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Induction of herpes simplex virus type 1 cell-to-cell spread inhibiting antibodies by a calcium phosphate nanoparticle-based vaccine

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Abstract

Herpes simplex viruses 1 and 2 are among the most ubiquitous human infections and persist lifelong in their host. Upon primary infection or reactivation from ganglia, the viruses spread by direct cell–cell contacts (cell-to-cell spread) and thus escape from the host immune response. We have developed a monoclonal antibody (mAb 2c), which inhibits the HSV cell-to-cell spread, thereby protecting from lethal genital infection and blindness in animal models. In the present study we have designed a nanoparticle-based vaccine to induce protective antibody responses exceeding the cell-to-cell spread inhibiting properties of mAb 2c. We used biodegradable calcium phosphate (CaP) nanoparticles coated with a synthetic peptide that represents the conformational epitope on HSV-1 gB recognized by mAb 2c. The CaP nanoparticles additionally contained a TLR-ligand CpG^{m} and were formulated with adjuvants to facilitate the humoral immune response. This vaccine effectively protected mice from lethal HSV-1 infection by inducing cell-to-cell spread inhibiting antibodies.

Key words: CaP nanoparticles; Herpes simplex virus; Antibodies; Cell-to-cell spread

Herpes simplex viruses belong to the most widespread human viruses worldwide and are responsible for a broad spectrum of diseases. The seroprevalence rate among adults for HSV-1 is approximately 90%,[1] and for HSV-2 roughly 16%.[2] HSV transmission occurs by close body contacts, mostly during early childhood from mother to child or after the onset of sexuality in intimate contacts.[3]

Upon primary infection of the oral or genital mucosa, HSV establishes a lifelong latent infection in trigeminal (HSV-1) or lumbosacral (HSV-1 or HSV-2) ganglia of the host, where it is protected from the host immune response.[4,5] After reactivation, the virus returns to the periphery to cause symptomatic ulcers or asymptomatic viral shedding. Recurrence of HSV infections contributes to painful lesions in the orofacial (cold sores) or

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genital area.[6] Asymptomatic viral shedding occurs frequently and facilitates the ability of the virus to spread efficiently and silently throughout the population.[7] Usually, recurrent infections are painful but self-limiting.[8] In contrast, serious complications can be observed in individuals suffering from ocular infections,[9,10] in immunocompromised patients with generalized infections affecting the central nervous system,[11] or in newborns who became infected during pregnancy or delivery.[12] Antiviral interventions are available but limited due to emerging drug-resistances.[13–15]

There are no licensed vaccines currently available for the prevention or treatment of HSV-1/2 infections. Numerous attempts to develop such a vaccine have been undertaken thus far, but did not lead to success.[16] However, the pipeline of vaccine candidates being evaluated in preclinical or clinical trials is rich and includes inactivated whole viruses, live or genetically attenuated viruses, recombinant subunit (glycoprotein) vaccines, replicating nonpathogenic vectors expressing HSV antigens, DNA plasmids expressing one or more HSV proteins, and epitope-based vaccines.[16,17] The largest clinical trials to date include the Chiron HSV vaccine trial using recombinant HSV-2 glycoproteins B and D (gB2/gD2) formulated with MF59[18] and the GlaxoSmithKline (GSK) Herpevac trial, which used recombinant gD2 with alum/MPL adjuvant.[19] Although both trials did not show efficacy against HSV-2 disease, the data suggest that vaccine-induced neutralizing antibodies are a correlate of anti-HSV immunity and a benchmark for an effective vaccine.[16] However, the induction of reliable antibody responses may be challenging, since HSV can move directly between cells without diffusing through the extracellular environment and thereby circumvents the host immune response. This mechanism is also known as "cell-to-cell spread" and used by HSV during primary infection to spread between epithelial cells and neurons (cell-to-neuron spread), after reactivation of the virus from ganglia to move between neurons and epithelial cells (neuron-to-cell spread), and during an ongoing infection to spread within infected peripheral tissues.[20] This intercellular route of viral transmission is crucial for effective viral spread within the host and usually is extremely difficult to access for neutralizing antibodies.[21] Importantly, we developed an antiviral monoclonal antibody mAb 2c that effectively inhibits the cell-to-cell spread of HSV-1 and HSV-2 thereby protecting from lethal generalized infection and ocular herpes in the corresponding mouse models.[22–25] Notably, this antibody and its humanized version mAb hu2c were also protective in highly immunocompromised NOD/SCID-mice characterized by an absence of functional T cells and B cells, highly reduced numbers of natural killer (NK) cells, macrophages, granulocytes and with defects in the complement system.[26,27] The humanized antibody mAb hu2c recognized the same epitope as the parental murine antibody mAb 2c.[27] The epitope recognized by this unique antibody was determined as a highly conserved, discontinuous conformational epitope on the glycoprotein B (gB) of HSV-1 and HSV-2.[24,28]

In the present study, we used a reverse vaccinology approach to induce protecting, cell-to-cell spread inhibiting antibody-responses in mice. Reverse vaccinology relies on the principle that the target antigen of a protective mAb will elicit the same antibody reaction when used as an immunogen.[29] Here, we used synthetic peptides consisting of either one or both gB subdomains recognized by mAb 2c for vaccination. We coupled the peptides to the surface of biodegradable calcium phosphate nanoparticles (CaP) with incorporated Toll-like receptor (TLR) 9 ligand CpG^m, which were described as a potent tool to induce strong antibody responses in a prior study.[30] Finally, we investigated whether the CaP-Peptide nanoparticles can induce cell-to-cell spread inhibiting antibodies in mice and thereby protect from lethal infection.

Methods

Ethics statement

Animal experiments were performed in strict accordance with the German regulations of the Society for Laboratory Animal Science (GV-SOLAS) and the European Health Law of the Federation of Laboratory Animal Science Associations (FELASA). The protocol was approved by the North Rhine-Westphalia State Agency for Nature, Environment and Consumer Protection (LANUV) (Permit number: G 1633/17). Preparation of murine sensory neurons was performed according to the German Animal Welfare Act. All efforts were made to minimize animal suffering.

Animals

Female BALB/c mice, 7-8 weeks of age, were purchased from Charles River Laboratories (Charles River Laboratories, Sulzfeld, Germany). All mice were maintained under pathogen-free conditions. Experiments were performed according to the German legal requirements with the approval of the University Hospital Essen's Animal Facility.

Viruses

The reporter virus HSV-1-ΔgE-GFP was kindly provided by Hartmut Hengel (Institute of Virology, Freiburg, Germany) and originally published by Farnsworth et al[31] HSV-1F and HSV-1-ΔgE-GFP were propagated on Vero cells as previously described.[27] A standard plaque assay was used to determine the plaque-forming units in the virus-containing supernatants.

Antibodies

The monoclonal antibody mAb 2c was produced and purified as described previously.[24] Antibody concentration was measured with a NanoDrop 2000 spectrometer (Thermo Fischer Scientific, Wilmington, USA). The purity was confirmed by fast protein liquid chromatography (FPLC) to be ≥95%. Polyclonal murine antibodies were purified from the sera of immunized mice using protein A/G agarose (Thermo Fisher Scientific) and dialyzed against Phosphate-buffered saline (PBS) for in vitro experiments.

Cells

Vero cells (American Type Culture Collection, ATCC, CCL81, Rockville, MD) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies Gibco, Darmstadt, Germany) containing 10% (v/v) fetal calf serum (FCS; Life Technologies Gibco), 100 U mL^{-1} penicillin and 0.1 mg mL^{-1} streptomycin.

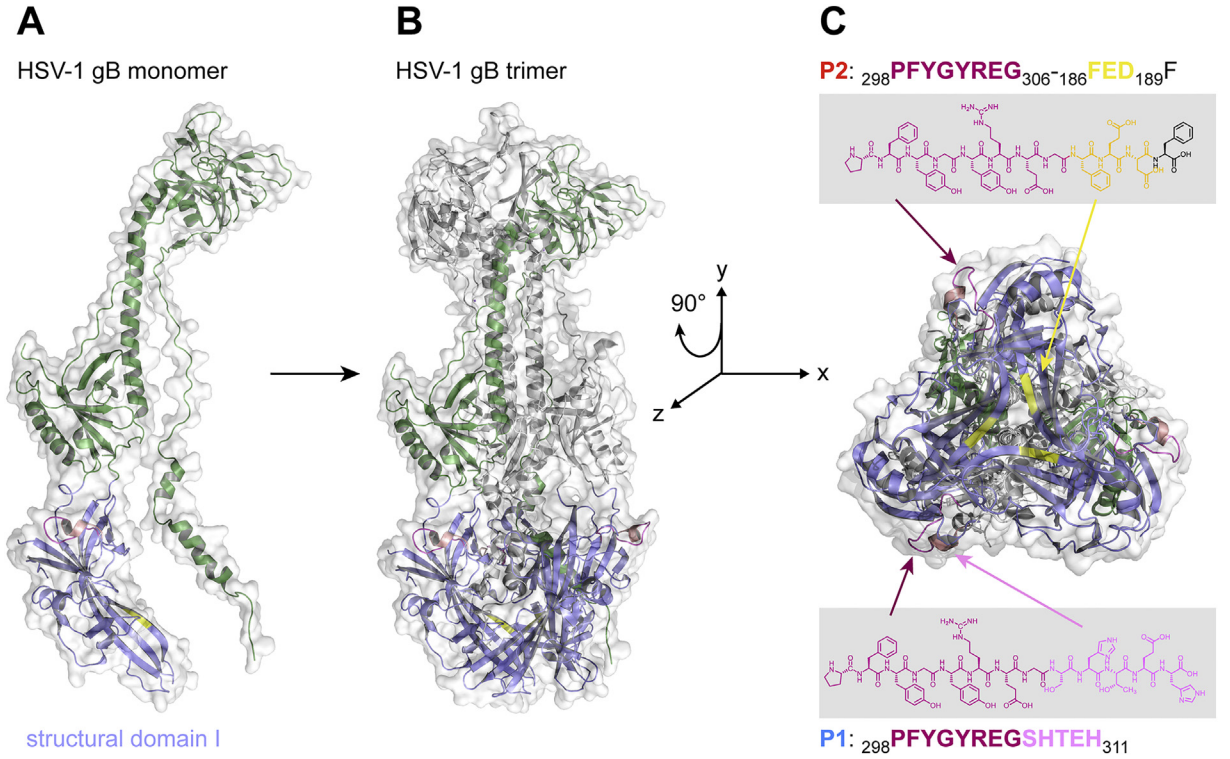

Figure 1. Localization of the P1 and P2 sequences on HSV-1 gB. The monoclonal antibody mAb 2c binds to a discontinuous epitope within the structural domain one on gB.[27,28] For vaccination, two synthetic peptides, P1 ($_{298}$PFYGYREGSHTEH$_{311}$) and P2 ($_{298}$PFYGYREG$_{306\text{-}186}$FED$_{189}$F), were selected upon their relative binding strength towards mAb 2c as reported by Däumer et al[28] The P1 sequence (aa 298-311 on gB; bordeaux and pink) comprises the epitope subdomain B sequence recognized by mAb 2c.[27] P2 is a fusion peptide consisting of the consensus sequences of the mAb 2c epitope subdomains A (bordeaux) and B (yellow) flanked by a phenylalanine at the C-terminal end.[27] The sequences of P1 and P2 are mapped to the crystal structure HSV-1 gB shown as monomer **(A)**, trimer **(B)** and for more details, the gB-base **(C)**. Out of the five structural domains of HSV-1 gB,[47] the domain I, which includes the mAb 2c epitope sequences, is highlighted in purple. Arrows indicate the position of P1 and P2 substructures within the structural domain I.

Peptides

The peptides P1 ($_{298}$PFYGYREGSHTEH$_{311}$) and P2 ($_{298}$PFYGYREG$_{306\text{-}186}$FED$_{189}$F) were selected by their relative binding strength towards mAb 2c as previously described[28] and purchased from *peptides&elephants* (Henningsdorf, Germany).

Immunization and infection of mice

The peptides or CaP nanoparticles were solved in PBS and formulated with 12.5 μg of Monophosphoryl Lipid A (MPL; Sigma-Aldrich, St. Louis, USA) and Alum (Thermo Fisher Scientific) at a ratio of 1:1. Mice were immunized with the synthetic peptides P1 or P2 (n = 10) or CaP nanoparticles coated with P1 or P2 (CaP_P1; CaP_P2) (n = 5) on days 0, 14 and 28. The vaccine was delivered by intramuscular injection (i.m.) of 30 μL vaccine formulation in each of the hind legs. The antigen concentration was 10 μg peptide (synthetic peptides or when immobilized on CaP nanoparticles) per immunization. Mock immunized mice served as control. One week after the last immunization, mice were treated with medroxyprogesterone acetate (Depo-Provera, Pfizer, NY, USA) to facilitate HSV infection.[32] One week after progesterone pre-treatment, mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (20 mg kg^{-1}) and the murine vagina was cleaned with a sterile cotton swab immediately before being challenged with a 20 μL inoculum of 1 × 10^5 TCID$_{50}$ of HSV-1 F per mouse. Skin glue (Epiglu; Meyer-Haake Medical Innovations, Wehrheim, Germany) was applied to the vulva to prevent discharge of the virus inoculum. Mice were assessed for infection by determining viral loads in vaginal irrigations harvested on days 2, 4, 6, and 8 after infection by endpoint dilution.[24]

Inhibition of HSV-1 cell-to-cell spread by vaccine-induced antibodies

Mice were immunized three times with peptides (P1 or P2) or CaP nanoparticles (CaP_P1 or CaP_P2) every 14 days as described above. Retro-orbital blood collection was performed before each vaccination on days 0, 14 and 28 and additionally two weeks after the third booster immunization on day 42. Antibodies were purified from sera and tested for the inhibition of the HSV-1 cell-to-cell spread. Therefore, Vero cells were seeded in 24-well microtiter plates. At a confluence of approximately 80% the cells were infected with 400 TCID$_{50}$ HSV-1-ΔgE-GFP reporter virus. At 4 h post infection, the inoculation medium was removed and the cultures were inoculated with medium containing antibodies (100 μg mL^{-1}) isolated from the immunized mice. Medium, purified IgG from naïve mice or mAb 2c at 500 nM (75 μg mL^{-1}) was used as a control. Plaque formation was examined by fluorescence microscopy at 48 h post infection.

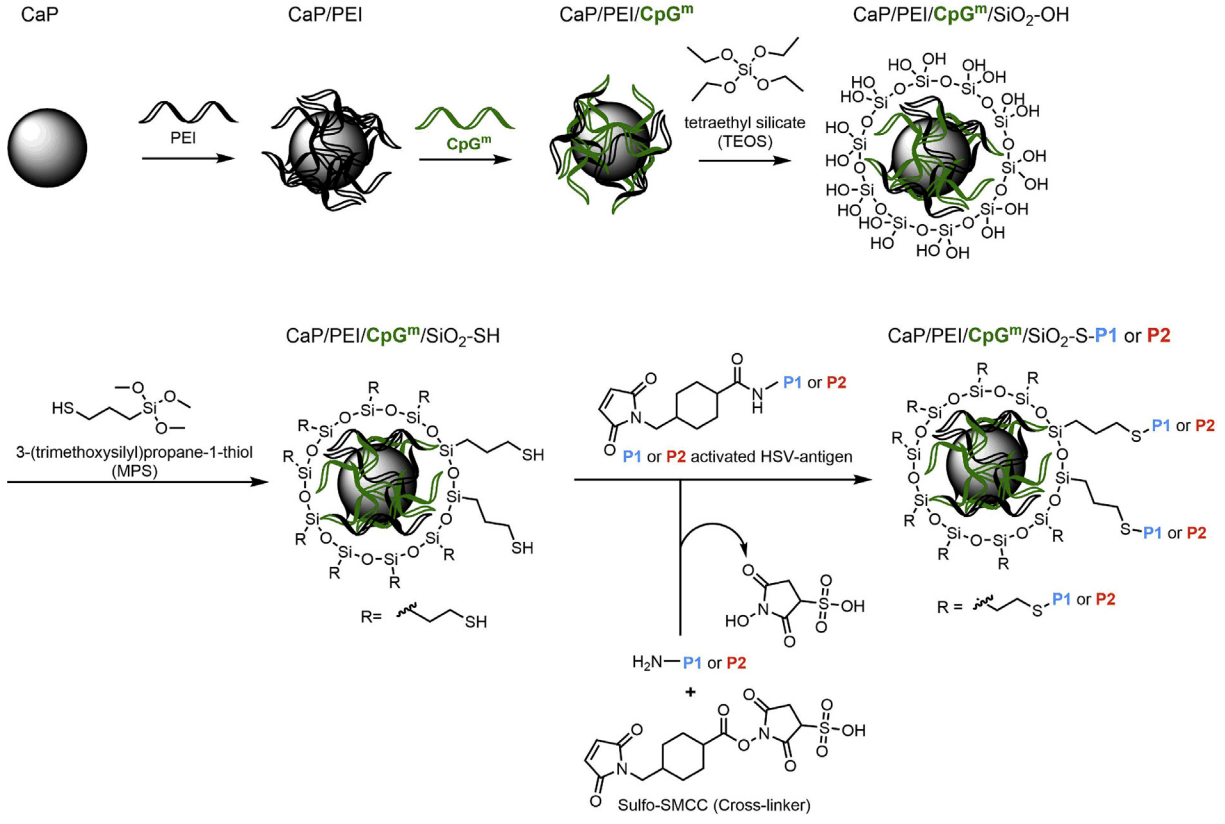

Figure 2. Synthesis of the antigen-coated calcium phosphate nanoparticles (CaP/PEI/CpG^m/SiO_2-S-P1 or -P2).Calcium phosphate nanoparticles are obtained by the precipitation of calcium lactate and diammonium hydrogen phosphate, forming a calcium phosphate core (CaP). This core is stabilized by a positively charged polyelectrolyte PEI (CaP/PEI). The positively charged dispersion is then loaded with the immuno-modulative agent CpG^m (CaP/PEI/CpG^m). The silanization of the dispersion is achieved by the Stöber method in which the nanoparticles are coated with a thin silica layer (CaP/PEI/CpG^m/SiO_2-OH). Addressable thiol-groups are obtained by a further derivatization with MPS, giving thiol terminated calcium phosphate nanoparticles (CaP/PEI/CpG^m/SiO_2-SH). After the activation of the peptide with sulfo-SMCC, the antigen-functionalized calcium phosphate nanoparticles are prepared by conjugating the peptide (antigen) to the nanoparticle surface.

Images were taken with a Zeiss Observer Z1 fluorescence microscope at a 100-fold magnification.

Statistical analysis

GraphPadPrism 5 (GraphPadPrism Software, La Jolla, CA, USA) was used to analyze the data. The differences in the viral loads between the mock and the vaccinated groups were analyzed using a nonparametric Mann–Whitney t-test. Changes of vaccine-induced, HSV-1 specific antibody concentrations upon vaccinations were analyzed with a two-way ANOWA and a Bonferroni post-test. Differences were considered significant at $*P < 0.05$ and $**P < 0.01$. Ns = not significant. The differences between the number of mice with lethal HSV-related symptoms and healthy mice were determined using the Log-rank (Mantel–Cox) test. Comparisons were considered significant at $*P < 0.05$.

Results

Neutralizing antibodies like mAb 2c or its humanized version mAb hu2c, which can inhibit the cell-to-cell spread of HSV-1, turned out to be highly protective against severe infections. We used a reverse vaccinology approach to elicit antibody responses with the same cell-to-cell spread inhibiting properties like mAb 2c. The antigens P1 ($_{298}$PFYGYREGSHTEH$_{311}$) and P2 ($_{298}$PFYGYREG$_{306\text{-}186}$FED$_{189}$F) used in this study were identified previously when characterizing the gB-epitope recognized by mAb 2c.[28] Both peptides mimic the conformational epitope recognized by mAb 2c, and consist of either one (P1) or both (P2) subdomains of the conformational epitope on HSV-1/2 gB recognized by mAb 2c (Figure 1). We immobilized each of the peptides on CpG^m-carrying CaP nanoparticles for immunization studies in mice as described in the experimental section (Figure 2).

The non-functionalized and peptide-loaded calcium phosphate nanoparticles were characterized by differential light scattering (DLS), scanning electron microscopy (SEM) and UV-spectroscopy (Figure 3). The PDI and zeta potential indicate a stable dispersion of fairly monodisperse nanoparticles (PDI < 0.3). The measured diameter by DLS was increased after the peptide conjugation, indicating a successful attachment of the peptides to the surface of the nanoparticles. SEM images showed a uniform and spherical shape of the nanoparticles (Figure 3).

To determine the number of peptide and CpG molecules on the nanoparticles that were applied in mice, the number of each molecule on one single nanoparticle was calculated as reported earlier (see experimental section and Table 1).[33] Table 1 gives all

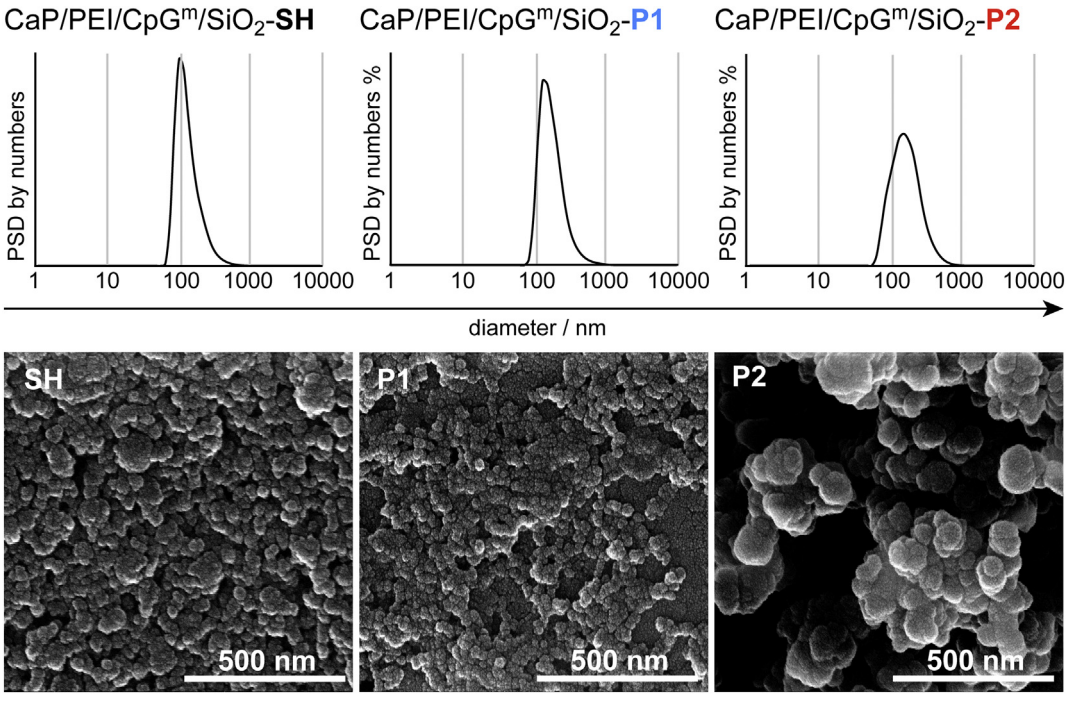

Figure 3. DLS and SEM data of the functionalized calcium phosphate nanoparticles.Top: DLS graphs of CpG^m-loaded and thiol-functionalized calcium phosphate nanoparticles with a hydrodynamic diameter of ~59 nm, CpG^m-loaded and P1-functionalized calcium phosphate nanoparticles with a hydrodynamic diameter of ~50 nm, and CpG^m-loaded and P2-functionalized calcium phosphate nanoparticles with a hydrodynamic diameter of ~106 nm. Bottom: The corresponding SEM data showed a spherical morphology. Scale bar 500 nm.

Table 1
Characterization of antigen-functionalized calcium phosphate nanoparticles.

Particles (CaP/PEI/CpG^m/SiO_2-S-X; X =)	H	P1	P2
Parameter			
Solid core diameter by SEM / nm	59 ± 10	50 ± 7	106 ± 29
Volume (one nanoparticle; only CaP) / m^3	$1.08 \cdot 10^{-22}$	$6.54 \cdot 10^{-23}$	$6.24 \cdot 10^{-22}$
Surface area (one nanoparticle; only CaP) / m^2	$1.09 \cdot 10^{-14}$	$7.85 \cdot 10^{-15}$	$3.53 \cdot 10^{-14}$
Mass (one nanoparticle; only CaP) / kg	$3.37 \cdot 10^{-19}$	$2.05 \cdot 10^{-19}$	$1.96 \cdot 10^{-18}$
w (Ca^{2+}) by AAS / kg m^{-3}	0.033	0.0102	0.0089
w ($Ca_5(PO_4)_3OH$) / kg m^{-3}	0.083	0.026	0.022
Number (nanoparticles) / m^{-3}	$2.45 \cdot 10^{17}$	$1.25 \cdot 10^{17}$	$1.14 \cdot 10^{16}$
w (CpG^m)/ kg m^{-3}	0.038	0.038	0.038
Number (CpG^m molecules) / m^{-3}	$3.60 \cdot 10^{21}$	$3.6 \cdot 10^{21}$	$3.60 \cdot 10^{21}$
Mass (CpG^m) per nanoparticle/kg	$1.6 \cdot 10^{-19}$	$3.0 \cdot 10^{-19}$	$3.3 \cdot 10^{-18}$
Number (CpG^m molecules) per nanoparticle	$1.5 \cdot 10^{4}$	$2.9 \cdot 10^{4}$	$3.2 \cdot 10^{5}$
wt.-ratio CpG^m to calcium phosphate	1:2.17	1: 0.67	1: 0.59
$w_{initial}$ (peptide) / kg m^{-3}		0.047	0.034
w (surface-bound peptide) / kg m^{-3}	-	0.040 (yield 86%)	0.023 (yield 67%)
Number (peptide molecules) / m^{-3}	-	$1.5 \cdot 10^{22}$	$9.1 \cdot 10^{21}$
Mass (peptide molecules) per nanoparticle / kg	-	$3.2 \cdot 10^{-19}$	$2.0 \cdot 10^{-18}$
Number (peptide molecules) per nanoparticle	-	$1.2 \cdot 10^{5}$	$7.9 \cdot 10^{5}$
Density of peptide molecules on the nanoparticle surface / nm^{-2}	-	16	23
wt.-ratio peptide to calcium phosphate	-	1: 0.64	1: 0.97
Hydrodynamic nanoparticle diameter by DLS / nm	232	296	270
PDI by DLS	0.283	0.334	0.270
Zeta potential by DLS / mV	+33	+26	+24
Particle composition			
CaP / wt.-%	68	25	27
CpG^m / wt.-%	32	37	46
Peptide / wt.-%	-	39	28

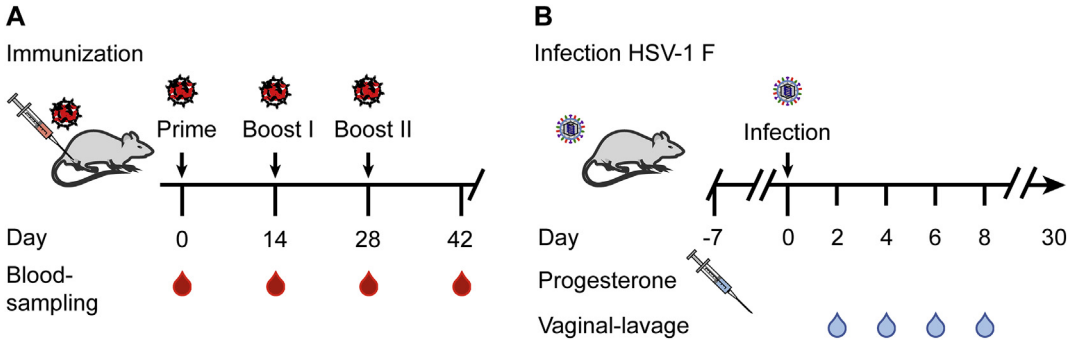

Figure 4. Vaccination and infection procedure. Mice were immunized with MPL/Alum adjuvanted synthetic peptides (P1 or P2) or MPL/Alum adjuvanted CaP nanoparticles (CaP_P1 or CaP_P2) on day 0 and booster-immunized every two weeks on days 14 and 28. The peptides and CaP nanoparticles were formulated with MPL and Alum. To monitor humoral immune responses, serum was taken as indicated in the scheme and examined for the presence of HSV-specific and cell-to-cell spread inhibiting antibodies **(A)**. To investigate the protective effect of the tested vaccine candidates, mice were infected with HSV-1 F and observed over a period of 30 days for HSV-associated disease **(B)**. Mice were pre-treated with progesterone one week after the last immunization (day 7 before infection), and intravaginally challenged with a lethal dose of 1×10^5 $TCID_{50}$ HSV-1 F. Vaginal lavages (20 µl PBS) were harvested on days 2, 4, 6 and 8 to determine the viral loads by microtitration.

nanoparticle characterization data, including the content of the biomolecules P1, P2, and CpG^m.

Induction of HSV-1 specific and cell-to-cell spread inhibiting antibodies

Prior studies indicated that vaccine-induced neutralizing antibodies are a correlate of protection against genital herpes.[16] Furthermore, neutralizing antibodies, which can additionally block the cell-associated viral transmission over the cell-to-cell spread, revealed to be most protective against HSV infections.[25] In line with these data, we could demonstrate that a humanized monoclonal antibody mAb hu2c capable of blocking the cell-to-cell spread of HSV-1 and HSV-2 can mediate complete protection from lethal infection and severe ocular manifestations. Hence, we investigated whether the CaP nanoparticle-based vaccine, which displays the short peptides P1 or P2 on the surface, thereby mimicking the HSV-gB epitope recognized by mAb 2c and mAb hu2c, can induce cell-to-cell spread inhibiting antibody responses in mice. Therefore, we immunized BALB/c mice with CpG^m-carrying CaP nanoparticles that were surface-decorated with either P1 or P2. As controls, the synthetic peptides P1 or P2 were used. Peptides and CaP nanoparticles were formulated with Alum/MPL adjuvants, respectively. The applied dose of soluble or CaP-bound peptides was 20 µg per vaccination. Vaccine-induced polyclonal antibodies were purified from the sera of the immunized mice harvested before each vaccination on days 0, 14, 28 and two weeks after the third vaccination on day 42 (Figure 4, *A*). The polyclonal antibodies were tested for the presence of HSV-1 specific antibodies using an ELISA assay. HSV-1 specific antibodies could be detected in samples derived from mice immunized with synthetic peptides and CaP nanoparticles. The highest amounts of HSV-1 specific antibodies could be observed in mice being three times vaccinated with CaP_P2 (Figure 5). Next, the antibodies were adjusted to a final concentration of 100 µg mL^{-1} and tested for the inhibition of the HSV-1 cell-to-cell spread in Vero cell cultures. The cell cultures were infected with 400 $TCID_{50}$ HSV-1 GFP reporter virus for 4 h to infect single cells within the cell layer. The inoculation medium was then removed and the cultures were inoculated with medium containing purified antibodies from the immunized mice. Medium, purified IgG from naïve mice or mAb 2c at 500 nM (75 µg mL^{-1}) was used as control. 48 h after infection, the cells were analyzed for plaque formation, which reflects the infection of cells via the cell-to-cell spread. Purified antibodies from naïve mice had no impact on the cell-to-cell spread, since plaque formation was observed and the number of infected cells was similar to the medium control without antibodies (Figure 6, *A* and *B*). In contrast, mAb 2c completely inhibited the cell-to-cell spread, since the infection was limited to the initially infected single cells (Figure 6, *A* and *B*). Polyclonal antibodies from mice immunized with synthetic peptides P1 or P2 had only a modest effect on the cell-to-cell spread. The plaque size was slightly reduced and the number of HSV-infected infected cells per slide was lower when compared

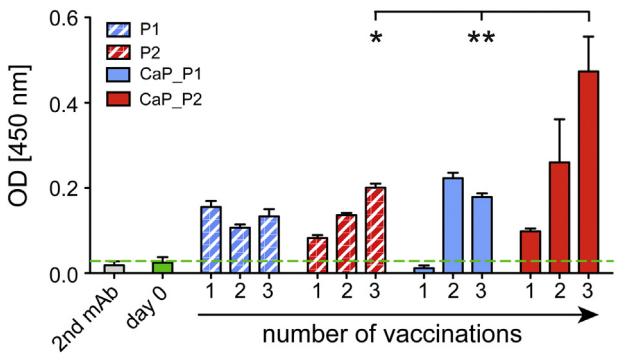

Figure 5. Antibody responses to CaP_P1 and CaP_P2 nanoparticles. Mice were immunized with MPL/Alum adjuvanted synthetic peptides (P1 or P2) or MPL/Alum adjuvanted CaP nanoparticles (CaP_P1 or CaP_P2) at days 0, 14 and 28. All vaccine candidates were formulated with alum/MPL. Antibodies were purified before each vaccination, adjusted at a final concentration of 0.2 µg/ml and tested for the presence of HSV-1 specific antibodies by ELISA. As control, purified antibodies from naïve mice (day 0) were used. Differences in the HSV-1 specific antibody levels were analyzed with a two-way ANOVA and a Bonferroni post-test and considered significant at $*P < 0.05$ and $**P < 0.01$.

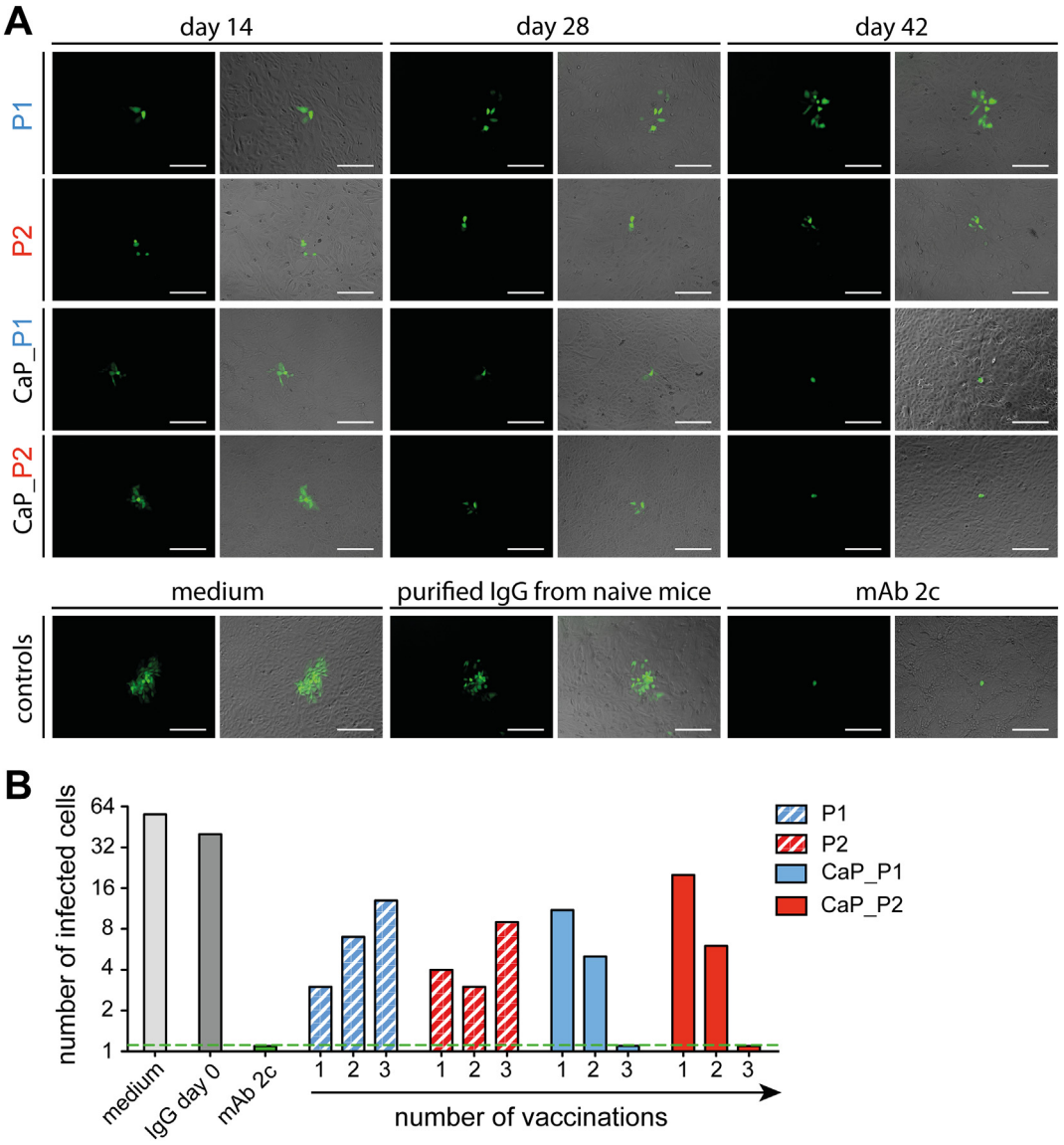

Figure 6. Inhibition of HSV-1 cell-to-cell spread by vaccine-induced antibodies. Mice were immunized three times with MPL/Alum adjuvanted peptides (P1 or P2, n = 10) or MPL/Alum adjuvanted CaP nanoparticles (CaP_P1 or CaP_P2, n = 5). Sera were collected before each vaccination on days 0, 14 and 28 and additionally two weeks after the third booster immunization on day 42. Antibodies were purified from pooled sera and tested for the inhibition of the HSV-1 cell-to-cell spread. Confluent Vero-cells were infected with 400 $TCID_{50}$ HSV-1 GFP reporter virus. At 4 h post infection, inoculation medium was removed and the cultures inoculated with medium containing purified antibodies (100 μg mL^{-1}) from the immunized mice. Medium, purified IgG from naïve mice or mAb 2c at 500 nM (75 μg mL^{-1}), was used as control. Pictures were taken 24 h post infection. **(A)** Magnification = 100×. Scale bar = 200 μm. **(B)** Semiquantitative analysis of the inhibition of HSV-1 cell-to-cell spread by vaccine induced antibodies as shown in Fig. 6A. HSV-1 GFP infected cells were counted and are shown as a bar diagram. The dotted line indicates the cut-off for the complete inhibition of the cell-to-cell spread (single-infected cell).

to the medium control or IgG from naïve mice (Figure 6, *A* and *B*). No difference could be observed in plaque-reducing effects of the antibodies harvested at different time points (days 14, 28 or 42 after immunization).

Notably, CaP nanoparticles loaded with P1 or P2 induced antibody responses that completely inhibited the HSV-1 cell-to-cell spread (Figure 6, *A* and *B*). Plaque reduction was observed when the HSV-1 infected cell cultures were treated with purified antibodies derived from CaP_P1 or CaP_P2 immunized mice 14 days after the first vaccination and before each booster vaccination. Strikingly, the antiviral effect of the polyclonal antibodies was clearly enhanced by the number of vaccinations. Antibodies purified from serum harvested after the second immunization had a better effect on the cell-to-cell spread than antibodies derived after the initial immunization. Remarkably, antibodies isolated two weeks after the third vaccination completely blocked the HSV-1 cell-to-cell spread (Figure 6, *A* and *B*, day 42).

Taken together, these data demonstrate that CpG^{m}-carrying CaP nanoparticles loaded with the peptides P1 or P2 can induce HSV-1 cell-to-cell spread inhibiting antibodies. Furthermore, three immunizations are needed to achieve a sufficient antibody-

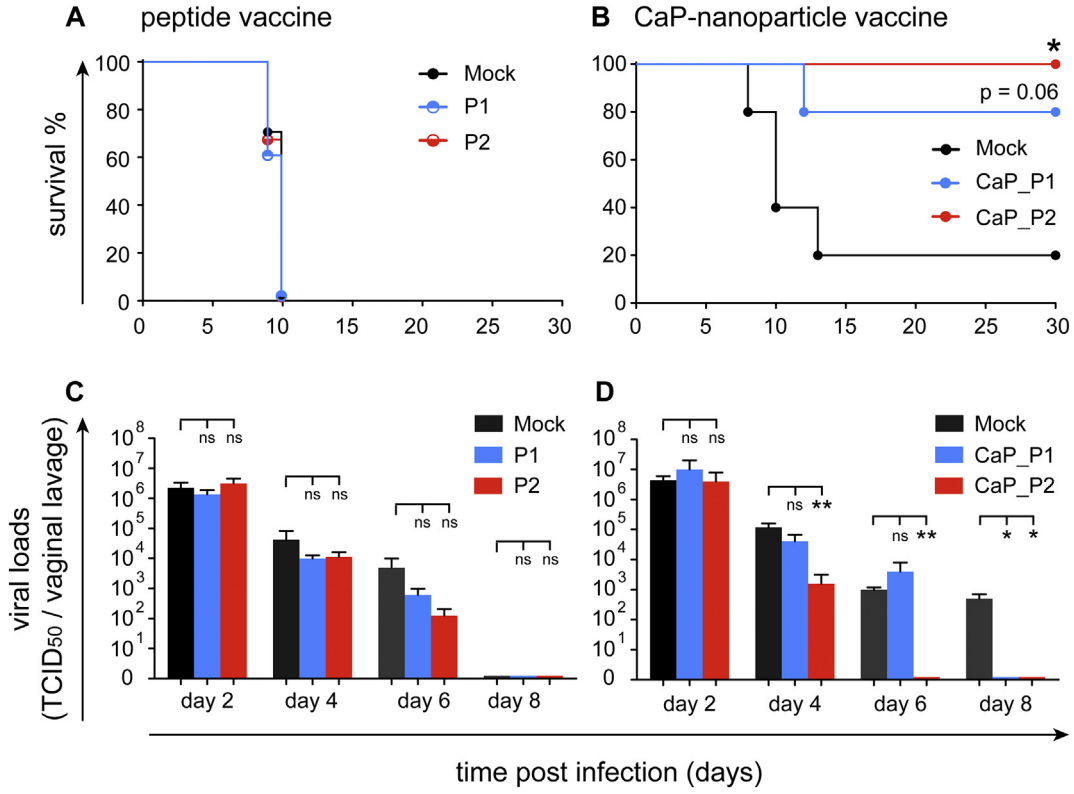

Figure 7. Protection against lethal HSV-1 infection after CaP nanoparticle immunization. Mice were immunized three times with peptides **(A)** and **(C)** (P1 or P2; n = 8; 20 μg peptide per dose; mock n = 6) or CaP nanoparticles **(B)** and **(D)** (CaP_P1 or CaP_P2; n = 5; 10 μg peptide per dose; mock n = 5) on days 0, 14 and 28. Peptides and CaP nanoparticles were adjuvanted with 12.5 μg MPL and formulated with Alum at 1:1 ratio. Two weeks after the third immunization, mice were intravaginally challenged with a lethal dose of 1×10^5 $TCID_{50}$ HSV-1 F. Mice were observed over a period of 30 days and analyzed for the appearance of HSV-1 associated disease. Mice were sacrificed upon the appearance of severe neurological symptoms. The statistical significances were determined using the Log-rank (Mantel–Cox) test. Comparisons were considered significant at $^*P < 0.05$. Viral lavages of peptide **(C)** or CaP nanoparticle **(D)** -immunized mice were harvested on days 2, 4, 6 and 8 post infection and analyzed for viral loads by microtitration. Differences in the viral loads between the mock and vaccinated groups were analyzed for each day using a nonparametric Mann–Whitney *t*-test and differences were considered significant at $^*P < 0.05$ and $^{**}P < 0.01$. ns = not significant.

concentration required for complete cell-to-cell spread inhibition in cell culture.

Protection from lethal HSV-1 genital infection

The cell-to-cell spread inhibiting monoclonal antibody mAb 2c was protective in several mouse models. Since the CaP nanoparticles loaded with P1 or P2 induced cell-to-cell spread inhibiting antibodies, we next investigated whether these antibody responses were sufficient to mediate protection from a lethal genital HSV-1 infection. Mice were immunized three times either with MPL/Alum adjuvanted synthetic peptides P1 or P2 or MPL/Alum adjuvanted CpG^m-carrying CaP nanoparticles loaded with P1 or P2 (Figure 4, *A*). Mock-immunized mice served as control. All vaccine candidates were formulated with MPL/Alum standard adjuvant.

One week after the third immunization, mice were pre-treated with progesterone to facilitate genital HSV-1 infection. One week later, mice were challenged with a lethal dose of 1×10^5 $TCID_{50}$ HSV-1 F. Mice were monitored daily for symptoms typical for HSV-1 disease, such as swollen vaginal mucosa or paralysis of the hind legs. Additionally, vaginal lavages (20 μL PBS) were harvested on days 2, 4, 6 and 8 after infection to determine the viral loads by microtitration (Figure 4, *B*). Monitoring of the viral loads allows estimating the neutralizing effect of antiviral antibodies on HSV-1 replication at the site of infection.

Vaccination with soluble peptides P1 or P2 had no impact on the lethal outcome of infection. On days 9 to 10, all mice showed symptoms of paralysis related to HSV-1 manifestation of the central nervous system and were sacrificed (Figure 7, *A*). Furthermore, no significant difference in viral loads in the vaginal mucosa could be observed between peptide and mock-immunized groups (Figure 7, *C*).

Remarkably, mice immunized with CaP nanoparticle vaccines were almost completely protected from a lethal outcome of infection (Figure 7, *B*). Mock-immunized mice developed HSV-1 related symptoms between day 8 and 13 and had to be sacrificed, except for one mouse (Figure 7, *B*). In contrast, all CaP_P2 and 4 out of 5 CaP_P1 immunized mice were free of symptoms and survived the otherwise lethal HSV-1 infection. Accordingly, HSV-1 infection could be completely eradicated in CaP_P1 or CaP_P2 immunized mice. The average viral loads in the CaP_P1 immunized group were similar to those of the mock-immunized group on days 2, 4 and 6. In contrast to the mock

group, there was no detectable virus load in the vaginal lavages from CaP_P1 immunized mice taken on day 8. Interestingly, CaP_P2 showed a better efficacy than CaP_P1, because the virus was eliminated more rapidly from the vaginal mucosa. CaP_P2 immunized mice showed significantly reduced viral loads in vaginal lavages taken on day 4, and the virus was eliminated completely by day 6 (Figure 7, *D*).

Taken together, mice could successfully be reverse-immunized with a peptide vaccine mimicking the mAb 2c epitope on HSV-1 gB. CpG^m-carrying CaP nanoparticles loaded with synthetic peptides P1 or P2 induced HSV-1 cell-to-cell spread inhibiting antibodies in mice. Thereby, immunized mice showed reduced viral loads in the vaginal mucosa and were almost completely protected from lethal HSV-1 infection.

Discussion

In the past few years, numerous efforts have been undertaken to develop a potent vaccine against HSV-1 and -2. Although some vaccine candidates were effective in animal models, to date none of those vaccines has been effective in humans. Lastly, a gD2 subunit vaccine failed in a Phase III study performed by GlaxoSmithKline.[19] The analysis of sera derived from study participants revealed that vaccine-induced antibodies targeting neutralizing epitopes on viral glycoproteins were of key importance to mediate protection from disease.[34] Furthermore, a recent retrospective analysis investigating the antibody responses of the participants of the Herpevac trial and gD2 immunized guinea pigs demonstrated that neutralizing and cell-to-cell spread inhibiting antibodies are a correlate of immune protection against symptomatic HSV-infections.[35] Unfortunately, participants of the Herpevac trial were shown to produce antibodies targeting a significantly lower number of neutralizing gD2 epitopes than guinea pigs, including those being associated with cell-to-cell spread. Based on these findings, there is clearly a need to generate an optimized vaccine, which specifically can induce neutralizing and cell-to-cell spread inhibiting antibodies.

In the present study, we used a reverse vaccination strategy to generate a vaccine capable of inducing antibody responses with cell-to-cell spread inhibiting properties. We have synthesized biodegradable CaP nanoparticles displaying synthetic peptides P1 and P2 on their surface to induce protecting antibody-responses in mice. The peptides mimic a neutralizing epitope on the HSV-1/2 gB, which is known to elicit a protective antibody response in mice. We have demonstrated that the CaP_P1 and CaP_P2 vaccine candidates induced cell-to-cell spread inhibiting antibodies in mice thereby protecting from lethal HSV-1 infection.

The selection of a proper antigen, a reliable delivery system and a potent adjuvant system are crucial for generating a protective vaccine. The antigens used in this study consist of either one (P1) or both (P2) subdomains of the gB-epitope recognized by the humanized antiviral antibody mAb hu2c and its parental murine counterpart mAb 2c.[27] In prior studies we have demonstrated that these antibodies mediated complete protection from HSV-disease in the murine models for immunosuppression and severe ocular infections.[22,25,27,36] The unique feature of both antibodies is the ability to inhibit the cell-to-cell spread of HSV-1 and HSV-2.[27] This cell-associated route of viral transmission is known as a mechanism of immune evasion and used by HSV to move from infected cells to non-infected neighboring cells to facilitate infection.[20] By this means the virus remains inaccessible to neutralizing antibodies. In animal models, mAbs 2c and hu2c showed superior effects when compared with polyclonal neutralizing antibodies.[22,27,36] Based on these findings, we reasoned that a vaccine that is capable of inducing antibodies with the same properties as mAb 2c should be highly protective in preventing HSV disease. To achieve this goal, we used a reverse vaccinology approach. The target antigens P1 and P2 used for vaccination experiments in this study were small peptides consisting of either one (P1) or both (P2) subdomains of the conformational epitope recognized by mAb 2c and mimic the original conformational epitope localized on HSV-1/2 gB.[28] Prior studies showed that immunization with small peptides induced virus-neutralizing and protective immune responses against other viruses like the Respiratory Syncytial Virus (RSV) or Measles virus.[37,38] However, peptide-based vaccines alone, even those comprising optimal B-cell epitopes, are poorly immunogenic and require adjuvants and appropriate delivery systems to be effective.[39]

Hence, we used biodegradable calcium phosphate nanoparticles (CaP) with incorporated Toll-Like receptor (TLR) 9 ligand CpG^m as a delivery system for the antigens P1 and P2. These nanoparticles were described as a potent tool to induce strong antibody responses in a prior study.[30] Additionally, Alum and MPL, also known as the GlaxoSmithKline adjuvant system AS04, were used as adjuvants to additionally modulate the adaptive immune response.[40] The major advantages of CaP nanoparticles over other nanoparticle-based delivery systems such as virus-like particles are their biodegradability and biocompatibility as CaP is the inorganic component of human hard tissues i.e. bone and teeth.[41–43] The calcium phosphate nanoparticles are taken up by the cells via endocytosis and end up in an endolysosome. There, dissolution of the calcium phosphate core due to the acidic environment occurs, and the cargo is released. The silica shell is very thin (a few nm in thickness) and will not dissolve in the endolysosome. However, this very small amount of silica will probably be excreted by the cell without causing any biological effect. It is also noteworthy that calcium phosphate nanoparticles do not pose a risk to cells or animals if applied in reasonably low doses.[44] The prepared calcium phosphate nanoparticles form a monodisperse and stable colloidal dispersion. All dispersions can be assumed as stable due to the positive zeta potential larger than >+20 mV and due to the positively charged PEI below the silica shell. The number of biomolecules per particle on the surface of the calcium phosphate nanoparticles agrees well with earlier studies[45,46] It is noteworthy that P1 and P2 peptides have more than one free amine moiety, and therefore their conjugation to the particle surface can occur not only through the terminal amine moiety but also via amino acid in the center of their sequence (via side chain amine). Thereby, the peptide conjugation might impair the efficacy of these peptides in inducing cell-to-cell spread inhibiting antibodies. However, the successful induction of such antibodies as well as protection from disease by vaccination with CaP_P1 or

CaP_P2 indicates that the conformation of P1 and P2 may remain unaffected when bound to CaP nanoparticles. This strongly highlights the potential of calcium phosphate nanoparticles as suitable antigen carriers for small peptides. Structural studies may help understand the NP's structural-functional relationship which will be a future topic in the lab.

In conclusion, we have generated CaP nanoparticles, which were loaded with the CpG^{m} adjuvant and further functionalized by covalent attachment of peptides P1 or P2, which mimic a neutralizing epitope on HSV-1 gB at the nanoparticle surface. Booster-immunization of mice with the CaP_P1 or CaP_P2 vaccine resulted in the production of cell-to-cell spread inhibiting antibodies and protection from lethal genital infection. The present study may contribute towards developing a novel vaccine candidate (e.g. as a CaP-nanoparticle based multi-epitope vaccine), which specifically induces protective cell-to-cell spread inhibiting antibodies in humans.

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Author contributions

MR, UD and AK designed the study. MK and ME designed and synthesized CaP nanoparticles for vaccination. MK, UA, MA, BG and AK performed the experiments. AMEH contributed reagents. All authors analyzed the data. MK and AK wrote the manuscript. All authors have approved the final version of the manuscript.

References

1. Bernstein DI, Bellamy AR, Hook III EW, Levin MJ, Wald A, Ewell MG, et al. Epidemiology, clinical presentation, and antibody response to primary infection with herpes simplex virus type 1 and type 2 in young women. *Clin Infect Dis* 2013;**56**:344-51.
2. Looker KJ, Garnett GP, Schmid GP. An estimate of the global prevalence and incidence of herpes simplex virus type 2 infection. *Bull World Health Organ* 2008;**86**:805-12 [A].
3. Anzivino E, Fioriti D, Mischitelli M, Bellizzi A, Barucca V, Chiarini F, et al. Herpes simplex virus infection in pregnancy and in neonate: status of art of epidemiology, diagnosis, therapy and prevention. *Virol J* 2009;**6**:40.
4. Whitley R, Kimberlin DW, Prober CG. Pathogenesis and disease. In: Arvin A, Campadelli-Fiume G, Mocarski E, Moore PS, Roizman B, Whitley R, Yamanishi K, editors. *Human herpesviruses: biology, therapy, and immunoprophylaxis*; 2007. [Cambridge].
5. Vrabec JT, Alford RL. Quantitative analysis of herpes simplex virus in cranial nerve ganglia. *J Neurovirol* 2004;**10**:216-22.
6. Balasubramaniam R, Kuperstein AS, Stoopler ET. Update on oral herpes virus infections. *Am* 2014;**58**:265-80.
7. Ramchandani M, Kong M, Tronstein E, Selke S, Mikhaylova A, Magaret A, et al. Herpes simplex virus type 1 shedding in tears and nasal and oral mucosa of healthy adults. *Sex Transm Dis* 2016;**43**:756-60.
8. Sen P, Barton SE. Genital herpes and its management. *BMJ* 2007;**334**:1048-52.
9. Lau CH, Missotten T, Salzmann J, Lightman SL. Acute retinal necrosis features, management, and outcomes. *Ophthalmology* 2007;**114**:756-62.
10. Burrel S, Boutolleau D, Azar G, Doan S, Deback C, Cochereau I, et al. Phenotypic and genotypic characterization of acyclovir-resistant corneal HSV-1 isolates from immunocompetent patients with recurrent herpetic keratitis. *J Clin Virol* 2013;**58**:321-4.
11. Meyding-Lamade U, Strank C. Herpesvirus infections of the central nervous system in immunocompromised patients. *Ther Adv Neurol Disord* 2012;**5**:279-96.
12. Straface G, Selmin A, Zanardo V, De Santis M, Ercoli A, Scambia G. Herpes simplex virus infection in pregnancy. *Infect Dis Obstet Gynecol* 2012;**2012**385697.
13. Darville JM, Ley BE, Roome AP, Foot AB. Acyclovir-resistant herpes simplex virus infections in a bone marrow transplant population. *Bone Marrow Transplant* 1998;**22**:587-9.
14. Duan R, de Vries RD, Osterhaus AD, Remeijer L, Verjans GM. Acyclovir-resistant corneal HSV-1 isolates from patients with herpetic keratitis. *J Infect Dis* 2008;**198**:659-63.
15. van Velzen M, van de Vijver DA, van Loenen FB, Osterhaus AD, Remeijer L, Verjans GM. Acyclovir prophylaxis predisposes to antiviral-resistant recurrent herpetic keratitis. *J Infect Dis* 2013;**208**:1359-65.
16. Johnston C, Corey L. Current concepts for genital herpes simplex virus infection: diagnostics and pathogenesis of genital tract shedding. *Clin Microbiol Rev* 2016;**29**:149-61.
17. Dasgupta G, Chentoufi AA, Nesburn AB, Wechsler SL, BenMohamed L. New concepts in herpes simplex virus vaccine development: notes from the battlefield. *Expert Rev Vaccines* 2009;**8**:1023-35.
18. Corey L, Langenberg AG, Ashley R, Sekulovich RE, Izu AE, Douglas Jr JM, et al. Recombinant glycoprotein vaccine for the prevention of genital HSV-2 infection: two randomized controlled trials. Chiron HSV Vaccine Study Group. *JAMA* 1999;**282**:331-40.
19. Belshe RB, Leone PA, Bernstein DI, Wald A, Levin MJ, Stapleton JT, et al. Efficacy results of a trial of a herpes simplex vaccine. *Med* 2012;**366**:34-43.
20. Sattentau Q. Avoiding the void: cell-to-cell spread of human viruses. *Nat Rev Microbiol* 2008;**6**:815-26.
21. Favoreel HW, Van Minnebruggen G, Van de Walle GR, Ficinska J, Nauwynck HJ. Herpesvirus interference with virus-specific antibodies: bridging antibodies, internalizing antibodies, and hiding from antibodies. *Vet Microbiol* 2006;**113**:257-63.
22. Bauer D, Alt M, Dirks M, Buch A, Heilingloh CS, Dittmer U, et al. A therapeutic antiviral antibody inhibits the anterograde directed neuron-to-cell spread of herpes simplex virus and protects against ocular disease. *Front Microbiol* 2017;**8**:2115.
23. Krawczyk A, Dirks M, Kasper M, Buch A, Dittmer U, Giebel B, et al. Prevention of herpes simplex virus induced stromal keratitis by a glycoprotein B-specific monoclonal antibody. *PLoS One* 2015;**10**e0116800.
24. Krawczyk A, Krauss J, Eis-Hubinger AM, Daumer MP, Schwarzenbacher R, Dittmer U, et al. Impact of valency of a glycoprotein B-specific monoclonal antibody on neutralization of herpes simplex virus. *J Virol* 2011;**85**:1793-803.
25. Eis-Hubinger AM, Schmidt DS, Schneweis KE. Anti-glycoprotein B monoclonal antibody protects T cell-depleted mice against herpes simplex virus infection by inhibition of virus replication at the inoculated mucous membranes. *J Gen Virol* 1993;**74**(Pt 3):379-85.
26. Meyerrose TE, Herrbrich P, Hess DA, Nolta JA. Immune-deficient mouse models for analysis of human stem cells. *Biotechniques* 2003;**35**:1262-72.
27. Krawczyk A, Arndt MA, Grosse-Hovest L, Weichert W, Giebel B, Dittmer U, et al. Overcoming drug-resistant herpes simplex virus (HSV) infection by a humanized antibody. *Proc Natl Acad Sci USA* 2013;**110**:6760-5.
28. Daumer MP, Schneider B, Giesen DM, Aziz S, Kaiser R, Kupfer B, et al. Characterisation of the epitope for a herpes simplex virus glycoprotein B-specific monoclonal antibody with high protective capacity. *Med Microbiol Immun* 2011;**200**:85-97.
29. Morris TAML. Broadly neutralizing antibodies. In: K.M. a., Koff WC, editors. *Human vaccines—emerging technologies in design and development*. Elsevier; 2017.
30. Zilker C, Kozlova D, Sokolova V, Yan H, Epple M, Uberla K, et al. Nanoparticle-based B-cell targeting vaccines: tailoring of humoral

immune responses by functionalization with different TLR-ligands. *Nanomedicine* 2017;**13**:173-82.
31. Farnsworth A, Goldsmith K, Johnson DC. Herpes simplex virus glycoproteins gD and gE/gI serve essential but redundant functions during acquisition of the virion envelope in the cytoplasm. *J Virol* 2003;**77**:8481-94.
32. Parr MB, Kepple L, McDermott MR, Drew MD, Bozzola JJ, Parr EL. A mouse model for studies of mucosal immunity to vaginal infection by herpes simplex virus type 2. *Lab Invest* 1994;**70**:369-80.
33. Anthis NJ, Clore GM. Sequence-specific determination of protein and peptide concentrations by absorbance at 205 nm. *Protein Sci* 2013;**22**:851-8.
34. Whitbeck JC, Huang ZY, Cairns TM, Gallagher JR, Lou H, Ponce-de-Leon M, et al. Repertoire of epitopes recognized by serum IgG from humans vaccinated with herpes simplex virus 2 glycoprotein D. *J Virol* 2014;**88**:7786-95.
35. Hook LM, Cairns TM, Awasthi S, Brooks BD, Ditto NT, Eisenberg RJ, et al. Vaccine-induced antibodies to herpes simplex virus glycoprotein D epitopes involved in virus entry and cell-to-cell spread correlate with protection against genital disease in Guinea pigs. *PLoS Pathog* 2018;**14**e1007095.
36. Bauer D, Keller J, Alt M, Schubert A, Aufderhorst UW, Palapys V, et al. Antibody-based immunotherapy of aciclovir resistant ocular herpes simplex virus infections. *Virology* 2017;**512**:194-200.
37. Chargelegue D, Obeid OE, Hsu SC, Shaw MD, Denbury AN, Taylor G, et al. A peptide mimic of a protective epitope of respiratory syncytial virus selected from a combinatorial library induces virus-neutralizing antibodies and reduces viral load in vivo. *J Virol* 1998;**72**:2040-6.
38. Steward MW, Stanley CM, Obeid OE. A mimotope from a solid-phase peptide library induces a measles virus-neutralizing and protective antibody response. *J Virol* 1995;**69**:7668-73.
39. Li W, Joshi MD, Singhania S, Ramsey KH, Murthy AK. Peptide vaccine: Progress and challenges. *Vaccine* 2014;**2**:515-36.
40. Didierlaurent AM, Morel S, Lockman L, Giannini SL, Bisteau M, Carlsen H, et al. AS04, an aluminum salt- and TLR4 agonist-based adjuvant system, induces a transient localized innate immune response leading to enhanced adaptive immunity. *J Immunol* 2009;**183**:6186-97.
41. Dorozhkin SV, Epple M. Biological and medical significance of calcium phosphates. *Angew Chem* 2002;**41**:3130-46.
42. Sokolova VV, Radtke I, Heumann R, Epple M. Effective transfection of cells with multi-shell calcium phosphate-DNA nanoparticles. *Biomaterials* 2006;**27**:3147-53.
43. Knuschke T, Bayer W, Rotan O, Sokolova V, Wadwa M, Kirschning CJ, et al. Prophylactic and therapeutic vaccination with a nanoparticle-based peptide vaccine induces efficient protective immunity during acute and chronic retroviral infection. *Nanomedicine* 2014;**10**:1787-98.
44. Epple M. Review of potential health risks associated with nanoscopic calcium phosphate. *Acta Biomater* 2018;**77**:1-14.
45. Sokolova V, Shi Z, Huang S, Du Y, Kopp M, Frede A, et al. Delivery of the TLR ligand poly(I:C) to liver cells in vitro and in vivo by calcium phosphate nanoparticles leads to a pronounced immunostimulation. *Acta Biomater* 2017;**64**:401-10.
46. Kopp M, Rotan O, Papadopoulos C, Schulze N, Meyer H, Epple M. Delivery of the autofluorescent protein R-phycoerythrin by calcium phosphate nanoparticles into four different eukaryotic cell lines (HeLa, HEK293T, MG-63, MC3T3): highly efficient, but leading to endolysosomal proteolysis in HeLa and MC3T3 cells. *PLoS One* 2017;**12**e0178260.
47. Heldwein EE, Lou H, Bender FC, Cohen GH, Eisenberg RJ, Harrison SC. Crystal structure of glycoprotein B from herpes simplex virus 1. *Science* 2006;**313**:217-20.