

1 Immunization by replication-competent controlled herpesvirus 2 vectors

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1 **ABSTRACT**

2 Replication-competent controlled virus vectors were derived from virulent HSV-1
3 wildtype strain 17syn+ by placing one or two replication-essential genes under the
4 stringent control of a gene switch that is co-activated by heat and an antiprogestin.
5 Upon activation of the gene switch, the vectors replicate in infected cells with an efficacy
6 that approaches that of the wildtype virus from which they were derived. Essentially no
7 replication occurs in the absence of activation. When administered to mice, localized
8 application of a transient heat treatment in the presence of systemic antiprogestin
9 results in efficient but limited virus replication at the site of administration. The
10 immunogenicity of these viral vectors was tested in a mouse footpad lethal challenge
11 model. Unactivated viral vectors - which may be regarded as equivalents of inactivated
12 vaccines - induced detectable protection against lethality caused by wildtype virus
13 challenge. Single activation of the viral vectors at the site of administration (rear
14 footpads) greatly enhanced protective immune responses, and second immunization
15 resulted in complete protection. Once activated vectors also induced far better
16 neutralizing antibody and HSV-1-specific T cells responses than unactivated vectors.
17 To find out whether the immunogenicity of a heterologous antigen was also enhanced in
18 the context of efficient transient vector replication, a virus vector constitutively
19 expressing an equine influenza virus hemagglutinin was constructed. Immunization of
20 mice with this recombinant induced detectable antibody-mediated neutralization of
21 equine influenza virus as well as a hemagglutinin-specific T cell response. Single
22 activation of viral replication resulted in a several-fold enhancement of this immune
23 response.

1 **IMPORTANCE**

2 We hypothesized that vigorous replication of a pathogen may be critical for eliciting the
3 most potent and balanced immune response against it. Hence, attenuation/inactivation
4 (as in conventional vaccines) should be avoided. Instead, necessary safety should be
5 provided by placing replication of the pathogen under stringent control and of activating
6 time-limited replication of the pathogen strictly in an administration region in which
7 pathology cannot develop. Immunization will then occur in the context of highly efficient
8 pathogen replication and uncompromised safety. We found that localized activation in
9 mice of efficient but limited replication of a replication-competent controlled herpesvirus
10 vector resulted in a greatly enhanced immune response to the virus or an expressed
11 heterologous antigen. This finding supports the above hypothesis as well as suggests
12 that the vectors may be promising novel agents worth exploring for the
13 prevention/mitigation of infectious diseases for which efficient vaccination is lacking, in
14 particular in immunocompromised patients.

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KEYWORDS

16 Herpesvirus vector, immunization, vaccination, regulation of viral genes, regulation of
17 viral replication, two-component gene switch.

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1 INTRODUCTION

2 Vaccination has been spectacularly successful, but there remain important infections for
3 which therapeutic or preventative vaccines either do not exist or are of insufficient
4 efficacy. One such example are infections caused by the herpes simplex viruses HSV-1
5 and HSV-2. Recent estimates have the world-wide prevalence of HSV-1 at 67% and of
6 HSV-2 at 11% (1,2). Perinatal herpes infection as well as infection in immune-
7 compromised persons can be life-threatening. HSV infection has many complications,
8 resulting in significant morbidity and even mortality. Currently, there is no vaccine for
9 treating or preventing HSV-1 or HSV-2 infections. There are many ongoing research
10 projects directed toward developing effective vaccines, of which some are being/have
11 been tested in the clinic (3,4), with GEN-003 (Genocera) (5), HSV529 (Sanofi) (6) and
12 HSV-2 0ΔNLS (Rational Vaccines) (7) receiving the most attention. Another example
13 are influenza infections. Annual infection rates for influenza are between 5 and 20%,
14 and the yearly death rate worldwide lies between 250,000 and 500,000 according to
15 WHO estimates. At this time, the vaccines remain seasonal, i.e., they need to be
16 updated/reformulated for every influenza season. Typical subunit vaccines are at least
17 trivalent, comprising hemagglutinins from H1N1 and H3N2 influenza A strains and an
18 influenza B strain (referred to as TIV). More recently, live attenuated influenza virus
19 vaccines (LAIV) were introduced. An updated systematic review and meta-analysis
20 published in 2012 documented a mean efficacy for TIV of 62% against RT-PCR or
21 virally confirmed influenza (all age groups) (8). Limited data were available for adults 65
22 years of age and older. Mean efficacy for LAIV in young children was higher, but little or
23 no protection was found in adults (18 years and older). A recent study reported a mean

1 overall vaccine efficacy of 48% for the 2015-2016 season, and lower efficacies for
2 persons 50 years of age and older (9). LAIV was ineffective in this season, even in
3 children. Notable is a study on young children that was carried on over two seasons in
4 both of which there was a good match between vaccine (TIV) and circulating strains
5 (10). Vaccine efficacy was 66% in the first season and -7% in the second season. Thus,
6 currently available influenza vaccines provide moderate protection against virologically
7 confirmed disease, but protection is not long-lasting and is especially tenuous in the
8 senior population. Ongoing preclinical and clinical work to develop better influenza
9 vaccines includes formulation of adjuvanted vaccines, MVA-vectored T cell vaccines
10 expressing conserved viral proteins, and hemagglutinin stalk-based vaccines for
11 obtaining broadly protective antibody responses (11-13). A third example concerns
12 *Mycobacterium tuberculosis* infections that affect about one third of the world population
13 with yearly death rates of over one million. Of concern in the industrialized world is the
14 rise of drug-resistant forms of the disease (14). The only vaccine in use is the (live)
15 Bacillus Calmette-Guérin (BCG) vaccine developed in 1921 whose efficacy appears to
16 be variable in adults. Candidate vaccines currently under development include virally
17 vectored vaccines presenting BCG antigens, adjuvanted subunit vaccines and whole-
18 cell vaccines based on *M. tuberculosis* or closely related mycobacterial species (15-17).
19 Further examples include malaria and HIV/AIDS. As these examples reveal, adequate
20 protection against many important infections is not yet available. Research continues,
21 and new immunization approaches appear to be urgently needed.

22 The question whether live attenuated vaccines that have retained some capacity for
23 replication provide more robust protective immunity than inactivated non-replicating

1 vaccines was already debated more than 50 years ago. In the case of poliomyelitis
2 vaccines, i.e., the live attenuated vaccine of Sabin vs. the inactivated vaccine of Salk,
3 and also in the case of measles vaccines, a live attenuated vaccine based on the
4 Edmonston B strain vs. an inactivated vaccine, the live attenuated vaccines eventually
5 gained the upper hand (although safety concerns result in the predominant use of the
6 Salk vaccine in regions in which the disease is considered eradicated) (18-21). It is
7 noted that the latter historical debates were not conclusive because the vaccines
8 compared differed in more than just their capacity to replicate (different routes of
9 administration, modification of surface antigens by the inactivation process, etc.).
10 Several more recent studies readdressed the issue in a more rigorous fashion. One
11 such study compared immune responses to an HIV envelope antigen expressed from
12 an attenuated replicating and a replication-defective adenovirus vector in a chimpanzee
13 model (22). Inoculation with the replicating recombinant resulted in a greater frequency
14 of HIV envelope-specific interferon-gamma-secreting peripheral blood lymphocytes,
15 better priming of T cell proliferative responses, higher anti-envelope binding and
16 neutralizing antibody titers and better antibody-dependent cellular cytotoxicity. In
17 another study, an attenuated vaccinia virus (MVTT) expressing the spike protein (S) of
18 SARS-CoV (MVTT-S) was compared with a replication-defective vaccinia virus (MVA)
19 expressing the same protein (MVA-S). Intranasal or intraoral vaccination of mice with
20 MVTT-S produced 20-100-fold higher neutralizing antibody levels than MVA-S (23,24).
21 Yet another study employed a mouse ocular challenge model to demonstrate that an
22 attenuated replicating HSV-1 strain (ICP0⁻) but not a replication-defective HSV-1 strain
23 (ICP4⁻) elicited a protective immune response (25). These findings support the theory

1 that attenuated replicating viruses induce more complete and more potent immune
2 responses to autologous or heterologous antigens than corresponding non-replicating
3 viruses.

4 We hypothesized that a virus vector that could replicate in a controlled fashion with
5 nearly the same efficiency as the respective wildtype virus (referred to herein as
6 “replication-competent controlled virus vector”) would induce an even more potent and
7 complete immune response to itself or an expressed heterologous antigen than a
8 corresponding attenuated vector (26). Our hypothesis is in part based on the rational
9 expectation that an efficiently replicating virus will produce a stronger inflammatory
10 response than an attenuated virus, which inflammatory response will result in a potent
11 activation of the innate immune system and, consequently, in strong and lasting B and T
12 cell responses (27). To realize such an immunization strategy, a regulation system must
13 be employed that reliably and stringently controls viral replication as well as is capable
14 of being turned on and off at will. However, virus disseminates after administration.
15 Simply restricting the number of replication cycles will not be enough: depending on the
16 number of cycles allowed, there will be a more or less pronounced manifestation of the
17 toxicity typical for the viral vector used. For example, allowing an HSV-1 vector to
18 replicate for a certain period of time may cause unacceptable neurotoxicity. Hence, the
19 regulation system must be capable of exerting regional control over viral replication so
20 that the immunizing virus only replicates in a locale in which it is certain not to cause a
21 disease phenotype.

22 To facilitate construction of a replication-competent controlled virus vector, advantage
23 may be taken of gene switches developed for eukaryotic cells (28). This choice implies

1 that the backbone virus must be selected from the group of viruses having a double-
2 stranded DNA genome (e.g., herpes viruses) or producing a double-stranded DNA
3 intermediate during replication and utilizing the host machinery for transcription. It
4 appears that the above-described requirements are best met by promoters of certain
5 heat shock (HSP) genes that are stringently controlled by reversibly heat-activated
6 endogenous transcription factor HSF1. A preferred choice may be the promoter of the
7 human HSP70B (HSPA7) gene, which promoter has been found to be highly inducible
8 and to exhibit very low basal expression wherever this has been tested (28-30). A virus
9 in which one or, better, two replication-essential genes are placed under the control of
10 this promoter could be activated to undergo one round of localized replication by
11 administration of a mild heat dose to the inoculation region. Further rounds may be
12 induced by repeat heat treatment. However, virus replication may be inefficient because
13 heat activation of HSP promoters is rapidly reversible as well as may be unsafe
14 because unintended weak activation could be triggered by high fever, intoxication, etc.,
15 in an inoculated subject. We previously described a two-component HSP promoter-
16 based gene switch that lacks the latter unwanted properties (31) (Fig. 1A). The first
17 component comprises a gene for a small-molecule regulator (SMR)-activated
18 transactivator (TA). The TA gene is controlled by a promoter cassette containing an
19 HSP70B promoter and a TA-responsive promoter (TRP). The second component is a
20 TRP promoter for driving a selected viral gene (VG). Heat treatment prompts expression
21 of the TA that is inactive unless activated by its SMR. Activated TA mediates expression
22 of the viral gene as well as autoactivates its own expression. The gene switch is
23 inactivated by withdrawal of the SMR. The present report presents a first study of

1 immune responses induced by replication-competent controlled HSV-1 vectors in which
2 one or two replication-essential genes are subjected to regulation by such a heat- and
3 SMR-dependent gene switch.

4 **RESULTS**

5 **Replication-competent controlled HSV-1 vectors.** The two-component HSP
6 promoter-based gene switch employed in the vectors of the present study relied on
7 transactivator GLP65 that is activated by a narrow class of antiprogestins including
8 mifepristone and ulipristal (31-34). GLP65 is a chimeric protein consisting of a DNA-
9 binding domain from yeast transcription factor GAL4, a truncated ligand-binding domain
10 from a human progesterone receptor and a transcriptional activation domain from
11 human NFkappaB protein P65. A DNA segment containing a promoter cassette
12 consisting of a human HSP70B promoter and a GAL4-binding site-containing (GAL4-
13 responsive) promoter and a functionally linked GLP65 gene was inserted in between the
14 UL43 and UL44 genes of HSV-1 wildtype strain 17syn+. This intermediate vector was
15 utilized for the construction of vectors HSV-GS3 and HSV-GS7. In vector HSV-GS3,
16 replication-essential immediate early gene RS1/ICP4 and early gene UL29/ICP8 had
17 been placed under gene switch control by replacing the resident ICP4 and ICP8
18 promoters in the latter intermediate vector with GAL4-responsive promoters (Fig. 1B). In
19 vector HSV-GS7, the replication-essential late gene UL38/VP19c had been subjected to
20 gene switch control by substitution of its promoter with a GAL4-responsive promoter.

21 Replication of HSV-GS3 had previously been shown to be strictly dependent on
22 activation by heat and antiprogestin in single-step growth experiments (34). When

1 activated, the recombinant replicated nearly as efficiently as wildtype virus 17syn+.
2 When administered to footpads of mice, HSV-GS3 only replicated subsequent to an
3 activating treatment by locally administered heat in the presence of systemic
4 antiprogesterone. The results of a single-step growth experiment with HSV-GS7 are shown
5 in Fig. 1C. The recombinant replicated similarly well as HSV-GS3 following heat
6 treatment in the presence of antiprogesterone ulipristal. No replication was observed after
7 either heat or antiprogesterone treatment, or in the absence of any treatment. To verify that
8 HSV-GS7 replication was also tightly controlled *in vivo*, two of three groups of mice
9 were administered HSV-GS7 virus (50,000 pfu per mouse) to the footpad, and the mice
10 of one of the latter groups were given ulipristal intraperitoneally (i.p.) as well as, 3 h
11 later, were subjected to a heat treatment to the footpads at 45°C for 10 min. One day
12 later, all mice were euthanized, and DNA and RNA were extracted from their feet and
13 analyzed by qPCR and RT-qPCR, respectively. Substantially larger amounts of HSV-1
14 DNA were detected in the feet of heat/ulipristal-treated mice than in not-treated mice
15 (Fig. 1D). Expression of several viral genes was observed for activated virus but not for
16 unactivated virus, strongly suggesting that viral replication and gene expression only
17 occurred subsequent to heat/ulipristal activation.

18 It is noted that stocks of the recombinant viruses were prepared in cells that were
19 subjected to daily heat treatment in the presence of antiprogesterone until maximal
20 cytopathic effect was reached. Titrations of the viruses were carried out on cells that
21 provided missing proteins in *trans*.

1 **Protective immunity induced by activated HSV-GS3.** Induction of protective
2 immunity was evaluated in a mouse footpad lethal challenge model (35). In a first
3 experiment, virus vectors were administered under anesthesia to the plantar surfaces of
4 both rear feet of adult Swiss Webster outbred female mice (50,000 pfu per animal; 20
5 animals per group). Concurrently, and again 24 h later, the animals of one group
6 received an intraperitoneal injection of 0.5 mg/kg of mifepristone. Three hours after
7 inoculation, the mice of the latter group were subjected to heat treatment (43.5^oC for 30
8 min) by immersion of their hindfeet in a temperature-controlled water bath. Twenty-two
9 days later, all animals were challenged by a 20-fold lethal dose of HSV-1 wildtype strain
10 17syn+ administered by the same route as the original virus inoculum. Survival of the
11 animals was followed until no more lethal endpoints were reached, i.e., until all surviving
12 animals had fully recovered (Fig. 2). ICP4(-) replication-incompetent HSV-1
13 recombinant KD6 (36) induced a modest level of immunity. As had been expected,
14 because it did not replicate and also did not express the major transcriptional regulator
15 ICP4, unactivated HSV-GS3 provided a comparable degree of protection. Activated
16 HSV-GS3 produced a far greater protective effect. Therefore, one round of efficient
17 replication and/or expression of master regulator ICP4 resulting in expression of all viral
18 proteins produced a dramatic enhancement of protective immunity. It is noted that the
19 heat and antiprogestin treatment had no systematic effect on the level of protection
20 induced by replication-defective virus KD6 (see under “Statistical evaluation of data
21 from all comparable experiments”).

22 To determine whether immunization with activated HSV-GS3 reduced replication of the
23 challenge virus more effectively than unactivated HSV-GS3 or replication-defective KD6

1 virus, additional groups of animals (5 animals per group) were immunized and
2 challenged by wildtype virus as described above. Four days after challenge, the animals
3 were euthanized, feet were dissected and homogenized, and virus present in the
4 homogenates was titrated. Results revealed that activated HSV-GS3 virus reduced
5 challenge virus replication by nearly two orders of magnitude (Table 1). Unactivated
6 recombinant virus or KD6 were far less effective.

7 We next examined in a similar experiment whether induction of enhanced protective
8 immunity was caused by an activation of the heat- and antiprogestin-dependent gene
9 switch that controls the expression of ICP4 and ICP8 in the HSV-GS3 recombinant. In
10 this and all subsequent experiments, ulipristal was utilized instead of mifepristone,
11 because the former compound is a more active gene switch co-activator than the latter
12 compound (34). Furthermore, the heat treatment regimen was changed to heat
13 exposure at 45⁰C for 10 min to achieve greater reproducibility between experiments.
14 Results revealed that enhanced protection was only achieved following activation of
15 HSV-GS3 by heat treatment in the presence of ulipristal (Fig. 3). Heat treatment alone
16 or ulipristal alone did not significantly increase the protective effect over that produced
17 by the unactivated virus. These findings strongly suggested that it was the activation of
18 the dual-responsive gene switch in HSV-GS3 which prompted the expression of the
19 regulated replication-essential genes as well as efficient virus replication that in turn
20 resulted in the observed enhanced protective response. It is noted that in the latter
21 experiment the virus inoculum was 500,000 pfu rather than 50,000 pfu as in most other
22 experiments. This may account for the somewhat elevated level of protection induced
23 by unactivated HSV-GS3.

1 **HSV-GS3 and HSV-GS7 induce comparable protective responses.** In recombinant
2 HSV-GS3, immediate early gene ICP4 and early gene ICP8 are subject to dual-
3 responsive gene switch control. The greatly enhanced protective response that resulted
4 from transient activation of the gene switch in HSV-GS3-immunized animals could have
5 been due to the efficient virus replication triggered by the activation and/or to the
6 activation of the genes for major transcription regulator ICP4 that in turn stimulated
7 expression of all other viral genes in primarily infected cells. Another issue related to
8 viral gene expression in cells that were secondarily (nonproductively) infected by HSV-
9 GS3. Since the ICP4 genes are regulated in the recombinant virus, little expression of
10 viral genes was expected to occur in the absence of another activating treatment.
11 Abundant viral gene expression in secondarily infected cells (that may include antigen-
12 presenting cells) conceivably may further enhance immune responses. Recombinant
13 HSV-GS7 in which the late VP19c gene had been subjected to regulation had been
14 constructed to examine these issues. HSV-GS3 and HSV-GS7 were compared in the
15 mouse footpad challenge model. Results revealed that activated HSV-GS3 and HSV-
16 GS7 provided similar levels of protection (Fig. 4). In the absence of activation, the
17 protective effect of either recombinant was modest. Taking data from other experiments
18 into account, the average protective effect for unactivated HSV-GS3 was 22% and that
19 for unactivated HSV-GS7 was 13% (see under “Statistical evaluation of data from all
20 comparable experiments”). The fact that the two recombinants which lacked expression
21 of different proteins in the absence of activation induced comparable (modest)
22 protective responses strongly suggested that it was not the absence of one or the other
23 viral protein that limited the immune response to the recombinants. It was indeed the

1 vigorous replication of the activated recombinants that enhanced the protective
2 responses against them. In secondarily infected cells (in which HSF1 is not activated),
3 HSV-GS7 was expected to express all viral proteins except the regulated capsid protein
4 at approximately normal levels. In contrast, HSV-GS3 all but lacks ICP4 expression in
5 the absence of activation and exhibits dramatically curtailed expression of other viral
6 proteins (34). The finding that HSV-GS3 and HSV-GS7 induced comparable protective
7 responses suggested that viral proteins expressed in secondarily infected cells did not
8 contribute in a relevant fashion to the immune response. However, we cannot exclude
9 the possibility that the immune system was sufficiently sensitive to detect the very low
10 levels of viral antigen expression occurring in cells secondarily infected with HSV-GS3.

11 **Second immunization further enhances protective immunity.** We next investigated
12 whether a second activation treatment applied two days after the first treatment (i.e., at
13 a time at which the initial round of replication of HSV-GS7 should have been
14 completed), or a second immunization would further enhance protection against
15 wildtype virus challenge. The second immunization was administered three weeks after
16 the initial virus application. Booster virus was either activated or left unactivated. In the
17 part of an experiment reported on in Fig. 5A, groups of mice were immunized with
18 50,000 pfu/animal of recombinant HSV-GS7. Activation of the virus induced an immune
19 response that protected 75% of animals after challenge with wildtype virus. After second
20 immunization with a similar dose of HSV-GS7, all animals were protected. However, this
21 only occurred if the booster virus was also subjected to an activating treatment. Second
22 activation had no apparent effect. As effects of re-immunization may be more apparent
23 in mice immunized with lower quantities of virus, the experiment also featured parallel

1 groups of animals that were immunized and boosted with 5,000 pfu/animal of
2 recombinant HSV-GS7. As Fig. 5B documents, second immunization followed by virus
3 activation had an even more pronounced effect at the 10-fold reduced virus doses. It is
4 noted that in this part of the experiment second activation appeared to modestly
5 enhance the protective immune response.

6 **Statistical evaluation of data from all comparable experiments.** Relevant data from
7 all immunization/challenge experiments that were conducted using essentially identical
8 protocols are assembled in Table 2. Results of a meta-analysis of the HSV-GS7 data
9 are presented graphically in Fig. 6. The difference in immunization efficacy between
10 activated and unactivated HSV-GS7 was found to be highly significant. Also significant
11 was the increase in protection afforded by second immunization with an activated HSV-
12 GS7 vector. The effect of second activation two days after virus administration and first
13 activation was not statistically relevant. Second activation tended to modestly enhance
14 protective immunity in a majority of experiments in which this was addressed. However,
15 in some experiments, e.g., in the experiment reported in Fig. 5A, an effect was not
16 apparent. It is noted that the conditions for second activation had not been optimized.

17 **Immune correlates of protection against challenge.** The presence and levels of
18 antibodies capable of neutralizing HSV-1 strain 17syn+ were assessed in sera of mice
19 that had been treated with activated or unactivated HSV-GS vectors, or with replication-
20 incompetent virus KD6 (all at 50,000 pfu per animal) three weeks earlier. As expected,
21 neutralizing antibodies could be detected subsequent to immunization with KD6 or
22 unactivated HSV-GS viruses (Fig. 7A). Antibody levels in sera from KD6-immunized and
23 unactivated HSV-GS vector-immunized animals were comparable. Both activated HSV-

1 GS3 and HSV-GS7 induced clearly superior neutralizing antibody responses when
2 compared with unactivated HSV-GS3 or HSV-GS7, or KD6. To assess cellular immune
3 responses, HSV-1-specific T cells present in peripheral blood mononuclear cells
4 (PBMCs) of mice that had been immunized three weeks prior were quantified by a
5 modified limiting dilution lymphoproliferation assay. Both unactivated HSV-GS vectors
6 and KD6 virus were found capable of inducing detectable levels of HSV-1-specific T
7 cells (Fig. 7B). Substantially better responses were produced by activated HSV-GS3
8 and HSV-GS7.

9 **Immune responses to a heterologous antigen presented in the context of**
10 **vigorous viral replication.** To evaluate immune responses to a heterologous antigen
11 expressed from a HSV-GS vector, recombinant HSV-GS11 was constructed. The
12 recombinant was derived from HSV-GS3 by the insertion in the UL37/UL38 intergenic
13 region of an expression cassette comprising a gene for an equine influenza
14 hemagglutinin (EIV Prague/56 HA) functionally linked to a CMV (immediate early)
15 promoter (Fig. 8A). Expression of the influenza gene was verified in groups of adult
16 female BALB/c mice inoculated on the rear footpads with either saline, HSV-GS3 or
17 HSV-GS11 (50,000 pfu per animal). The HSV-GS3 group as well as one of the HSV-
18 GS7 groups were subjected to activation treatment. Another HSV-GS7 group underwent
19 an activation treatment twice, the second treatment administered two days after the first
20 treatment. One day after the last treatment, animals were euthanized, and RNA was
21 extracted from one hindfoot and protein from the other. Results from an RT-qPCR
22 analysis of HA gene expression are shown in Fig. 8B, and results from an EIV
23 Prague/56 HA-specific ELISA in Fig 8C. HA RNA and HA could be detected in samples

1 from HSV-GS11-inoculated animals, most abundantly when the animals had been
2 subjected to activation treatment. Both HA RNA and protein levels appeared to be
3 somewhat higher in twice-activated animals than in once-activated animals.

4 To assess immune responses (three weeks after immunization), additional groups of
5 mice were inoculated with saline (mock immunization), or with 50,000 pfu of HSV-GS3
6 or HSV-GS11 (three groups). As in the above-described part of the experiment, all
7 animals in the HSV-GS3 group as well as in two of the HSV-GS11 groups were
8 subjected to an activation treatment. The animals of one of the latter groups received a
9 second activation treatment two days later. Serum samples were tested for their ability
10 to neutralize EIV Prague/56. As expected, neutralizing antibodies were not detected in
11 unimmunized (not shown), mock-immunized or vector-immunized animals (Fig. 8D).
12 Unactivated HSV-GS11 was capable of inducing a neutralizing antibody response.
13 Activation of HSV-GS11 shortly after inoculation resulted in a several-fold magnified
14 response. It is noted that twice-activated HSV-GS11 elicited an only marginally better
15 response than once-activated virus. HA-specific T cells present in PBMCs were
16 quantified by the same type of responder cell frequency assay that had been used to
17 assess numbers of HSV-1-specific T cells. HA-specific T cells were not detected in
18 unimmunized (not shown) or mock-immunized animals (Fig. 8E). Induction of a T cell
19 response was observed in animals immunized with HSV-GS11 but not subjected to an
20 activation treatment. Activated vector (HSV-GS3) produced a similar response. Far
21 greater numbers of HA-specific T cells were found in animals immunized with once or
22 twice activated HSV-GS11.

23

1 **DISCUSSION**

2 Once-activated replication-competent controlled HSV-1 recombinants HSV-GS3 and
3 HSV-GS7 induced considerably more potent protective immune responses than
4 replication-defective HSV-1 strain KD6. Twice immunized animals were completely
5 protected against lethal challenge. Levels of partial protection engendered by
6 unactivated HSV-GS3, unactivated HSV-GS7 and KD6 (lacking expression of different
7 viral proteins) were comparable, which strongly suggests that the much-enhanced
8 protection provided by the activated HSV-GS recombinants was a consequence of their
9 ability to efficiently replicate. Both better neutralizing antibody and better T cell
10 responses were induced by the activated HSV-GS vectors, indicating that the replicating
11 vectors broadly enhanced immune responses and not only boosted cellular immunity.
12 Generalizing these findings, it appears that replication-competent controlled vectors are
13 promising new agents that may better protect against diseases caused by the viruses
14 from which they were derived than replication-incompetent/inactivated vaccines and,
15 possibly, also live attenuated vaccines. What is perhaps even more important, our
16 observations suggest that replication-competent controlled vectors can be excellent
17 immunization platforms that may be exploited for the elaboration of new “vaccines”.
18 Expression of an influenza antigen induced far more potent neutralizing antibody and
19 antigen-specific T cell responses when the antigen was expressed in the context of
20 vector replication than in the absence of vector replication. To the extent that such a
21 comparison is valid, neutralizing antibody responses as well as antigen-specific T cell
22 responses against the influenza antigen elicited by activated HSV-GS11 were both
23 stronger than the corresponding responses against vector antigens.

1 A second activation treatment administered two days after the initial activation of a
2 replication-competent controlled HSV-1 vector did not consistently enhance the
3 protective immune response against challenge by the wildtype virus from which the
4 vector was derived. Also, humoral and cellular immune responses against an influenza
5 virus antigen expressed from a replication-competent controlled HSV-1 vector were only
6 marginally enhanced by a second activation treatment. At this time, we cannot
7 distinguish the possibilities that the second activation was only marginally effective
8 because the immune system was already maximally engaged by the initial immunization
9 or because optimal conditions for the second activation treatment had not been
10 identified. Additional research is required to resolve this question.

11 Immunization by replication-competent controlled vectors represents a novel paradigm
12 that may be elaborated in various ways. Therefore, the work presented herein should be
13 regarded as a first illustration of the principle. In general, any of a number of DNA
14 viruses including members of all herpesvirus subfamilies may be employed for the
15 construction of replication-competent controlled vectors. We selected a fully virulent
16 HSV-1 strain as the backbone of the GS vectors for several reasons, including that HSV
17 have a double-stranded DNA genome that does not integrate in the host genome and
18 are tolerant of sizeable insertions, support stable expression of heterologous genes,
19 replicate and lyse cells efficiently as well as respond to antiviral drugs (37-40). Although
20 HSV are endemic in the human population, available data indicate that pre-existing
21 immunity is not an obstacle to vaccination/immunization uses of HSV vectors
22 (discussed in ref. 34). Furthermore, latent infection was considered a manageable issue
23 because replication of replication-competent controlled vectors can be stringently

1 controlled, and reactivation should not be capable of occurring in the absence of
2 activation.

3 Regarding the two-component HSP promoter-based gene switch that is used for
4 controlling viral replication, several ligand-dependent transactivators are known that
5 may be considered for incorporation in such a gene switch. They include transactivators
6 that are derived from the bacterial tetracycline repressor or other bacterial repressors as
7 well as chimeric transactivators that exploit ligand-binding domains of various steroid
8 receptors, e.g., human progesterone, human estrogen or insect ecdysone receptors, or
9 are activated by dimerization (28). We previously constructed and analyzed two-
10 component HSP promoter-based gene switches that are co-activated by antiprogestins,
11 ecdysteroid ponasterone A and rapamycin/rapalog, respectively (31,41). For regulating
12 the HSV-GS vectors, we made use of a heat- and antiprogestin-activated gene switch
13 that is strictly dependent on both heat and antiprogestin. While it has been more
14 thoroughly characterized than any other (31), this gene switch also recommends itself
15 for several other reasons. It comprises transactivator GLP65 that is activated by a
16 narrow spectrum of antiprogestins but not progestins (34). Since it only includes a
17 truncated ligand-binding domain of a progesterone receptor, GLP65 should not be
18 susceptible to ligand-independent activation; in fact, after many years of experimental
19 use, no antiprogestin-independent activation has been reported. At the low doses at
20 which they activate GLP65, antiprogestins such as mifepristone and ulipristal are not
21 expected to have any negative effects on the immune system (26). The latter
22 antiprogestins also have been safety-tested in human subjects as well as are readily
23 available. They are not considered essential active ingredients by the WHO, and their

1 primary use has been in emergency contraception. To maintain maximal safety in a
2 human immunization scenario, i.e., to preclude any possibility of reactivation from
3 latency, antiprogestins should not be administered subsequent to immunization with a
4 HSV-GS-type vector. Therefore, the vectors may not be employed for immunization of
5 healthy female subjects of reproductive age or younger, or the subjects may forego later
6 use of antiprogestins for emergency contraception. In contrast, the HSV-GS vectors
7 may be well suited as immunization agents or platforms in middle-aged or elderly
8 subjects. It is noted that it may be feasible to disable replication of fully activated HSV-
9 GS vectors in latently infected cells through further engineering.

10 Replication-competent controlled virus vectors may be capable of making a unique
11 contribution to immunization of immunocompromised patients. A recent study estimated
12 that in 2012 over 3% of the adult populations of the United States and the United
13 Kingdom were immunocompromised (42). Frequencies in other upper-income countries
14 are expected to be comparable. These patients are particularly vulnerable to vaccine-
15 preventable disease and would greatly benefit from effective vaccination. Owing to their
16 condition, their immune response to inactivated or subunit vaccines may be inadequate.
17 Unfortunately, use of live attenuated vaccines that may produce a more balanced and
18 more persistent immune response is problematic because of the perceived danger that
19 such vaccines may be insufficiently controlled by the weakened immune system and
20 may cause the disease they are intended to prevent. According to most relevant
21 guidelines, live attenuated vaccines are contraindicated in patients under
22 immunosuppressive therapy or HIV patients with low CD4 count (43). Hence, use of
23 currently marketed vaccines against tuberculosis (BCG), measles, mumps and rubella,

1 varicella-zoster, rotavirus, influenza (nasal) and yellow fever is universally discouraged.
2 Newer more effective vaccines under development, e.g., new pertussis vaccine BPZE1
3 (44), also may not become available to the immunocompromised. Replication-
4 competent controlled virus vectors expressing antigens of the latter microbes in the
5 context of efficient viral replication can be expected to elicit similarly or more potent
6 immune reactions as the contraindicated live attenuated vaccines. Escape from immune
7 control will not be an issue because expression of replication-essential genes is under
8 stringent dual control. Hence, replication of the vectors can be activated and safely
9 deactivated independent of the immune status of a patient.

10 In this report, we have focused on immunization effects of once-activated replication-
11 competent controlled vectors that undergo one round of replication. We note that viral
12 vectors that were intended to be limited to one cycle of replication were advanced
13 before as potential vaccines (45). These so-called disabled infectious single-cycle
14 (DISC) viruses typically were deleted for the gene for glycoprotein H which is critical for
15 infection. Juxtaposing DISC viruses and replication-competent controlled viruses, the
16 latter viruses replicate nearly as efficiently as the wildtype virus that had been used for
17 their construction. No comparable information is available for the DISC viruses that
18 were advanced as vaccine candidates. Replication of DISC viruses is not localized but
19 occurs throughout the host. Depending on the dose of virus administered, a single
20 round of replication of disseminated virus can be expected to result in significant
21 toxicity. Not surprisingly, intracranial administration of DISC virus TA-HSV resulted in
22 neurotoxicity that was only about two orders of magnitude lower than that of the
23 wildtype virus from which the strain was derived (46). Replication-competent controlled

1 viruses should be devoid of such toxicity because they are virtually incapable of
2 replicating unless deliberately activated. It is noted that the stringency of the replication
3 block can be increased further by subjecting additional replication-essential genes to
4 gene switch control. Furthermore, even though progeny virus produced by a DISC virus
5 upon immunization is not supposed to be capable of re-infecting cells, latent infection
6 has been observed (46). Latent infection is known to involve spread from cell to cell
7 (47). Hence, as has been pointed out before (48), DISC viruses such as TA-HSV
8 appear to be leaky for replication *in vivo*. The above properties of DISC viruses are not
9 desirable in an immunization agent or vector administered to healthy people and would
10 disqualify such viruses from use in persons with impaired immune systems. It is noted
11 that a clinical trial was conducted that investigated a DISC virus as a therapeutic
12 vaccine against recurrent genital herpes (49). Results were negative. The DISC virus
13 (TA-HSV) studied had been derived from a minimally virulent HSV-2 wildtype strain
14 (46,50). Moreover, the virus was administered at a relatively low dose, apparently to
15 avoid toxicity. Failure of the trial should not be very surprising.

16 Unlike a conventional vaccine, a replication-competent controlled vector will need to be
17 activated after administration to a subject to be immunized. Activation will need to be
18 neither costly nor inconvenient. A single ulipristal dose can be administered orally at the
19 time of immunization. Regarding heat treatment, we have developed small pads that
20 conduction-heat the inoculation region by crystallization of a supercooled salt solution
21 (51). The pads are capable of maintaining a temperature of 45⁰C for 15 min. This heat
22 dose was shown to be sufficient for strong activation in all layers of the skin of human

- 1 subjects of the HSP70B promoter, which is the promoter used in the two-component
- 2 gene switch that controls the HSV-GS vectors.

3

4

1 MATERIALS AND METHODS

2 **Cells, plasmids and viruses.** Rabbit skin (RS) cells were a gift from E. Wagner and
3 were propagated in minimal essential medium Eagle's salts (MEM) (Life
4 Technologies/Thermo Fisher Scientific) supplemented with 5% heat-inactivated calf
5 serum (Atlanta Biologicals, Lawrenceville, GA), 292 mg/ml L-glutamine, 250 U/ml of
6 penicillin and 250 µg/ml of streptomycin (Life Technologies). Vero cells (procured from
7 the American Type Culture Collection (ATCC) and Vero-derived E5 cells (52) (obtained
8 from N. DeLuca) were propagated in Dulbecco's modified Eagle's medium (DMEM) with
9 10% heat-inactivated fetal calf serum, 250 U/ml of penicillin and 250 µg/ml of
10 streptomycin. HSV-1 strain 17syn+ and ICP4 deletion strain KD6 (36) were obtained
11 from J. Stevens. It is noted that stocks of KD6, HSV-GS3 and HSV-GS7 were
12 propagated on E5 cells. For HSV-GS3 and HSV-GS7, medium was supplemented with
13 10 nM mifepristone, and infected cultures were subjected to daily heat treatment at
14 43.5°C for 30 min for three successive days. EIV Prague/56 was a gift of T. Lengsfeld
15 and was propagated on Madin-Darby canine kidney (MDCK) cells (ATCC) in DMEM
16 supplemented with 10% heat-inactivated horse serum as previously described (53).
17 Virus stocks of EIV were titrated using TCID50 assays in MDCK cells. All cells were
18 cultured at 37°C under 5% CO₂.

19 **Chemical reagents.** Mifepristone was obtained from Sigma-Aldrich, and ulipristal
20 acetate was from D-Innovation Pharmaceutical Inc., Chengdu, Sichuan, PR China.
21 Both compounds were USP grade.

1 **Construction of viruses.** All viruses were constructed using wild type HSV-1 strain
2 17syn+ as the backbone. Viral recombinants were generated by homologous
3 recombination of engineered plasmids along with purified virion DNA in RS cells
4 transfected by the calcium phosphate precipitation method as previously described (40).
5 Plasmids used to engineer the insertions of the transactivator cassette or the GAL4-
6 responsive promoters comprised HSV-1 sequences cloned from strain 17syn+. HSV-
7 GS3 (Fig. 1B) was constructed as previously described (3,34). HSV-GS7 was derived
8 from intermediate vector HSV-17GS43 (34) that contains an insertion between the UL43
9 and UL44 genes of a transactivator cassette containing a GLP65 gene under the control
10 of a promoter cassette that combined a human HSP70B promoter and a GAL4-
11 responsive promoter (31). To place the UL38/VP19c gene under regulation of a GAL4-
12 responsive promoter, plasmid pBS-KS:GAL4-UL38 was constructed. This plasmid
13 contains a GAL4 promoter inserted in between the HSV-1 UL38 recombination arms of
14 plasmid pBS-KS:UL38 Δ promoter. A GAL4-responsive promoter comprising six copies of
15 the yeast GAL4 UAS (upstream activating sequence), the adenovirus E1b TATA
16 sequence and the synthetic intron lvs8 was excised from plasmid pGene/v5-HisA
17 (Invitrogen, now Life Technologies) with AatII and HindIII, and the resulting 473 bp
18 fragment was gel-purified. For the vector, pBS-KS:UL38 Δ promoter was digested with
19 AatII and HindIII, and the resulting 4285 bp fragment was gel-purified and shrimp
20 alkaline phosphatase-treated. Ligation of the latter two fragments placed the GAL4
21 promoter in front of the UL38 transcriptional start site. To produce recombinant HSV-
22 GS7, RS cells were co-transfected with plasmid pBS-KS:GAL4-UL38 and purified HSV-
23 17GS43 virion DNA. Subsequent to the addition of mifepristone to the medium, the co-

1 transfected cells were exposed to 43.5⁰C for 30 min and then incubated at 37⁰C.
2 Subsequently, on days 2 and 3, the cells were again incubated at 43.5⁰C for 30 min and
3 then returned to 37⁰C. Picking and amplification of plaques, screening and plaque
4 purification was performed essentially as described for HSV-GS3 (3,34). The resulting
5 plaque-purified HSV-GS7 was verified by Southern blot as well as by PCR and DNA
6 sequence analysis of the recombination junctions. Plasmid pBS-KS:UL38 Δ promoter
7 was constructed by deletion of the region from -1 to -47 of the UL38 promoter, i.e., by
8 synthesizing two PCR fragments (one 437 bp and the other 550 bp) on either side of the
9 deletion and cloning these into pBS KS+. HSV-GS11 was derived from the vector HSV-
10 GS3 (34) and contains an insertion between the UL37 and UL38 genes of a gene
11 cassette expressing the EIV Prague/56 HA gene driven by the CMV IE promoter. A
12 recombination plasmid was constructed by the following sequential steps. First, a 814
13 bp fragment containing the region spanning the HSV-1 UL37/UL38 intergenic region
14 from nt 83,603-84,417 from plasmid NK470 was subcloned into pBS that had had the
15 MCS removed (digestion with KpnI/SacI) to yield pBS:UL37/38. A cassette containing a
16 synthetic CMV IE promoter flanked by the pBS-SK+ MCS was ligated into pBS:UL37/38
17 digested with BspE1/AflIII, which enzymes cut between the UL37 and UL38 genes, to
18 yield the plasmid pIN:UL37/38. The EIV Prague/56 HA gene was PCR cloned from
19 cDNA prepared from EIV Prague/56. Briefly, RNA was prepared by Trizol extraction of
20 a stock of EIV Prague 56 and was reverse transcribed using Omni-Script Reverse
21 Transcriptase (Qiagen) according to the manufacturer's instructions. The cDNAs were
22 cloned into pBS, and the clone containing the HA gene (pBS-EIVPrague56/HA) was
23 confirmed by sequence analysis. The Prague/56 HA gene was subcloned from this

1 plasmid and inserted behind the CMV promoter in the plasmid pIN:UL37/38 to yield
2 plasmid pIN:37/38-Prague56/HA. To produce recombinant HSV-GS11, RS cells were
3 co-transfected with plasmid pIN:37/38-Prague56/HA and purified HSV-GS3 virion DNA.
4 Subsequent to the addition of mifepristone to the medium, the co-transfected cells were
5 exposed to 43.5⁰C for 30 min and then incubated at 37⁰C. Subsequently, on days 2 and
6 3, the cells were again incubated at 43.5⁰C for 30 min and then returned to 37⁰C.
7 Picking and amplification of plaques, screening and plaque purification was performed
8 essentially as described for HSV-GS3 (3,34). The resulting plaque-purified HSV-GS11
9 was verified by Southern blot as well as by PCR and DNA sequence analysis of the
10 recombination junctions.

11 **Construction of expression plasmid pVP19c.** Plasmid pVP19c was constructed by
12 PCR- cloning of the promoter and coding sequence of the HSV-1 VP19c gene into pBS
13 KS+ using HSV-1 strain 17syn+ virion DNA as the PCR target.

14 **Single step growth analysis.** Confluent monolayers of Vero cells were infected with
15 HSV-GS7 at a multiplicity of infection (MOI) of 3. Virus was allowed to adsorb for 1 h at
16 37⁰C; then the inoculum was removed, and the cells were overlaid with complete
17 medium. Ulipristal treatment (10 nM) was initiated at the time of the initial infection. Heat
18 treatment was performed either immediately after infection or 4 h later by floating the
19 sealed dishes in a 43.5⁰C water bath for 30 min. The dishes were then incubated for 72
20 h at 37⁰C. At 0, 4 or 8, 12 or 16, and 24 or 28 h post-infection, two dishes were
21 removed, and the cells were scraped into medium for harvesting and subjected to two
22 freeze-thaw cycles. Infectious virus was then determined by titrating the lysate of each
23 dish in triplicate on 24 well plates of confluent E5 cells transfected 24 h prior to infection

1 with expression vector pVP19c using Lipofectamine 2000 (Life Technologies). Plaques
2 were visualized 2 days after infection using an antibody plaque assay, essentially as
3 previously described (34).

4 **Immunization.** Briefly, 4-6-week old female outbred Swiss Webster mice (Envigo,
5 Tampa, FL) (or BALB/c mice (Envigo, Tampa, FL) for experiments involving HSV-GS11)
6 were inoculated with the appropriate viral vector or phosphate-buffered saline (pH 7.3)
7 (50 μ l/mouse) on the lightly abraded plantar surfaces of both rear feet as previously
8 described (40). To facilitate efficient uptake of vector, and to minimize the amount of
9 abrasion required, the feet were saline-treated prior to infection. The following
10 procedure was employed: mice were first anesthetized with isoflurane by inhalation.
11 Flunixin meglumine (1.1 mg/kg) was then administered intramuscularly (IM) to alleviate
12 any pain associated with the procedure. Thereafter, 25-50 μ l (no more than 50 μ l) of
13 sterile 10% saline were injected sub-epidermally under both rear footpads. The mice
14 were then returned to their cages. Four h later, the mice were anesthetized by IM
15 injection of 10-20 μ l of a cocktail of acepromazine (2.5-3.75 mg/kg), xylazine (7.5-11.5
16 mg/kg) and ketamine (30-45 mg/kg). Both rear footpads of the anesthetized mice were
17 then lightly abraded with an emery board to scratch the keratinized layer of the skin to
18 allow the virus to adsorb efficiently. The anesthetized mice were rested on their backs,
19 and 50 μ l of the appropriate dilution of viral vector were placed on the footpads using a
20 pipette. The viral vector was allowed to adsorb until the mice awoke. To activate vector
21 replication, a combination of heat and mifepristone/ulipristal treatment was used. Heat
22 treatment was performed by immersion of hindlimbs in a temperature-controlled water
23 bath 3 h after virus administration. Mice were allowed to recover at 37⁰C for 15 min.

1 Mifepristone (0.5 mg/kg) or ulipristal (50 µg/kg) in DMSO was administered
2 intraperitoneally (IP) at the time of immunization and, again, 24 h later.

3 **HSV-1 challenge in mice.** Immunized and control mice were challenged by infection
4 on both rear footpads with 10,000 pfu, typically, of HSV-1 strain 17syn+. The procedure
5 for the saline pre-treatment, anesthesia and application of virus was the same as that
6 described above for application of the viral vector, except that no mifepristone/ulipristal
7 or heat treatment was administered. For the efficacy determination, a modified
8 endpoint analysis was used. Mice were monitored daily (with cages coded in a masked
9 fashion). When mice reached clinical endpoints indicating severe CNS infection
10 (bilateral hindlimb paralysis, inability to move when touched, or trembling), they were
11 euthanized.

12 **Blood collection, and serum and lymphocyte isolation.** For serum neutralization
13 studies, blood was collected by retro-orbital bleeding prior to immunization. For retro-
14 orbital bleeding, mice were anesthetized with ketamine (90 mg/kg) and xylazine (10
15 mg/kg) IP. For the collection of serum and lymphocytes at 3 weeks after immunization,
16 mice from each group were anesthetized by inhalation of 2-3% isoflurane. The total
17 blood volume of each mouse was collected, and the mice were euthanized by cervical
18 dislocation. PBMCs were isolated by Ficoll gradient separation using Lymphoprep
19 (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's
20 protocol.

21 **Neutralizing antibody assay.** After collection, the blood was allowed to clot for 30 min.
22 After centrifugation at 800 x g, the serum was collected. Serum samples were heated

1 to 56°C for 1 h to inactivate complement and were then diluted 1:10 in complete DMEM
2 containing 10% heat-inactivated FBS. For HSV-1 neutralization, 50 µl of a suspension
3 containing approximately 100 pfu of HSV-1 strain 17syn+ were added to each dilution of
4 serum to a final volume of 100 µl. For EIV neutralization, 50 µl of a suspension
5 containing approximately 100 TCID50 units of EIV Prague/56 were added to each
6 dilution of serum to a final volume of 100 µl. Initial serum dilution therefore was 1:20.
7 Serum-virus mixtures were placed on a rocker at room temperature for 1 h, and the
8 amount of virus that was not neutralized at a given concentration of serum was titrated
9 on RS (HSV-1) or MDCK (EIV) cell monolayers in order to calculate the neutralizing
10 antibody titers.

11 **Responder cell frequency (RCF) assay.** HSV-1- or EIV-specific T cells were
12 quantified by a modified limiting dilution lymphoproliferation assay (54), using either cell-
13 free HSV-1 or EIV Prague/56 HA protein lysate and control antigen lysate. Briefly, wells
14 of 96 well plates were coated with 20 µl/well of antigen extract (HSV-1 or RS cell control
15 lysate; or EIV Prague/56 HA or Vero cell control lysate) and were allowed to air-dry in a
16 laminar flow hood. Dilutions of mouse PBMCs in DMEM were added to each well such
17 that each well contained a minimum of 1 and a maximum of 10 lymphocytes per well in
18 a volume of 100 µl complete medium (with serum). The plates were then incubated at
19 37°C. After 24 h, medium containing 10 µCi of H3-thymidine was added to each plate
20 for 12 hours, medium was replaced and the plates were incubated for an additional 72
21 h. The wells were harvested, and the DNA was precipitated in 20 volumes of cold 10%
22 trichloroacetic acid, transferred onto glass fiber discs (Whatman GF/C) by filtration,
23 rinsed with 95% ethanol, and dried using a heat lamp. The filters were then transferred

1 to scintillation vials with Scintiverse (Fisher Scientific) and counted. The counts per
2 minute (cpm) of H3-thymidine were converted to RCF using the maximum likelihood
3 estimate method of Levin et al. (55).

4 **Viral DNA replication and transcription.** Groups (n=5) of 4-6-week old female outbred
5 Swiss Webster mice were anesthetized and inoculated with an HSV-GS vector on both
6 rear footpads as described above. Ulipristal (50 µg/kg) was administered IP at the time
7 of infection. Three h after virus administration, heat treatment was performed by
8 immersion of rear feet in a 45⁰C water bath for 10 min. Mice were recovered at 37⁰C for
9 15 min. Mice were sacrificed 24 h after heat induction, and the feet were dissected and
10 snap-frozen in RNAlater (Sigma-Aldrich). DNA and RNA were extracted by grinding the
11 tissues in TRIzol (Thermo Fisher Scientific), and back-extracting the DNA from the
12 interface. Extracted DNA was subjected to Taqman real-time PCR (qPCR) for
13 quantitative analysis of HSV-1 DNA (using HSV-1 DNA polymerase primers/probe) (56).
14 RNA was analyzed by reverse transcription and Taqman real-time PCR (RT-qPCR) for
15 the presence of ICP4, thymidine kinase (TK), capsid protein VP19c and glycoprotein C
16 (gC) transcripts. Normalization of DNA and RNA quantities was relative to the cellular
17 gene APRT. Sequences of primers and probes used are provided in Table 3.

18 **EIV Prague/56 HA ELISA.** In order to produce the antigen for the ELISA, the insert
19 from plasmid pBS-EIVPrague56/HA (described above) was subcloned into expression
20 vector pET-31b (Millipore), and the EIVPrague56/HA protein was expressed in *E. coli* in
21 accordance with the manufacturer's instructions. After induction and growth, the
22 bacteria were lysed, proteins were isolated in the presence of protease inhibitors, and
23 the proteins were used to coat a 96 well ELISA plate which was allowed to air-dry.

1 Dilutions (1:10 to 1:200) of murine serum were applied to the wells, and the plate was
2 incubated at room temperature for 30 minutes. The serum was removed, and the wells
3 were washed 2 x with PBS. Horseradish peroxidase-conjugated anti-IgG antibody was
4 added to the wells, the plate was incubated for 30 min at room temperature, and the
5 wells were washed 2 x with PBS. 3,3',5,5'-Tetramethylbenzidine was added to the
6 wells, and the plate was incubated for 10 minutes and then read with a
7 spectrophotometer plate reader. Results were evaluated based on the mean of sample
8 to negative control mean (S/N) ratio.

9 **Statistical analyses.** Data sets were analyzed using a two-way ANOVA analysis (no
10 RM). Unless otherwise stated in the figure legends, data are presented as mean values
11 with standard deviation. Kaplan-Meier survival curves were analyzed for significance
12 using the log rank test. No data sets that were analyzed for statistical significance varied
13 in sample size by more than 3-fold.

14 **Ethics statement.** The animal studies were approved by the University of Florida
15 Institutional Animal Care and Use Committee (IACUC) and were performed in
16 accordance with the approved protocol (Protocol #0541). All experiments were carried
17 out under adherence with the Guide for the Care and Use of Laboratory Animals of the
18 National Institutes of Health and the American Veterinary Medical Association
19 Guidelines on Euthanasia.

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8 TABLES

9

10 **TABLE 1.** Infectious virus (pfu) in the feet of mice 4 days post challenge

Mock	KD6	HSV-GS3 (unactivated)	HSV-GS3 (activated)
$5.5 \times 10^5 \pm 1.2 \times 10^4$	$4.0 \times 10^4 \pm 8.3 \times 10^2$	$9.4 \times 10^4 \pm 3.1 \times 10^4$	$6.2 \times 10^3 \pm 1.5 \times 10^2$

11 Groups of 5 mice were immunized, treated and challenged as described in Fig. 2. Four
12 days post challenge, the mice were euthanized, and feet dissected and homogenized.
13 The tissue homogenates were diluted and titrated on rabbit skin (RS) cells.

14

15

1 **TABLE 2.** Number/fraction of survivors in immunization/challenge experiments.

Virus	Dose (x10 ³ pfu)	Activa-tion	Second activa-tion	Boost	Boost/ activa-tion	Exp. 1	Exp. 2 ⁺⁺	Exp. 3	Exp. 4	Exp. 5 ⁺⁺⁺	Exp. 6	Exp. 7	Exp. 8	No./fraction surviving mice	
-		-	-	-	-	0 (10)	2 (10)	1 (10)	0 (10)	0 (10)	0 (10)	0 (10)	1 (10)	4 (80) 0%	
KD6	50	-	-	-	-	4 (10)	3 (10)		3 (10)					10 (30) 33%	
		+	-	-	-	2 (10)								2 (10) 20%	
	500	-						2 (10)						2 (10) 20%	
		+	-	-	-			4 (10)						4 (10) 40%	
HSV-GS3	50	-	-	-	-	3 (10)	4 (10)		3 (10)			1 (20)		11 (50) 22%	
		+	-	-	-	6 (10)	7 (10)		6 (10)			14 (20)		33 (50) 66%	
		+	+	-	-		8 (10)							8 (10) 80%	
	500	-	-	+	-			4 (10)							4 (10) 40%
		+	-	+	-			8 (10)							8 (10) 80%
		+	+	+	-			8 (10)							8 (10) 80%
HSV-GS7	5	-	-	-	-					0 (10)		0 (10)	0 (10)	0 (30) 0%	
		+	-	-	-					5 (10)	5 (10)	2 (10)	4 (10)	16 (40) 40%	
		+	+	-	-						7 (10)	4 (10)		11 (20) 55%	
		+	-	-	+							17 (20)		17 (20) 85%	
		+	+	-	+						8 (10)	7 (10)		15 (20) 75%	
	50	-	-	-	-							2 (20)	2 (10)	4 (30) 13%	
		+	-	-	-						7 (10)	15 (20)	8 (10)	30 (40) 75%	
		+	+	-	-							13 (20)		13 (20) 65%	
		+	-	+	-						8 (10)	7 (10)		15 (20) 75%	
		+	-	+ ⁺	-						8 (10)			8 (10) 80%	
		+	-	-	+						10 (10)	10 (10)		20 (20) 100%	
		+	+	-	+							20 (20)		20 (20) 100%	
	500	-	-	-	-						6 (10)			6 (10) 60%	
		+	-	-	-						9 (10)			9 (10) 90%	

2 Group sizes are shown in parentheses. Activation: regional heat treatment at 45°C for
3 10 min in the systemic presence of an anti-progestin (50 µg/kg ulipristal in most
4 experiments.). Challenge: 10,000 pfu HSV-1 strain 17syn+. Boost/second immunization
5 was with same virus dose that was used for the initial immunization. ⁺Boost with

1 500,000 pfu HSV-GS7; **challenge dose: 1,000 pfu; ***activation at 44.5°C for 10 min.
 2 Data relating to incomplete activation (heat treatment only, or antiprogestin only) were
 3 omitted.

4

5 **TABLE 3:** Primers and probes used for qPCR.

HSV DNA Pol	F	5' AGAGGGACATCCAGGACTTTGT 3'
	R	5' CAGGCGCTTGTGGTGTAC 3'
	P	5' ACCGCCGAACTGAGCA 3'
ICP4	F	5' CACGGGCCGCTTCAC 3'
	R	5' GCGATAGCGCGCGTAGA 3'
	P	5' CCGACGCGACCTCC 3'
gC	F	5' CCTCCACGCCCAAAGC 3'
	R	5' GGTGGTGTGTTCTTGGGTTTG 3'
	P	5' CCCACGTCCACCCC 3'
TK	F	5' CACGCTACTGCGGGTTTATATAGAC 3'
	R	5' GGCTCGGGTACGTAGACGATAT 3'
	P	5' CACCACGCAACTGC 3'
VP19c	F	5' CTTGGTTCGACGAGCTGTTTG 3'
	R	5' GCCCCGTTGCGTACAG 3'
	P	5' CCGTCCGCGTTCATG 3'
EIV Prague/56 HA	F	5' GGAACATTAGAATTCACAGCAGAGG 3'
	R	5' CCTGTTCTCAATTTAACATATCCCC 3'
	P	5' GGGGATATGTTAAAT 3'
Mouse APRT	F	5' CTCAAGAAATCTAACCCCTGACTCA 3'
	R	5' GCGGGACAGGCTGAGA 3'
	P	5' CCCACACACACCTC 3'

6 F, forward; R, reverse; P, probe.

7

1 **FIGURE LEGENDS**

2 **FIG 1** Two-component HSP70B promoter-based gene switch and replication-
3 competent, controlled HSV-1-derived viral vectors. (A) Illustration of the operation of the
4 dual-responsive gene switch employed to control replication of the viral vectors of the
5 present study. (Copyright © American Society for Microbiology, (34)) eHSF1:
6 endogenous heat shock transcription factor; HSP70B: HSP70B/HSPA7 promoter; TRP:
7 transactivator-responsive promoter; TA: transactivator; VG: replication-essential viral
8 gene; SMR: small-molecule regulator. (B) Diagrams of viral vectors HSV-GS3 (top of
9 panel) and HSV-GS7 (bottom of panel). Both vectors contain, inserted into the
10 intragenic region between the HSV-1 UL43 and UL44 genes, a GLP65 transactivator
11 gene functionally linked to a promoter cassette consisting of a human HSP70B
12 promoter and a GAL4-responsive promoter. In HSV-GS3, the promoters of the
13 replication-essential genes encoding ICP4 and ICP8 were replaced with GAL4-
14 responsive promoters. In HSV-GS7, the promoter of the replication-essential gene for
15 VP19c was replaced with a GAL4-responsive promoter. TR_L, TR_S: long and short
16 terminal repeats; U_L, U_S: long and short unique regions; IRL, IRS: long and short
17 internal repeats. (C) Single step growth experiment for HSV-GS7 in Vero cells. Heat:
18 exposure to 43.5⁰C for 30 min immediately after infection (i.e., immediately after
19 removal of the viral inoculum). Ulipristal: 10 nM ulipristal was added to the medium at
20 the time of initial infection. Each data point represents the mean of three individual
21 assays. Error bars are not visible due to their range. pfu/ml values are shown. For
22 details see under Materials and Methods. (D) Replication of HSV-GS7 (top graph) and
23 expression of viral genes (bottom graph) in rear feet of mice (group size (n) of 5)

1 determined 24 h after administration of the recombinant vector (50,000 pfu) to the
2 plantar surfaces of the rear feet. HSV-GS7 replication was either not activated or was
3 activated three h after infection by immersion of the infected rear feet in a 45⁰C water
4 bath for 10 min in the presence of ulipristal. Ulipristal (50 µg/kg) had been administered
5 IP at the time of initial infection. Twenty-four h after infection, animals were euthanized,
6 and DNA and RNA were isolated from the infected rear feet. Levels of viral DNA were
7 assessed by qPCR, and levels of ICP4, TK, gC and VP19c transcripts by RT-qPCR.
8 Relative values and standard deviations are shown. *** $p \leq 0.05$ for the comparison of
9 “HSV-GS7 activated” and “HSV-GS7 unactivated” or “mock”.

10 **FIG 2** Immunization with HSV-GS3 protects against lethal challenge with HSV-1 17syn+
11 in the mouse footpad model. Groups of mice were inoculated on the plantar surfaces of
12 both rear feet with 50,000 pfu/mouse of either the ICP4(-) HSV-1 replication-
13 incompetent recombinant KD6 or the HSV-1 replication-competent controlled
14 recombinant HSV-GS3 (two groups), or were mock-immunized with saline. Animals of
15 one of the two HSV-GS3 groups received a 43.5⁰C/30min heat treatment 3 h after virus
16 inoculation in the systemic presence of mifepristone (0.5 mg/kg administered IP at the
17 time of inoculation) (“HSV-GS3 activated”), whereas animals of the other group were left
18 untreated (“HSV-GS3 unactivated”). At 22 days post immunization, all mice were
19 challenged with a 20-fold lethal dose of wild type HSV-1 strain 17syn+ applied to both
20 rear feet. Data are presented as percent survival for each treatment group (n=20 for
21 each treatment). *** $p \leq 0.05$. See Materials and Methods for further details, and Table
22 2 and Fig. 6 for comparable experiments.

1 **FIG 3** Gene switch-controlled immunization by recombinant HSV-GS3. Mice in four
2 groups (n=10) were administered on both rear feet 500,000 pfu/mouse of HSV-GS3.
3 The first HSV-GS3 group received a 45⁰C/10min heat treatment 3 h after inoculation in
4 the systemic presence of ulipristal (50 µg/kg administered IP at the time of inoculation)
5 (“HSV-GS3 (activated)”), the second group only received heat treatment (“HSV-GS3
6 (heat only)”), the third group was only administered ulipristal (“HSV-GS3 (uli only)”), and
7 the fourth group was left untreated (“HSV-GS3 unactivated”). A further group was mock-
8 immunized with saline. At 21 days, animals in the HSV-GS3 groups were boosted with
9 another 500,000 pfu/mouse dose of HSV-GS3, and 10 days later all mice were
10 challenged with a 20-fold lethal dose of wild-type HSV-1 strain 17syn+ applied to both
11 rear feet. The data are presented as percent survival for each treatment group. *** p ≤
12 0.05. See Materials and Methods for further details. Comparable data were obtained
13 from a second experiment (not shown), in which mice were immunized with 50,000
14 pfu/mouse of HSV-GS3 and were not boosted.

15 **FIG 4** Immunization with HSV-1 replication-competent controlled recombinant HSV-GS7
16 and comparison with HSV-GS3. Mice were inoculated on both rear feet with 50,000
17 pfu/mouse of either HSV-GS3 or HSV-GS7 or were mock-immunized with saline. For
18 each recombinant, one group of animals was subjected to heat treatment in the
19 presence of ulipristal as described under Fig. 3 (“activated”), and another group did not
20 receive this treatment (“unactivated”). At 21 days post inoculation, all mice were
21 challenged with a 20-fold lethal dose of wild-type HSV-1 strain 17syn+ applied to both
22 rear feet. The data are presented as percent survival for each treatment group (n = 20

1 for each HSV-GS3 or HSV-GS7 group; n = 10 for mock; *** p ≤ 0.05). See Table 2 and
2 Fig. 6 for comparable experiments.

3 **FIG 5** Effects of second immunization and second activation on HSV-GS7 efficacy. (A)
4 Mice were inoculated on both rear feet with 50,000 pfu/mouse of HSV-GS7 or saline
5 (mock; n = 10). HSV-GS7 groups: untreated (“unactivated”; n = 20), activated on day 1
6 (3 h after virus inoculation) by heat treatment at 45⁰C for 10 min in the presence of
7 ulipristal (50 µg/kg administered IP at the time of inoculation) (“activated”; n = 20),
8 activated on day 1 and reactivated 2 days later (“activated + 2nd activation”; n = 20),
9 activated on day 1 and readministered 50,000 pfu/mouse of HSV-GS7 21 days later
10 (“activated + boost”; n = 10), and activated on day 1, readministered 50,000 pfu/mouse
11 of HSV-GS7 21 days after the first inoculation and reactivated 3 h later (“activated +
12 boost + 2nd activation”; n = 10). Twenty-one days after the last treatment, all mice were
13 challenged with a 20-fold lethal dose of wild-type HSV-1 strain 17syn+ applied to both
14 rear feet. ** p ≤ 0.05; *** p < 0.01. (B) A similar experiment except that both initial and
15 second immunizations were with 5,000 pfu/mouse of HSV-GS7 (n = 10; for second
16 immunization n = 20; *** p ≤ 0.05). The data are presented as percent survival for each
17 treatment group. See Table 2 and Fig. 6 for comparable experiments.

18 **FIG 6** Meta-analysis of efficacy of HSV-GS7 in protecting mice against lethal HSV-1
19 challenge across comparable experiments. Selected data from Table 2 showing
20 percent survival for different treatment conditions examined for KD6, HSV-GS3 and/or
21 HSV-GS7 are graphically depicted. Data sets for HSV-GS7 were analyzed using a two-
22 way ANOVA analysis. *** p ≤ 0.05.

1 **FIG 7** Anti-HSV-1 humoral and cellular immune responses. (A) Neutralizing antibodies
2 induced by immunization. Dilutions of mouse serum samples (n = 5 for each
3 experimental group) were tested for their ability to neutralize HSV-1 as described in
4 Materials and Methods. Values are presented as averaged percentages of HSV-1 pfu
5 neutralized for each group. ANOVA: *** $p \leq 0.05$. See Materials and Methods for
6 experimental detail. (B) Responder cell frequency. The number of HSV-1-specific
7 lymphocytes induced three weeks after immunization was determined by a limiting
8 dilution lymphocyte proliferation assay. The data are presented as averaged responder
9 cell frequency for each experimental group. Three blood/PBMC samples were analyzed
10 per group. ANOVA: *** $p \leq 0.05$. See Materials and Methods for experimental detail.

11 **FIG 8** Immunization with HSV-GS11 induces a robust immune response against EIV
12 Prague/56 HA. (A) HSV-GS11 vector containing the EIV Prague/56 (H7N7) HA gene
13 driven by the CMV IE promoter and inserted into the intergenic region between UL37
14 and UL38. The HSP70/GAL4-GLP65 TA cassette is inserted into the intergenic region
15 between UL43/44. The promoters of the essential HSV-1 genes ICP4 and ICP8 are
16 replaced with GAL4-responsive promoters. (B) Detection of EIV HA RNA expression
17 following immunization of mice with HSV-GS11. Groups (n = 5) of 6-8-week old female
18 BALB/c mice were inoculated on both rear footpads with either saline (mock), HSV-GS3
19 (50,000 pfu) or HSV-GS11 ((50,000 pfu) as described under Materials and Methods.
20 Vector replication was activated in some treatment groups by administration of heat and
21 ulipristal as described in Fig. 3. One treatment group received a second activation
22 treatment that was administered two days after the first activation treatment. Mice feet
23 were harvested 24 hours after the last treatment, and RNA was isolated and cDNA

1 prepared. Samples were analyzed by qPCR with EIV Prague/56 HA-specific Taqman
2 primers/probe (Table 3). Data are presented in relative units of fluorescence. ANOVA:
3 *** = $p \leq 0.05$. (C) Detection of EIV HA antigen by ELISA following immunization of mice
4 with HSV-GS11. Groups (n = 5) of 6-8-week old female BALB/c mice were inoculated
5 on both rear footpads with either saline (mock), HSV-GS3 (50,000 pfu) or HSV-GS11
6 ((50,000 pfu) and were subjected to the same treatments as in B. Mice feet were
7 harvested 24 hours after the last treatment, and protein homogenates were analyzed
8 using an EIV Prague/56 HA-specific ELISA as described under Materials and Methods.
9 Data are presented in relative units of fluorescence. ANOVA: *** = $p \leq 0.05$. (D)
10 Neutralizing antibodies induced by immunization. Groups (n = 5) of 6-8-week old
11 female BALB/c mice were administered HSV-GS3, HSV-GS7 or saline (mock) and were
12 treated as in B. Twenty-one days post immunization, mouse serum samples were
13 tested for their ability to neutralize EIV Prague/56. Values are presented as percent of
14 EIV Prague/56 HA pfu neutralized. (E) Responder cell frequency assay. Immunizations
15 and treatments were as in D. Twenty-one days post immunization, the number of EIV
16 Prague/56 HA-specific lymphocytes was determined by a limiting dilution lymphocyte
17 proliferation assay. The data are presented as responder cell frequency of each
18 experimental group.

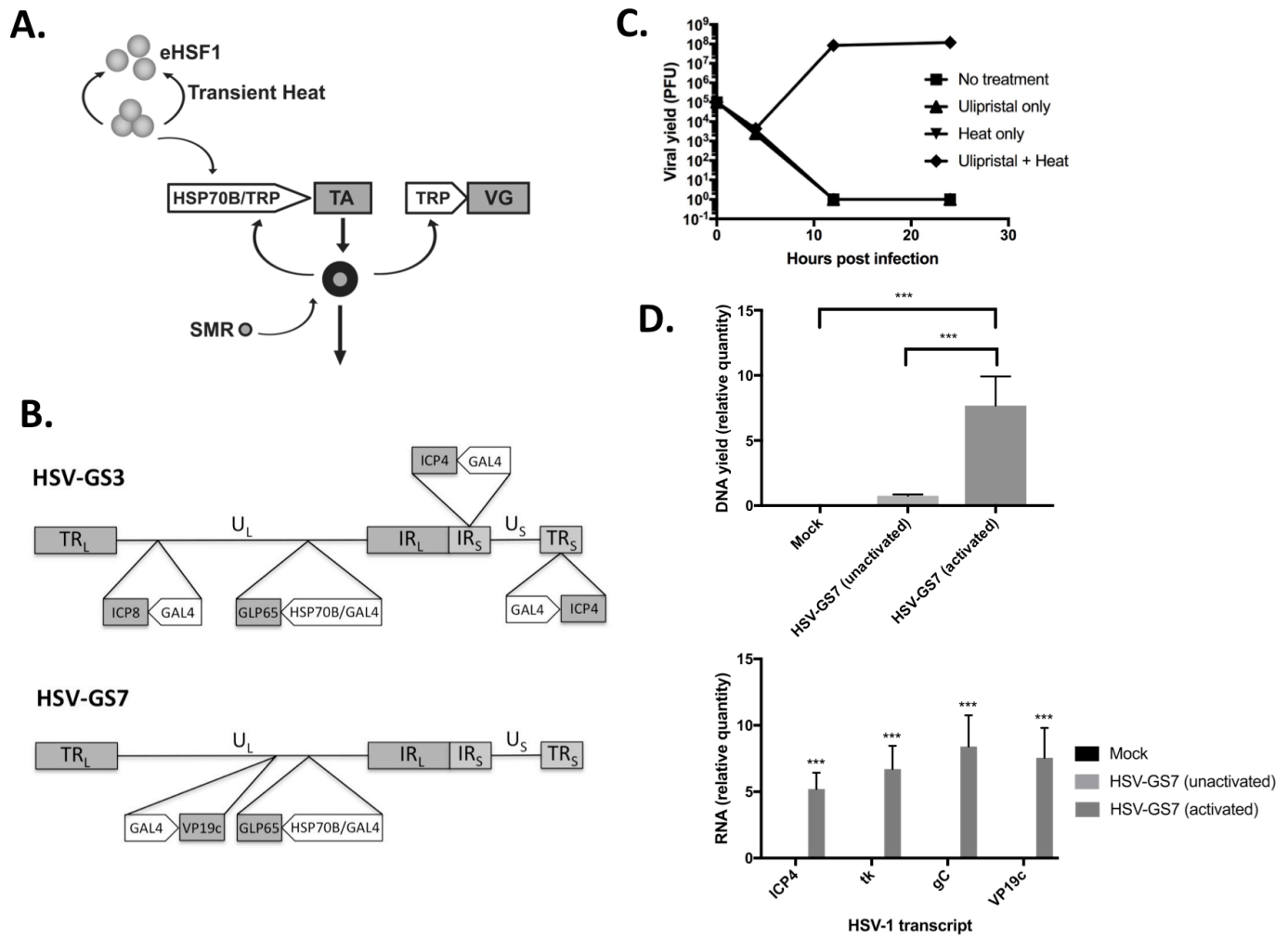
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1 Figure 1

2

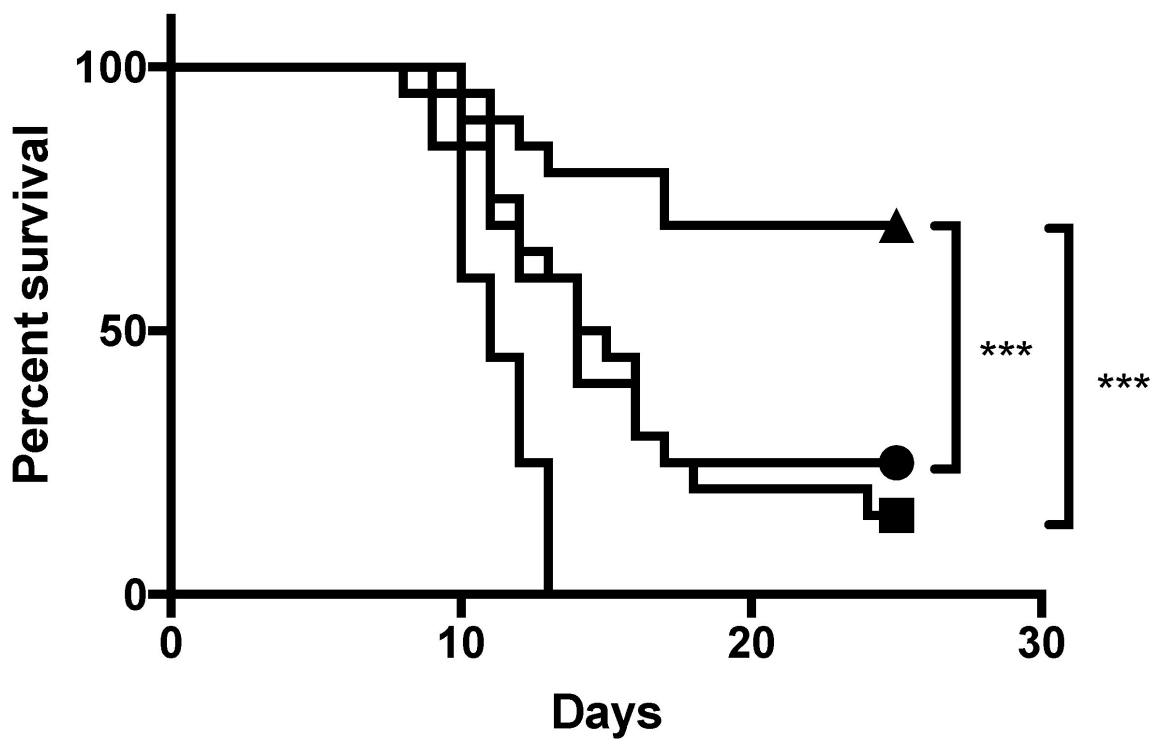


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4

1 Figure 2

2

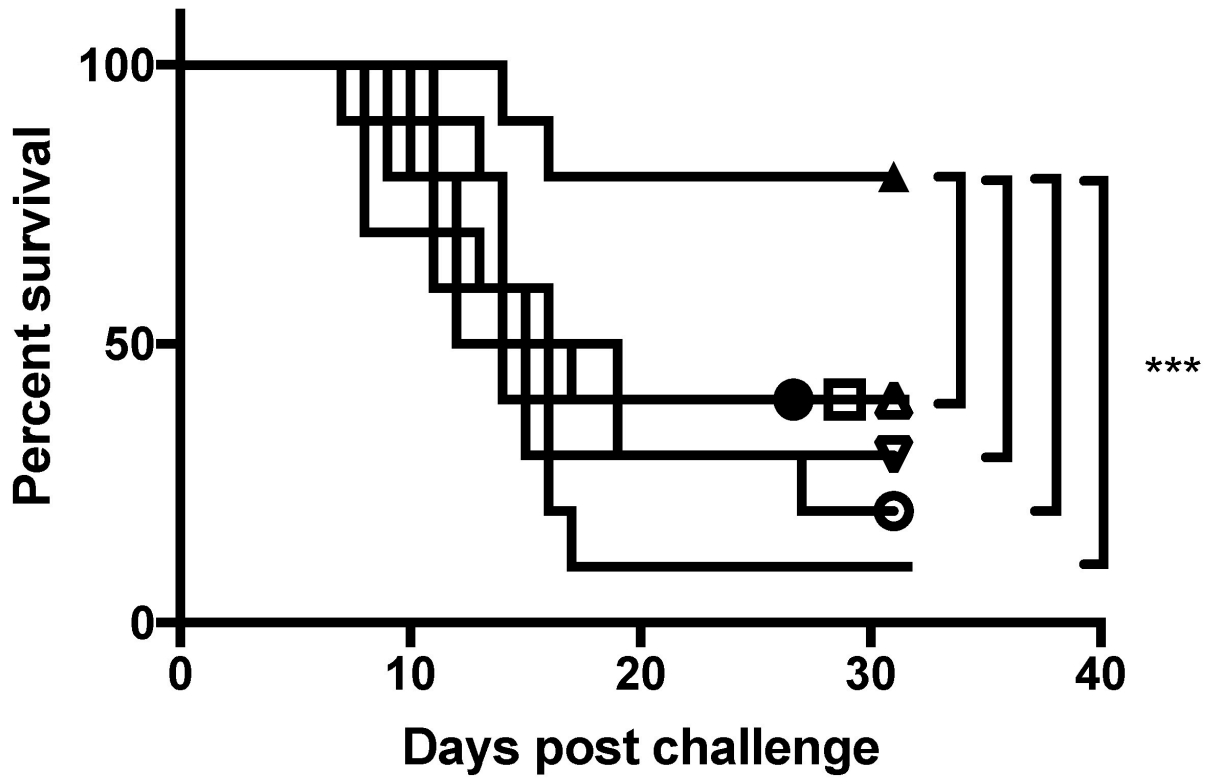


- Mock
- KD6
- HSV-GS3 (unactivated)
- ▲ HSV-GS3 (activated)

3

1 Figure 3

2

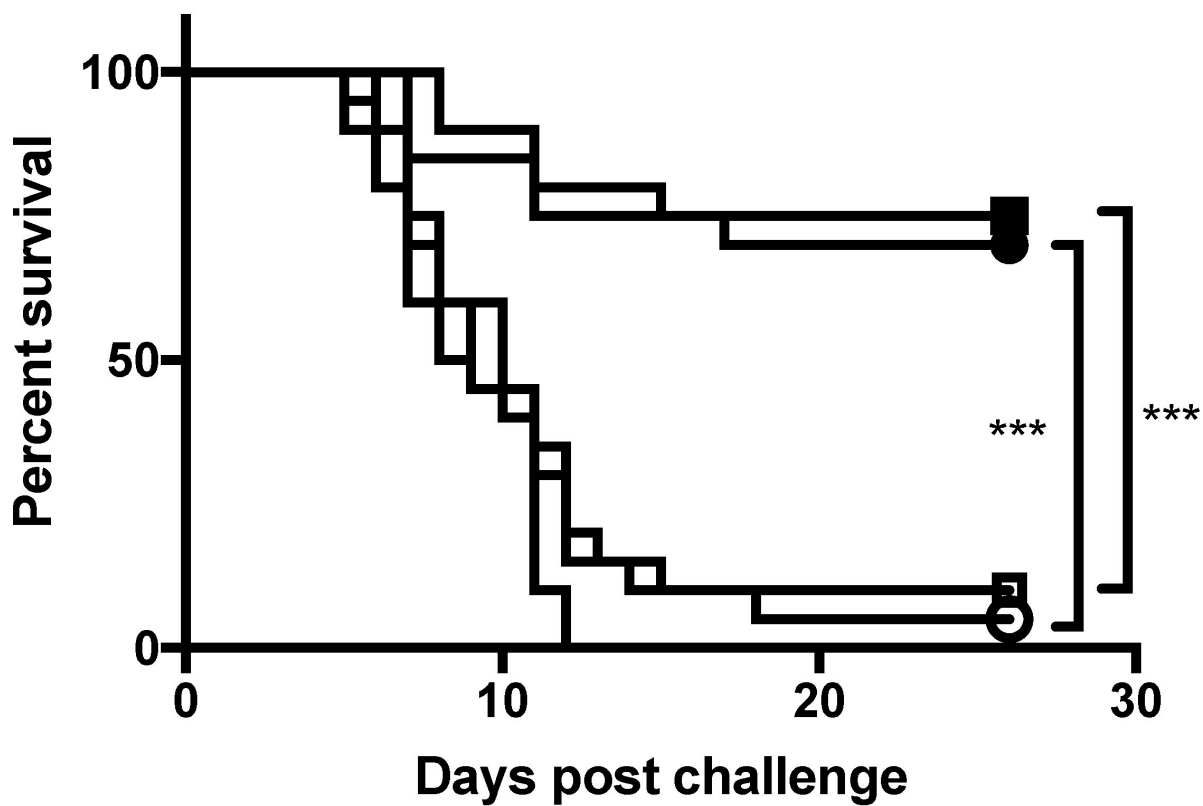


- Mock
- KD6 (unactivated)
- KD6 (activated)
- HSV-GS3 (unactivated)
- △ HSV-GS3 (heat only)
- ▽ HSV-GS3 (uli only)
- ▲ HSV-GS3 (activated)

3

1 Figure 4

2



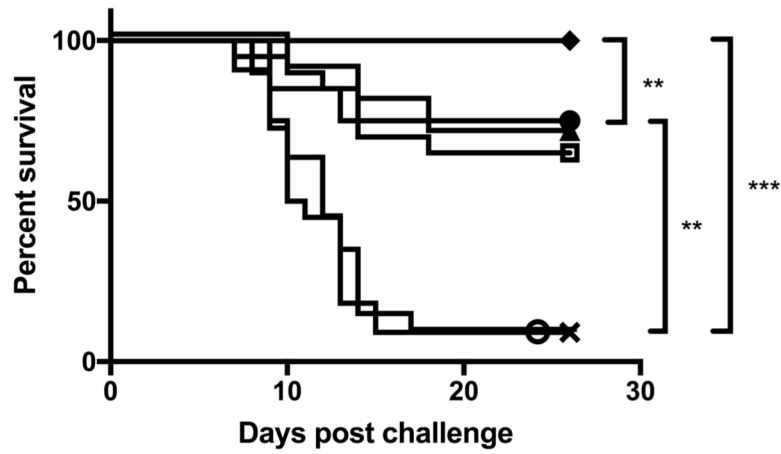
- Mock
- ⊖ HSV-GS3 (unactivated)
- HSV-GS3 (activated)
- ⊖ HSV-GS7 (unactivated)
- HSV-GS7 (activated)

3

4

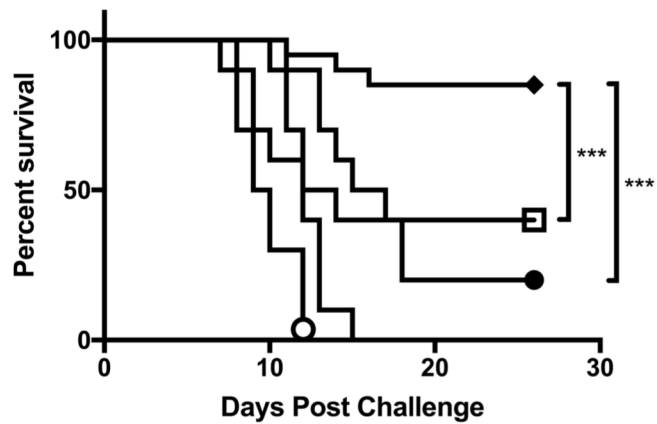
1 Figure 5

A.



- ✕ Mock
- HSV-GS7 (unactivated)
- HSV-GS7 (activated)
- ◻ HSV-GS7 (activated + 2nd activation)
- ▲ HSV-GS7 (activated + boost)
- ◆ HSV-GS7 (activated + boost + 2nd activation after boost)

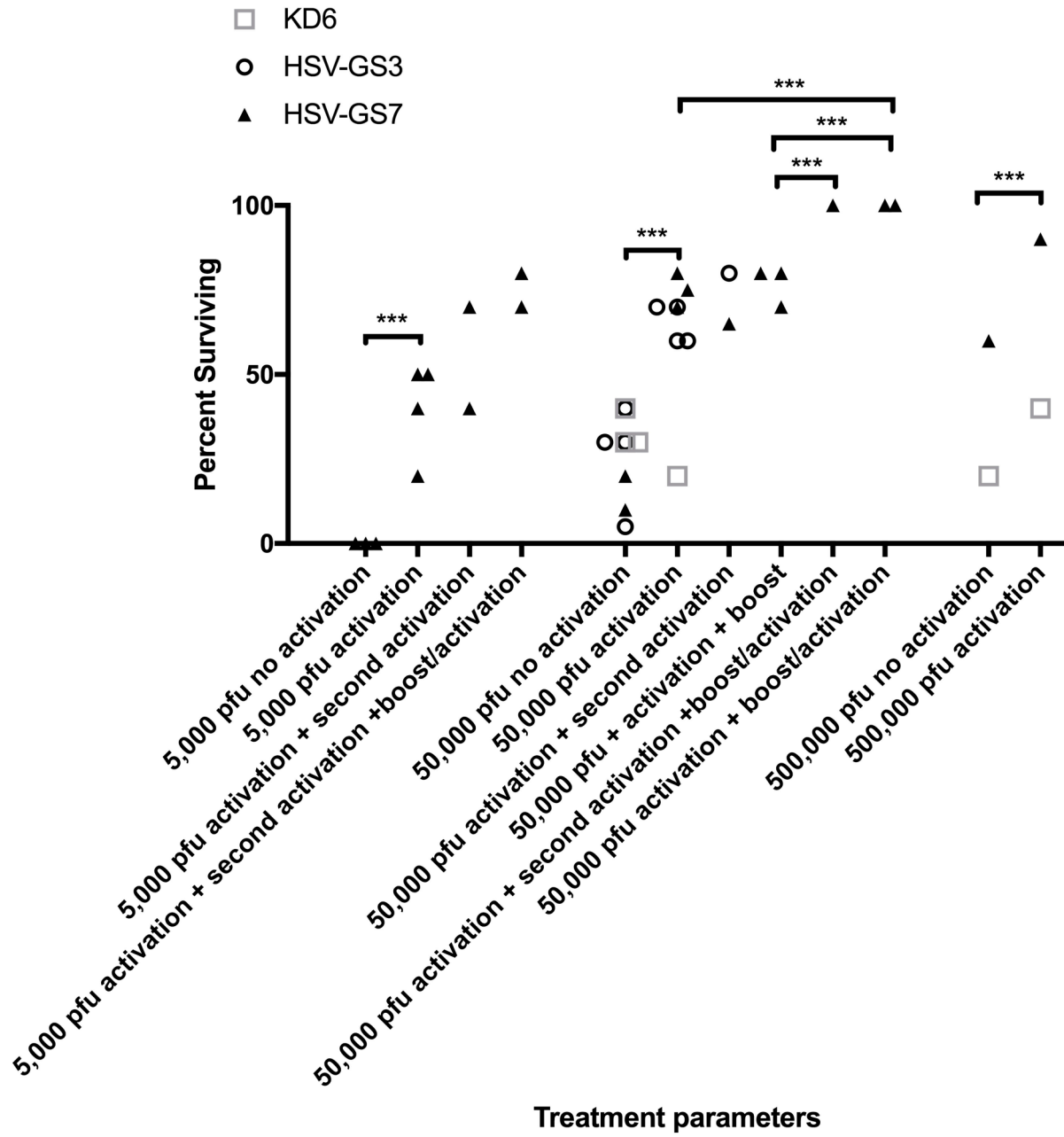
B.



- Mock
- HSV-GS7 (unactivated)
- HSV-GS7 (activated)
- ◻ HSV-GS7 (activated + 2nd activation)
- ◆ HSV-GS7 (activated + boost + 2nd activation after boost)

2

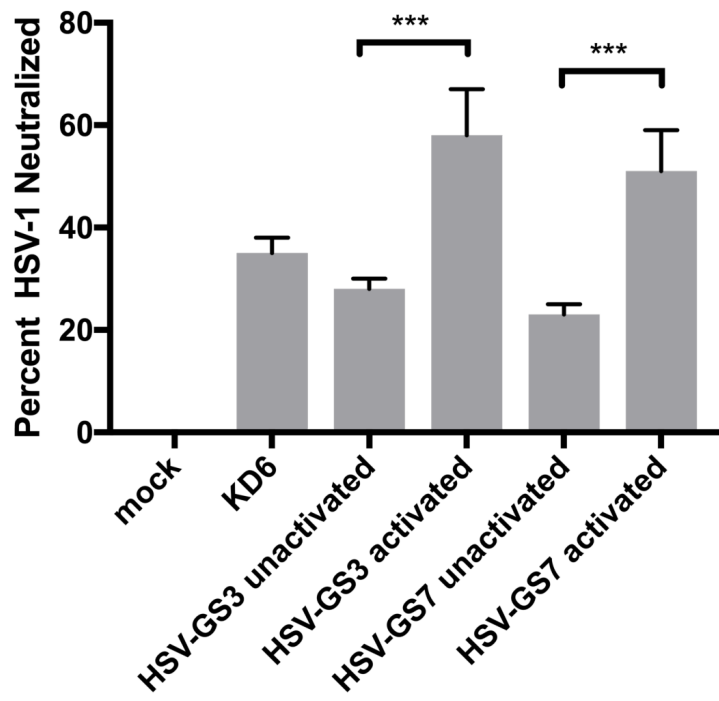
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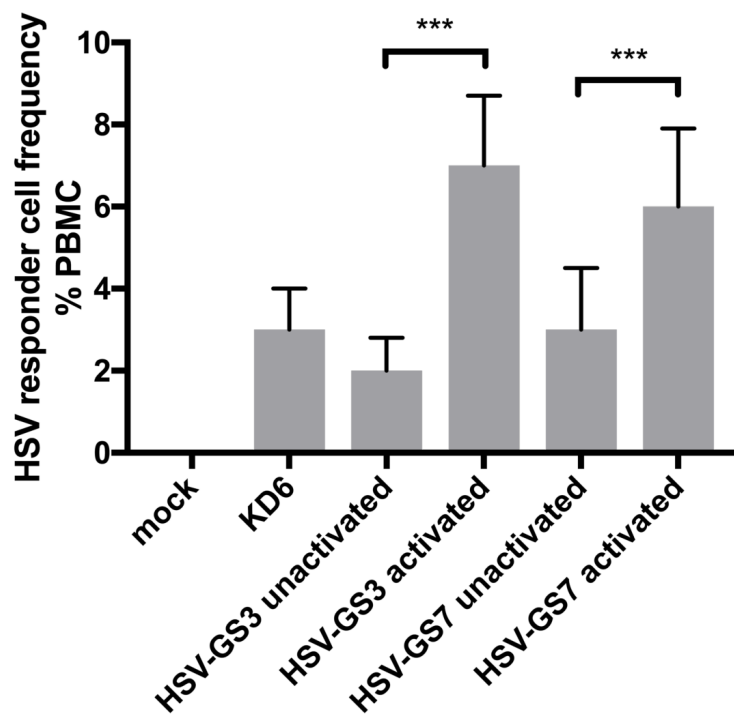
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1 Figure 7

A.

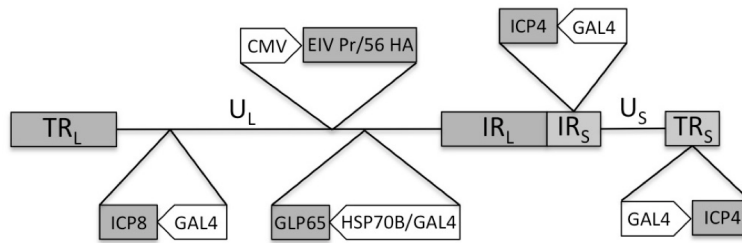


B.

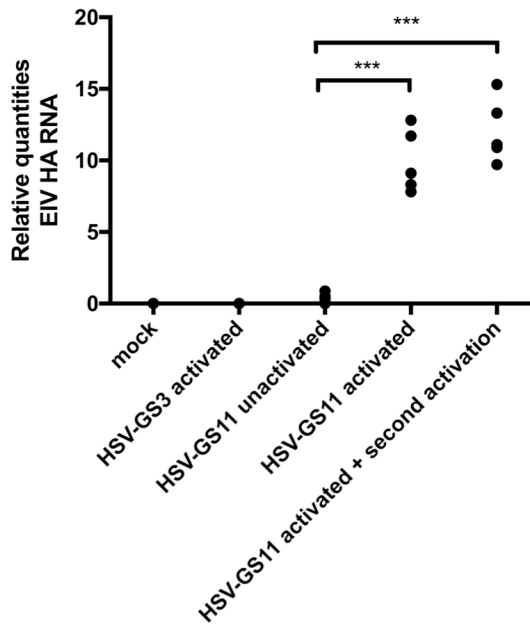


1 Figure 8

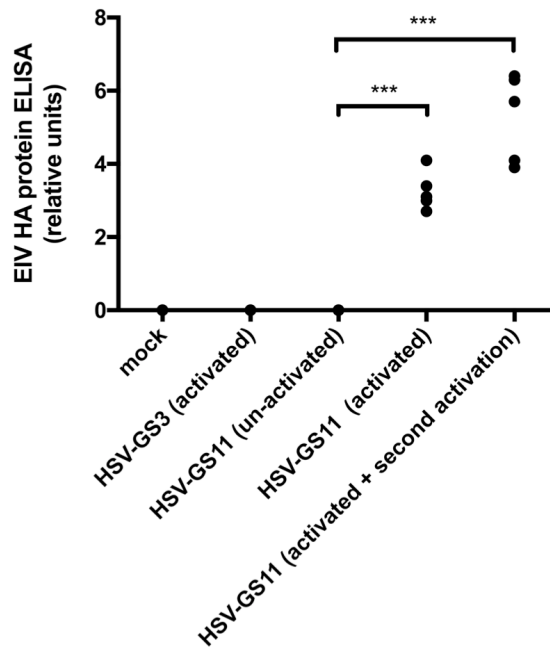
A.



B.



C.



2
3

1 Figure 8 (continued)

