# A recombinant herpes virus expressing influenza hemagglutinin confers protection and induces antibody-dependent cellular cytotoxicity

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Contributed by William R. Jacobs Jr., July 14, 2021 (sent for review June 9, 2021; reviewed by Adolfo Garcia-Sastre and Jeffrey V. Ravetch)

**Despite widespread yearly vaccination, influenza leads to significant morbidity and mortality across the globe. To make a more broadly protective influenza vaccine, it may be necessary to elicit antibodies that can activate effector functions in immune cells, such as antibody-dependent cellular cytotoxicity (ADCC). There is growing evidence supporting the necessity for ADCC in protection against influenza and herpes simplex virus (HSV), among other infectious diseases. An HSV-2 strain lacking the essential glycoprotein D (gD), was used to create ΔgD-2, which is a highly protective vaccine against lethal HSV-1 and HSV-2 infection in mice. It also elicits high levels of IgG2c antibodies that bind FcγRIV, a receptor that activates ADCC. To make an ADCC-eliciting influenza vaccine, we cloned the hemagglutinin (*HA*) gene from an H1N1 influenza A strain into the ΔgD-2 HSV vector. Vaccination with ΔgD-2::$HA_{PR8}$ was protective against homologous influenza challenge and elicited an antibody response against HA that inhibits hemagglutination ($HAI^+$), is predominantly IgG2c, strongly activates FcγRIV, and protects against influenza challenge following passive immunization of naïve mice. Prior exposure of mice to HSV-1, HSV-2, or a replication-defective HSV-2 vaccine (*dl5-29*) does not reduce protection against influenza by ΔgD-2::$HA_{PR8}$. This vaccine also continues to elicit protection against both HSV-1 and HSV-2, including high levels of IgG2c antibodies against HSV-2. Mice lacking the interferon-α/β receptor and mice lacking the interferon-γ receptor were also protected against influenza challenge by ΔgD-2::$HA_{PR8}$. Our results suggest that ΔgD-2 can be used as a vaccine vector against other pathogens, while also eliciting protective anti-HSV immunity.**

influenza vaccine | viral vector | herpes simplex virus | ADCC

Influenza remains a global health threat. Seasonal strains of influenza A and B cause an estimated 5 million cases of severe infections and 500,000 deaths per year (1). Influenza pandemics have caused even greater morbidity and mortality. During the H1N1 pandemic of 1918 to 1919, 500 million people, approximately one-third of the world's population at that time, were estimated to have been infected with this strain, leading to 50 million deaths (2). The H1N1 pandemic of 2009 is estimated to have caused up to 575,000 deaths (2). Currently, three types of influenza vaccines are offered annually in the United States: a recombinant virus expressing influenza proteins, chemically inactivated virus, and live attenuated virus (3). Regardless of the vaccine type, multiple strains are included to increase the chances of developing sufficient protection against major circulating influenza strains. However, these vaccines primarily elicit a neutralizing antibody response that is sensitive to changes in the influenza virus due to antigenic drift and shift (4). Antigenic drift results from an accumulation of random mutations in influenza antigens, like hemagglutinin (HA), altering sites recognized by the immune system (4). Influenza A strains can also undergo antigenic shift, whereby two different influenza strains infect the same cell to form a reassortant virus with new antigenic properties (4). Due to limited immunity in the population, these new strains are highly virulent, causing widespread epidemics and disease (4). With antigenic drift and shift, vaccine-mediated protection against circulating strains has been insufficient (5). Influenza vaccines that elicit more robust and long-term protection are therefore needed. Notably, if an influenza vaccine with ≥75% efficacy were to be broadly used in the United States, an estimated 19,500 deaths a year could be prevented and direct healthcare costs reduced by $3.5 billion (6).

For many years, efforts to improve influenza vaccines have focused on eliciting an immune response for full, broad protection against both circulating and future strains of the virus. These studies have shown that, in general, neutralizing antibodies are sufficient for homologous protection (7). However, achieving heterologous

## Significance

Despite decades of research, we lack an effective vaccine against influenza, a deadly virus that costs the United States nearly $90 billion annually. Current strategies do not translate into highly protective immunity against circulating and novel influenza strains. Here, we demonstrate that a herpes simplex viral (HSV) vector expressing hemagglutinin can be used to elicit a protective response against influenza. The efficacy of this vector is not abrogated by preexisting immunity to HSV, protects against lethal HSV challenge, and elicits highly functional FcγRIV-binding antibodies that can activate immune cell effector function. Expanding the use of ΔgD-2 as a viral vector in general could generate vaccines that are highly protective, quickly synthesized, and simultaneously effective against multiple pathogens.

Author contributions: K.K., J.D., K.A.W., and W.R.J. designed research; K.K., J.D., A.P.d.O., K.A.W., R.L., J.K., L.L., S.R., S.W., and L.A. performed research; D.M.K. and G.B. contributed new reagents/analytic tools; K.K. analyzed data; and K.K. and W.R.J. wrote the paper.

Reviewers: A.G.-S., Icahn School of Medicine at Mount Sinai; and J.V.R., The Rockefeller University.

Competing interest statement: The laboratories of W.R.J. receive financial support for sponsored research from X-Vax Technology, Inc., which holds licenses to several patents and patent applications related to ΔgD-2 vaccines, antibodies, and their use. W.R.J. serves as scientific advisor and consultant for the company. W.R.J. has equity interests in X-Vax Technology, Inc. W.R.J. is a coinventor on US patent no. 9,999,665 B2 "RECOMBINANT HERPES SIMPLEX VIRUS 2 (HSV-2) VACCINE VECTORS" and other patents related to ΔgD-2 vaccines, antibodies, and their use. D.M.K. is a coinventor on a patent on HSV-2 dl5-29 vaccine technology that is licensed by Harvard University to Sanofi Pasteur.



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This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2110714118/-/DCSupplemental.

Published August 20, 2021.

protection may require more broadly neutralizing antibodies or nonneutralizing antibodies able to activate effector immune cells (5). Previous studies have found that passively transferred nonneutralizing monoclonal antibodies can be potently protective in a mouse influenza challenge model (8–10). Several novel strategies have attempted to generate a nonneutralizing response against influenza. For example, vaccines have been created to specifically target the conserved stem region of HA (11–13).

Nonneutralizing antibodies stimulate effector cell mechanisms, including antibody-mediated phagocytosis and antibody-dependent cellular cytotoxicity (ADCC), both of which require activation of the Fcγ receptors (FcγRs) (14). Specific isotypes of IgG antibodies are associated with FcγR modulation and subsequent ADCC activation, including the IgG1 and IgG3 subtypes in humans, as well as IgG2a and IgG2c subtypes in mice (15–19). IgG2a and IgG2c isotypes are functionally equivalent and mouse strain-dependent, with IgG2c present in C57BL/6J mice (20). Recent studies have demonstrated that natural infection by influenza and vaccination elicit nonneutralizing antibodies with effector functions that contribute to protection (5, 9, 21–27). In mouse and nonhuman primate challenge models, ADCC-mediating antibodies have demonstrated protection against both homologous and heterologous influenza challenge (9, 28).

Recently, we developed a single-cycle herpes simplex virus (HSV) vaccine that completely protects against vaginal, skin, and ocular challenges by HSV-1 and HSV-2 (29, 30). Protection elicited by this vaccine, designated ΔgD-2 for its lack of the essential glycoprotein D (*gD*) gene, is transferable via passive infusion of immune sera to naïve wild-type mice but not to mice lacking the Fcγ common chain (30). The immune response elicited by ΔgD-2 primarily elicits nonneutralizing antibodies with high levels of FcγRIV-activating function.

We asked whether ΔgD-2 could be used as a vaccine platform to induce broadly protective FcγRIV-activating antibodies against a heterologous antigen, such as influenza HA. In this study, we demonstrate that our recombinant vaccine, ΔgD-2::$HA_{PR8}$, elicits protection against influenza with a high proportion of FcγRIV-activating antibodies. Additionally, anticipating the use of ΔgD-2 as a vaccine vector against other pathogens, we tested whether our construct would still be protective in mice lacking interferon (IFN) function. Many humans have inborn errors in their IFN signaling pathways, leading to more lethal outcomes in infection (31). Patients with such deficiencies are disproportionately represented among HSV encephalitis cases and are often diagnosed only after presenting with serious symptoms (32–38). This at-risk population underscores the importance of eliciting protection against HSV in the absence of a functional IFN-α/β response. Additionally, many pathogens, such as dengue virus, require mouse models lacking IFN function, and for ease of testing, an efficacious vaccine should remain functional in these mice (39–41). In this study, we demonstrate that ΔgD-2 is a versatile, immunogenic vaccine vector that provides a strong FcγRIV-activating immune response against heterologous pathogens, while maintaining its protective benefit against HSV, in both wild-type and IFN-deficient mice.

## Results

**Cloning and Expression of Influenza PR8 HA into ΔgD-2.** The goal of this study was to show that expression of a foreign viral antigen by an HSV vector can elicit a protective immune response. We therefore generated a ΔgD-2 construct expressing the *HA* gene from the H1N1 A/Puerto Rico/8/1934 (PR8) strain of influenza A. First, ΔgD-2::RFP was derived from ΔgD-2::GFP (29) by homologous recombination with the pYUB2167 plasmid in VD60 cells (Fig. 1*A*). A similar process was then used to create the ΔgD-2::$HA_{PR8}$ construct, using ΔgD-2::RFP DNA and the pJHA3 plasmid (Fig. 1*B*). The $HA_{PR8}$ gene was codon-optimized, according to HSV-2 codon usage bias for *gD* (*SI Appendix*, Tables S4 and S5). Sequence analysis of the resulting virus revealed that the complete codon-optimized $HA_{PR8}$ sequence was present and in the correct viral genome location (*SI Appendix*, Fig. S1*B*). All ΔgD-2 constructs were expressed in the complementing VD60 cell line, which are Vero cells engineered to express the *gD* gene from the KOS strain of HSV-1 to generate single-cycle replication viruses (42). VD60 cells infected with the recombinant ΔgD-2::$HA_{PR8}$ expressed the $HA_{PR8}$ protein both in the cytoplasm and on the cell surface (Fig. 1 *D* and *E*). Western blot analysis confirmed that cells infected with ΔgD-2::$HA_{PR8}$ expressed both HSV glycoprotein B and $HA_{PR8}$ proteins (Fig. 1*F*).

**HSV ΔgD-2::$HA_{PR8}$ Is Protective Against Homologous, but Not Heterologous, Influenza Challenge.** To test if ΔgD-2::$HA_{PR8}$ is an effective influenza vaccine, female C57BL/6J mice were immunized by a prime-boost regimen, based on previous studies, with either ΔgD-2::$HA_{PR8}$, formalin-inactivated PR8 virus (FI-PR8), or negative control (VD60 lysate or ΔgD-2::RFP) (29). Three weeks after the boost, mice were bled to obtain serum and then challenged intranasally with a lethal 10×$LD_{50}$ dose of PR8 influenza. Mice were weighed and monitored daily for 2 wk. Any mouse that lost more than 25% of its initial weight was humanely killed.

In one challenge experiment, mice immunized with ΔgD-2::$HA_{PR8}$ had a higher survival rate ($P < 0.001$), with all 10 subjects surviving the challenge, whereas none of the mice that received VD60 cell lysate survived (Fig. 2*A*). Of the FI-PR8–vaccinated mice, 7 of 10 of them survived, which was not a significant difference with ΔgD-2::$HA_{PR8}$-vaccinated mice ($P = 0.07$) (Fig. 2*A*). Additionally, over the course of the challenge, mice given ΔgD-2::$HA_{PR8}$ lost an average of 14.5% less weight than those that received the negative control, VD60 lysate ($P < 0.0001$) and 6.2% less weight then those that received FI-PR8 ($P < 0.0001$) (Fig. 2*B*). Similarly, in a separate challenge with ΔgD-2::RFP (a different negative control used in influenza challenges), four of five mice survived after vaccination with the ΔgD-2::$HA_{PR8}$ or FI-PR8 and lost 7.3% and 4.3% less weight, respectively ($P < 0.0001$ and $P = 0.0002$) (Fig. 2*D*). None of the ΔgD-2::RFP-vaccinated mice survived ($P = 0.0094$) (Fig. 2*C*). In additional challenges, ΔgD-2::$HA_{PR8}$ vaccination protected mice significantly better than negative control (either ΔgD-2::RFP or VD60 lysate) from 6x$LD_{50}$ to 20x$LD_{50}$ doses of PR8 virus (*SI Appendix*, Fig. S4 *C–F*), whereas there was no such difference at lower challenge doses (*SI Appendix*, Fig. S4 *A* and *B*). Male mice vaccinated with ΔgD-2::$HA_{PR8}$ were also protected from doses of 10x$LD_{50}$ PR8, unlike the negative controls used in the challenge (*SI Appendix*, Fig. S4 *G* and *H*). Therefore, expression of HA from the ΔgD-2 vector elicits protection against varying doses of homologous influenza challenge in both female and male mice.

To test if ΔgD-2::$HA_{PR8}$ would elicit broad protection against heterologous strains of influenza, mice were immunized in a prime-boost regimen with either ΔgD-2::$HA_{PR8}$, FI-PR8, or negative control (ΔgD-2::RFP). The same vaccination protocol was followed as above. At week 6, mice were challenged with a lethal dose (10x$LD_{50}$) of either A/California/04/2009 H1N1 (Cal09) or A/Hong Kong/1/68 H3N2 (HK68). Mice were weighed and monitored daily for 2 wk. Survival and weight loss were similar in all three groups, indicating that neither FI-PR8 nor ΔgD-2::$HA_{PR8}$ protected against heterologous challenge by Cal09 or HK68 (Fig. 3).

**Mice Vaccinated with ΔgD-2::$HA_{PR8}$ Elicit High Levels of Total IgG and IgG2c Antibodies That Activate FcγRIV.** To characterize the immune response elicited by ΔgD-2::$HA_{PR8}$, we analyzed the humoral response. Mice were immunized as above and bled at 6 wk postprime. Compared with the negative control (VD60 lysate), $10^{-2}$ dilutions of sera from mice immunized with ΔgD-2::$HA_{PR8}$ showed 7-, 5-, 16-, and 2-fold higher levels of anti-HA total IgG, IgG2c, IgG2b, and IgA, respectively (Fig. 4 *A* and *C* and *SI Appendix*, Fig. S2 *D* and *F*). Immunization with ΔgD-2::$HA_{PR8}$ induced 3.5-fold lower levels of IgG1 compared to FI-PR8 ($P = 0.0002$) (Fig. 4*B*), while

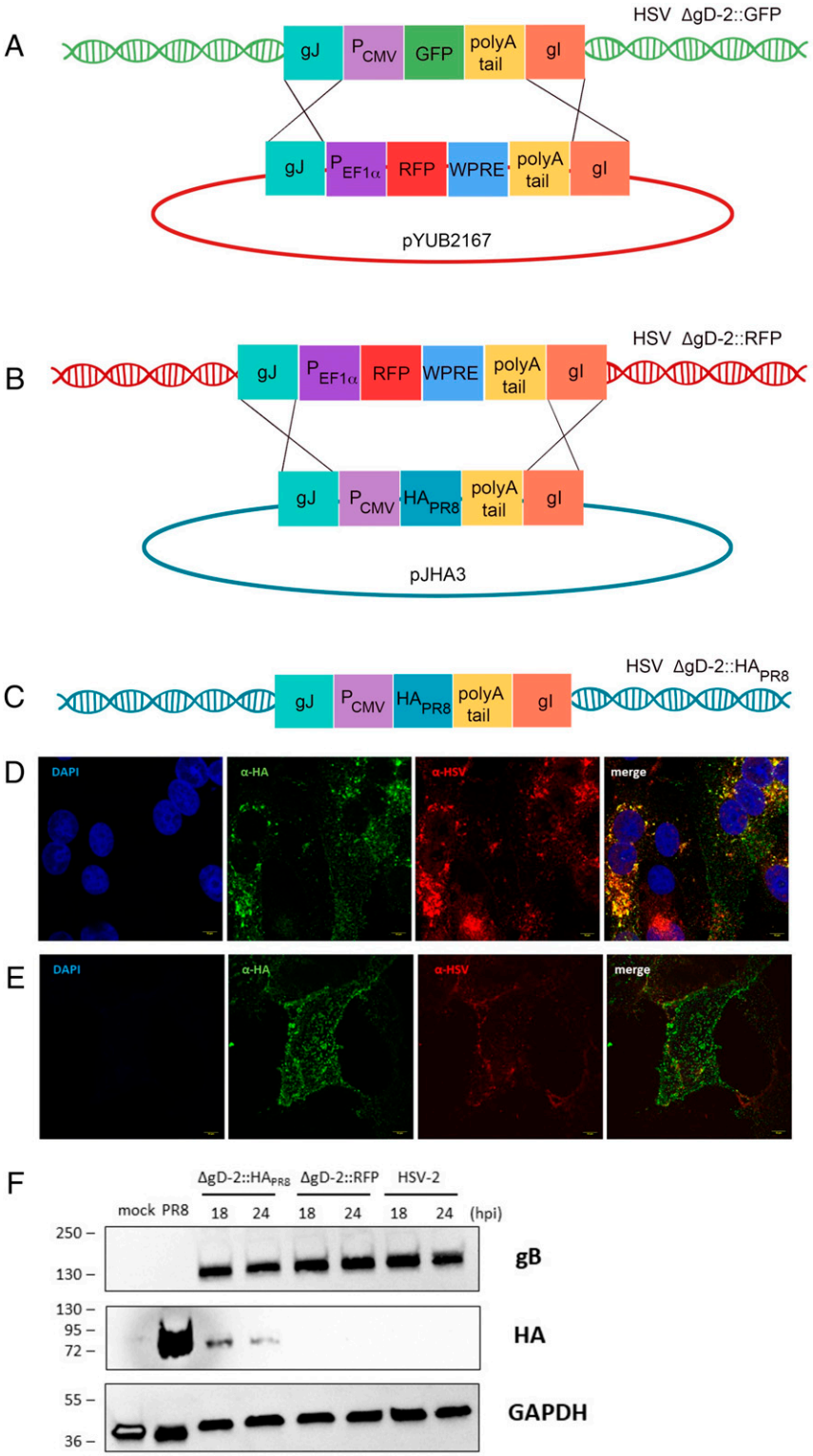


**Fig. 1.** $HA_{PR8}$ is expressed on the surface of ΔgD-2::$HA_{PR8}$-infected cells. The pYUB2167 plasmid expressing RFP underwent homologous recombination with ΔgD-2::GFP to generate ΔgD-2::RFP (*A*). Plasmid pJHA3 expressing $HA_{PR8}$ was cotransfected with ΔgD-2::RFP (*B*) to create the ΔgD-2::$HA_{PR8}$ virus (*C*). For immunofluorescence analysis, VD60 cells ($5 \times 10^4$) were infected with ΔgD-2::$HA_{PR8}$ (5 PFU per cell) for 18 h. Cells were stained with DAPI, an H1N1 HA-specific antibody (M145, Takara) and an HSV-1/HSV-2 ICP-specific antibody (ab226858, Abcam). The staining was analyzed in both permeabilized (*D*) and non-permeabilized cells (*E*). Images were acquired using a Leica TCS SP5 Confocal microscope with settings specific for DAPI, AF488, and AF594. (Scale bars, 10 μm.) For Western blot analysis (*F*), VD60 cells ($5 \times 10^5$ cells) infected with ΔgD-2::$HA_{PR8}$ (5 PFU per cell). As a positive control, MDCK cells were infected with PR8 (10 PFU per cell). At 18- and 24-h postinfection, cell lysates were analyzed by Western blotting for expression of viral proteins: HSV-1/2 gB (sc-56987, Santa Cruz Biotechnology) and H1N1 HA (GTX127357, GeneTex), and an anti-GAPDH antibody (AM4300, Invitrogen) as a control.

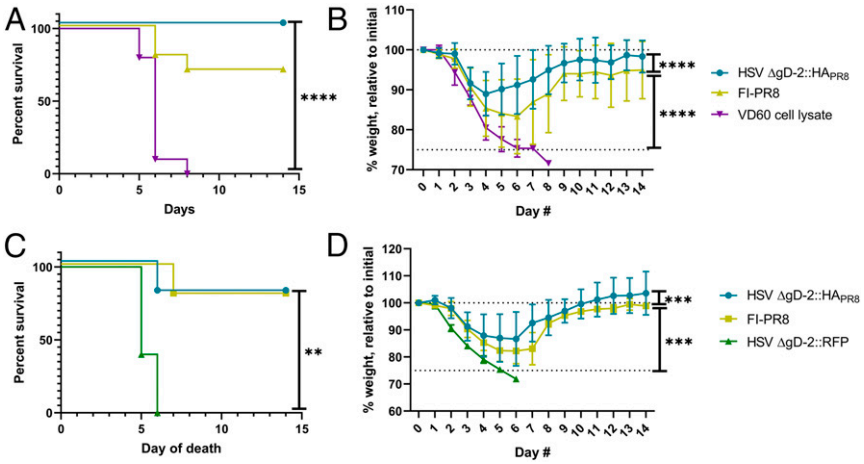


**Fig. 2.** ΔgD-2::HA$_{PR8}$ completely protects mice from a lethal challenge by a homologous strain of influenza. Mice were primed and boosted at week 3 with ΔgD-2::HA$_{PR8}$ ($5 \times 10^6$ PFU), FI-PR8 ($10^3$ hemagglutination units, HAU), ΔgD-2::RFP ($5 \times 10^6$ PFU), or VD60 lysate and challenged at week 6 intranasally with a 10xLD$_{50}$ dose of PR8 virus. Efficacy against challenge was measured using survival (*A* and *C*) and weight loss (*B* and *D*) over the subsequent 2-wk period. Survival was analyzed using a Gehan–Breslow–Wilcoxon test and weight loss was analyzed with two-way ANOVA (mean ± SD), main column effect (**$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$); $n = 10$ mice per group for *A* and *B* and $n = 5$ mice per group for *C* and *D*.

there was no significant difference in IgG2c levels between the two vaccines ($P = 0.6523$) (Fig. 4*C*). No significant differences were observed for IgG3, IgE, and IgM levels elicited by the different immunization protocols (*SI Appendix*, Fig. S2 *E*, *G*, and *H*). Sera from mice vaccinated with either ΔgD-2::HA$_{PR8}$ or FI-PR8 had comparable titers of HA inhibition (HAI), 20- and 29-fold higher than the negative control, respectively ($P = 0.0011$) (Fig. 4*D*).

The FcγRIV assay (Promega) was conducted similarly to our previous publications (30). Briefly, Madin-Darby canine kidney (MDCK) cells were seeded on plates and infected with PR8 influenza virus (3 PFU per cell). After 12 h, sera from immunized mice were added with effector cells supplied in the Promega Mouse FcγRIV assay kit. These engineered Jurkat T cells express the murine FcγRIV receptor. When the receptor is activated, signal transduction pathways induce expression of luciferase. Quantification of luminescence indicated that FcγRIV activation was 4- and 8-fold higher in sera from ΔgD-2::HA$_{PR8}$-vaccinated mice, compared to FI-PR8– or VD60-vaccinated mice, respectively ($P = 0.0027$ and $P = 0.001$) (Fig. 4*E*).

**ΔgD-2::HA$_{PR8}$ Vaccination Is Protective in Mice with Prior Exposure to HSV.** We tested if prior immunity to HSV would decrease protection elicited by ΔgD-2::HA$_{PR8}$ against PR8 influenza challenge. Mice were exposed to a sublethal dose of HSV-1, HSV-2, or negative control (Vero lysate) on their right hind flanks, as previously described (29, 30). On the same day, an additional group received an intramuscular injection of *dl5-29*, a well-characterized replication-defective HSV-2 strain lacking the *UL5* and *UL29* genes,

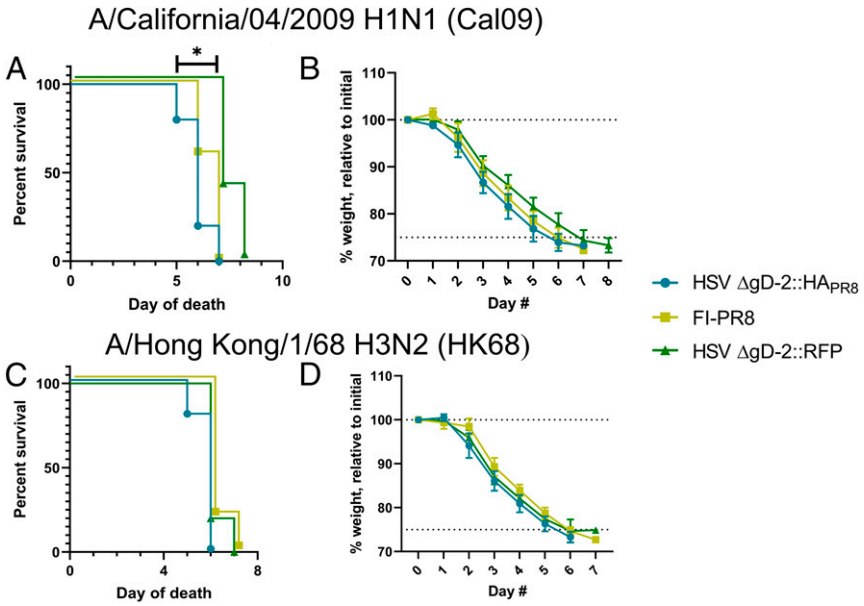


**Fig. 3.** ΔgD-2::HA$_{PR8}$ does not protect against heterologous challenge by influenza A viruses. Mice were primed and boosted at week 3 with ΔgD-2::HA$_{PR8}$ ($5 \times 10^6$ PFU), FI-PR8 ($10^3$ HAU), or ΔgD-2::RFP ($5 \times 10^6$ PFU). Six weeks later, they were challenged intranasally with 10xLD$_{50}$ of either A/California/04/2009 H1N1 (Cal09; *A* and *B*) or A/Hong Kong/1/68 H3N2 (HK68; *C* and *D*) and monitored over a 2-wk period. Survival was analyzed using a Gehan–Breslow–Wilcoxon test and weight loss was analyzed with two-way ANOVA (mean ± SD), main column effect (*$P < 0.05$); $n = 5$ mice per group.

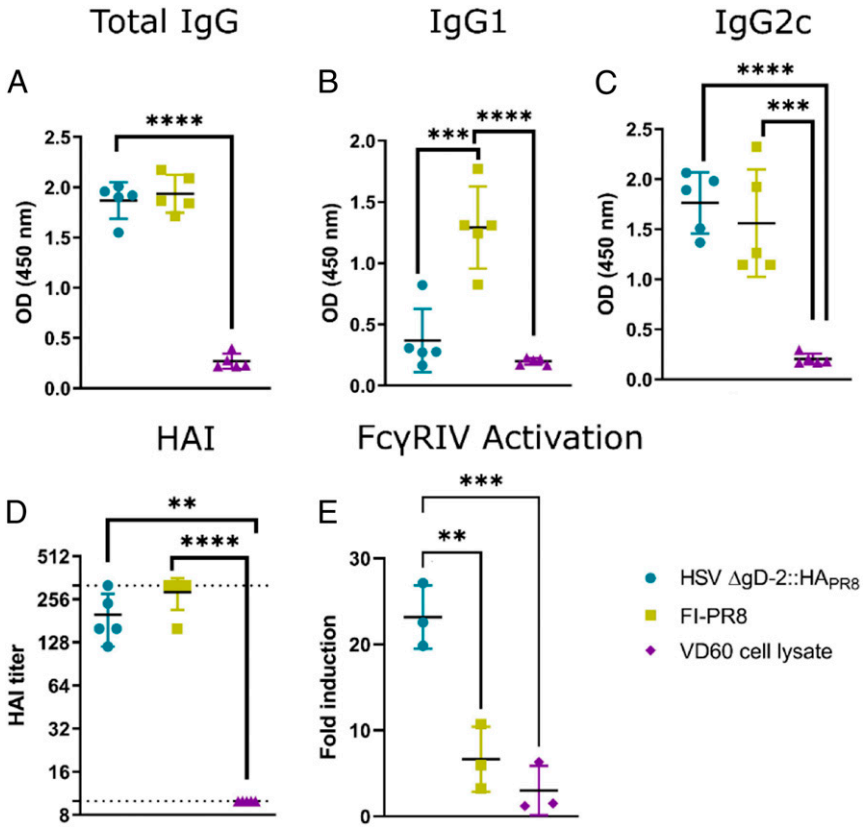


**Fig. 4.** ΔgD-2::HA$_{PR8}$ elicits high levels of HAI$^{+}$ IgG antibodies that activate FcγRIV signaling. Mice were primed and boosted after 3 wk with ΔgD-2::HA$_{PR8}$ ($5 \times 10^6$ PFU), FI-PR8 ($10^3$ HAU), or negative control ΔgD-2::RFP ($5 \times 10^6$ PFU) and bled 6 wk after the initial prime. Sera ($10^{-2}$ dilution) were analyzed by ELISA to quantify total IgG (*A*), IgG1 (*B*), IgG2c (*C*), and HAI levels (*D*). Sera were also analyzed using an FcγRIV activation assay (*E*). Antibody levels and FcγRIV fold-induction were analyzed using ANOVA (**$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$) and represented as mean ± SD; $n = 5$ mice per group in *A*–*D* and $n = 3$ mice per group in *E*.

that has been used in clinical trials (43–46). Over the course of 2 wk, mice were weighed and monitored daily for epithelial and neurological disease. Sera taken at 2 wk postinfection comprised 4-, 2-, and 5-fold higher total IgG antibody levels in HSV-1–, HSV-2–, and *dl5-29*–exposed mice, respectively, compared to the negative control (Fig. 5*A*). This indicated that we induced humoral immunity against the HSV-2 strain used to make ΔgD-2. Within the HSV-1– and HSV-2–exposed groups, there was variability in the magnitude of the anti–HSV-2 IgG response, likely due to the inherent variability of sublethal exposure. However, there were no significant differences in anti–HSV-2 total IgG antibody levels between cages in the various exposure groups (*SI Appendix*, Fig. S5).

These mice were then subjected to a prime-boost regimen with either ΔgD-2::HA$_{PR8}$, FI-PR8, or negative control (VD60 cell lysate). Because only limited numbers of HSV-1–exposed mice were available, that group did not contain an FI-PR8 vaccination arm. Blood was obtained from the mice at 6 wk postprime. Mice were then challenged 2 d later with PR8 influenza virus (10xLD$_{50}$) and weighed daily for 2 wk. Prior immunity to HSV induced by any of the exposure methods did not impact the levels of total anti-HA IgG (Fig. 5 *B*–*E*) or IgG2c (Fig. 5 *F*–*I*) elicited by ΔgD-2::HA$_{PR8}$ or FI-PR8. Importantly, there were no significant differences in total IgG (Fig. 5 *B*–*E*) and IgG2c (Fig. 5 *F*–*I*) between any of the groups that received ΔgD-2::HA$_{PR8}$. This demonstrates that prior exposure to HSV—even with high levels of anti–HSV-2 humoral immunity—does not affect the humoral response against influenza induced by ΔgD-2::HA$_{PR8}$.

Additionally, despite prior anti–HSV-2 immunity, all of the mice that received ΔgD-2::HA$_{PR8}$ or FI-PR8 survived the 10xLD$_{50}$ PR8 influenza challenge, whereas none of the mice given VD60 cell lysate survived (Fig. 5 *J*–*M*). All mice receiving ΔgD-2::HA$_{PR8}$ or FI-PR8 had significantly less weight loss throughout the challenge than did mice receiving VD60 cell lysate ($P < 0.0001$) (Fig. 5 *N*–*Q*). In the control group, mice vaccinated with ΔgD-2::HA$_{PR8}$ lost 3.1% less weight than did the FI-PR8–vaccinated group ($P = 0.0064$) (Fig. 4*Q*), similar to what we observed previously (Fig. 2 *B* and *D*). In the *dl5-29*– and HSV-2–exposed groups, there was no significant difference in weight loss between the ΔgD-2::HA$_{PR8}$ and FI-PR8 ($P = 0.9007$ and $P = 0.7541$, respectively) (Fig. 5 *O* and *P*). Overall, these data indicate that prior exposure to HSV does not decrease the ability of ΔgD-2::HA$_{PR8}$ to elicit a protective response against influenza challenge.

**Passive Transfer of Serum from ΔgD-2::HA$_{PR8}$-Vaccinated Mice into Naïve Mice Is Protective.** To determine if antibodies alone are responsible for protection, mice were injected intraperitoneally with heat-inactivated sera from animals vaccinated with ΔgD-2::HA$_{PR8}$, FI-PR8, or negative control (ΔgD-2::RFP). The next day, mice were challenged intranasally with PR8 (5xLD$_{50}$) and monitored for 2 wk. Naïve, wild-type mice that received serum from ΔgD-2::HA$_{PR8}$- or FI-PR8–vaccinated mice had survival rates of 80% and 60%, respectively, whereas none of the mice that received ΔgD-2::RFP serum survived (Fig. 6*A*). Additionally, mice given ΔgD-2::HA$_{PR8}$ and FI-PR8 serum lost 6.1% and 5.1% less weight, respectively, when compared with the negative control (Fig. 6*B*). Therefore, ΔgD-2::HA$_{PR8}$ and FI-PR8 immune sera offer significant protection against homologous influenza challenge in passive transfer conditions.

**ΔgD-2::HA$_{PR8}$ Is Effective Against Both HSV-1 and HSV-2 Challenge.** HA is a well-described immunodominant antigen in influenza, so we sought to determine if ΔgD-2::HA$_{PR8}$ was as protective against HSV challenge as a ΔgD-2 strain without HA. Mice were vaccinated with ΔgD-2::HA$_{PR8}$, ΔgD-2::RFP, or negative control (VD60 cell lysate). At 6 wk, animals were challenged with HSV-1

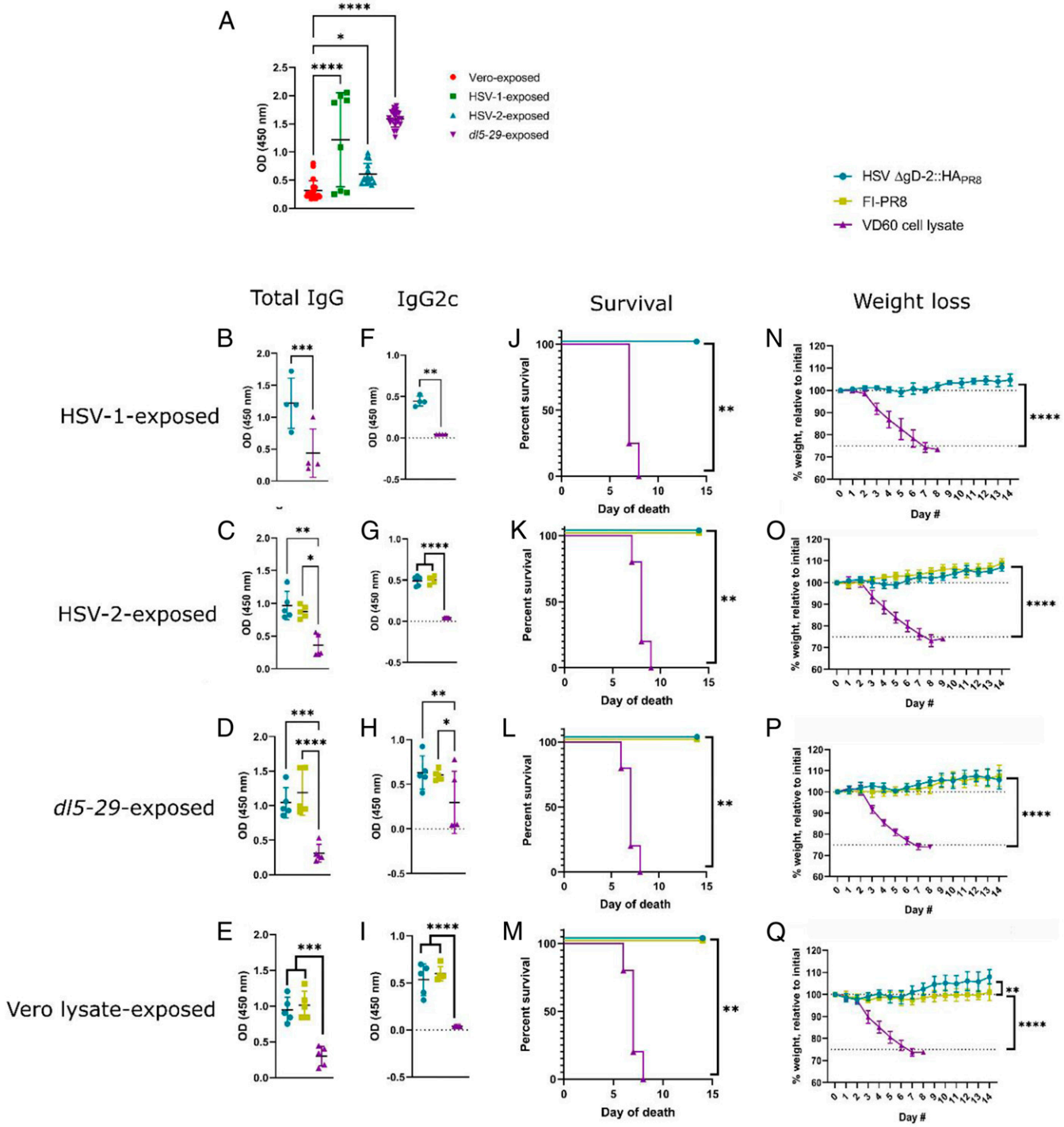


**Fig. 5.** ΔgD-2::HA$_{PR8}$ is still protective in mice with humoral immunity to HSV. Mice were exposed to HSV-1.1 ($10^6$ PFU), of HSV-2 ($2.5 \times 10^6$ PFU), or Vero cell lysate using an epithelial challenge protocol. On the same day, an additional group was immunized with *dl5-29* ($10^6$ PFU) by intramuscular injection. Mice were bled 3 wk after the initial exposure, but prior to immunization, to measure total anti–HSV-2 IgG levels by ELISA using $10^{-2}$ dilutions of sera (*A*). These mice were then primed and boosted 3 wk later with ΔgD-2::HA$_{PR8}$ ($5 \times 10^6$ PFU), FI-PR8 ($10^3$ HAU), or VD60 lysate. Six weeks after the prime, total anti–HA IgG (*B–E*) and IgG2c (*F–I*) were measured by ELISA using $10^{-2}$ dilutions of sera. Two days later, mice were challenged intranasally with PR8 virus (10xLD$_{50}$). Survival (*J–M*) and weight loss (*N–Q*) were quantified daily over the next 2-wk period. Antibody levels were analyzed using ANOVA, survival was analyzed using a Gehan–Breslow–Wilcoxon test, and weight loss was analyzed with mixed-effects analysis, main column effect (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$); $n = 15$ mice per most groups and $n = 8$ for the HSV-1-exposed mice in *A* and $n = 4$ to 5 mice per group in *B–Q*. Antibody analyses and weight loss are expressed as mean ± SD.

or HSV-2 (10xLD$_{90}$) using a previously established skin scarification challenge model (29, 30). ΔgD-2::HA$_{PR8}$ and ΔgD-2::RFP completely protected mice against these lethal challenges (Fig. 7 *A* and *D*). No significant differences in survival, epithelial disease, or neurological disease were detected between mice given either ΔgD-2::HA$_{PR8}$ or ΔgD-2::RFP (Fig. 7 *A*–*F*). These mice did develop transient epithelial disease during the HSV-2 challenge (Fig. 7*E*) but not the HSV-1 challenge (Fig. 7*B*). Neither group developed neurological disease in the HSV-1 or HSV-2 challenges (Fig. 7 *C* and *F*). Mice vaccinated with VD60 lysate all developed epithelial and neurological disease that reached the humane endpoint (Fig. 7 *A*–*F*). Additionally, in comparison to the negative control (VD60 lysate), mice vaccinated with ΔgD-2::HA$_{PR8}$ elicited 3- and 18-fold higher levels of total IgG and IgG2c antibodies, respectively (Fig. 7 *C* and *D*). Mice receiving ΔgD-2::RFP had similar but slightly increased levels of IgG2c antibodies than the mice immunized with ΔgD-2::HA$_{PR8}$ ($P = 0.0051$) (Fig. 7*D*). Despite expressing an immunodominant antigen from another pathogen (HA), ΔgD-2::HA$_{PR8}$ was still able to generate a protective immune response against HSV.

**ΔgD-2::HA$_{PR8}$ Is Protective in Mice Lacking the IFN-α/β Receptor or the IFN-γ Receptor.** To establish ΔgD-2 as a possible immunization vector for use in people with IFN-pathway deficiencies and in animal models for other pathogens, we tested ΔgD-2::HA$_{PR8}$ in mice lacking the IFN-α/β receptor (IFNAR$^{-/-}$) and mice lacking the IFN-γ receptor (IFNγR$^{-/-}$). Both of these strains have a C57BL/6J background, so wild-type C57BL/6J mice were used as an IFN$^{+}$ control. Mice from each strain were vaccinated with either ΔgD-2::HA$_{PR8}$ or negative control (ΔgD-2::RFP), bled at week 6, and challenged with a lethal dose PR8 (10xLD$_{50}$). All strains of mice vaccinated with ΔgD-2::HA$_{PR8}$ were completely protected against PR8 challenge and lost significantly less weight than did ΔgD-2::RFP-vaccinated mice (Fig. 8*A*). None of the IFNAR$^{-/-}$ and IFNγR$^{-/-}$ mice, and only 20% of the C57BL/6J mice, survived after vaccination with ΔgD-2::RFP (Fig. 8*A*). All ΔgD-2::HA$_{PR8}$-vaccinated mice also lost significantly less weight than did the ΔgD-2::RFP-vaccinated mice, ranging from a difference of 7.2% between the C57BL/6J groups and 14.9% between the IFNγR$^{-/-}$ groups ($P < 0.0001$) (Fig. 8*B*). When tested against a lethal (10xLD$_{90}$) HSV-2 challenge, ΔgD-2::RFP vaccination was completely protective in both C57BL/6J and IFNAR$^{-/-}$ mice (*SI Appendix*, Fig. S3*A*). In this experiment, the IFNAR$^{-/-}$ and C57BL/6J mice that received VD60 lysate demonstrated maximal epithelial and neurological disease, whereas mice vaccinated with ΔgD-2::RFP developed no neurological disease and some epithelial disease that completely resolved by the end of the experiment (*SI Appendix*, Fig. S3 *B* and *C*). All mice that received the ΔgD-2::HA$_{PR8}$ vaccine had significantly higher levels of anti-HA total IgG and IgG2c antibodies compared with the ΔgD-2::RFP-vaccinated mice (Fig. 8 *C* and *D*). Additionally, ΔgD-2::RFP elicited 17- and 8-fold higher levels of anti-HSV-2 IgG2c in the C57BL/6J and IFNAR$^{-/-}$ strains, respectively (*SI Appendix*, Fig. S3*D*). ΔgD-2 therefore does not require IFN-α/β function to elicit protective immunity against HSV (using ΔgD-2::RFP) or influenza (using ΔgD-2::HA$_{PR8}$), and it does not require IFN-γ to elicit anti-HA immunity. The ΔgD-2 vector may be able to function as a vaccination in people that have IFN deficiencies, even protecting against multiple pathogens.

## Discussion

Quick production of effective vaccines is a critical need in global health. Despite decades of research, we are still lacking sufficiently effective vaccines against many pathogens, including influenza. The immune response elicited by natural infection or current vaccines is inadequate for broad protection against emerging strains. A method to direct the type of desired immune response is the use of viral vectors. Various HSV strains have been used for gene therapy and immunizations against cancer and heterologous infectious diseases. HSV is an ideal vector for a number of reasons, including its wide range of host cell tropism and its large cloning capacity (47).

In this report, we demonstrated that a viral vector derived from an attenuated strain of HSV can be used to elicit immunity against another pathogen. By expressing the *HA* gene from influenza in the ΔgD-2 vector, we created a vaccine that effectively protects against otherwise lethal homologous PR8 influenza, HSV-1, and HSV-2 challenge. Mice with high levels of humoral immunity against HSV-2 were just as protected against influenza challenge by ΔgD-2::HA$_{PR8}$ as unexposed mice. Therefore, one advantage of using the ΔgD-2 vector is that preexisting immunity does not impede its ability to function as a vaccine. Another study showed specifically that exposure to HSV-1 does not impact ΔgD-2 vaccine efficacy (48), while others have also indicated a similar benefit to using HSV as a vaccine vector (49, 50). The same cannot be said for other viral vectors, such as adenovirus (51–53). This finding broadly expands the population of people that can receive a recombinant ΔgD-2 vaccine directed against a heterologous pathogen, like influenza. Additionally, we have found that sera from ΔgD-2::HA$_{PR8}$-vaccinated mice yields protection via passive transfer against PR8 challenge, indicating the antibody response is largely responsible for its effectiveness. We have yet to explore the contribution to protection against viral challenge of cell-mediated immunity elicited by ΔgD-2::HA$_{PR8}$. Previous studies have indicated that adoptive transfer of T cells from mice immunized with ΔgD-2 will not protect naïve mice from HSV challenge (29). However, we have not yet assessed the contribution of T cells to the protection mediated by ΔgD-2::HA$_{PR8}$. We note that ΔgD-2::HA$_{PR8}$ is not protective against heterologous strains of influenza A, including Cal09 (H1N1) and HK68 (H3N2). Incorporating conserved antigens, such as M1 or M2, or multiple influenza HA variants may elicit more broadly protective immunity. To accomplish this, the cloning capacity of ΔgD-2 may have to be increased,

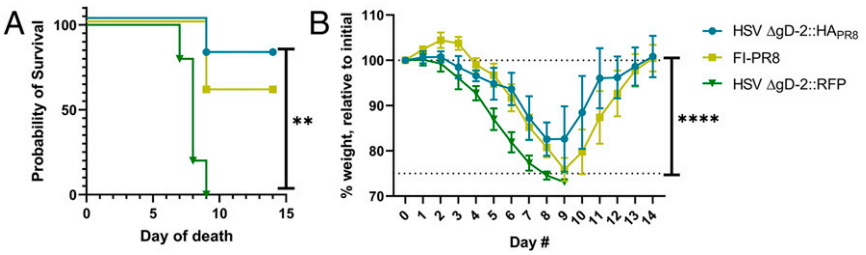


**Fig. 6.** Passive transfer of ΔgD-2::HA$_{PR8}$ and FI-PR8 immune sera protects against PR8 influenza challenge. Mice were primed and boosted at week 3 with ΔgD-2::HA$_{PR8}$ ($5 \times 10^6$ PFU), FI-PR8 ($10^3$ HAU), or ΔgD-2::RFP ($5 \times 10^6$ PFU) and bled at week 6. Serum from each group was injected into naïve mice, which were then challenged with PR8 (5xLD$_{50}$). Survival (*A*) and weight loss (*B*) were measured over 2 wk. Weight loss was analyzed with two-way ANOVA (mean ± SD), main column effect, and survival analyzed using a Gehan–Breslow–Wilcoxon test (**$P < 0.01$, ****$P < 0.0001$); $n = 5$ mice per group.

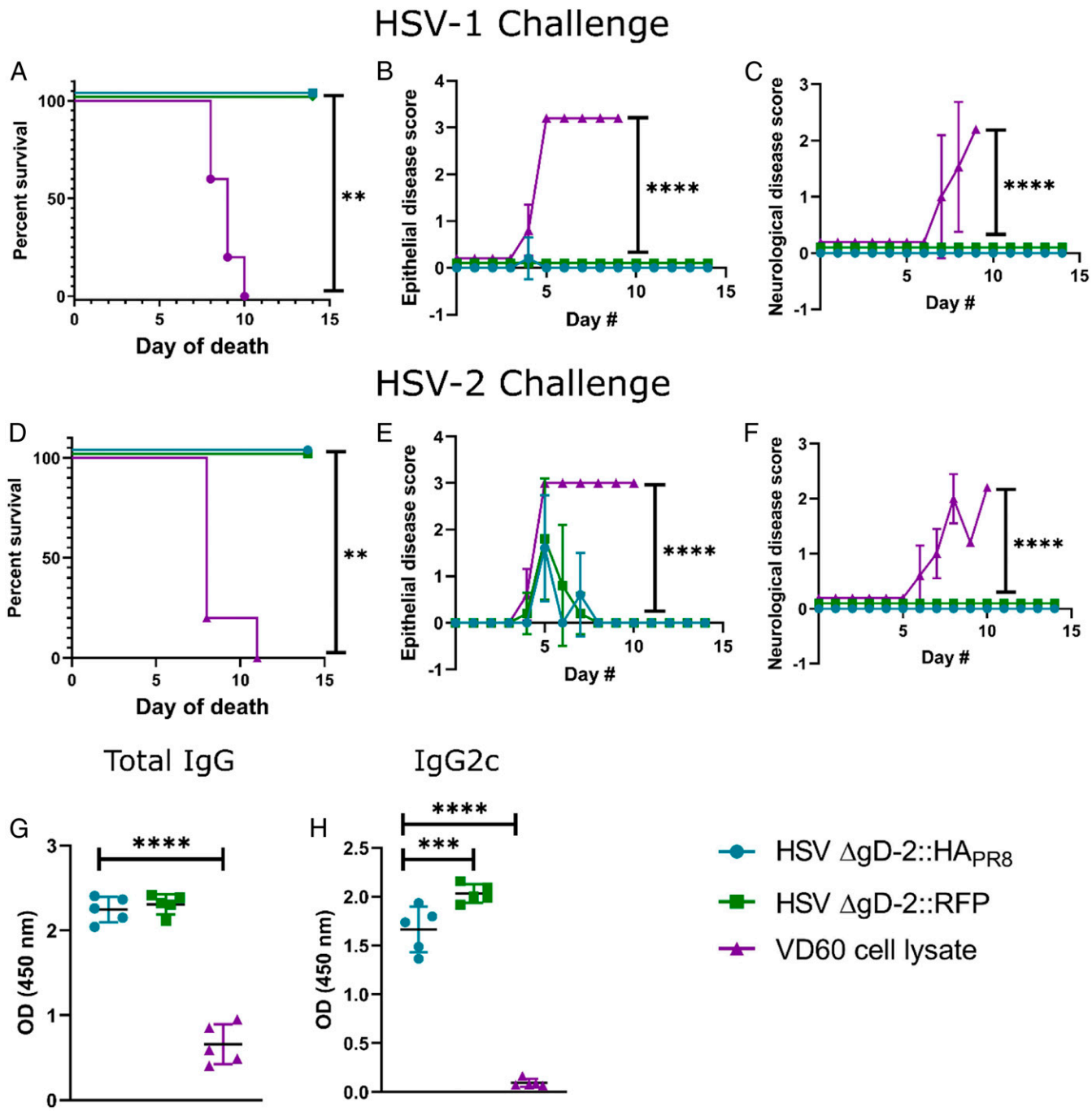


**Fig. 7.** ΔgD-2::HA$_{PR8}$ protects against HSV-1 and HSV-2 and elicits high levels of IgG. Mice were primed and boosted 3 wk later with ΔgD-2::HA$_{PR8}$ ($5 \times 10^6$ PFU), ΔgD-2::RFP ($5 \times 10^6$ PFU), or VD60 lysate. Mice were challenged with either HSV-1 ($10^6$ PFU) (*A*–*C*) or HSV-2 ($2.5 \times 10^6$ PFU) (*D*–*F*). Survival (*A* and *D*), epithelial disease (*B* and *E*), and neurological disease (*C* and *F*) were measured over 2 wk. Sera were obtained 2 d prior to challenge and analyzed by anti–HSV-2 ELISA for total IgG (*G*) and IgG2c (*H*). Survival was analyzed using a Gehan–Breslow–Wilcoxon test, epithelial and neurological disease was analyzed with two-way ANOVA (mean ± SD), main column effect, and antibody levels were analyzed using ANOVA (mean ± SD; $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$); $n = 5$ mice per group.

along with optimization of various promoters and signal sequences that will ensure robust expression of foreign antigens.

Given the association between broad protection against multiple influenza strains and ADCC, finding a vaccine that enhances this arm of immunity should be a priority. ADCC is initiated by immune cells, including natural killer cells and macrophages, when an Fc receptor is engaged by the Fc region of an antibody (54). This interaction activates a downstream signaling cascade that results in a cytotoxic response against the infected target cell (54). Among FcγRs, FcγRIV seems to have a central role in mediating ADCC (55). In mice, the FcγRIV binds to IgG2a, IgG2c, IgG2b, and IgE antibodies to activate neutrophils, monocytes, and macrophages (56). The ΔgD-2::HA$_{PR8}$ vaccine elicits high titers of HAI$^+$ IgG antibodies with a bias toward the IgG2c subtype. Our positive control, a formalin-inactivated influenza virus (FI-PR8), elicited a less protective immune response against PR8 challenge than ΔgD-2::HA$_{PR8}$. FcγRIV activation was significantly higher in ΔgD-2::HA$_{PR8}$-immunized animals, although both vaccines elicited

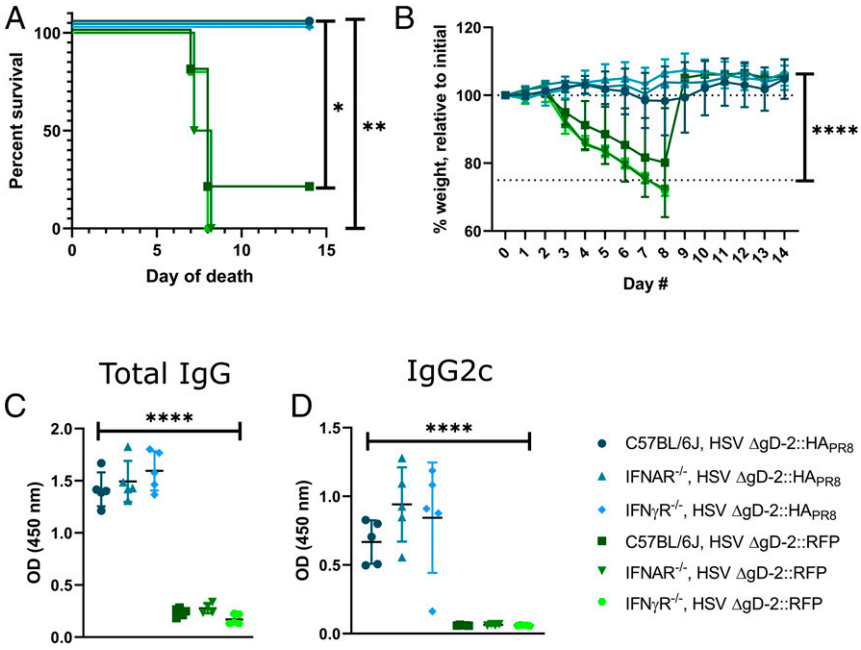


**Fig. 8.** Mice lacking the IFN-α/β receptor or the IFN-γ receptor are protected by ΔgD-2::HA$_{PR8}$ against homologous influenza challenge. Mice lacking the IFN-α/β receptor (IFNAR$^{-/-}$), mice lacking the IFN-γ receptor (IFNγR$^{-/-}$), and wild-type C57BL/6J mice were primed and boosted after 3 wk with either ΔgD-2::HA$_{PR8}$ ($5 \times 10^6$ PFU) or ΔgD-2::RFP ($5 \times 10^6$ PFU). Mice were monitored for 2 wk for survival (*A*) and weight loss (*B*). Sera were obtained from the mice 2 d before challenge and analyzed by ELISA for total IgG (*C*) and IgG2c (*D*). Survival was analyzed using a Gehan–Breslow–Wilcoxon test, weight loss was analyzed with two-way ANOVA (mean ± SD), main column effect, and antibody levels were analyzed using ANOVA (mean ± SD; $*P < 0.05$, $**P < 0.01$, $****P < 0.0001$); $n = 4$ to 5 mice per group.

similar levels of IgG2b, IgG2c, and IgE antibodies. The reason for the difference in FcγRIV activation between these two vaccines is unknown and may be contributing to the improved survival we see with ΔgD-2::HA$_{PR8}$. Nevertheless, the contribution of the HAI$^+$ antibodies in influenza virus neutralization and subsequent protection against challenge cannot be excluded.

To investigate potential mechanisms that contribute to the elicitation of ADCC-mediating antibodies by ΔgD-2::HA$_{PR8}$, we interrogated its requirement for IFN signaling pathways. The functions of IFN-α/β and IFN-γ receptors do not contribute to the protection afforded by the ΔgD-2::HA$_{PR8}$ vaccine. The similarity in protection against influenza that we observed in IFNAR$^{-/-}$, IFNγR$^{-/-}$, and wild-type C57BL/6J mice vaccinated with ΔgD-2::HA$_{PR8}$ has important clinical significance for people with deficiencies in IFN function. Our data suggest that this population could mount a protective immune response after vaccination with ΔgD-2 recombinant viruses. Additionally, many pathogens, like dengue virus, will not replicate in wild-type mice and so require specific models, such as IFN-α/β– and IFN-γ–deficient mice for testing vaccines (39–41). ΔgD-2::HA$_{PR8}$ is still protective in mice lacking the IFN-α/β receptor and mice lacking the IFN-γ receptor, making it a valuable tool for generating and testing vaccines against a range of pathogens.

The observation that protection by ΔgD-2::HA$_{PR8}$ is not dependent on IFN-γ function was surprising, given that the antibody response is predominated by IgG2c. Typically, the induction of IgG2a or IgG2c antibodies is linked to initiation of a Th1 helper response and release of IFN-γ (57). However, it has also been previously reported that IgG2a antibodies can be produced against multiple pathogens in IFNγR-deficient mice (58). Another study found that the activation of Toll-like receptor 9 (TLR9) in B cells could mediate the class switch to IgG2a antibodies without IFN-γ (59). This is of particular interest, as TLR9 is known to recognize double-stranded DNA in the endosome, which occurs in HSV infection (60, 61). Of note, one group found that adding an adjuvant with TLR9 agonism to a monovalent inactivated split influenza vaccine led to FcγR-dependent heterologous protection against influenza challenge (62). TLR9 is linked to MyD88 initiation of proinflammatory downstream signaling within immune cells (63, 64), which then contributes to class switching to the IgG2a or IgG2c subtype (65–69). MyD88 is an adaptor protein connecting both TLRs and interleukin-1 receptor families to inflammatory signaling cascades, including NF-κB (64). MyD88 is essential for generating long-term, protective humoral responses in mice (67, 69). Therefore, rather than relying on IFN-γ to mediate the IgG2c class switch, ΔgD-2::HA$_{PR8}$ might be inducing the class switch by recognition via TLR9 and the MyD88 signaling pathway. Knowing more about this class switch pathway and the mechanism of production of ADCC-mediating antibodies could improve vaccines and also generate more effective cancer immunotherapies.

In conclusion, the ΔgD-2 vector is a robust and adaptable tool for initiating an ADCC response in vivo. Additionally, the ΔgD-2::HA$_{PR8}$ vaccine is unique as a hybrid vaccine that elicits a strong, predominantly IgG2c antibody response, and remains protective, even in IFN-deficient mice, against two different pathogens.

## Materials and Methods

All animal procedures were approved by the Albert Einstein College of Medicine Animal Care and Use Committee under animal protocols 20170519, 20170601, 00001290, and 00001291. Detailed descriptions of influenza strains, the generation of the recombinant HSV strains, isolation and sequencing of DNA from the recombinant HSV strains, Western blot analysis, immunofluorescence, immunizations of mice, strains of mice used, generation of recombinant HA protein, influenza and HSV challenges, passive transfer of immune serum, ELISA, HAI, FcγRIV activation assay, and statistical analysis are included in *SI Appendix*.

**Data Availability.** All study data are included in the article and supporting information.

**ACKNOWLEDGMENTS.** We acknowledge Drs. Stephen Harrison, Garnett Kelsoe, and Masayuki Kuraoka for helpful discussions. We thank our funding, including NIH grants National Institute of Allergy and Infectious Diseases (NIAID) R01 AI117321 (W.R.J.), NIAID P01 AI098681 (D.M.K.), and National Institute of General Medical Sciences T32 GM007288 (K.K.). J.D. was supported by a grant from the Howard Hughes Medical Institute.

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