Protection from Bacterial Infection by a Single Vaccination with Replication-Deficient Mutant Herpes Simplex Virus Type 1

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Adaptive immune responses in which CD8+ T cells recognize pathogen-derived peptides in the context of major histocompatibility complex class I molecules play a major role in the host defense against infection with intracellular pathogens. Cells infected with intracellular bacteria such as Listeria monocytogenes, Salmonella enterica serovar Typhimurium, or Mycobacterium tuberculosis are directly lysed by cytotoxic CD8+ T cells. For this reason, current vaccines for intracellular pathogens, such as subunit vaccines or viable bacterial vaccines, aim to generate robust cytotoxic T-cell responses. In order to investigate the capacity of a herpes simplex virus type 1 (HSV-1) vector to induce strong cytotoxic effector cell responses and protection from infection with intracellular pathogens, we developed a replication-deficient, recombinant HSV-1 (rHSV-1) vaccine. We demonstrate in side-by-side comparison with DNA vaccination that rHSV-1 vaccination induces very strong CD8+ effector T-cell responses. While both vaccines provided protection from infection with L. monocytogenes at low, but lethal doses, only rHSV-1 vaccines could protect from higher infectious doses: HSV-1 induced potent memory cytotoxic T lymphocytes that, upon challenge by pathogens, efficiently protected the animals. Despite the stimulation of relatively low humoral and CD4-T-cell responses, rHSV-1 vectors are strong candidates for future vaccine strategies that confer efficient protection from subsequent infection with intracellular bacteria.

Major histocompatibility complex (MHC) class I-restricted CD8+ T cells recognizing antigenic peptides derived from pathogens play major roles in protection against intracellular bacteria like Mycobacterium tuberculosis, Salmonella enterica serovar Typhimurium, and Listeria monocytogenes (for a review, see references 15 and 16). Vaccines utilizing inactivated or recombinant bacteria have been demonstrated to elicit both CD4- and CD8-T-cell activation (16), but they seem to be inefficient stimulators of effector (25) and memory (34) T cells if compared side by side with live bacteria (25) or recombinant viral vaccines (34). In this respect, inactivated intracellular bacterial vaccines are similar to natural M. tuberculosis (44) and L. monocytogenes (33) infections, which fail to induce sufficient immunological memory to prevent recurrent infections. DNA vaccines have been demonstrated to induce protection against infections with M. tuberculosis (43) and L. monocytogenes (9). However, it was demonstrated that combining DNA vaccines with attenuated bacteria (12), protein antigen (42), or modified vaccinia virus (28) in heterologous prime-boost vaccination protocols could further optimize protection. In other words, DNA vaccines alone are not sufficient to induce maximal protective immunity and, in a clinical setting, might require complex vaccination arrangements. Similarly, recombinant vaccinia virus could elicit only limited protection against intracellular bacteria and delayed, rather than prevented death after infection (2). Thus, as stated previously elsewhere (16), efficacious “single-shot” vaccines to intracellular infection have yet to be developed.

While most intracellular bacteria replicate in maturation-arrested phagosomes (17), L. monocytogenes bacteria egress from the phagosome and gain access to the cytoplasm of infected cells (37). This led initially to the assumption that, because their antigens have access to the cytotoxic MHC class I presentation pathway, only the latter would induce CD8-T-cell responses. In contrast, bacteria localized in phagosomes were thought to preferentially trigger CD4-T-cell responses via the phagosomal MHC class II presentation pathway. However, it has been demonstrated in mice (39) as well as in humans (41) that cytotoxic T lymphocytes (CTL) are important players in the control of both M. tuberculosis and L. monocytogenes infections (24, 29), and human MHC class I peptides derived from M. tuberculosis were recently identified (6). In addition to lysis of infected cells, CTL expressing granulysin have been shown to directly kill extracellular bacteria (11, 40). CD8+ T cells seem to be the effector cells of choice for intracellular bacteria and are currently major targets for vaccination studies (for review see (16)). Replication-deficient (10, 30) or disabled infection with sin-
gle-cycle herpes simplex virus (HSV) (3,27) has been shown to induce strong immune responses against HSV-derived antigens. Besides vaccination against HSV infections (26), the application of replication-deficient HSV vectors has been largely restricted to suicide (20) or cytokine (22) gene therapy against tumors. Vaccination with replication-defective recombinant HSV (rHSV) encoding simian immunodeficiency virus Env and Nef proteins could also be shown to induce partial protection against simian immunodeficiency virus in rhesus monkeys (32). However, neither in vivo analysis of humoral and cellular responses following recombinant HSV type 1 (rHSV-1) vaccination nor results of rHSV-1-based vaccination against bacterial infection has been reported.

In order to examine the potential of rHSV-1 vaccines for vaccination against intracellular bacteria, we used recombinant replication-defective HSV-1, which was modified to encode OVA as a model antigen. We demonstrate that in contrast to gene gun DNA vaccination, rHSV-1 induce neither strong humoral responses nor CD4+ T-cell responses specific for the model antigen OVA. However, when ex vivo CD8+ T-cell responses were compared, rHSV-1 was much more potent than DNA vaccination and resulted in immediate as well as long-term memory protection against infection with recombinant L. monocytogenes expressing OVA. Thus, rHSV-1 represents a promising “single-shot” vaccine with the potential to induce long-term protective CTL responses.

MATERIALS AND METHODS

Mice. All mice were bred and maintained under standard conditions in the animal facilities of the Institute for Immunology, Ludwig-Maximilians-University Munich; the Institute for Microbiology, Immunology and Hygiene, Technical University Munich; or the Department of Pharmacy, University of Ferrara. DO11.10 mice (expressing transgenic T-cell receptors [TCR] specific for OVA257-264/MHC class II I-A^d) were obtained from Jackson Laboratory, Bar Harbor, Maine.

Plasmid construction and preparation of recombinant, replication-defective HSV-1 vaccines. A BamHI/XhoI fragment of rabbit IgG light chain was cloned into a BamHI/XhoI-opened pcDNA3 vector (Invitrogen) to create pcDNA3-IgG-l. The pcDNA3-OVA vector encoding the secreted form of chicken ovalbumin (OVA) was constructed by cloning a 1.9-kb EcoRI fragment from the plasmid pcAc-neo-OVA (provided by F. Carbone, Melbourne, Walter and Eliza Hall Institute, Australia), which contained the entire coding sequence of OVA, into the Ul41 locus of HSV-1. The cDNA under the HSV early-early genes such as ICP4, ICP22, and ICP27 sub-

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RESULTS

Induction of CTL responses by rHSV-1 vaccines. The goal of the present study was to investigate the capacities of rHSV-1 vectors to induce protective primary and long-term immune responses against intracellular bacterial infection in vivo. Earlier attempts to use HSV-1-derived vectors in gene therapy approaches were complicated by viral cytotoxicity and transient expression of transgenes. Replication-defective HSV-1 strains, in which nonreverting mutations have been incorporated into mandatory viral genes, retain the immunogenicity of wild-type HSV but are much safer. Deletion of a series of viral immediate-early genes such as ICP4, ICP22, and ICP27 substantially reduces cytotoxicity and enhances long-term trans-
gene expression (21). Further studies have shown that these or similar mutant vectors do not interfere with MHC class I expression in the infected neurons (20) or fibroblasts (46), an important consideration with respect to antigen presentation. For our studies, a replication-incompetent, low-cytotoxicity, ICP4−, ICP22−, and ICP27− triple mutant HSV-1 virus was modified to express the model antigen chicken OVA under control of the human cytomegalovirus (CMV) promoter. This virus (T0H-OVA) was used to vaccinate mice. As positive vaccination control, we employed gene gun DNA vaccination, a method previously proven to confer CD8-T cell-mediated protective immunity against bacterial challenge (9). The plasmid used for this approach was pcDNA3-OVA, containing the same CMV-OVA-expression cassette as T0H-OVA.

Using OVA as a model antigen, we first investigated the capacity of the different vaccines to induce OVA-specific CD8-T-cell activation and expansion in vivo. With tetrameric H2-Kb/OVA257-264 complexes (Tet), we determined that the frequency of OVA-specific Tet+ CD8+ T cells in normal nonimmune C57BL/6 mice was, on average, 0.05% of all peripheral blood lymphocytes (Fig. 1a). This percentage did not increase upon gene gun (0.03%) (Fig. 1b) or control rHSV-1 (TO-GFP) vaccination (0.02%) (Fig. 1d). In contrast, T0H-OVA induced a 10-fold expansion of OVA-specific CTL compared to the background (0.59%) (Fig. 1c). When the kinetics of CD8+ Tet+ T-cell expansion in peripheral blood was analyzed as a percentage of total CD8+ T cells (Fig. 1e), a weak, but statistically significant (Student’s t test, \( P = 0.0026 \)) expansion was detectable at day 7 after gene gun vaccination (0.43% ± 0.15%) compared to negative-control mice vaccinated with T0-GFP (0.15% ± 0.03%). However, a significant expansion after gene gun vaccination could not be observed in all of our experiments (see Fig. 4). In contrast, T0H-OVA vaccination induced >21-fold expansion of Tet+ CD8+ CTL over background (Fig. 1e) (3.03% ± 0.57%) (except as noted, values are presented as means ± standard deviations). At the peak of the response (day 7), the frequency of Tet+ CD8+ T cells induced by T0H-OVA was sevenfold larger than following gene gun vaccination. While levels of Tet+ CD8+ CTL remained elevated in T0H-OVA-vaccinated mice for more than 50 days, the expansion in gene gun-vaccinated mice was transient and short-lived, declining rapidly to background levels (Fig. 1e). As CD8-T-cell expansion in animals vaccinated with T0H-OVA was variable between different experiments, we could not observe elevated levels of Tet+ CD8+ CTL for such a long period in each experiment performed (see Fig. 4b). When the peripheral blood of the same animals was analyzed for nonspecific (Tet−) augmentation of the CD8− T-cell compartment, TOH-OVA and T0-GFP induced an approximately twofold increase of Tet− CD8+ T cells (data not shown). This effect was probably due to a proinflammatory response in rHSV-1-vaccinated mice and was not observed in the DNA-vaccinated group. These results show that T0H-OVA induce a severalfold stronger expansion of antigen-specific CD8+ T cells compared to gene gun vaccination.

Recombinant HSV-1 vaccination protects against L. monocytogenes infection. While infection of mice with sublethal doses of L. monocytogenes leads to rapid clearance of the bacterium and long-lived protective immunity, larger doses result in death of the animals within a few days. Despite a relatively weak induction of antigen-specific CTL expansion, as demonstrated in Fig. 1, gene gun DNA vaccination has been reported to provide protective immunity against L. monocytogenes infection in mice (9). We wondered if vaccination with T0H-OVA would have similar protective properties. To directly compare both vaccination strategies, we challenged rHSV-1 or gene gun-vaccinated mice with a lethal dose (5 × 10⁶, two times the 50% lethal dose [LD₅₀]) of L. monocytogenes genetically modified to express the model antigen OVA (L.m.-OVA [36]). In order to determine the expansion of OVA-specific CTL after vaccination and L.m.-OVA challenge, the experimental protocol was designed as shown in Fig. 2a, including Tet analysis at two stages during the experiment. Tet analysis at day 7 postvaccination confirmed the findings shown in Fig. 1; a strong increase of Tet+ CD8+ CTL could be detected in T0H-OVA immunized mice, while DNA vaccination induced a weak, but significant (\( P < 0.05 \)) increase com-

![FIG. 1. Visualization of antigen-specific CTL expansion after vaccination. PBL were stained with anti-CD8-APC and H-2Kb/OVA257-264 tetramers-phycocerythrin and analyzed by flow cytometry before (a) and 7 days after vaccination with pcDNA3-OVA (b), T0H-OVA (c), or T0-GFP (4 × 10⁶ virus particles i.v.) (d). The percentage of CD8+ Tet+ CTL among PBL is indicated on each plot. (e) The frequency of antigen-specific cells among peripheral blood CD8+ T cells was determined by H-2Kb/OVA257-264 tetramer staining for pcDNA3-OVA-, T0H-OVA-, and T0-GFP-immunized mice as shown (a to d). Data represent average values ± standard deviations (error bars) obtained from five mice per group at each time point. p.i., postimmunization.](http://jvi.asm.org/Downloaded from)
pared to nonimmunized mice (Fig. 2b). Despite the relatively weak expansion of Tet$^+$ CTL following gene gun vaccination, all mice from this group were protected from the subsequent L.m.-OVA challenge, whereas all control animals (unimmunized or control-vaccinated with T0-GFP) died between day 3 and day 6 postinfection (Fig. 2c). T0H-OVA-vaccinated mice were also fully protected (Fig. 2c). These results demonstrate that under these conditions of bacterial challenge (low dose) the strength of the CD8$^+$-T-cell response did not correlate with protection. As expected, elevated levels of Tet$^+$ CD8$^+$ CTL were detectable in both gene gun- and T0H-OVA-vaccinated mice 18 days postvaccination (10 days postinfection, Fig. 2d). While the average of Tet$^+$ CD8$^+$ CTL was higher in DNA-vaccinated mice than in the T0H-OVA-vaccinated group, the difference was not statistically significant (Fig. 2d).

To control the specificity of protection, we infected gene gun- and T0H-OVA-vaccinated mice with wild-type L. monocytogenes not expressing the model antigen OVA (Fig. 2e). As expected, neither group was protected, and all animals died from infection. Together with the results from control vaccination using T0-GFP (Fig. 2c), where mice were not protected from lethal infection with L.m.-OVA, these data exclude the possibility that protection induced by T0H-OVA is due to unspecified HSV-1-mediated effects. These findings indicate that T0H-OVA induce strong CTL responses specific for recombinant antigen and are sufficient to protect mice from subsequent bacterial infection.

**Protection from infection with a high dose of L. monocytogenes** is induced by HSV-1 vaccines but not by DNA vaccination. Although DNA vaccination induced much lower numbers of circulating antigen-specific Tet$^+$ CTL compared to T0H-OVA, both groups were protected from a lethal (two times the LD$_{50}$) L.m.-OVA challenge (Fig. 2). We wondered, however, if DNA vaccination would also provide protection from higher doses of L. monocytogenes. We therefore repeated the experiment shown in Fig. 2a, infecting the mice with a twofold-higher dose of L.m.-OVA ($10^5$ cells, four times the LD$_{50}$). When spleens and livers were analyzed 3 days postinfection for presence of viable *Listeria*, striking differences between gene gun- and T0H-OVA-vaccinated mice became evident: spleens from two out of three T0H-OVA-vaccinated animals were completely free of bacteria, while bacterial counts in the third were approximately 6,000-fold lower than those from gene gun-vaccinated mice (Fig. 3a). The latter had bacterial numbers similar to the control-vaccinated (T0-GFP) group. Livers of the same mice showed a similar picture, with an average of 430-fold-lower bacterial counts in livers of T0H-OVA-vaccinated mice than in gene gun- or control virus (T0-GFP)-vaccinated mice (Fig. 3b).

In order to test if these differences in bacterial numbers correlate with survival, we infected vaccinated mice with 10-fold more L.m.-OVA ($5 \times 10^5$ cells, 20 times the LD$_{50}$) than the amounts used for the experiment depicted in Fig. 2, and then we monitored survival (Fig. 3c). With this high dose, a direct relationship between the frequency of antigen-specific Tet$^+$ CTL induced by the vaccine and protection from infection became clear. The T0H-OVA-vaccinated mice survived without signs of disease (data not shown), while all gene gun- and T0-GFP-vaccinated mice died.
survival was monitored for 13 days.

The induction of antigen-specific CD8+ CTL observed in T0H-OVA- versus gene gun-vaccinated mice (Fig. 4b) is a systemic phenomenon, observed not only in the peripheral blood, but also in spleens of surviving mice (day 49) (Fig. 4d). The mesenteric lymph nodes of T0H-OVA-immunized mice also contained significantly more Tet+ CD8+ CTL than detected in gene gun-vaccinated animals ($P < 0.005$), although the difference was not as great as observed in spleen or blood (Fig. 4d). This analysis excludes the possibility that the differences in frequencies of Tet+ CD8+ CTL detected in peripheral blood lymphocytes (PBL) following T0H-OVA or gene gun vaccination were a result of differential homing properties of the stimulated T cells.

Taken together this analysis shows that T0H-OVA-derived vaccines induce potent long-term protection against infection with the facultative intracellular bacterium L. monocytogenes and generate a large memory CTL pool capable of clearing even large bacterial loads very efficiently. In contrast, gene gun vaccination induces lower frequencies of specific CTL sufficient for the protection of mice from challenge with lower but lethal Listeria-doses but are less efficient at achieving complete bacterial clearance.

**Induction of antigen-specific CD4+ T helper responses and antibodies by recombinant replication-deficient HSV-1.** In order to directly monitor the capacity of T0H-OVA to induce CD4+ T-cell responses against their encoded recombinant antigen in vivo, we vaccinated mice that had previously received adoptively transferred TCR-transgenic OVA-specific DO11.10 CD4+ T cells (18). Since DO11.10 transgenic T cells recognize the OVA323-339 peptide in the context of MHC class II I-A$^d$ and CD8+ CTL 7 days postvaccination (Fig. 4b). This increase was transient and after the contraction of Tet+ CTL, lower levels of Tet+ CD8+ CTL could be detected in the vaccinated mice 5 weeks after vaccination (day 39) (Fig. 4b). Following challenge with L.m.-OVA, changes were first detectable at day 5 (day 47) (Fig. 4b), with significantly elevated levels of Tet+ CD8+ CTL in T0H-OVA-vaccinated animals compared to the gene gun group or controls ($P < 0.001$); more than 20% of all CD8+ T cells in the blood of T0H-OVA-vaccinated mice were Tet+. Elevated levels of antigen-specific Tet+ CD8+ CTL in gene gun-vaccinated mice could be detected at day 49, 7 days postinfection, where they represented approximately 10% of all CD8+ CTL, an expansion sixfold lower than that of the T0H-OVA group (Fig. 4b).

Despite these differences in antigen-specific CTL frequency, both T0H-OVA- and gene gun DNA-vaccinated mice were well protected from low-dose ($5 \times 10^4$ cells) L.m.-OVA challenge, showing no signs of serious illness. In contrast, all control TO-GFP-vaccinated mice died within 6 days postinfection (Fig. 4c).

At day 7 postinfection, we sacrificed the surviving mice and analyzed spleens and livers for presence of L. monocytogenes. The spleens of all animals from both surviving groups were bacteria free (data not shown). However, while the livers of T0H-OVA-vaccinated mice were also free of L. monocytogenes, 50% of the gene gun-vaccinated mice had not yet cleared the bacteria ($3 \times 10^4$ to $9 \times 10^4$ bacteria per liver; data not shown). These data indicate that despite survival of all mice from both groups, the T0H-OVA vaccine results in more complete or, alternatively, more rapid bacterial clearance.

The different frequencies of antigen-specific CTL observed in T0H-OVA- versus gene gun-vaccinated mice (Fig. 4b) is a systemic phenomenon, observed not only in the peripheral blood, but also in spleens of surviving mice (day 49) (Fig. 4d). The mesenteric lymph nodes of T0H-OVA-immunized mice also contained significantly more Tet+ CD8+ CTL than detected in gene gun-vaccinated animals ($P < 0.005$), although the difference was not as great as observed in spleen or blood (Fig. 4d). This analysis excludes the possibility that the differences in frequencies of Tet+ CD8+ CTL detected in peripheral blood lymphocytes (PBL) following T0H-OVA or gene gun vaccination were a result of differential homing properties of the stimulated T cells.

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can be detected with the clonotypic TCR-specific MAb KJ1-26 facilitating the monitoring of their activation and expansion following vaccination with the specific antigen. While control vaccinated mice contained very few CD4^+ T cells (T0-GFP) (Fig. 5a and d), the percentages increased 5- to 10-fold and total numbers increased 10- to 30-fold in the draining lymph nodes in gene gun-vaccinated animals (Fig. 5b and d) 5 days after vaccination. In contrast, vaccination with T0H-OVA did neither augment the frequencies (Fig. 5c) nor cell numbers (Fig. 5d) of antigen-specific DO11.10 T cells. Simultaneously, we monitored the activation status of DO11.10 T cells with a MAb specific for CD44, a cell surface marker modulated from moderate levels on naive T cells to high expression on activated T cells (data not shown). While CD4^+ KJ1^− 26^+ T cells demonstrated a highly activated phenotype.
following gene gun vaccination, in lymph nodes of control (T0-GFP), and T0H-OVA-immunized mice they did not gain an activated phenotype (data not shown). A CD4-T-cell response, following i.v. T0H-OVA vaccination would be expected primarily in the spleen. Surprisingly, the kinetics of DO11.10 expansion in the spleen following T0H-OVA vaccination, in lymph nodes of control mice. Sera were obtained from mice immunized with pcDNA3-OVA, T0H-OVA, or T0-GFP (4 × 10⁶ virus particles i.v.) at the indicated time points postvaccination, and OVA-specific antibody serum levels were determined by enzyme-linked immunosorbent assay. Preimmune serum (day 0) from each group was determined with a pool of sera. Results are expressed as the mean of optical density at 450 nm (O.D. 450 nm) ± standard deviation (error bars) from at least three individual mice per group.

FIG. 6. OVA-specific antibody responses in immunized BALB/c mice. Sera were obtained from mice immunized with pcDNA3-OVA, T0H-OVA, or T0-GFP (4 × 10⁶ virus particles i.v.) at the indicated time points postvaccination, and OVA-specific antibody serum levels were determined by enzyme-linked immunosorbent assay. Preimmune serum (day 0) from each group was determined with a pool of sera. Results are expressed as the mean of optical density at 450 nm (O.D. 450 nm) ± standard deviation (error bars) from at least three individual mice per group.

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low-dose challenge will be sufficient to protect the animals from high doses of infectious material. Thus, as a consequence of the low frequencies of memory precursor CTL generated in gene gun-vaccinated mice, the response to a large infectious challenge was insufficient to offer protection (Fig. 4). These data are in line with findings that gene gun vaccination can be further enhanced by combining it in heterologous prime-boost protocols with other vaccines (12, 28, 42); rHSV-1 vaccine, on the other hand, is by itself a very efficient vaccine against even high doses of infection with intracellular bacteria. Interestingly, TOh-OVA, unlike DNA vaccination, did not induce significant CD4 T helper cell or antibody responses specific for the recombinant antigen. This may be irrelevant, as protection against intracellular bacterial pathogens such as L. monocytogenes is largely CTL mediated (17, 39). Although antibody responses could be important to neutralize L. monocytogenes immediately after bacterial entry into the host and might therefore be considered a prerequisite for the establishment of an efficient long-term vaccination effect against intracellular bacteria (8), a lack of pathogen-specific antibodies did not negatively affect the ability of HSV-1-derived vaccines to provide long-term memory protection in our system.

Many different recombinant viral vector systems, including alpha-, adeno-, pox- and poliovirus systems, have been developed for vaccination. One concern common to all these vaccines is that their efficiency might be negatively affected by preexisting immunity to the viruses, as has proven true in the case of adenovirus (38) and poxvirus vectors (14). It has recently been demonstrated that HSV-1-derived vectors are not affected by preexisting immunity to HSV-1 (4). This aspect is currently being investigated in our laboratory for the specific rHSV-1 vaccine presented here. In the light of 75% of the adult human population have been previously exposed to HSV-1, this is an important prerequisite for the efficient usage of these vaccines (13). In addition, the strength of CTL response we observed upon vaccination with TOh-OVA indicates that, in contrast to other vaccines tested in the infectious Listeria model (for a review, see reference 16), further homologous or heterologous prime-boost protocols may be superfluous in the case of rHSV-1.

We have evaluated the efficacy of rHSV-1 vaccines in mice and demonstrate in the present report that a single vaccination is sufficient to elicit a protective response specific for a vaccine-encoded, nonviral protein. We have directly compared the cellular and humoral responses elicited by rHSV-1 vaccines and gene gun DNA vaccination and demonstrate significant differences in the strength and quality of the elicited responses. To our knowledge this is the first report analyzing the capacity of a recombinant HSV-1 vaccine to directly induce primary and memory CTL, CD4 T helper cells, and humoral responses specific for a nonviral antigen. We demonstrate for the first time that HSV-1-derived vectors induce strong CTL responses, making them a promising candidate for vaccines against intracellular bacterial infection. Further experiments with HSV-1-derived vaccines will be necessary to evaluate if our findings with the model antigen OVA can be extended to more relevant bacterial antigens, which may have different immunogenic properties. Experiments will have to be performed to confirm the usefulness of HSV-1 as a vector for human immunization.

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