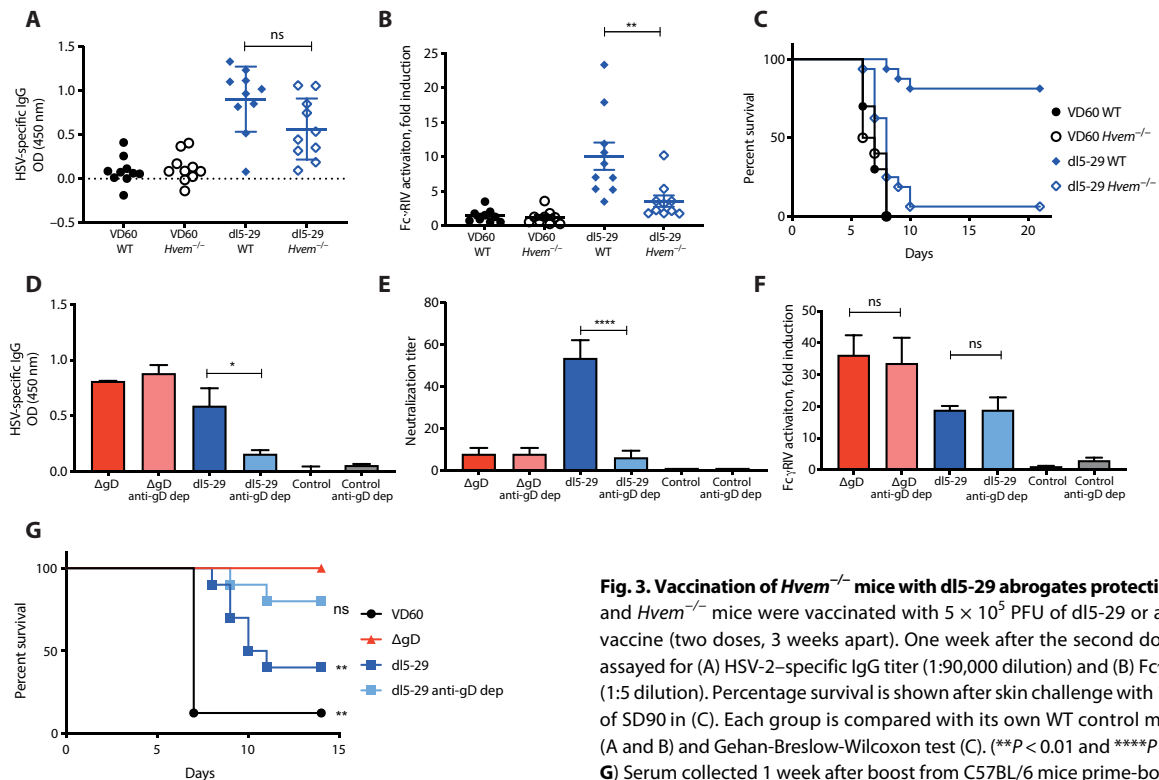


Fig. 3. Vaccination of *Hvem*^{-/-} mice with dl5-29 abrogates protection. (A to C) WT and *Hvem*^{-/-} mice were vaccinated with 5 × 10⁵ PFU of dl5-29 or a VD60 control vaccine (two doses, 3 weeks apart). One week after the second dose, serum was assayed for (A) HSV-2–specific IgG titer (1:90,000 dilution) and (B) FcγRIV activation (1:5 dilution). Percentage survival is shown after skin challenge with 10x LD90 dose of SD90 in (C). Each group is compared with its own WT control mice by ANOVA (A and B) and Gehan-Breslow-Wilcoxon test (C). (***P* < 0.01 and *****P* < 0.0001) (D to G) Serum collected 1 week after boost from C57BL/6 mice prime-boost vaccinated with 5 × 10⁶ PFU of ΔgD-2, dl5-29, or VD60 control lysate was depleted of gD-specific

antibody using biotinylated gD protein–coated streptavidin magnetic beads. Uncoated beads were used as a control for depletion. After depletion, serum was assessed for (D) total HSV-2–binding IgG by ELISA, (E) neutralization titer, or (F) FcγRIV activation. (G) Seven hundred fifty micrograms of total IgG from gD- or control-depleted immune serum was administered (intraperitoneally) to naïve mice challenged on the skin with an LD90 dose of HSV-2 (4674) 24 hours later and monitored for 14 days. (A to C) ***P* < 0.01; ANOVA; two independent experiments. (D to F) **P* < 0.05 and *****P* < 0.0001; paired *t* test; one representative experiment (of two) is shown. (G) **P* < 0.05 and ***P* < 0.01 compared with ΔgD-2 immune serum transfer by Gehan-Breslow-Wilcoxon test with Bonferroni correction for multiple testing. *n* = 10 mice per group from two independent experiments.

in the glycoprotein-specific antibody and ADCC responses but not nAb titer comparing *Hvem*^{-/-} and WT mice (fig. S2, A to C).

Deletion of the HVEM binding domain of gD results in decreased FcγRIV-activating Ab response to sublethal infection

Deletion of the gD HVEM binding domain results in a fully infectious virus that is not a vaccine candidate because nectin is the primary gD receptor for entry and spread (38, 39). Thus, rather than vaccinating, we applied a recently optimized sublethal intranasal infection model (36) to evaluate the immune response to HSV-2/gD-Δ7-15 (W260; a mutant deleted in the HVEM binding domain) or its WT repair virus (W176) (40). There was little difference in the end-point dilution of HSV-specific Ab or nAb titer, but mice infected with W260 generated relatively more IgG2 and ADCC Abs compared with mice infected with W176 (*P* < 0.05) (Fig. 4, A to D).

LIGHT, but not BTLA, contributes to the generation of FcγRIV-activating responses

To test whether competition between gD and BTLA or LIGHT for HVEM binding (17, 28–30) contributed to the reduction in ADCC after vaccination with ΔgD-2, studies were conducted in *Btla*^{-/-} and *Light*^{-/-} mice. There were no significant differences in any of the Ab responses (total, neutralizing, or ADCC) or in protection against

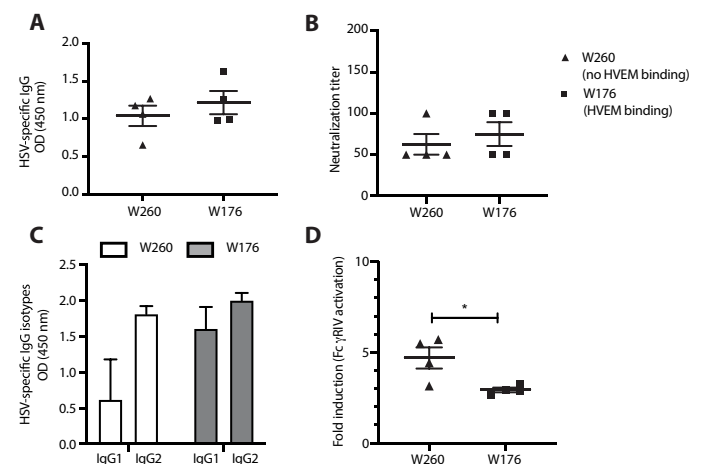


Fig. 4. Infection with an HVEM binding–deficient HSV-2 induces higher IgG2 and FcγRIV activating antibodies. Female C57BL/6 mice were infected intranasally with a sublethal dose (5 × 10⁴ PFU per mouse) of HSV-2 W260 (gD-Δ7-15; lacking HVEM binding domain) or the repaired strain HSV-2 W176 (WT gD). Serum was collected 14 days after infection, and seropositive mice were tested for (A) HSV-specific IgG (1:90,000 dilution), (B) neutralization titer, (C) HSV-specific IgG isotypes (1:1000 dilution), and (D) FcγRIV activation (1:5 dilution) (**P* < 0.05; *n* = 4 mice per group).

lethal challenge in *Btla*^{-/-} mice (Fig. 5, A to D). However, immunization of *Light*^{-/-} mice resulted in a significant decrease in ADCC and a reduction in immune protection (Fig. 5, E to H).

Effector cells in HVEM^{-/-} mice are also impaired in mediating ADCC responses

Transfer of immune serum from Δ gD-2-vaccinated *Hvem*^{-/-} mice into WT naïve mice failed to protect, consistent with the decreased ADCC (Fig. 2H). Unexpectedly, however, when the converse experiment was conducted and immune serum from Δ gD-2-vaccinated WT mice, which completely protects WT mice, was transferred into *Hvem*^{-/-} mice, no significant protection was observed after skin or vaginal challenge (Fig. 6, A and B). A similar reduction in protection was also observed when the immune serum was transferred into *Light*^{-/-} but not *Btla*^{-/-} mice (Fig. 6, C and D). These results suggest that LIGHT-HVEM signaling contributes not only to generation of ADCC but also to effector cell function.

Similar results were obtained when comparing the killing activity of bone marrow-derived immune cells harvested from *Hvem*^{-/-} with WT mice using green fluorescent protein (GFP)-expressing HSV-2

(333ZAG) as the target in flow cytometry-based ADCC assays to identify virally infected cells. HVEM expression in different cell subpopulations was assessed by flow cytometry (fig. S3). ADCC assays were conducted using total bone marrow or CD11c⁺ cells because the latter displayed potent activity in pilot studies. There was a significant reduction in killing (percentage of dead, GFP⁺ cells) when the effector cells were isolated from *Hvem*^{-/-} versus WT mice ($P < 0.05$; Fig. 7, A and B), which did not reflect differences in Fc γ R expression (fig. S4).

To further evaluate the role of CD11c⁺ cells and Fc γ RIV, passive transfer studies were conducted in CD11c-DTR (express diphtheria toxin receptor) and Fc γ RIV^{-/-} mice (41). Although intraperitoneal administration of Δ gD-2 immune serum into untreated or diphtheria toxin-treated WT mice provided complete protection against lethal skin challenge, protection was lost when serum was transferred into diphtheria toxin-treated CD11c-DTR mice (Fig. 7C). Protection was also lost when immune serum was transferred into Fc γ RIV^{-/-} mice (Fig. 7D).

Recombinant or viral gD or anti-HVEM blocks Fc γ RIV activation

The observation that *Hvem*^{-/-} cells were impaired in mediating ADCC suggests that gD, by binding to HVEM, may inhibit Fc γ RIV activation. Therefore, Fc γ RIV activation reporter assays were conducted in the presence of soluble gD protein or anti-HVEM Abs. The addition of gD reduced Fc γ RIV activity in a dose-dependent manner, whereas addition of gD deleted for the HVEM binding domain (Δ 7-32) did not (Fig. 8A, left and middle). Similarly, the addition of anti-HVEM Abs, but not an isotype control, to the effector cells also reduced the Fc γ RIV response (Fig. 8A, right, and fig. S5A). Moreover, there was an increase in Fc γ RIV activity when the target cells were infected with Δ gD-2 (no gD was expressed by the targets) compared with target cells infected with WT virus, an effect that was overcome by the addition of gD protein (Fig. 8B, left). There was also an increase in Fc γ RIV activity when target cells were infected with W260 (no HVEM binding) versus W176 (repaired virus) (Fig. 8B, right). The same inhibitory effect of soluble gD-2 or anti-HVEM Abs was observed when serum from HSV-2-seropositive individuals was used as the antibody source in a human ADCC reporter assay. Although the fold induction of the nuclear factor of activated T cell (NFAT) reporter was substantially lower with human immune serum compared with vaccinated mouse serum, preincubating human Fc γ RIIIa-expressing effector cells with soluble gD or anti-HVEM resulted in a significant decrease in effector cell activation (Fig. 8C). Anti-HVEM Abs, but not an isotype control, also reduced the ability of murine anti-CD20 to activate Fc γ RIV when Raji cells were used as the target in the assay (Fig. 8D and fig. S5B), indicating that this effect is not HSV specific.

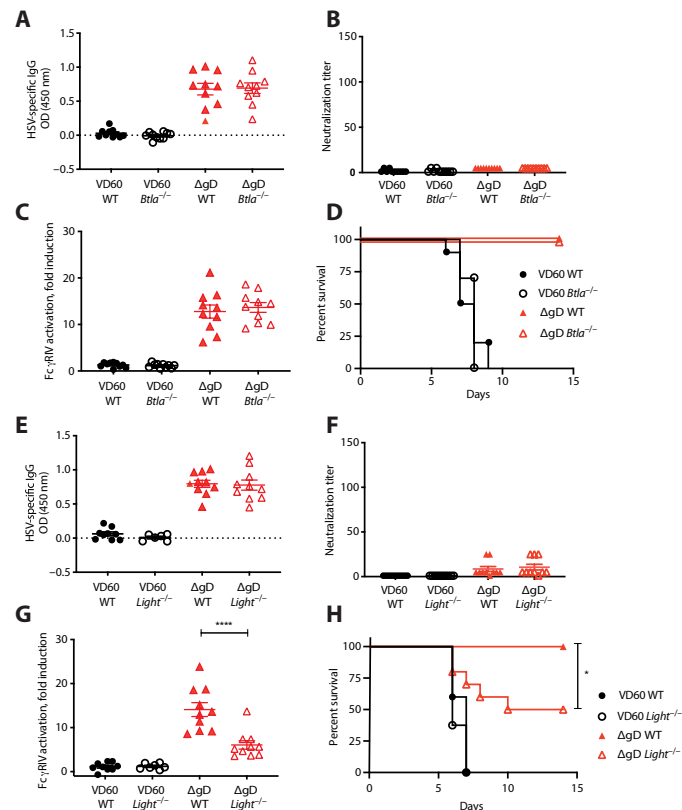


Fig. 5. Vaccination of *Light*^{-/-} but not *Btla*^{-/-} mice leads to reduction in Fc γ R-activating antibodies and protection. WT, BTLA, or LIGHT (E to H) knock-out mice were vaccinated with 5×10^5 PFU per mouse of Δ gD-2 or control VD60 lysates, and 1 week after the second vaccine dose, serum was assayed for (A and E) HSV-2-specific IgG titer (1:90,000 dilution), (B and F) neutralization titer, (C and G) Fc γ RIV activation (1:5 dilution), or (D and H) survival after skin challenge with $10 \times$ LD90 dose of SD90 virus. Responses were compared between WT and knockout mice by one-way ANOVA or, for survival curves, were compared with WT Δ gD-2-vaccinated mice by Gehan-Breslow-Wilcoxon test with Bonferroni correction for multiple testing (* $P < 0.05$ and **** $P < 0.0001$); $n = 10$ animals per group.

DISCUSSION

The current studies highlight the importance of ADCC in mediating a fully protective active or passive immune response against skin or vaginal high-dose challenge with clinical isolates of HSV in mice. Results with Δ gD-2 are in contrast to those obtained with gD-2/AS04 and a similar formulation of gD-2 combined with alum and MPL, which provided significantly less protection in the current and other studies in HSV-1-seropositive mice (36). The findings are, however, consistent with clinical trial outcomes, where nAbs elicited to

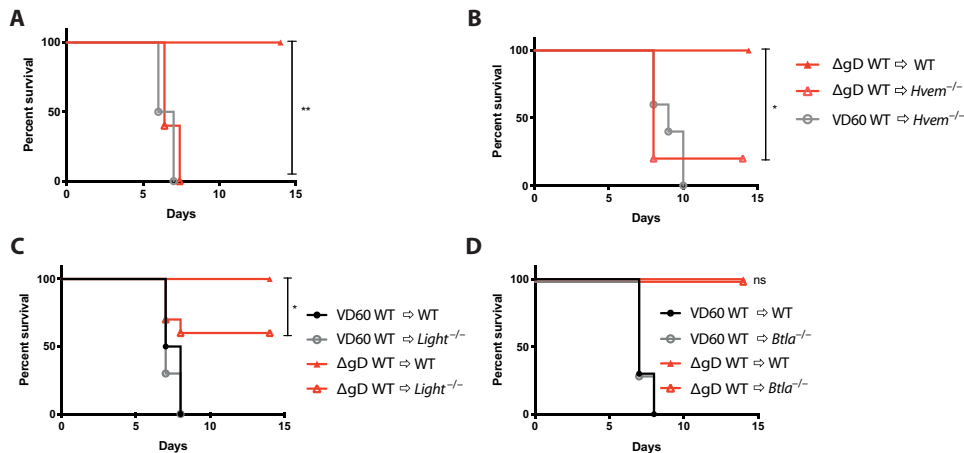


Fig. 6. A role for HVEM and LIGHT but not BTLA expression in mediating effector responses. Immune serum containing 750 μg of total IgG pooled from C57BL/6 WT mice vaccinated with ΔgD -2 or VD60 control lysate was transferred intraperitoneally into WT, *Hvem*^{-/-} (A and B), *Light*^{-/-} (C), or *Btla*^{-/-} (D) mice 1 day before challenge on the skin (A, C, and D) or intravaginally (B) with an LD90 dose of HSV-2 (4674). Survival is compared between WT and knockout mice by Gehan-Breslow-Wilcoxon test (**P* < 0.05 and ***P* < 0.01); *n* = 10 animals per group across two independent experiments.

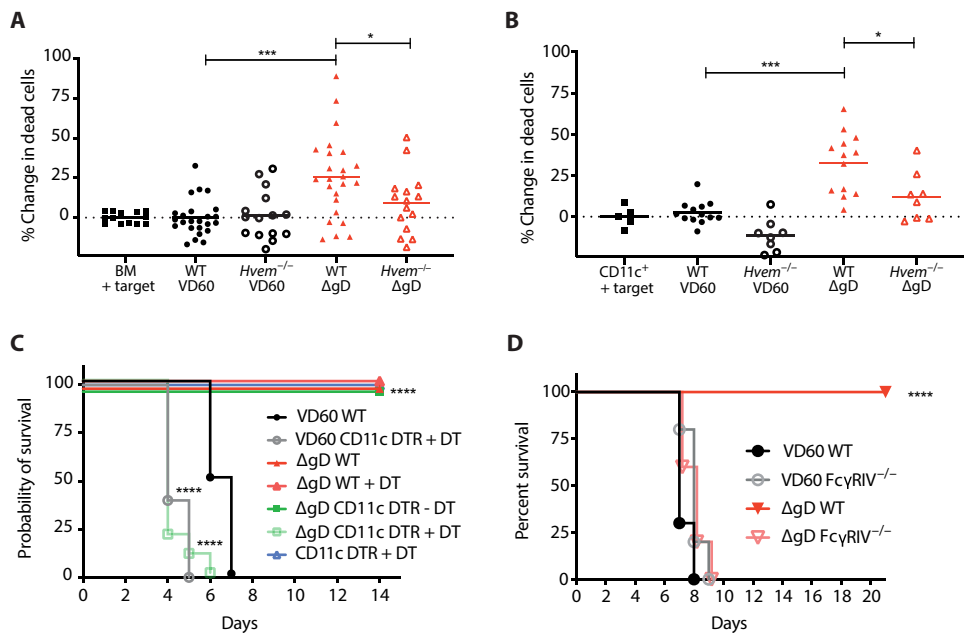


Fig. 7. Cells derived from *Hvem*^{-/-} mice are impaired in mediating protective ADCC responses. HaCAT cells were infected with HSV-2 (333-ZAG) (expressing GFP) for 4 hours, incubated with immune serum from ΔgD or VD60 control-immunized mice (1:5 dilution), and then cultured with either (A) total bone marrow or (B) bone marrow-derived CD11c⁺ cells (cultured with granulocyte-macrophage colony-stimulating factor) isolated from WT or *Hvem*^{-/-} mice as effector cells. ADCC was measured by quantifying dead GFP⁺ infected target cells by flow cytometry and is expressed as percentage change in dead cells compared with a “no serum” control. The line represents the median of individual results; data were analyzed by Mann-Whitney test (**P* < 0.05 and ****P* < 0.001); *n* = 10 to 15 per group; three independent experiments. Immune serum normalized to 750 μg of total IgG from ΔgD -2 or VD60 control-vaccinated mice was transferred to (C) naïve WT or CD11c-DTR mice treated or not with diphtheria toxin (DT) 24 hours before skin with an LD90 dose of HSV-2 (4674) or (D) naïve WT or Fc γ RIV^{-/-} mice. The asterisks, ****, indicate significant protection (*P* = 0.0001) relative to WT mice that received VD60 control immune serum (Gehan-Breslow-Wilcoxon test). *n* = 5 to 10 per group; two independent experiments.

gD-2/AS04 correlated poorly with vaccine efficacy against HSV-2 (4, 5, 12). ADCC responses were not reported for any of the *gD*-2/AS04 trials.

In contrast to the nAb response elicited by *gD*-2/AS04 and natural infections in mice and humans (42, 43), ΔgD -2 primarily induces an IgG2, Fc γ RIV-activating ADCC response in mice with little or no neutralizing or *gD*-specific Abs. These Fc γ RIV-activating Abs are sufficient to protect WT (but not Fc γ RIV^{-/-}) mice from subsequent HSV challenge in passive transfer studies and provide greater protection than nAb responses elicited by *gD*-2/AS04 or sublethal infection (44). The efficacy of active or passive ΔgD -2 vaccination was independent of whether mice were infected vaginally or on the skin. Although vaginal challenge has been used in most preclinical vaccine studies, it has not proven predictive of clinical outcomes. The skin challenge offers the advantage of being applicable to both males and females, does not require hormonal pretreatment, and is potentially more reflective of some aspects of human disease because most of the primary genital HSV lesions are observed on the skin, although murine and human genital skin may differ in immune cell populations (45). We observed no sex differences in the outcomes in WT or *Hvem*^{-/-} mice.

The absence of a significant nAb response to ΔgD -2 presumably reflects loss of the primary neutralizing target because *gD* is not produced during the replication of the single-cycle vaccine strain. The dominance of *gD* as a nAb target in mice is further evidenced by the reduction in neutralizing but not ADCC responses when the immune serum from d15-29-vaccinated mice was depleted of *gD*-specific Abs.

The observation that ΔgD -2 induces potent Fc γ RIV-activating responses, which is not observed after sublethal infection with WT viruses in mice (36, 44), suggests that *gD*, through its interactions with HVEM, interferes with ADCC as an immune evasion strategy. The reduction in ADCC responses in *Hvem*^{-/-} and *Light*^{-/-} mice and the increase in IgG2c, Fc γ RIV-activating Ab responses after sublethal infection with W260 (no HVEM binding domain) compared with the repaired virus support this hypothesis and demonstrate that HVEM-LIGHT

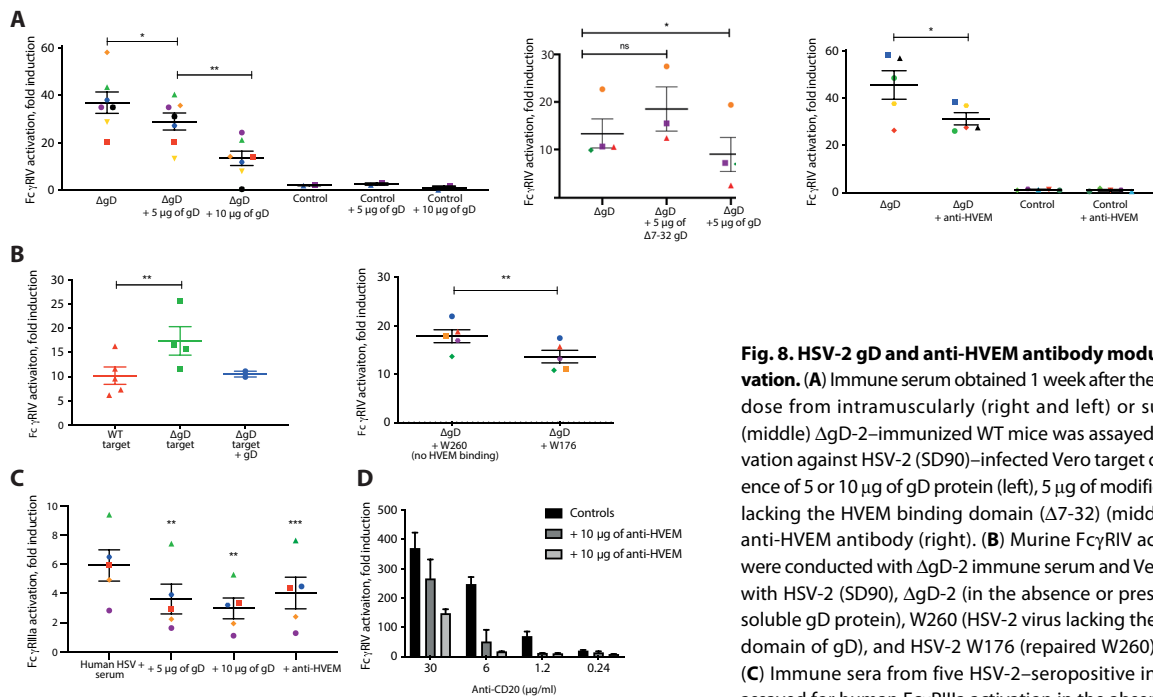


Fig. 8. HSV-2 gD and anti-HVEM antibody modulate FcγR activation. (A) Immune serum obtained 1 week after the second vaccine dose from intramuscularly (right and left) or subcutaneously (middle) ΔgD-2-immunized WT mice was assayed for FcγRIV activation against HSV-2 (SD90)-infected Vero target cells in the presence of 5 or 10 μg of gD protein (left), 5 μg of modified gD-2 protein lacking the HVEM binding domain (Δ7-32) (middle), or 10 μg of anti-HVEM antibody (right). (B) Murine FcγRIV activation assays were conducted with ΔgD-2 immune serum and Vero cells infected with HSV-2 (SD90), ΔgD-2 (in the absence or presence of 5 μg of soluble gD protein), W260 (HSV-2 virus lacking the HVEM binding domain of gD), and HSV-2 W176 (repaired W260) as target cells. (C) Immune sera from five HSV-2-seropositive individuals were assayed for human FcγRIIIa activation in the absence or presence of increasing doses of soluble gD protein or anti-HVEM antibody.

(D) Increasing amounts of anti-HVEM antibody were added to the positive-control Raji cells with anti-CD20 antibody in the murine FcγRIV activation assay. Results are from two independent experiments; $n = 5$ to 7 per group; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, paired Student's t test.

signaling promotes, and gD-HVEM interactions interfere with, IgG2 subclass switching. The inhibition likely depends both on the quantity of gD (dl5-29 is replication defective and expresses less gD than WT virus) and whether gD is soluble or membrane bound. HSV-infected cells, cell-free virions, and gD shed by infected cells could all be a source of this inhibitory effect.

Precisely what regulates IgG subclass switching is not fully understood, although recent work suggests that interferon- γ (IFN- γ) could provide a link between HVEM signaling and subclass switch. Mature B lymphocytes undergo recombination to produce different IgG subclasses in response to several extracellular signals, and IFN- γ appears to selectively stimulate production of IgG2a/c (46). IgG2a and IgG2c are functionally similar; C57BL/6 mice express IgG2c, whereas BALB/c mice express IgG2a (47). Expression of the T-box transcription factor, T-bet, by B cells plays an important role in IFN- γ -mediated IgG2a/c switch, and T-bet-deficient B cells were impaired in production of IgG2a/c transcripts in a murine lupus model (48). HVEM-LIGHT signaling stimulates IFN- γ -producing T cells, as well as IFN- γ production by type 3 innate lymphoid cells (ILC-3) (48–51). We speculate that, in addition to the recently described restrictive effects of HVEM-BTLA signaling on B cell proliferation (52), activating signaling mediated by LIGHT binding to HVEM promotes IFN- γ production and subclass switch to IgG2a/c Abs. Precisely which cell subpopulations are involved in this process will require future study; activated T cells and innate immune cells express HVEM, BTLA, and LIGHT, whereas dendritic cells and B cells do not express LIGHT (53). The notion that HVEM-LIGHT signaling promotes subclass switching to IgG2 is consistent with the observed reduction in IgG2c/ADCC Abs when HVEM is absent or when its interactions with LIGHT are inhibited by viral gD. Presumably, HVEM binding

partners other than LIGHT (such as CD160 or LT α) contribute to this activation pathway because the reduction in protection was not as complete in *Light*^{-/-} as in *Hvem*^{-/-} mice.

HVEM signaling was required not only for mounting an ADCC response but also for mediating killing. Passive transfer of immune serum from ΔgD-2-immunized WT mice failed to protect *Hvem*^{-/-} and only partially protected *Light*^{-/-} mice from subsequent viral challenge. Moreover, total bone marrow and, specifically, CD11c⁺ cells isolated from *Hvem*^{-/-} mice were impaired from their ability to mediate ADCC. Passive transfer studies with CD11c-DTR and FcγRIV^{-/-} mice confirmed a dominant role for murine FcγRIV and CD11c⁺ cells in mediating ADCC. The absence of CD11c⁺ cells resulted in significantly faster mortality in HSV-infected mice, consistent with a previous study, which found that ablation of CD11c⁺ cells increased the susceptibility to HSV infection (54). A central role for CD11c⁺ cells in mediating ADCC is not unexpected because murine natural killer (NK) cells express little or no FcγRIV, and other studies have found that murine CD11c⁺ cells contribute to antibody-mediated cell killing (35, 55, 56). In humans, NK cells play a major role in mediating ADCC (57).

The decrease in FcγRIV activation when gD protein or anti-HVEM Abs were added to the in vitro ADCC assay with mouse or human immune sera or when target cells do (WT virus) or do not (ΔgD-2) express gD uncovers an additional gD-mediated immune evasion strategy. The interference was mapped to the HVEM binding domain on gD because rgD protein lacking the HVEM binding region (Δ7-32) did not block FcγRIV activation. Conversely, FcγRIV activation was increased when the target cells in the assay were infected with either ΔgD-2 or W260 (missing the HVEM binding domain). Thus, by interacting with HVEM, gD mediates a two pronged

immune evasion strategy; it reduces the generation of IgG2c Abs and blocks their activity by interfering with FcγRIV activation. This would be especially relevant within HSV lesions where levels of gD are likely high. The high titer of ADCC Abs elicited by ΔgD-2 overcomes this interference.

Few studies have quantified ADCC responses to natural HSV infection in humans, although ongoing studies (including samples in Fig. 8C) indicate a more limited ADCC response consistent with gD-mediated interference. The low titers of ADCC generated in response to sublethal HSV infection in female mice (despite high nAb responses) failed to protect their pups from subsequent viral challenge (44) and also failed to protect HSV-1-seropositive mice from subsequent HSV-2 challenge (36). In contrast, the high-titer ADCC responses elicited by ΔgD-2 fully protected pups and protected HSV-1-seropositive mice from subsequent HSV-2 challenge (44). The ability of individuals to overcome this immune evasion strategy may depend on viral exposure and heterogeneity in immune responses. A small clinical study of neonatal HSV disease found that, after controlling for the nAb titer, higher titers of maternally acquired ADCC Abs were protected against viral dissemination (58, 59). Why some women exhibited higher ADCC than others will require future study.

The finding that HVEM contributes to both arms of ADCC-mediated immunity (generation of the ADCC Abs and effector cell function) was not restricted to ΔgD-2. *Hvem*^{-/-} mice exhibited reduced ADCC Ab titers compared with WT mice when vaccinated with a replication-defective HSV viral vaccine (dl5-29), which generates lower levels of gD than natural infection, or an rVSV-EBOV GP vaccine. Moreover, in a human ADCC assay, antibodies to HVEM, but not an isotype control Ab, inhibited the response mediated by anti-CD20 against Raji target cells. Other pathogens may also interfere with HVEM signaling to block the generation of ADCC responses and/or the ability of effector cells to activate FcγRs. This mechanism might be particularly relevant for microbes that escape nAbs (60). For example, cytomegalovirus (CMV) UL144 protein is an ortholog of HVEM that targets BTLA (61). The function of UL144 in CMV pathogenesis is unknown, but it may have a role in immune evasion (62). Although we did not identify a phenotype in *Btla*^{-/-} mice with respect to the ΔgD-2 vaccine, BTLA signaling may contribute to ADCC for other pathogens. In conclusion, these studies uncovered a role for HVEM signaling in both generating and mediating ADCC vaccine responses. By engaging HVEM, HSV gD interferes with both of these processes, providing evidence for a previously unrecognized viral immune evasion strategy. These results may have implications for promoting ADCC responses to other pathogens or, more broadly, in promoting or interfering with ADCC in other immune-modulated diseases.

MATERIALS AND METHODS

Study design

These studies were designed to assess the role of HVEM signaling in the generation and effector function of ADCC antibody responses in vitro and in vivo mouse models of HSV vaccination and challenge. For in vivo studies, at least five mice per group per experiment were used, and where possible, these experiments were completed at least twice as indicated in the figure legends. For in vitro studies, each sample was analyzed in duplicate; figure legends indicate the number of independent experiments. Mice were randomly assigned

to vaccination groups; researchers were blinded for HSV challenge and disease scoring. Human samples for in vitro analysis were selected on the basis of HSV seropositivity and HSV-specific activation of FcγRIIIa.

Ethics statement

The use of animals was approved by the Institutional Animal Care and Use Committee at the Albert Einstein College of Medicine under protocols 2015-0805, 2017-0518, and 2018-0504.

Statistical analysis

Analyses were performed using GraphPad Prism version 8.3 software (GraphPad Software Inc., San Diego, CA). A *P* value of 0.05 was considered statistically significant. Survival curves were compared using the Gehan-Breslow-Wilcoxon test; other results were compared using analysis of variance (ANOVA), paired *t* tests, or Mann-Whitney tests with multiple testing as indicated. All data are shown as means ± SEM unless otherwise indicated.

SUPPLEMENTARY MATERIALS

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Materials and Methods

Fig. S1. ΔgD-2 vaccination rapidly clears challenge virus.

Fig. S2. Absence of HVEM reduces FcγR-activating antibody responses in an rVSV-EBOV GP vaccination model.

Fig. S3. HVEM expression on immune cell populations in WT and *Hvem*^{-/-} mice.

Fig. S4. FcγR expression on immune cells isolated from WT or *Hvem*^{-/-} mice.

Fig. S5. Anti-HVEM, but not an isotype control antibody, reduces FcγRIV activation.

Table S1. Raw data file (Excel spreadsheet).

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[View/request a protocol for this paper from Bio-protocol.](#)

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HVEM signaling promotes protective antibody-dependent cellular cytotoxicity (ADCC) vaccine responses to herpes simplex viruses

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Subversion of ADCC by herpes viruses

Antibodies to viruses produced after infection or vaccination can protect the host by virus neutralization or through antibody-dependent cellular cytotoxicity (ADCC). The strong ADCC response elicited by an HSV-2 vaccine strain lacking glycoprotein D (Δ gD-2) provides robust protection against wild-type HSV-2 challenge in mice. Burn Aschner *et al.* tested the hypothesis that HSV-2 gD binding to the HVEM entry receptor interferes with host immunity by blocking an HVEM-dependent signaling pathway needed to achieve a protective ADCC response. They found that the Δ gD-2 vaccine –induced ADCC response in mice requires HVEM and its ligand LIGHT during the inductive phase and HVEM and the Fc γ V receptor during the effector phase. This research provides a deeper understanding of the host signaling pathways that support the establishment of ADCC.

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