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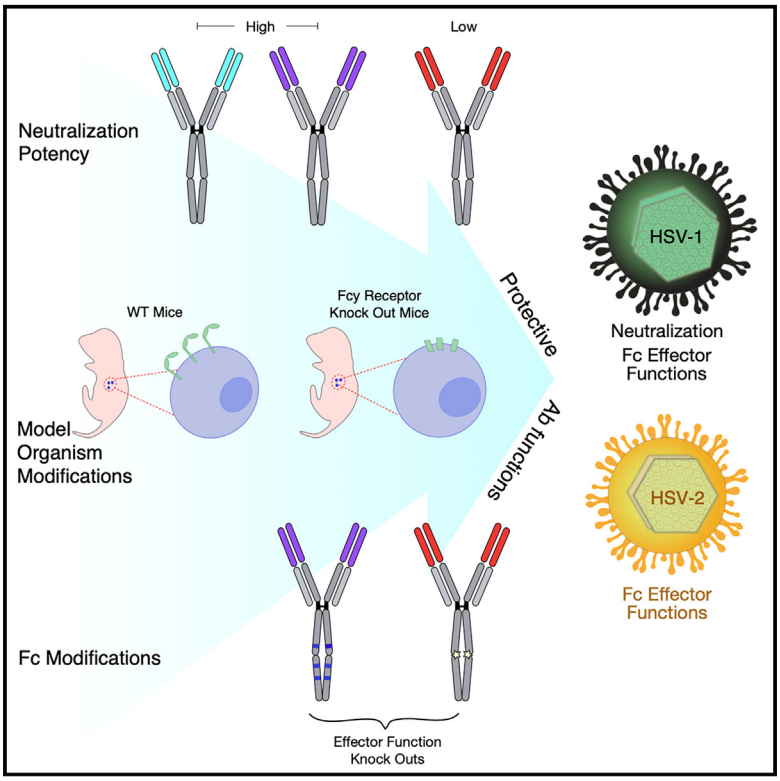
Effector functions are required for broad and potent protection of neonatal mice with antibodies targeting HSV glycoprotein D

Graphical abstract



Highlights

- gD-specific mAbs mediate protection of neonatal mice against both HSV-1 and -2
- Neutralization and effector functions contribute to protection against HSV-1
- Effector functions contribute to the control of HSV dissemination *in vivo*
- For these mAbs, effector functions alone contribute to protection against HSV-2

Authors

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In brief

Slein et al. describe the mechanisms by which a panel of gD-specific mAbs protect neonatal mice from HSV-mediated mortality and morbidity. Neutralization and Fc effector functions mediate protection against HSV-1 mortality and viral dissemination *in vivo.* Fc effector functions alone are sufficient for protection against HSV-2 by these gD-specific mAbs.

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Effector functions are required for broad and potent protection of neonatal mice with antibodies targeting HSV glycoprotein D

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SUMMARY

Multiple failed herpes simplex virus (HSV) vaccine candidates induce robust neutralizing antibody (Ab) responses in clinical trials, raising the hypothesis that Fc-domain-dependent effector functions may be critical for protection. While neonatal HSV (nHSV) infection results in mortality and lifelong neurological morbidity in humans, it is uncommon among neonates with a seropositive birthing parent, supporting the hypothesis that Ab-based therapeutics could protect neonates from HSV. We therefore investigated the mechanisms of monoclonal Ab (mAb)-mediated protection in a mouse model of nHSV infection. For a panel of glycoprotein D (gD)-specific mAbs, neutralization and effector functions contributed to nHSV-1 protection. In contrast, effector functions alone were sufficient to protect against nHSV-2, exposing a functional dichotomy between virus types consistent with vaccine trial results. Effector functions are therefore crucial for protection by these gD-specific mAbs, informing effective Ab and vaccine design and demonstrating the potential of polyfunctional Abs as therapeutics for nHSV infections.

INTRODUCTION

When encountered during the neonatal period, herpes simplex virus (HSV) infections can result in loss of life or long-term neurological disability.[1–3] Neonatal infections can present as skin, eye, and mouth disease, which is amenable to antiviral therapy, or more invasive disseminated and/or central nervous system disease. While new treatment regimens with acyclovir and its derivatives have improved outcomes, mortality following disseminated disease remains unacceptably high.[4,5] Most neonatal HSV (nHSV) infections are vertically transmitted during birth from a recently infected birthing parent who has not yet developed a mature antibody (Ab) response to HSV type 1 or type 2 (HSV-1 or HSV-2).[6] Given the severity of neonatal infection resulting from primary maternal infection,[6,7] birthing parent seropositivity is believed to be protective due to the transfer of HSV-specific Abs via the placenta.[2,5,8] High titers of neutralizing or Ab-dependent cellular cytotoxicity (ADCC)-inducing Abs in infected neonates have been associated with less severe disease.[9–11] Animal studies support the notion that neutralization and Fc effector functions, such as ADCC, Ab-dependent cellular phagocytosis, and Ab-dependent complement deposition, can aid in the clearance of acute HSV infection.[12–15] Further insights into how Abs exert direct and indirect antiviral activities to protect against infection could aid in the design of both passive and active immunization strategies for HSV.

To this end, whether neutralization or effector functions play a dominant role in protection from HSV-mediated disease has long been unclear, as conflicting results have been reported in animal models.[13,14,16–18] Previous studies differentiated effector functions from neutralization by treating with digested Ab fragments (Fabs).[19] However, digestion is known to compromise neutralization potency and half-life, which confounds interpretation of study results. Other studies have sought to answer this question using polyclonal Ab or monoclonal Abs (mAbs) that could either neutralize or carry out specific effector functions.[10,20] While such approaches have contributed to our understanding of the potential contributions of Ab effector functions, disparities in protection from disease could also be attributed to the specific epitope(s) targeted, differences in Ab affinity or avidity, or other factors. Ab Fc engineering strategies that allow separation of Fc-dependent effector functions from neutralization provide a platform to improve experimental resolution in defining Ab-dependent mechanisms of protection,[21–24] which can inform both vaccine design and therapeutic mAb development.

Like other consequential early-life pathogens, most studies of HSV have focused on adult animal models. There is therefore a dearth of information on how Abs protect in the neonatal period. Given this knowledge gap, we sought to investigate the

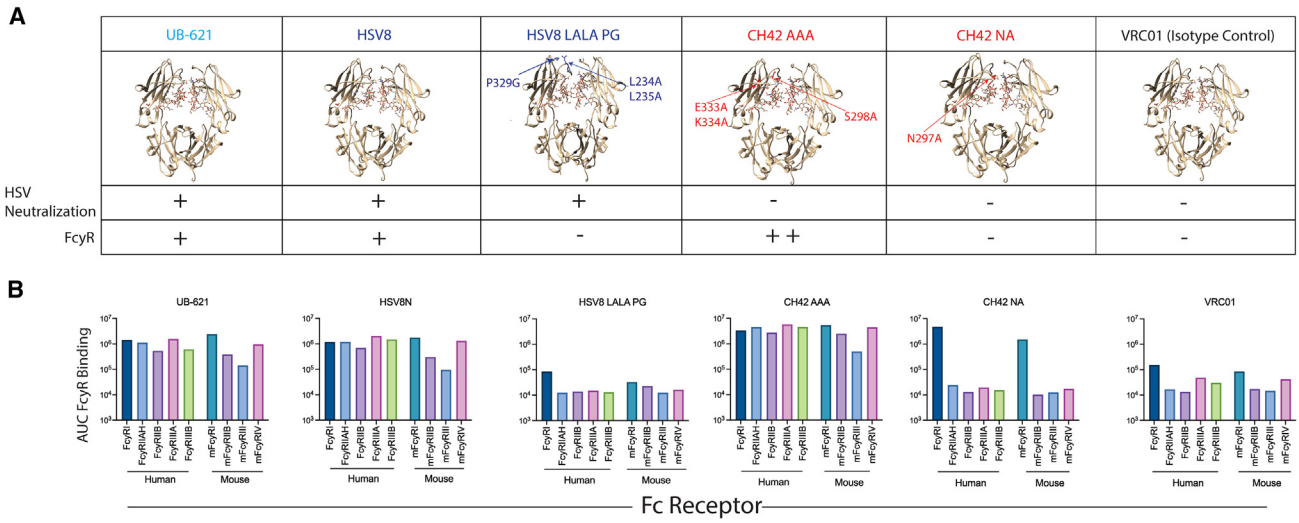

Figure 1. Biophysical characterization of HSV gD-specific mAbs

(A) Visualization of the Fc domains of mAbs used in this study. For reference purposes, mutated positions in the HSV mAbs are superimposed on the crystal structure of the Fc domain of the HIV-specific mAb b12 (PDB: 1HZH). Reported neutralization potencies of each mAb and the expected ability of each Fc domain to bind FcγRs are indicated.

(B) FcγR binding profiles of the mAbs used in this study. Bar graphs present the area under the curve (AUC) for the binding of each mAb to recombinant human (left) and mouse (right) Fc receptors. Orthologous human and mouse Fc receptors are color matched.

See also Figure S1 and Table S1.

mechanism(s) by which Abs that target glycoprotein D (gD) mediate protection against nHSV-1 and nHSV-2 infections. Using a mouse model of nHSV infection, we demonstrate that there are distinct mechanisms of Ab-mediated protection that differ between viral types, motivating the optimization of Ab therapeutics that could ameliorate nHSV. Given the short time window of vulnerability to nHSV, this work could facilitate the design of effective therapeutic mAbs, whose timely administration could yield tremendous benefit for this devastating disease.

RESULTS

Characterization of HSV-gD-specific mAbs

The mAbs used in this study protect both adult and neonatal mice from HSV-1- and HSV-2-induced mortality[15,25–27] and are currently being evaluated in human clinical trials in adults (ClinicalTrials.gov: NCT04714060, NCT02346760, and NCT02579083), but the mechanisms by which they mediate protection have not been defined. In order to better understand the contribution of neutralization and other Fc-mediated functions, we studied UB-621, HSV8, and CH42 AAA, mAbs that exhibit different neutralization potencies and effector function activity (Figure 1A; Table S1). To probe the contributions of effector functions *in vivo*, HSV8 and CH42 AAA were expressed with Fc domain point mutations that serve as functional Fcγ receptor (FcγR) and C1q binding knockouts (KOs). UB-621 and HSV8 are unmodified human immunoglobulin G1 (IgG1) mAbs, while CH42 AAA has been engineered with S298A/E333A/K334A mutations, which increase affinity for FcγRIIIA.[28] For construction of FcγR KO mAbs, we incorporated LALA PG[29] mutations into HSV8 and the N297A[30] substitution into CH42. VRC01,[31] an HIV-specific IgG1 mAb, was included as an isotype control. The FcR binding profiles of the engineered mAbs were evaluated *in vitro* (Figures 1B and S1). The binding patterns of all three Fc-intact Abs, UB-621, HSV8, and CH42 AAA, were comparable, with CH42 AAA exhibiting the strongest binding to all human and mouse FcRs tested. As expected, the HSV8 LALA PG variant displayed diminished binding to both human and mouse FcRs as compared to HSV8. The CH42 NA variant also exhibited diminished binding to human and mouse FcRs, with the exception of murine FcγRI, to which binding was only slightly diminished. Importantly, given our use of these mAbs in mouse experiments, the Fc-modified and -unmodified forms of each HSV-specific mAb displayed similar binding profiles to the four mouse FcRs as to their human orthologs. These data indicate a high level of concordance between species.

To more directly assess the function of each mAb, *in vitro* assays of antigen recognition, neutralization, and effector function were performed (Figure 2). Each HSV-specific mAb bound to both HSV-2 gD (gD-2) that was recombinantly expressed and HSV-1 gD (gD-1) expressed on the surface of mammalian cells (Figures 2A, 2B, and S2B). In contrast, the isotype control, VRC01, showed no binding. Notably, while CH42, HSV8, and UB-621 exhibited different antigen-binding dose-response profiles from each other, the binding of Fc KO forms of HSV8 and CH42 to antigen was unchanged. Furthermore, direct antiviral activity afforded by antigen recognition again varied by mAb but not by Fc modification (Figures 2C and 2D). UB-621 and HSV8 potently neutralized both HSV-1 and HSV-2, while CH42 poorly neutralized both viruses. Consistent antigen binding and neutralization activities of unmodified and Fc KO mAbs permits the isolation of Fab- from Fc-dependent activities.

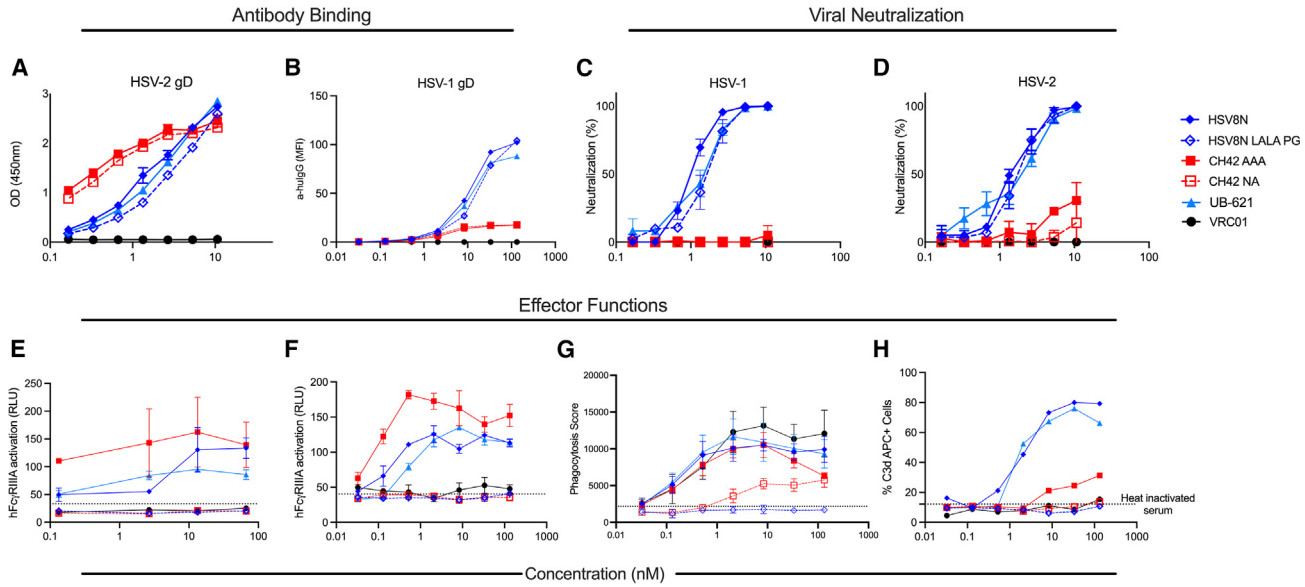

Figure 2. *In vitro* functional characterization of HSV gD-specific mAbs

(A and B) Ability of the HSV gD mAbs to bind recombinant HSV-2 gD (A) or cell-surface-expressed HSV-1 gD (B) via ELISA or flow cytometry, respectively.

(C and D) Ability of the HSV gD mAbs to neutralize HSV-1 (C) or HSV-2 (D) by plaque reduction assay.

(E–H) Effector function of HSV gD mAbs, including human FcγRIIIA stimulation of a reporter cell line in the context of antibody-bound HSV-2 gD on a microtiter plate (E), or HSV-1 gD-expressing cells (F) as surrogates for ADCC activity, phagocytosis using anti-human Fab beads (G), or complement deposition using HSV-1 gD-expressing cells (H). Error bars represent standard deviation from the mean. OD, optical density; MFI, mean fluorescent intensity; RLU, relative light units; APC, allophycocyanin. Unless otherwise labeled, the dashed lines represent no-antibody controls. Assays were performed in technical and biological replicates.

See also Figure S2.

Lastly, we tested the *in vitro* effector functions of these mAbs. We profiled their ability to promote FcγRIIIA activation upon recognition of recombinant gD-2 or cell-expressed gD-1 as a surrogate for ADCC activity (Figures 2E and 2F). We also measured their ability to induce complement deposition and phagocytosis (Figures 2G, 2H, S2A, and S2C). HSV8, UB-621, and CH42 AAA all mediated effector functions *in vitro*, whereas KO mAbs were unable to mediate FcγRIIIA activation, complement deposition, or phagocytosis. As may have been anticipated from stronger binding to FcγRIIA and FcγRIIIA, CH42 AAA exhibited the most potent ADCC and phagocytic activity, indicating that the AAA mutations enhanced the ability of CH42 to mediate Fc function. CH42 NA, which eliminates the conserved Fc glycan, maintained some phagocytic activity, presumably due to residual binding to human FcγRI. Consistent with this observation, others have reported that aglycosylated IgG1 mAbs retain phagocytic activity via FcγRI expressed on macrophages.[32,33] Taken together, these experiments demonstrated the divergent activities of the mAb panel, supporting its utility to define *in vivo* mechanisms of action.

Neutralization and Fc-mediated functions contribute to nHSV survival

To begin to understand the roles of viral neutralization and Fc effector functions in mediating protection against a nHSV challenge, 2-day-old C57BL/6J pups were injected intraperitoneally (i.p.) with 40 μg mAb and immediately challenged with 1.0×10^4 plaque-forming unit (PFU) HSV-1 intranasally (i.n.). Pups that received potently neutralizing mAbs, HSV8 or UB-621, had improved survival compared to pups that received non-neutralizing mAb (CH42 AAA) (Figures 3A and 3B). That said, all three HSV-specific Abs improved survival compared to isotype control (VRC01) (Figures 3A–3C). HSV-infected mice treated with CH42 NA, which lacks both neutralization and effector function activity, succumbed to infection (Figure 3B). In contrast, the mice that received the neutralizing but effector function KO HSV8 LALA PG survived HSV-1 infection (Figure 3A). These results indicate that for HSV8, neutralization alone was sufficient to mediate protection, while the moderate protection mediated by CH42 AAA was wholly Fc dependent.

As an orthogonal test to define the specific contribution of FcγR-dependent Fc effector functions in mediating protection, FcγR-deficient mice ($Fc\gamma R^{-/-}$)[34] were treated i.p. with mAbs and challenged i.n. with 1.0×10^4 PFU HSV-1. $Fc\gamma R^{-/-}$ mice that received neutralizing mAbs HSV8, HSV8 LALA PG, and UB-621 exhibited increased survival as compared to CH42 AAA and control IgG (Figures 3D–3F). They were, however, considerably more susceptible to HSV infection as compared to wild-type (WT) mice. In both challenge experiments, surviving pups gained weight normally through the end of the weaning period (Figure S3). Viral neutralization was highly protective in WT mice but not in $Fc\gamma R^{-/-}$ mice, indicating a role for Fc function in contributing to protection against HSV-1 in neonatal mice.

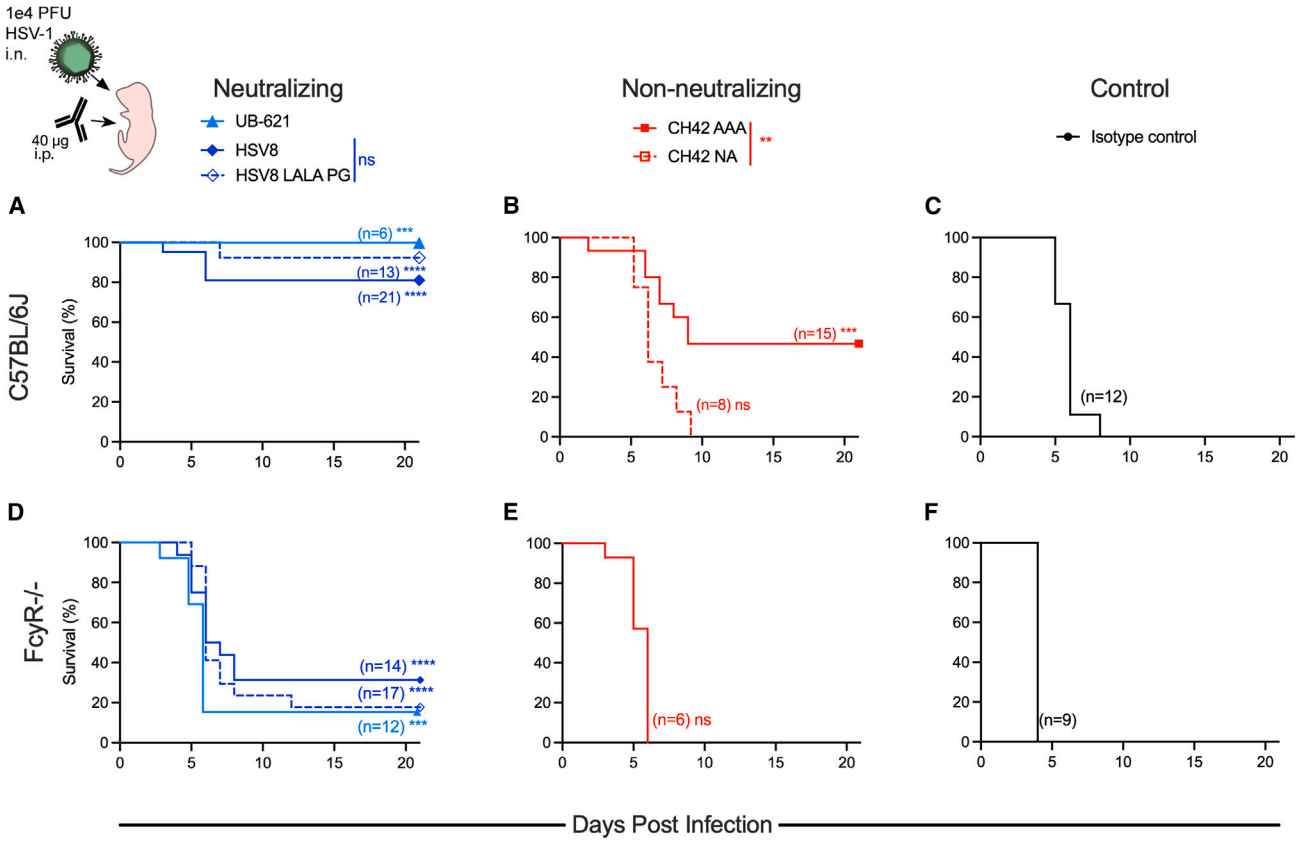

Figure 3. Both neutralization and effector function contribute to mAb-mediated protection from lethal HSV-1 challenge

Immediately before lethal intranasal (i.n.) challenge with 1 × 10^4 plaque-forming units (PFU) HSV-1, 2-day-old pups were administered 40 μg mAb by intraperitoneal (i.p.) injection.

(A–C) Survival of C57BL/6J pups receiving neutralizing mAbs UB-621, HSV8, or HSV8 LALAPG (A), non-neutralizing mAbs CH42 AAA or CH42 NA (B), or isotype control mAb VRC01 (C).

(D–F) Survival of *Fc*γ*R*$^{-/-}$ pups receiving neutralizing (D), non-neutralizing (E), or isotype control mAb (F). Number of mice in each condition and statistical significance as compared to isotype control in matched mouse strain determined by the log-rank (Mantel-Cox) test (***$p < 0.001$ and ****$p < 0.0001$) are reported in inset. Significance between HSV8 and HSV8 LALA PG or CH42 AAA and CH42 NA are reported in the top legend as determined by the log-rank (Mantel-Cox) test. See also Figure S3.

Fc functions provide partial protection in the absence of complete viral neutralization

Given the increased mortality observed in *Fc*γ*R*$^{-/-}$ mice treated with potently neutralizing mAbs, we next investigated the role of Fc functions under conditions of maximal viral neutralization. Achieving maximal neutralization activity was accomplished by pre-incubating excess mAb with 1.0 × 10^4 PFU HSV-1 prior to *in vivo* challenge. With this experimental design, both C57BL/6J and *Fc*γ*R*$^{-/-}$ mice were completely protected from disease by HSV8 and UB-621 (Figures 4A and 4D). In contrast, when virus was pre-incubated with 20 μg CH42 AAA, the majority of the pups succumbed to infection (Figure 4B), as did all animals treated with the isotype control mAb (Figure 4C). While increasing the CH42 AAA concentration 5-fold to 100 μg mAb/pup did improve survival (Figure 4B), it was unable to achieve the complete protection seen when mice were administered neutralizing mAbs. In contrast to neutralizing mAbs, even a 100 μg dose of CH42 AAA failed to protect *Fc*γ*R*$^{-/-}$ pups (Figure 4E) and resulted in survival comparable to the isotype control mAb (Figure 4F). These results provide evidence that FcγR-mediated activities are not necessary to provide protection in the context of fully neutralized HSV-1 but can be responsible for protection in the absence of complete neutralization.

The relative impacts of neutralization and Fc effector functions are mAb-dose dependent

Survival of pups was equivalent whether treated with HSV8 or HSV8 LALA PG mAbs at the 40 μg dose, and full protection of *Fc*γ*R*$^{-/-}$ mice with neutralizing mAb-opsonized virus was observed. Together, these data indicate the lack of a major role for Fc effector functions in mediating protection in the context of high levels of neutralizing activity. We wished, therefore, to assess the hypothesis that Fc effector functions may be more important at lower Ab doses.[35] To test this possibility, we treated C57BL/6J mice with 10 μg mAb delivered i.p. and subsequently challenged with a lethal dose of HSV-1. As

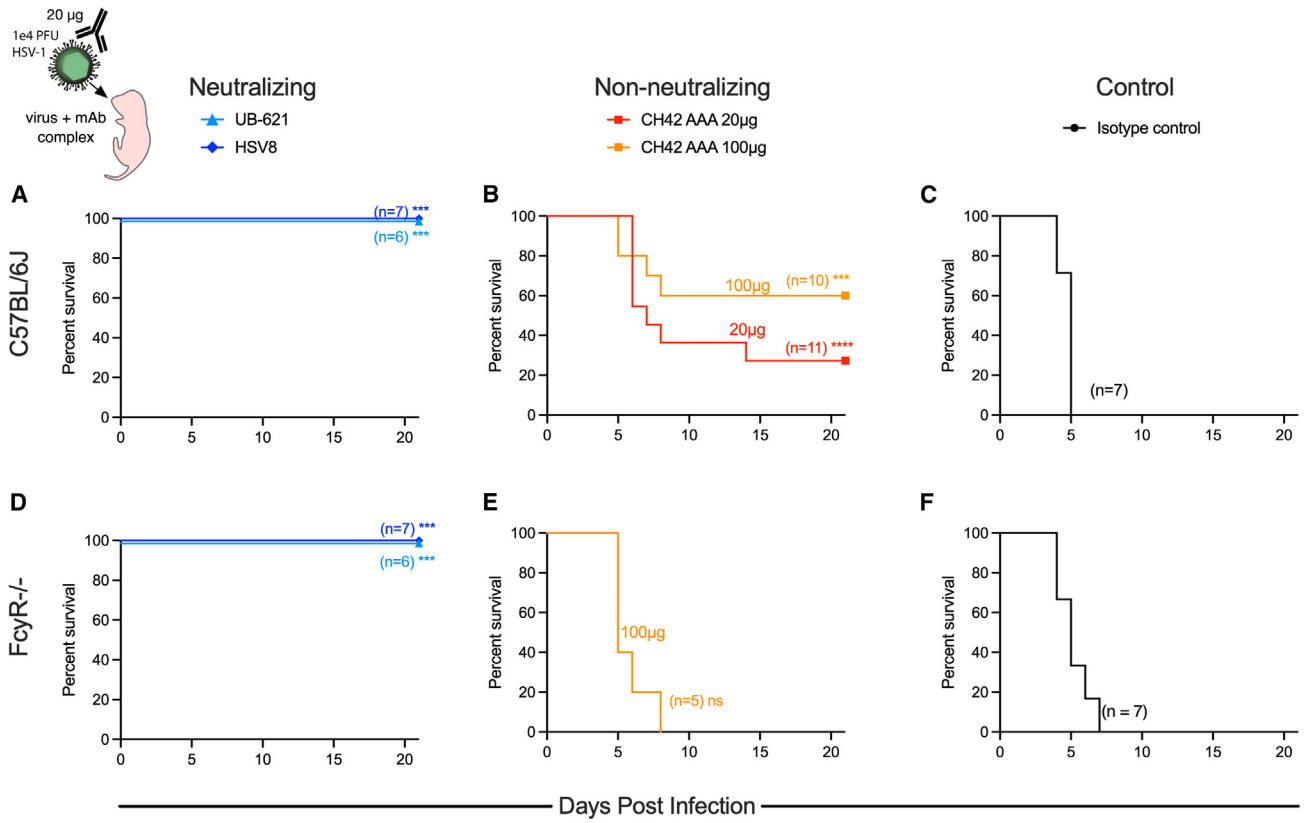

Figure 4. Fc functions are protective in the absence of complete viral neutralization

One hour before i.n. challenge of 2-day-old pups, immune complexes were formed by incubation of 1 × 10^4 PFU HSV-1 with mAb at 37°C (20 μg unless otherwise noted).

(A–C) Survival of C57BL/6J pups following immune complex challenge with virus opsonized with neutralizing mAbs UB-621 or HSV8 (A), non-neutralizing mAb CH42 AAA (20 or 100 μg) (B), or isotype control mAb (C).

(D–F) Survival of *Fc*γ*R*$^{-/-}$ pups following immune complex challenge with virus opsonized with neutralizing mAbs UB-621 or HSV8 (D), non-neutralizing mAb CH42 AAA (100 μg) (E), or isotype control mAb (F). Number of mice in each condition and statistical significance as compared to isotype control in matched mouse strain determined by the log-rank (Mantel-Cox) test (**p < 0.01, ***p < 0.001, and ****p < 0.0001) are reported in inset.

See also Figure S4.

expected, this dose of the WT HSV8 was less protective than the 40 μg dose. Indeed, at this lower dose, HSV8 LALA PG was completely unable to protect (Figure 5A), demonstrating that neutralization alone is insufficient to protect mice at lower mAb doses. Intriguingly, CH42 AAA provided comparable protection to HSV8 at the 10 μg dose (Figure 5B). In contrast, 10 μg CH42 NA and the isotype control failed to protect mice from HSV-mediated mortality (Figure 5C). Effector functions, therefore, were observed to mediate protection from HSV-1-induced mortality at low Ab concentrations, at which viral neutralization may be incomplete.

As an additional metric to explore the relative contributions of neutralization and effector functions in mediating protection, we assessed viral titers in various organs following 10 μg mAb treatment (Figures 5D–5F). At 5 days post-infection, both HSV8 and CH42 AAA significantly reduced viral burden in the brain, trigeminal ganglia (TGs), and visceral organs (liver, spleen, and lungs) as compared to the isotype control mAb. HSV8 LALA PG, however, only significantly reduced viral burden in the brain as compared to isotype control (Table S2). The viral burden in pups treated with CH42 NA was indistinguishable from pups given an isotype control mAb. While not statistically significant, pups treated with HSV8 had lower viral burden as compared to pups given HSV8 LALA PG, consistent with survival data in indicating a contribution of effector functions in mediating protection (Figure 5D). Further evidence for the role of effector functions in mediating protection was observed in the differences in viral burden in pups treated with CH42 AAA and CH42 NA. Pups treated with CH42 AAA had statistically significant lower viral burden in the brain, TGs, spleen, and lungs as compared to mice given CH42 NA (Figure 5E). Of note, some pups given HSV8 LALA PG, CH42 NA, or the isotype control died prior to day 5 post-infection, while no pups given HSV8 or CH42 AAA died prior to organ collection. Taken together, these data support a role for effector functions in protecting mice from HSV-1-mediated mortality and viral burden in the nervous system and viral dissemination.

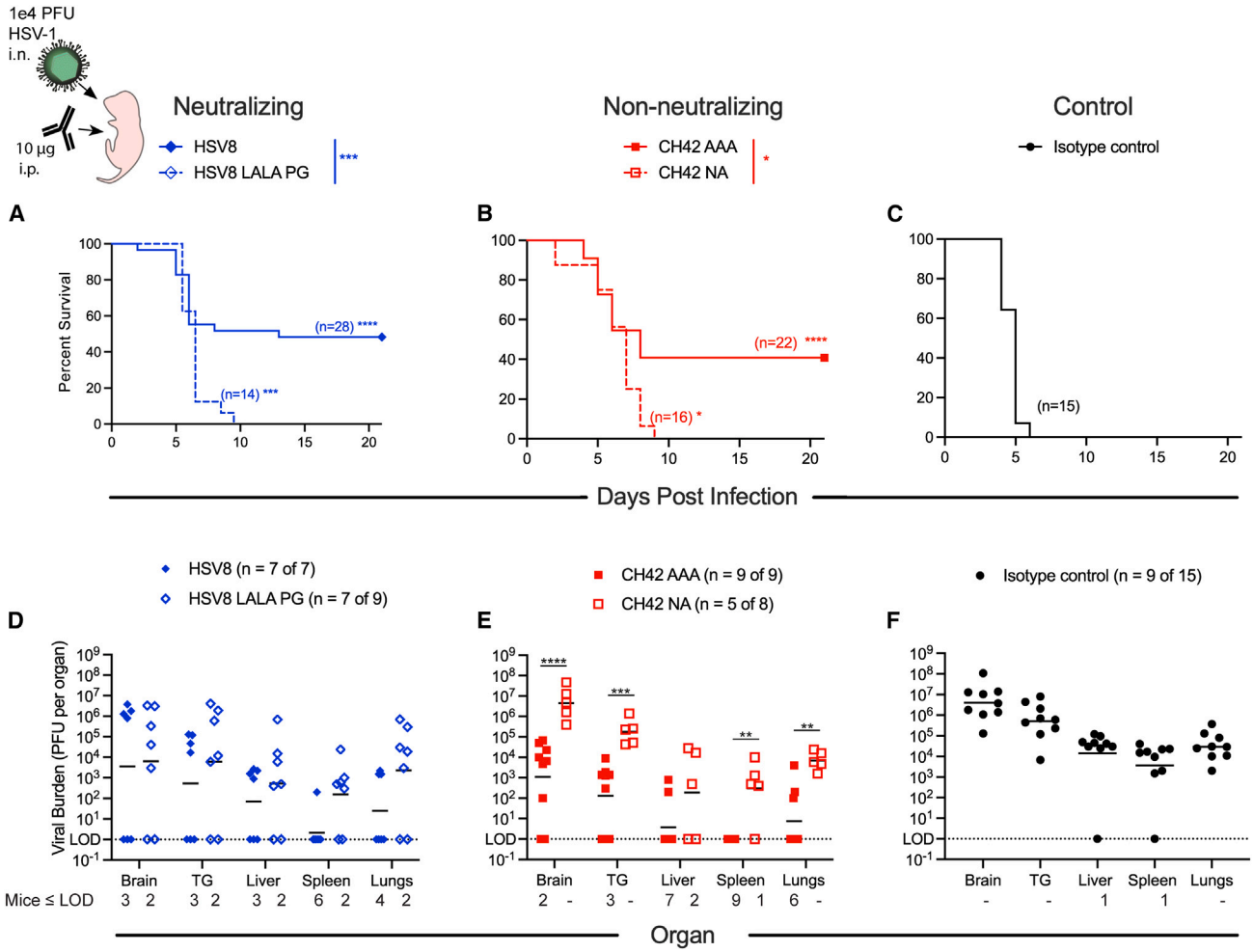

Figure 5. Relative contributions of neutralization and effector functions to protection from lethal challenge depending on antibody dose

Immediately before lethal i.n. challenge with 1 × 10^4 PFU HSV-1, 2-day-old pups were administered 10 μg mAb by i.p. injection.

(A–C) Survival of C57BL/6J pups receiving neutralizing mAb HSV8 or HSV8 LALA PG (A), non-neutralizing mAb CH42 AAA or CH42 NA (B), or isotype control mAb (C). Number of mice in each condition and statistical significance as compared to isotype control determined by the log-rank (Mantel-Cox) test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$) are reported in inset. Statistical significance between HSV8 and HSV8 LALA PG or CH42 AAA and CH42 NA is reported in the top legend as determined by the log-rank (Mantel-Cox) test (**$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$).

(D–F) Viral titers were determined 5 days post-infection (DPI). Data are shown as viral burden in perfused organs from surviving pups following 10 μg mAb treatment on DPI 0. Statistical significance was determined by two-way ANOVA with Bonferroni's test for multiple comparisons (**$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$). See also Table S2. Geometric mean of the viral burden in organ type per treatment group is displayed. In the legend, n = number of pups included in viral titer of the total number of pups treated with mAb to account for pups who died prior to the time point of organ collection.

See also Figure S5.

HSV-specific mAbs require effector functions for control of viral replication

To determine whether effector functions contribute to viral clearance, mouse pups were infected in a non-lethal challenge model utilizing a luciferase-producing recombinant HSV-1,[36] allowing real-time imaging of *in vivo* viral replication. Pups were challenged with HSV-1 17syn+dLux i.n. and 10 μg HSV8, HSV8 LALA PG, CH42 AAA, CH42 NA, or an isotype control mAb delivered i.p. the following day. Consistent with the results of survival and viral load experiments, mice that received a 10 μg dose of either HSV8 LALA PG or CH42 NA exhibited significantly greater levels of viral replication as measured by bioluminescence than mice treated with HSV8 or CH42 AAA starting at day 4 post-infection (Figures 6A and 6B). Bioluminescence in Fc KO mAb-treated mice persisted for significantly longer than in those that received mAbs with intact effector functions and was comparable to the animals that received the IgG control mAb (Figures 6A and 6B). Statistically significant differences in bioluminescence were observed in animals treated with Fc KO versus Fc-functional mAbs (Figure 6C). Given the equivalent neutralization profiles of HSV8 and HSV8 LALA PG, differences in viral replication and dissemination must be attributable to the lack of effector functions in the LALA PG variant. Moreover, CH42 AAA, which does not neutralize, cleared virus significantly faster than its Fc

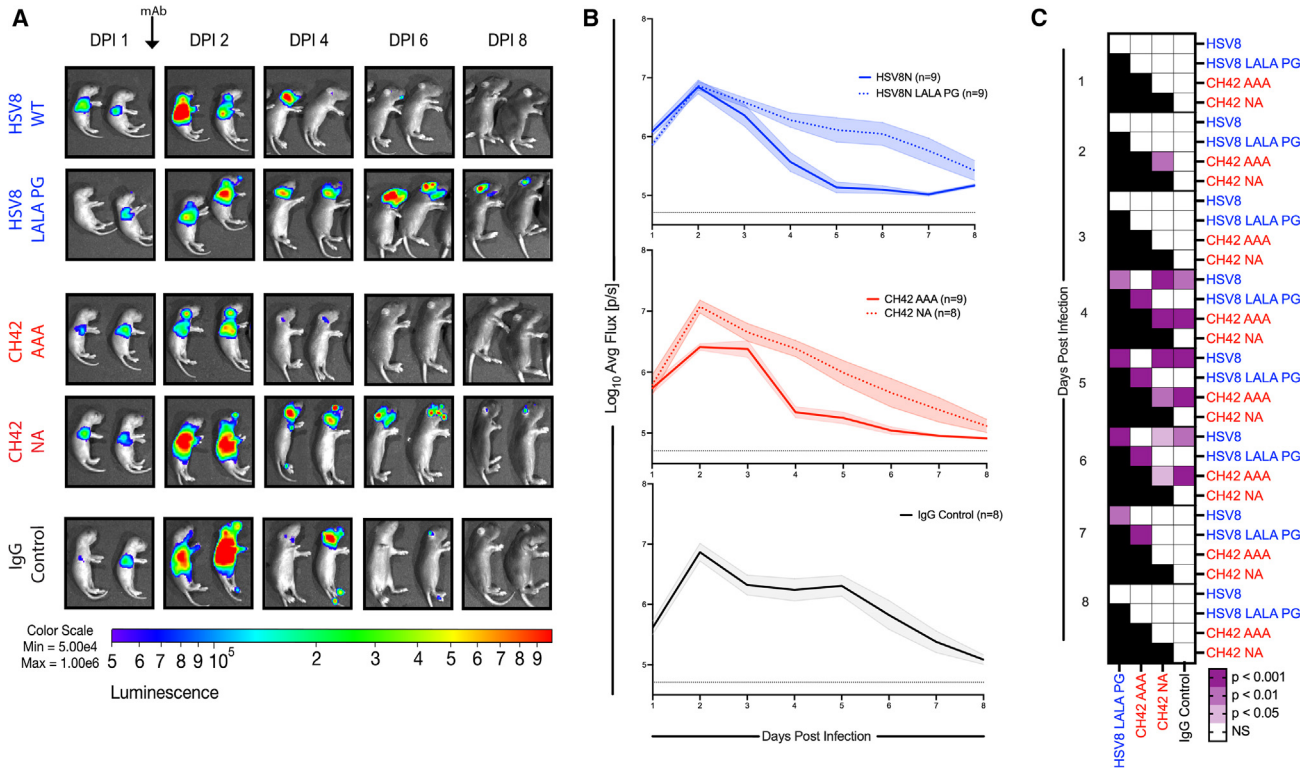

Figure 6. Effector functions accelerate control of viral replication after non-lethal HSV-1 challenge

One day post-i.n. infection with a luciferase-expressing HSV-1, 2-day-old pups were administered 10 μg mAb i.p., and viral replication, as represented by bioluminescence, was quantified daily.

(A) Representative bioluminescence images of viral infection and replication following mAb treatment are presented for the same two pups over time.

(B) Quantification of virally derived bioluminescence over time for HSV8 and HSV8 LALA PG (top), CH42 AAA and CH42 NA (middle), and isotype control (bottom). Lines and shaded regions represent the mean luminescence and standard error of the mean across pups (number listed in inset).

(C) Heatmap depicting statistical significance (two-way ANOVA with Tukey's test for multiple comparisons) between groups treated with indicated mAbs over time after infection.

KO counterpart. These results extend observations from the lethal challenge model and demonstrate that effector functions contribute to control of HSV-1 replication for these mAbs.

Ab functions contributing to protection differ between HSV serotypes

Since nHSV is caused by both HSV-1 and HSV-2, we next sought to examine whether the mechanism and patterns of protection were equivalent for both viruses. To test mechanism of protection against HSV-2, 2-day-old C57BL/6J mouse pups were treated with 40 μg HSV-specific mAb or isotype control and then challenged with 300 PFU HSV-2 strain G.[37] In contrast to HSV-1, and despite differences in neutralizing activities, both HSV8 and CH42 AAA provided equivalent protection against lethal challenge with HSV-2 (Figures 7A and 7B). Moreover, the Fc mutations in HSV8 LALA PG and CH42 NA completely ablated their protective activities, rendering them equivalent to the isotype control mAb (Figures 7A–7C). These results demonstrate that Fc-mediated effector functions, and not viral neutralization, are essential for protection against HSV-2 infection, exposing a dichotomy between viral subtypes. Together, for this panel of gD-specific mAbs, these data demonstrate that optimal Ab-mediated protection against HSV-1 in neonates is achieved by both neutralization and effector functions. In contrast, for protection against HSV-2, effector functions alone are sufficient.

DISCUSSION

Understanding the mechanism by which Abs provide protection has the potential to contribute to the development of mAb-based prevention and therapy, as well as to inform vaccine design. In this study, nHSV infection outcomes depended on mAb specificity, neutralization potency, effector functions, dose, and viral strain. Both neutralization and effector functions improved virological outcomes following HSV-1 challenge. At higher Ab doses, neutralizing mAbs afforded near-complete protection, whereas the non-neutralizing mAb afforded only moderate, Fc-dependent protection. In contrast, under the same dose and challenge conditions, the mAb with viral neutralization activity alone was unable to prevent significant mortality in $Fc\gamma R^{-/-}$ mice. Notably, pre-incubating the virus with neutralizing mAb prior to challenging $Fc\gamma R^{-/-}$ mice completely protected these mice from mortality. This apparent discrepancy in the protective contribution of Fc-dependent Ab functions observed with KO mAbs

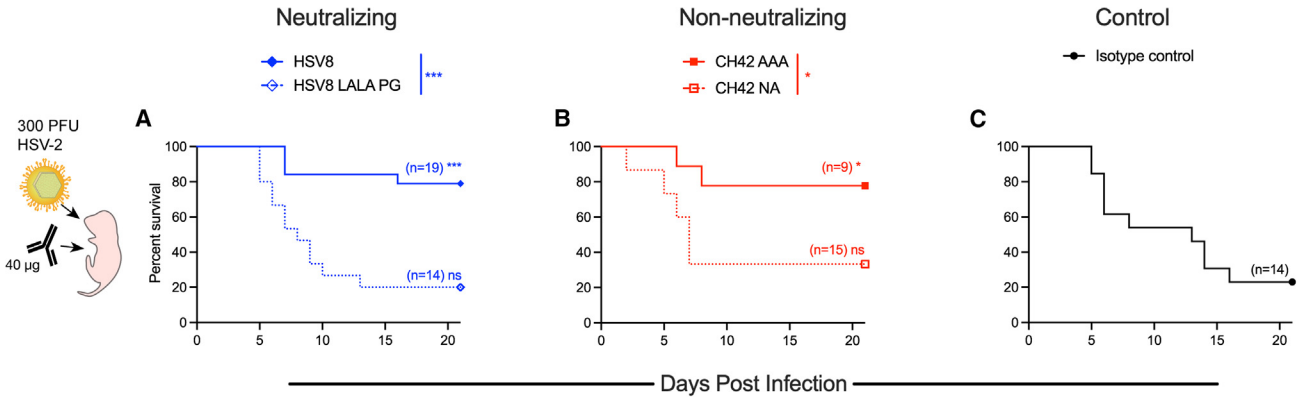

Figure 7. Antibody functions contributing to protection differ between HSV serotypes

Immediately before lethal i.n. challenge with 300 PFU HSV-2, 2-day-old pups were administered 40 μg mAb by i.p. injection.

Survival of C57BL/6J pups receiving neutralizing mAb HSV8 or HSV8 LALAPG (A), non-neutralizing mAb CH42 AAA or CH42 NA (B), or isotype control mAb (C). Number of mice in each condition and statistical significance as compared to isotype control determined by the log-rank (Mantel-Cox) test (*$p < 0.05$ and ***$p < 0.001$) are reported in inset. Statistical significance between HSV8 and HSV8 LALA PG or CH42 AAA and CH42 NA is reported in the top legend as determined by the log-rank (Mantel-Cox) test (*$p < 0.05$ and ***$p < 0.001$).

See also Figure S6.

versus $Fc\gamma R^{-/-}$ mice could be attributed to, for example, residual mAb effector function, differences in Ab biodistribution, and the intrinsic susceptibility of $Fc\gamma R^{-/-}$ mice,[21] among other possible factors.

When mAb was present at low concentrations, Abs with effector functions were more protective than Abs with neutralization activity alone. In contrast, when present at high levels systemically, or when pre-incubated with virus before nasal challenge, neutralization activity was sufficient for high efficacy. This functional shift suggests that Ab concentration and biodistribution are determinants of the dominant mechanism of protection. Our findings support the hypothesis that Ab-mediated protection against HSV-1 is driven primarily by neutralization at high doses, while at lower doses, both neutralization and Fc effector functions play a role, as has been previously hypothesized.[35] Evidence in support of this hypothesis has been seen for other viruses. At subneutralizing Ab doses, mAb effector functions can be associated with improved resistance to infection,[38,39] control of viremia,[40,41] and clearance of virions[42] during simian-human immunodeficiency virus (SHIV) infection in non-human primates. Additionally, optimal mAb-mediated protection against severe acute respiratory syndrome coronavirus 2 infection required effector functions in addition to viral neutralization, particularly when neutralization potency was compromised.[43–45] Although these viruses differ from HSV in their pathogenesis and immune evasion strategies, our data support the idea that mAb dose is a pivotal determinant of the mechanism of protection. Ab dose can also directly impact clinical outcomes associated with viral pathogenesis. Subneutralizing doses of Abs against Dengue virus can lead to FcR-driven, Ab-dependent enhancement of disease,[46] furthering the consideration of Ab dose as a determinant for mechanism of action.

Given the ability of HSV8 LALA PG to protect against HSV-1, its relative inability to protect against HSV-2 was unexpected. The inability of both CH42 NA and HSV8 LALA PG to protect against HSV-2 indicates that Fc-mediated effector functions, rather than viral neutralization, at least as assessed *in vitro* against cell-free virus, drive mAb-mediated protection against this serotype. This result may explain in part the failures of human HSV-2 vaccine trials.[47–49] A subunit vaccine containing gD and gB that induced high titers of neutralizing Abs but low titers of ADCC-inducing Abs[50] showed poor efficacy,[51] indicating that neutralizing activity was not sufficient for the prevention of genital disease and transmission of HSV-2. Similarly, a later gD-2 subunit vaccine candidate that induced robust neutralizing titers, but little to no ADCC activity,[52] had 58% efficacy in preventing HSV-1 genital disease but could not prevent HSV-2 genital disease.[47] In this trial, neutralization titers against HSV-2 did not correlate with protection and could not explain the lack of vaccine efficacy.[53] Overall, the lack of protection afforded by neutralization and the poor effector function of Abs raised by these vaccine candidates are consistent with the hypothesis that protection against HSV-2 requires effector functions. These observations may also be due in part to differences between HSV-1 and HSV-2 in relationship to Ab neutralization and evasion by surface glycoproteins.[54] In our study, protection against HSV-2-mediated mortality was independent of mAb neutralization potency in that CH42 AAA poorly neutralized HSV-2 and yet provided protection comparable to HSV8. Consistent with this result, a non-neutralizing but FcγR-activating mAb that targets gB mediated protection from HSV-2 *in vivo*.[55]

The importance of Ab effector functions was also observed in bioluminescent imaging experiments that quantified viral load. Effector functions played a clear role in contributing to viral control, as both HSV8 and CH42 AAA cleared the HSV-derived bioluminescence significantly faster than their KO equivalents. Moreover, the ability for an Ab to mediate effector functions also greatly contributed to reducing viral burden and dissemination. Pups that received HSV8 or CH42 AAA had lower viral

burden in tissues of the nervous system and in visceral organs as compared to their functional KO counterparts. This reduction in viral burden indicates a role for effector functions in the control of viral spread. HSV8 LALA PG was also able to slightly reduce viral burden in the brain as compared to the isotype control, indicating that neutralization still contributes to protection. Together, these pre-clinical studies highlight the importance of investigating non-neutralizing Ab functions in mediating protection against HSV disease, particularly HSV-2.

While there are caveats to direct translation of observations from animal models to humans, prior studies provide a high degree of confidence as to which murine FcγRs are engaged when introducing human IgG1 into a mouse.[23,56] The distribution of FcRs varies between human and murine innate immune cells, but the overall effector functions mediated by the differing cell types are conserved. ADCC activity mediated via human cells is generally a good predictor of murine ADCC (predominantly carried out by macrophages and polymorphonuclear neutrophils).[57] We focused on several mAbs specific for gD and tested a limited number of viral strains in a single mouse strain background, and our results may or may not generalize across other mAbs, target antigens, viruses, or host genetic backgrounds. Testing a human-specific pathogen in an incidental host invariably fails to faithfully recapitulate all aspects of host-pathogen interactions that may be relevant in humans. As examples, while HSV can establish latency in mouse neuronal ganglia, it does not appear to spontaneously reactivate efficiently.[58] Additionally, unlike the human IgG1 mAbs tested here, endogenous mouse IgG cannot bind to the HSV viral FcR (gE/gI) that plays a role in immune evasion.[59] Other caveats include when and where mAbs initially encounter virus, particularly in the context of differing hosts. Given that humans show a spectrum of anatomical, physiological, and immunological profiles, and based on the data of this study, Abs with broad functional activities are more likely to afford clinical efficacy. This idea is supported by clinical evidence: both neutralizing and ADCC Ab activities serve as biomarkers for protection of infants from disseminated HSV disease.[10,11] The inability of neutralizing activity alone to serve as a reliable biomarker of vaccine-mediated protection in adults, particularly for HSV-2, is also consistent with our results.

Additional variables include cell-to-cell spread of virus, which presents a path for the virus to evade neutralizing Ab responses.[60–63] For HSV, cell-to-cell transmission is critical for neuropathogenesis and the establishment of latency in the peripheral nervous system.[61] Naturally infected individuals have Abs that poorly limit cell-to-cell spread *in vitro*, which may partially explain viral reactivation and transmission even in the presence of robustly neutralizing Abs.[63] Of the Abs tested here, HSV8 limits cell-to-cell spread and syncytia formation *in vitro*.[64–67] While little is known about the role of Fc function in combating cell-to-cell spread, our data provide evidence that Fc effector functions mediated by these mAbs can prevent the spread of HSV *in vivo*. While *in vitro* neutralization activity, historically, has been shown to be the best predictor for Ab efficacy,[68] it is clearly an incomplete metric whereby to predict protection from HSV. Collectively, these data support the conclusion that polyfunctional mAbs able to mediate both neutralization and effector functions are the best candidates for therapeutic and prophylactic translation. Expanding the focus of vaccine research and development to include activities beyond viral neutralization has the potential to accelerate the quest for interventions to reduce the global burden of HSV infection.

Limitations of this study

We acknowledge some limitations of this study. Firstly, we have only investigated the mechanisms of mAbs targeting gD. HSV-1 and -2 encode multiple surface glycoproteins that are involved in viral entry, and Abs targeting these antigens may require different combinations of Ab functions to mediate optimal protection. Secondly, we globally knocked out effector function by using both Fc mutation of Abs and mice lacking expression of FcγRs. Therefore, while our data demonstrate a role for effector functions, the specific effector cell types and Ab effector functions that are required for protection have yet to be determined. Other mouse models and Fc mutations may elucidate specific cell types and pathways that are critical for mAb-mediated protection. Lastly, we used well-characterized laboratory strains of HSV-1 and HSV-2, which may differ from clinical and circulating viruses in their susceptibility to mAbs.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xcrm.2024.101417.

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AUTHOR CONTRIBUTIONS

M.D.S., I.M.B., D.A.L., and M.E.A. conceptualized the study. M.D.S., I.M.B., N.S.K., and C.R.G. performed experiments. D.A.L. and M.E.A. obtained funding and supervised research. M.D.S. and I.M.B. drafted the manuscript and generated figures. I.M.B., M.D.S., D.A.L., and M.E.A. finalized the manuscript, and all other authors read and edited it.

DECLARATION OF INTERESTS

I.M.B., D.A.L., and M.E.A. report a patent, WO2020077119A1, for mAbs used in this manuscript as a method for the treatment for nHSV infections.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Goat anti-human IgG Fc Cross-Adsorbed Secondary Antibody HRP	Invitrogen	Cat#31413 RRID: AB_429693
Goat anti-human IgG (H + L) Cross-Adsorbed secondary antibody AlexaFluorTM 647	ThermoFisher Scientific	Cat#A-21445 RRID: AB_2535862
Goat anti-guinea pig C3 – biotin	ICL Labs	Cat#GC3-60B-Z
CH42 AAA	Dr. M. Anthony Moody (Duke University)	N/A
CH42 NA	This manuscript	N/A
UB-621	United Biopharma	N/A
HSV8	ZabBio	N/A
HSV8 LALA PG	ZabBio	N/A
Bacterial and virus strains		
HSV-1 17syn+	Brown et al.[69]	N/A
HSV-2 G	Ejercito et al.[37]	N/A
HSV-1 17syn+/Dlux	Luker et al.[36]	N/A
DH5a	New England BioLabs	Cat #C2987H
NEB® Stable Competent E. coli (High Efficiency)	New England BioLabs	Cat #C3040H
Biological samples		
LowTox Guinea Pig Complement	CedarLane Labs	Cat # CL4051
Chemicals, peptides, and recombinant proteins		
D-luciferin potassium salt	Gold Biotechnology	Cat # LUCK-1g
HSV-2 gD-306 ectodomain	Dr. Gary Cohen, Nicola et al.[70]	N/A
Recombinant Human FcyRs	Duke Human Vaccine Institute, Boesch et al.[71]	N/A
Recombinant mouse FcyRI	Sino Biological	Cat # 50086-M27H-B
Recombinant mouse FcyRIIb	Sino Biological	Cat # 50030-M27H-B
Recombinant mouse FcyRIII	Sino Biological	Cat # 50326-M27H-B
Recombinant mouse FcyRIV	Sino Biological	Cat # 50036-M27H-B
Streptavidin-R-Phycoerythrin	Invitrogen	Cat #S866
eBioscience Cell Stim Cocktail (500x)	Invitrogen	Cat # 00-4970-93
Critical commercial assays		
QUANTI-Luc	Invivogen	Cat # rep-qlc1
Experimental models: Cell lines		
VeroE6	ATCC	RRID: CVCL_0574
THP-1 monocytes	ATCC	RRID: CVCL_0006
Jurkat-Lucia NFAT-CD16	Invivogen	Cat # jktl-nfat-cd16
Expi293F Cells	ThermoFisher	Cat # A14527; RRID: CVCL_D615
HEK293T	ATCC	Cat # CRL-3216; RRID: CVCL_0063
HSV-1 gD expressing HEK293Ts	This manuscript	N/A
Experimental models: Organisms/strains		
Mouse: C57BL/6J	The Jackson Laboratories	RRID: IMSR_JAX:000664
Mouse: B6.129P2-*Fcer1g*^{tm1Rav} N12	Taconic	Model 583; RRID: IMSR_TAC:583
Recombinant DNA		
pLenti-DsRed-IRES-EGFP vector	Rousseaux et al.[72]	RRID: Addgene_92194
HSV-1 st17 DNA	McGeoch et al.[73]	NCBI:txid10299

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
GraphPad Prism 9	GraphPad Software INC	RRID:SCR_002798
FlowJo 10	BD Life Sciences	RRID: SCR_008520
Other		
MagPlex microspheres	Luminex Corp	Cat # MC100XX

RESOURCE AVAILABILITY

Lead contact

Further information and requests for reagents and resources should be directed to and will be fulfilled by the lead contact, Dr. Margaret E. Ackerman (margaret.e.ackerman@dartmouth.edu)

Materials availability

Antibodies, cell lines, and plasmids generated for this study may be requested with a material transfer agreement.

Data and code availability

All data reported in this paper will be shared by the lead contact upon reasonable request.
This paper does not report original code.
Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Cell lines

Vero Cells (CCL-81) were purchased from American Type Culture Collection (ATCC) and were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C with 5% CO_2. HEK293Ts were purchased from ATCC and maintained in DMEM with 10% FBS at 37°C and 5% CO_2. The human monocytic cell line, THP-1, was purchased from ATCC and maintained in RPMI-1640 supplemented with 10% FBS and 55μM beta-mercaptoethanol at 37°C with 5% CO_2. EXPI293Fs were purchased from ThermoFisher and were maintained in Expi293F Media (Thermo Fisher). Cells were grown in a Thermo Scientific reach-in CO_2 incubator at 37°C with 8% CO_2 on an innOva 2300 platform shaker at 125 RPM. Jurkat-Lucia NFAT CD16 cells were purchased from Invivogen and grown in RPMI-1640 supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 1x non-essential amino acids, 1x penicillin/streptomycin, 100 μg/mL Normocin, 100 μg/mL Zeocin, and 10 μg/mL Blasticidin.

Animals

Naive male and female C57BL/6J (RRID: IMSR_JAX:000664) were either purchased from The Jackson Laboratories or bred in animal facilities at Dartmouth College in accordance with institutional animal care and use committee protocols (Dartmouth College IACUC 2151). C57BL/6J mice were bred according to IACUC protocols and 2-day-old offspring of both sexes were then used in challenge studies. Naive male and female B6.129P2-Fcer1gtm1Rav N12 (FcγR−/−) (model: 583) were purchased from Taconic Labs. FcγR−/− mice were bred in accordance with IACUC protocols and 2-day-old offspring of both sexes were used in challenge studies.

METHOD DETAILS

Mouse procedures and viral challenge

C57BL/6J (B6) mice were purchased from The Jackson Laboratory. FcγR−/− mice (B6.129P2-Fcer1gtm1Rav N12) were purchased from Taconic Labs.[34] Administration of mAbs was via the peritoneal route with a 25 μL Hamilton syringe in a 20 μL volume under 1% isoflurane anesthesia. The wild-type viral strains used in this study were HSV-1 17syn+,[69] HSV-2 G (kindly provided by Dr. David Knipe).[37] The bioluminescent luciferase-expressing recombinant virus HSV-1 17syn+/Dlux was constructed as previously described.[36] Viral stocks were prepared using Vero cells as previously described.[74,75] Newborn pups were infected i.n. on day 2 postpartum with indicated amounts of HSV in a volume of 5–10 μL under 1% isoflurane anesthesia. Pups were then monitored for survival, imaging, or viral burden analysis. For survival studies, pups were challenged with $1x10^4$ plaque-forming units (PFU) of HSV-1 (Strain 17), and 3×10^2 PFU of HSV-2 (Strain G), as indicated. Endpoints for survival studies were defined as excessive morbidity (hunching, spasms, or paralysis) and/or >10% weight loss (Figures 3, 4, 5, and 6). For bioluminescent detection, pups were injected i.p. with 20 μL of 15 mg/mL D-luciferin potassium salt (Gold Biotechnology), placed in isoflurane chamber, and moved into a Xenogen IVIS-200 with a warmed stage and continuous isoflurane. Pups were typically imaged beginning at 1 day post-infection and serially

imaged every day until 8 days post-infection to monitor bioluminescence. For viral titers of organs, tissues were harvested 5 days post infection following cardiac perfusion with at least 5 mL of ice-cold PBS. All tissues were collected in 1.7 mL tubes containing ~100 μL of 1mm sterile glass beads and 1 mL of DMEM containing 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Tissue homogenates were prepared via mechanical disruption using a Mini-Beadbeater-8 (BioSpec Products). Organ titers were measured via plaque assay on Vero cells.

Monoclonal antibodies

CH42[15] AAA plasmids were kindly provided by Dr. Anthony Moody (Duke University). When expressed *in vitro*, CH42 contained the Fc mutation known as AAA (S298A/E333A/K334A), which enhances antibody dependent cellular cytotoxicity.[28] The variable heavy chain sequence of CH42 was subcloned into a plasmid coded with an IgG1 heavy chain backbone containing the N297A[30] mutation via QuikChange Site-Directed Mutagenesis kit (Agilent). Antibodies were expressed through co-transfection of heavy and light chain plasmids in Expi293 HEK cells (Thermo Fisher) according to the manufacturer's instructions. Seven days after transfection, cultures were spun at 3000 x g for 30 min to pellet the cells, and supernatants were filtered (0.22 μm). IgG was affinity purified using a custom packed 5 mL protein A column with a retention time of 1 min (i.e., 5 mL/min) and eluted with 100 mM glycine pH 3, which was immediately neutralized with 1 M Tris buffer pH 8. Eluate was then concentrated to 2.5 mL for size exclusion chromatography on a HiPrep Sephacryl S-200 HR column using an AktaPure FPLC at a flow rate of 1 mL/min of sterile PBS. Fractions containing monomeric IgG were pooled and concentrated using spin columns (Amicon UFC903024) to approximately 2 mg/mL of protein and either used within a week or aliquoted and frozen at −80°C for later use. HSV8 mAb was kindly provided by ZabBio and Kentucky Bioprocessing, and a clinical grade antibody preparation of UB-621 was kindly provided by United BioPharma.

Measurement of antibody binding to mouse and human Fc receptors

Recombinant HSV-2 gD (gD-2) antigen,[70] kindly provided by Dr. Gary Cohen (UPenn), was coupled to MagPlex beads (Luminex) as previously described.[76] gD mAbs were serially diluted in 1x PBS with 0.1% bovine serum albumin (BSA) and 0.05% Tween 20 and incubated with antigen-coupled beads overnight at 4°C with constant shaking. Beads were washed before being incubated with recombinant biotinylated human Fc receptors[71] (Duke Human Vaccine Institute) or mouse Fc receptors (Sino Biologics) that were tetramerized with streptavidin-PE for 1 h. The beads were washed and analyzed on the xMap system. The median fluorescence intensity of at least 10 beads/region was recorded. An isotype control antibody and a buffer only control were used to determine antigen-specific binding and assay background signal. Area under the curve was calculated using Prism 9 (GraphPad).

Viral neutralization

Serially diluted mAb and 50 PFU of HSV-1 st17 or HSV-2 G were incubated for 1 h at 37°C before being added to confluent Vero cells grown in 6 well plates. Immune complexes were incubated with Vero cell monolayers for 1 h at 37°C with 5% CO_2 with shaking every 15 min. Methylcellulose overlay was added to the wells after the hour incubation. Plates were incubated for 48 (HSV-1) or 72 (HSV-2) hours at 37°C with 5% CO_2. Methylcellulose overlay was removed, Vero cells were fixed with 1:1 ethanol:methanol before being stained with 12% Giemsa overnight. Stain was removed and plaques were counted on a light box. Virus neutralization (%) was calculated as [(# of plaques in virus only - # of plaques counted at mAb dilution)/# of plaques in virus only well] x100.

Antigen binding ELISA

The ability for the HSV-specific mAbs to bind to gD-2 was evaluated via an ELISA. Briefly, the wells of a high-binding 96 well plate were coated with 1 μg/mL gD-2 in sodium bicarbonate buffer pH 9.4 and incubated overnight at 4°C. The plates were washed 5x with 1x PBS, 0.1% BSA, 0.05% Tween 20 and blocked with 1x PBS with 2.5% BSA overnight at 4°C. The plates were washed 5x. Antibodies were serially diluted in 1x PBS with 0.1% BSA over a seven point 2-fold dilution curve (10.66 nM–0.16 nM), added to the plates, and incubated at room temperature for 1 h. The wells were washed 5x and incubated with 100 μL/well with an HRP-conjugated anti-human IgG Fc antibody (1:10000 dilution, Invitrogen) for 1 h. Wells were washed a final time before being incubated with 100 μL/well 1-step Ultra TMB (Invitrogen) for 5 min. The reaction was halted with 100 μL/well 1N H_2SO_4. The plate was read at 450 nm on a SpectraMax Paradigm Plate Reader (Molecular Devices). Buffer only wells were used as a control and the assay was performed in technical replicate.

Antibody-dependent cellular cytotoxicity (ADCC)

A CD16 activation reporter assay was performed as previously described.[77] Briefly, the wells of a high-binding 96 well plate were coated with 1 μg/mL recombinant gD-2 protein in PBS and incubated overnight at 4°C. The plate was washed 3x with 1x PBS with 0.01% Tween 20 and blocked at room temperature with 1x PBS with 2.5% BSA for 1 h. Antibodies were serially diluted in growth medium and added to the washed plate with 100,000 Jurkat Lucia NFAT CD16 cells/well (Invivogen). Antibodies and cells were incubated for 24 h at 37°C with 5% CO_2. A 25 μL volume of the cell supernatant was removed and added to a new, opaque white 96 well plate. A 75 μL volume of the QuantiLuc (Invivogen) substrate was added to the supernatant and luminescence was immediately read on SpectraMax Paradigm plate reader (Molecular Devices) using a 1 s integration time. A kinetic read time of 0, 2.5 and 5 min was performed, and the reported values are the averages of the three reads. Buffer only wells were used as negative controls and a cell stimulation cocktail with 2 μg/mL ionomycin was used as a positive control. The assay was performed in technical replicate.

Antibody-dependent cellular phagocytosis (ADCP)

Antibody-dependent cellular phagocytosis was performed as previously described[78] with slight modifications. Briefly, goat-anti human IgG F(ab')2 (Invitrogen) was covalently coupled to yellow-green carboxylate beads (Thermofisher). Antibodies were diluted in culture medium to a starting concentration of 133 nM and serially diluted 4-fold 7 times. Diluted mAbs were incubated with anti-human IgG beads for 2 h at 37°C to form immune complexes. THP-1 (ATCC) cells (25,000/well) were added to the immune complexes and incubated at 37°C for 4 h. Cells were washed 2x with cold 1x PBS prior to being fixed with 4% paraformaldehyde. The cells were analyzed on a NovoCyte Advanteon flow cytometer (Agilent) (Figure S2C). A phagocytosis score was calculated as the (percentage of FITC+ cells) x (the geometric mean fluorescence intensity (gMFI) of the FITC+ cells)/100,000. Buffer only wells were used as negative controls and the assay was performed in technical replicate with two biological replicates.

Engineering HEK293Ts expressing HSV-1 gD as a surface antigen

The gD gene was PCR amplified from HSV-1 strain 17 DNA.[73] The gene was cloned into pLenti-DsRed-IRES-EGFP vector (Addgene plasmid number 92194)[72] by restriction digestion using Afe1 and BamH1 (New England BioLabs (NEB)). Restriction digestion was followed by ligation using T4 DNA ligase (NEB). The ligated PCR product was transformed into NEB Stable Competent *E. coli* (High Efficiency). The gene insertion into the vector was confirmed by using restriction digestion by SgrA1 (NEB) and plasmid sequencing (Azenta LifeSciences). The sequence confirmed plasmid (transfer plasmid) and packaging vector (VSVG, PSPAX2) were used at concentrations of 6 μg, 0.6 μg and 5.4 μg to transfect HEK293T cells at 60% confluency in a T150 flask. Transfer plasmid and packaging vector were mixed with Opti-MEM (ThermoFisher Scientific). In a separate tube, Opti-MEM and 109.38 μg Polyethylenimine (PEI) was added. The DNA:Opti-MEM and PEI:Opti-MEM mixtures were combined and incubated together for 15 min at room temperature prior to being added to the HEK-293Ts. Media was replenished the next day (day 1). On day 2, the viral supernatant was collected and Lenti-X GoStix Plus (Takara) was used to test presence of lentiviral p24. The viral supernatant was filtered using 0.45-micron filter and aliquots were stored at −80°C.

Adherent HEK293T cells were trypsinized and 500,000 cells were mixed in 1 mL of thawed viral supernatant, to which 0.8 μg of polybrene (Santa Cruz Biotechnology) was added. The mixture was incubated in a 6 well plate at 37°C, 5% CO_2. On the next day, old media was removed and was replaced with 2 mL fresh media. At 4 days post transduction, GFP positive cells were sorted using cell sorter (Sony Biotechnology, MA900) using a 100-micron sorting chip (Sony Biotechnology) and cultured in media containing 1X penicillin/streptomycin. Non-transfected HEK293T cells were used as a negative control to set the sort gates (Figure S2).

Measurement of binding of antibody to HEK293Ts expressing HSV-1 gD as a surface antigen

HEK293T cells expressing HSV-1 gD as a surface antigen and non-transfected HEK293T cells (control) were washed twice with PBS. Cells (200,000/well) were added to a 96 well V bottom plate (USA Scientific). gD-specific antibodies were diluted to 20 μg/mL and serially diluted 4-fold in PBS +1% BSA before being added to the cells. After a 1-h incubation on ice, the cells were washed twice with PBS +1% BSA and stained with 10 μg/mL Alexa Fluor 647 Goat anti-Human IgG (H + L) Cross-Adsorbed Secondary Antibody (ThermoFisher Scientific) diluted in PBS with 1% BSA. After a 30 min incubation in the dark, cells were washed twice with PBS +1% BSA and were resuspended in 100 μL of PBS prior to fixation with 4% paraformaldehyde. The antibody binding was measured by checking signal intensity of Alexa Fluor 647 using a MACSQuant Analyzer (Miltenyi) (Figure S2B). The experiment had two biological replicates. The data was analyzed using FlowJo version 10.8.2.

Antibody dependent complement deposition

HEK293T cells expressing HSV-1 gD as surface antigen and non-transfected HEK293T cells (control) were washed twice with PBS. Cells (200,000/well) were added to a 96 well V bottom plate (USA Scientific). Antibodies were diluted to 20 μg/mL and serially diluted 4-fold in PBS +1% BSA before being added to the cells. After 45 min, the cells were washed with PBS +1% BSA, followed by a wash with Gelatin Veronal Buffer (GVB++) (Complement Technology Inc). Low-tox Guinea Pig complement (Cedarlane) was reconstituted in 1 mL cold distilled water, a 500 μL volume of which was added to 9.5 mL GVB++. Diluted guinea pig complement (100 μL) was then added to each well prior to incubation with orbital shaking for 1 h at 37°C, with 5% CO_2. The cells were then washed with PBS +1% BSA prior to staining with 100 μL of 1 μg/mL biotinylated goat anti-guinea pig C3 antibody (ICL labs) at room temperature for 1 h. The cells were washed twice with PBS +1% BSA prior to addition of 100 μL of 1 μg/mL Streptavidin-APC (ThermoFisher) and incubation for 1 h at room temperature. After the incubation, the cells were washed twice and resuspended in PBS +1% BSA. Antibody-dependent activation of complement protein C3 was measured using a MACSQuant Analyzer (Miltenyi) quantifying the mean fluorescence intensity of APC (Figure S2A). The assay was performed with two biological replicates. Heat-inactivated guinea pig complement was used as a control. For heat inactivation, the serum was heated at 58°C for 30 min. VRC01 antibody was used as a negative control. The data was analyzed using FlowJo version 10.8.2.

CD16 activation assay (ADCC)

HEK293T cells expressing HSV-1 gD as surface antigen and non-transfected HEK293T cells were washed 2x with PBS before being added to a V bottom plate (USA Scientific) (200,000 cells/well). Into the same plate, 100,000 cells/well of Jurkat Lucia NFAT CD16 cells (Invivogen) were added, along with 180 μL of assay media (RPMI 1640 + 10% FBS + 1mM sodium pyruvate + non-essential amino acids + penicillin/streptomycin) and 20 μL of diluted gD-specific antibodies (in PBS +1% BSA). The plate was incubated

overnight at 37°C, 5% CO_2. After overnight incubation, the cells were centrifuged and 25 μL of supernatant was drawn from each well and transferred into 96-well white walled clear bottom polystyrene plate (Costar) and mixed with 75 μL of reconstituted QUANTI-Luc reagent (InvivoGen). Luminescence was immediately read on a SpectraMax Paradigm Plate reader (Molecular Devices) using 1s integration time. Kinetic reads at 0 min, 2.5 min and 5 min were measured, and the mean reading was noted. Cell Simulation Cocktail (eBioscience) was used as positive control. VRC01 was used as negative control. The assay was performed with two biological replicates.

Study approval

Procedures were performed in accordance with Dartmouth's Center for Comparative Medicine and Research policies and following approval by the institutional animal care and use committee.

QUANTIFICATION AND STATISTICAL ANALYSIS

Prism 9 (GraphPad) software was used for statistical tests. For survival studies, HSV-specific mAbs were compared to isotype controls using the Log rank Mantel-Cox test to determine p values. HSV-specific Fc-competent and KO mAbs were also compared to each other using the Log rank Mantel-Cox test to determine p values. For imaging studies, groups and time points were compared to each other via two-way ANOVA, with Tukey's test for multiple comparisons to determine p values. For viral burden analysis, mAbs were compared to each other within each organ group via an ordinary two-way ANOVA with Bonferoni's test for multiple comparison. Within each organ, HSV-specific mAbs were compared to the isotype control mAb via a two-way ANOVA with Dunnet's test for multiple comparisons.