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Vaccine-Derived Neutralizing Antibodies to the Human Cytomegalovirus gH/gL Pentamer Potently Block Primary Cytotrophoblast Infection

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ABSTRACT

Human cytomegalovirus (HCMV) elicits neutralizing antibodies (NAb) of various potencies and cell type specificities to prevent HCMV entry into fibroblasts (FB) and epithelial/endothelial cells (EpC/EnC). NAb targeting the major essential envelope glycoprotein complexes gB and gH/gL inhibit both FB and EpC/EnC entry. In contrast to FB infection, HCMV entry into EpC/EnC is additionally blocked by extremely potent NAb to conformational epitopes of the gH/gL/UL128/130/131A pentamer complex (PC). We recently developed a vaccine concept based on coexpression of all five PC subunits by a single modified vaccinia virus Ankara (MVA) vector, termed MVA-PC. Vaccination of mice and rhesus macaques with MVA-PC resulted in a high titer and sustained NAb that blocked EpC/EnC infection and lower-titer NAb that inhibited FB entry. However, antibody function responsible for the neutralizing activity induced by the MVA-PC vaccine is uncharacterized. Here, we demonstrate that MVA-PC elicits NAb with cell type-specific neutralization potency and antigen recognition pattern similar to human NAb targeting conformational and linear epitopes of the UL128/130/131A subunits or gH. In addition, we show that the vaccine-derived PC-specific NAb are significantly more potent than the anti-gH NAb to prevent HCMV spread in EpC and infection of human placental cytotrophoblasts, cell types thought to be of critical importance for HCMV transmission to the fetus. These findings further validate MVA-PC as a clinical vaccine candidate to elicit NAb that resembles those induced during HCMV infection and provide valuable insights into the potency of PC-specific NAb to interfere with HCMV cell-associated spread and infection of key placental cells.

IMPORTANCE

As a consequence of the leading role of human cytomegalovirus (HCMV) in causing permanent birth defects, developing a vaccine against HCMV has been assigned a major public health priority. We have recently introduced a vaccine strategy based on a widely used, safe, and well-characterized poxvirus vector platform to elicit potent and durable neutralizing antibody (NAb) responses targeting the HCMV envelope pentamer complex (PC), which has been suggested as a critical component for a vaccine to prevent congenital HCMV infection. With this work, we confirm that the NAb elicited by the vaccine vector have properties that are similar to those of human NAb isolated from individuals chronically infected with HCMV. In addition, we show that PC-specific NAb have potent ability to prevent infection of key placental cells that HCMV utilizes to cross the fetal-maternal interface, suggesting that NAb targeting the PC may be essential to prevent HCMV vertical transmission.

Human cytomegalovirus (HCMV) is the most common infectious cause of permanent births defects worldwide, often resulting in auditory and cognitive abnormalities and in rare cases even in multiorgan failure and death (1–4). Congenital HCMV infection occurs in 0.05 to 1% of all pregnancies, and 10 to 25% of congenitally infected newborns develop long-term developmental disabilities (2–6). The annual numbers of HCMV-infected infants at birth based on viral shedding range from 35,000 in Brazil to 40,000 in the United States and 250,000 in India (5). In fact, persistent newborn medical conditions are more frequently associated with congenital HCMV infection than with other well-known childhood diseases such as trisomy 21, spina bifida, or fetal-alcohol syndrome (2, 4, 7–10). Besides its leading role in permanent birth defects, HCMV is also a major cause of morbidity and mortality in hematopoietic stem cell and solid organ transplant recipients (11–13). Based on the societal and financial health burden and in the absence of effective treatment options, HCMV has been assigned as one of the highest priority vaccine targets (14, 15). However, incompletely defined correlates of protection, lack of animal models susceptible to HCMV infection, insufficiently powered vaccine trials, and general unawareness, are a number of obstacles that have hampered the development of an effective and safe HCMV vaccine (16).

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High-titer and durable neutralizing antibodies (NAb) that block glycoprotein complex-mediated entry into host cells are thought to be essential to prevent or control congenital HCMV infection. For many decades, HCMV subunit vaccine research has primarily focused on the stimulation of NAb targeting the major essential envelope glycoprotein, gB, culminating in the encouraging findings obtained with recombinant gB admixed in adjuvant MF59 (17). In phase II clinical trials, gB/MF59 has been shown to reduce viremia and the need for antiviral therapy in solid organ transplant recipients and provide moderate efficacy of 38 to 50% to prevent primary infection in young women of childbearing age (17–20). These findings have spurred interest to improve vaccine-mediated induction of NAb responses as an approach to improve protective efficacy beyond that observed with gB/MF59.

In recent years it has been recognized that HCMV entry into fibroblasts (FB) and epithelial/endothelial cells (EpC/EnC) occurs by alternate routes of entry that are blocked by NAb of various potencies and cell type specificities (21–23). HCMV infection of FB depends on the major essential envelope glycoprotein complexes (gC) gM/gN, gB, and gH/gL (22, 23). In contrast to FB entry, HCMV infection of EpC/EnC requires an additional complex formed by gH/gL, UL128, UL130, and UL131A (PC) (21, 24–26). A third gH/gL complex composed of gH/gL/gO appears necessary for entry into both FB and EpC/EnC, although this remains controversial (27–31). NAb targeting the major gC block both HCMV entry routes (32); however, NAb recognizing predominantly conformational epitopes formed by two or more of the UL128/UL130/UL131A (UL128/130/131A) subunits of the PC are unable to prevent FB entry, although they are able to interfere with EpC/EnC infection that dramatically exceeds that of NAb targeting the major gC (32, 33).

Many vaccine strategies based on either live-attenuated virus, viral vector systems or purified protein confirm that the PC is the major target of NAb blocking HCMV infection of EpC/EnC (33–36). All of these vaccine approaches consistently demonstrate in animal models that the PC has superior immunogenicity to elicit NAb against EpC/EnC entry compared to PC subunit subsets (gH/gL or UL128/130/131A) or gB (33–36). These studies also show that vaccine approaches employing the PC are equally or even more effective than gB-based vaccine strategies to induce NAb blocking FB entry (33, 34, 36). Consequently, PC subunit vaccines elicit high-titer EpC/EnC-specific NAb responses and less potent NAb against FB entry, which is consistent with the NAb response induced by HCMV during natural infection (37–39). These findings argue at minimum and when correlated with *in vitro* studies that the PC is sufficient to elicit NAb that potently and broadly interfere with HCMV host cell entry.

Although the mechanisms through which HCMV crosses the placenta are still debated, cytotrophoblasts (CTB), including their syncytial forms and progenitors, are thought to be the key mediators involved in all potential HCMV vertical transmission routes (40–44). These cells build a bridge at the fetal-maternal interface and can be efficiently infected by HCMV *in vitro* and *in vivo* (10, 43–46). In addition, infection of CTB in early gestation often results in placental developmental abnormalities (44, 46–48). However, NAb that interfere with HCMV infection of placental cells are only poorly characterized. A recent study has shown that HCMV infection of CTB progenitor cells can be efficiently blocked by NAb to gB, although NAb targeting the PC are unable to interfere with CTB progenitor infection (49, 50). Whether PC-specific NAb are able to prevent infection of differentiating CTB is unknown.

We recently developed a vaccine concept based on the delivery of a membrane-tethered PC by modified vaccinia virus Ankara (MVA) (36), a widely used, clinical viral vector platform that has been safely tested in over 120,000 humans (51, 52). As observed for other PC-based vaccine candidates and consistent with the Ab response in $HCMV^+$ individuals, mice and rhesus monkeys immunized with MVA-PC developed high-titer and sustained NAb against EpC/EnC entry and less-potent NAb that blocked FB infection (36–39). However, antibodies responsible for the vaccine-mediated neutralizing activity are incompletely characterized. Here, we demonstrate that MVA-PC elicits PC- and gH-specific NAb that have properties similar to previously described human NAb (32). In addition, we show that PC-specific NAb are significantly more potent than gH-specific NAb in preventing HCMV infection of primary CTB from term placentae, suggesting that NAb recognizing the PC may play a critical role in interfering with HCMV vertical transmission.

MATERIALS AND METHODS

Cells. ARPE-19 and MRC-5 (ATCC) cells were in maintained in Dulbecco minimal essential medium (DMEM; Corning, NY) or minimal essential medium (MEM; Corning), respectively, supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT). Human umbilical vein endothelial cells (HUVEC; American Type Culture Collection [ATCC]) were grown in VascuLife basal medium added with VascuLife EnGS LifeFactors (Lifeline Cell Technology, Frederick, MD). BHK-21 cells (ATCC) were maintained in MEM with addition of 10% FBS, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids (Life Technologies, Grand Island, NY).

Isolation and culture of CTB. With written informed consent, term (>37 weeks gestation) placentae from HIV-1-seronegative and hepatitis B-uninfected women (>18 years of age) were obtained immediately after elective caesarian section without labor from Emory Midtown Hospital in Atlanta, GA. Approval of the study was granted by the Emory University Institutional Review Board. Written informed consent was obtained from donors, and samples were deidentified prior to handling by laboratory personnel. In order to isolate CTB, membrane-free villous was dissected from the placenta, as previously described (53–55). The tissue was thoroughly washed and mechanically dispersed in Hanks' balanced salt solution (HBSS) to minimize peripheral blood contamination. Minced tissue fragments were then subjected to three sequential enzymatic digestions in a solution containing 0.25% trypsin (Mediatech, Inc., Manassas, VA), 0.2% DNase I (Roche Diagnostics, Mannheim, Germany), 25 mM HEPES, 2 mM $CaCl_2$, and 0.8 mM $MgSO_4$ in HBSS at 37°C. After each digestion, the undigested tissue was removed by passage through a gauze and 100-μm-pore-size cell strainer (BD Biosciences, Franklin Lakes, NJ) and washed with phosphate-buffered saline (PBS). Supernatants from the second and third digestions were collected, and the resulting cell pellets resuspended in 1:1 DMEM/F-12 supplemented with 10% FBS, 1 mM L-glutamine, and 1% penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO). The CTB were isolated on a discontinuous gradient of Percoll (GE Healthcare, Uppsala, Sweden; 50, 45, 35, and 30%) by centrifugation. Cells migrating to the 35%/45% Percoll interface were recovered and immunopurified by negative selection with simultaneous treatment with anti-CD9 (to exclude EnC, FB, platelets, smooth muscle, extravillous trophoblast cells, B cells, and monocytes) and anti-CD45RA (to exclude leukocytes) antibodies and magnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany) (56, 57). The purity of the CTB population was assessed by cytokeratin-7 staining and was on average >97%. Vimentin staining to quantify contamination from mesenchymal cells was on average <3% (56–58).

Antibodies. Cytogam (CMV-HIG, 50 mg/ml) was obtained from the manufacturer (Baxter-Healthcare Corp., Irvine, CA). The isolation of anti-gH Ab AP86, anti-pp65 Ab 28-103, and anti-HCMV IE1 Ab (p63-27) has been described elsewhere (59–61).

Viruses. MVA expressing all five PC subunits (MVA-PC), single PC subunits or subunit combinations were reconstituted from MVA-BAC as previously described (36, 62) and propagated on BHK-21 (63). For preparing MVA virus stocks, MVA was harvested from infected BHK-21, purified by 36% sucrose density ultracentrifugation, and resuspended in 1 mM Tris-HCl (pH 9) (36, 62, 64). MVA stocks were maintained at −80°C. Purified MVA was titrated on BHK-21 by standard procedure. HCMV strain TB40/E-GFP (TB40/E) was kindly provided by Christian Sinzger (Ulm University, Germany) (65). HCMV strain TR-GFP (TR) was a gift from Jay Nelson (Oregon Health and Sciences University, Portland, OR). HCMV strains Davis, Towne and AD169 were kindly provided by John Zaia (Beckman Research Institute of the City of Hope, Duarte, CA) (64). Generation of HCMV stocks was performed as previously described (36). Briefly, ARPE-19 were infected with HCMV and reseeded until 70 to 80% of the cells were green fluorescent protein (GFP) positive. Virus particles were concentrated from clarified medium by ultracentrifugation (70,000 × *g* for 1 h) over 20% sucrose (wt/vol) in Tris-buffered saline (0.1 M Tris-Cl [pH 7.4], 0.1 M NaCl). Concentrated virus was resuspended in Tris-buffered saline and stored at −80°C. Virus titration was performed by adding serial dilutions of the virus to ARPE-19, MRC-5, HUVEC, and CTB and by immunostaining for immediate early-1 protein (IE1) after 48 h of incubation. HCMV titer on CTB was on average three times lower than the one measured on ARPE-19.

Mouse immunization. The Institutional Animal Care and Use Committee of the Beckman Research Institute of City of Hope approved protocol 98004 assigned for this study. All study procedures were carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* and the *Public Health Service Policy on the Humane Care and Use of Laboratory Animals*. Methods of euthanasia followed "Report of the AVMA Panel on Euthanasia" (http://www.avma.org/kb/policies/documents/euthanasia.pdf). BALB/cJ mice (Jackson Laboratory, Bar Harbor, ME) were vaccinated with MVA-PC as previously described (36) and boosted 4 days before the spleens were removed for hybridoma production. PC-specific NAb were isolated from mice ($n = 3$) during a period of 57 to 70 weeks after initial immunization with MVA-PC (36).

Hybridoma derivation. Hybridomas were derived by conventional procedure (66). Briefly, myeloma partner cells (P3X63Ag8.653, ATCC) were maintained in RPMI 1640 (Corning) supplemented with 10% FBS. Splenocytes and myeloma cells were counted, and fusion was performed at a 1:5 ratio by adding 1 ml of PEG 1500 (Sigma-Aldrich). After centrifugation, fused cells were resuspended in RPMI 1640 supplemented with 15% FBS, 10% UltraCruz hybridoma cloning supplement (HCS; Santa Cruz Biotechnology, Santa Cruz, CA), and HAT medium supplement (Sigma-Aldrich) at a concentration of 5×10^5 splenocytes/ml. Cells were seeded in 96-well plates and incubated in a 5% CO_2, 37°C incubator. Selected hybridoma clones were grown in RPMI 1640 supplemented with 15% FBS and 10% HCS. Each clone underwent two rounds of single cell subcloning to ensure the clonality of the antibody (66). Collected hybridoma supernatant was added with 20 mM sodium phosphate buffer (pH 7.0) and NAb purified using a HiTrap Protein G HP 5-ml column (GE Healthcare) according to the manufacturer's instructions. The Ab concentration was verified with Bradford-Coomassie brilliant blue dye method using a bovine gamma globulin standard (Thermo Scientific/Pierce, Rockford, IL).

Neutralization assay. Cells were seeded at 1.5×10^4 cells/well (ARPE-19, HUVEC, and MRC-5) or 1.5×10^5 cells/well (CTB) in a clear-bottom 96-well plate (Corning). Approximately 24 h later, the medium in every plate was replaced with 50 μl per well of fresh growth medium. Neutralization assays were performed as previously described (36). Briefly, serial 2-fold dilutions of the purified NAb were prepared in complete growth medium in a final volume of 75 μl. NAb dilutions were mixed with 75 μl of complete growth medium containing ~9,000 PFU of HCMV TB40/E or TR and incubated for 2 h at 37°C. The mixture was transferred to the cells (50 μl each, duplicate wells). After 48 h, the cells were fixed and permeabilized with a methanol-acetone solution. Infected cells were identified by immunostaining with mouse anti-HCMV IE1 Ab (p63-27) and a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. The substrate was 3,3′-diaminobenzidine (Vector Laboratories). The plates were analyzed by an automated system using the Axio Observer Z1 inverted microscope equipped with a linear motorized stage (Carl Zeiss, Germany). IE1-positive nuclei were counted using ImagePro Premier (Media Cybernetics, Silver Spring, MD). The NAb concentration inhibiting 50% of the virus infectivity (IC_{50}) was calculated as previously described (36). Briefly, the average number of positive nuclei for each NAb dilution was calculated. The percentage of neutralization inhibition (IC) was calculated as: IC = [1 − (positive nuclei number with NAb)/(positive nuclei number without NAb)] × 100. The IC_{50} was calculated by determining the linear slope of the graph plotting IC versus NAb dilution by using the next higher and lower IC values that were closest to 50% neutralization.

NAb binding specificity. NAb subunit specificity was evaluated by staining BHK-21 cells infected with different MVA recombinants. One or more vectors were used to coinfect BHK-21 at a multiplicity of infection (MOI) of 5. The combinations analyzed were UL128, UL130, UL131A, UL128/130, UL128/131A, UL130/131, UL128/130/131A, gH, gH/gL, gH/gL/UL128, gH/gL/UL130, gH/gL/UL131A, gH/gL/UL128/130, gH/gL/UL128/131A, gH/gL/UL130/131A, and gH/gL/UL128/130/131A. At 4 h postinfection, the cells were fixed and permeabilized using Cytofix/Cytoperm solution (BD Biosciences). NAb (1 mg/ml) were diluted 1:500 in Perm/Wash buffer (BD) and added to the cells for 1 h at 4°C. After washing with Perm/Wash buffer, Alexa Fluor 647 goat anti-mouse IgG (Life Technologies) was added at a dilution of 1:2,000. The cells were washed again and resuspended in PBS–0.1% bovine serum albumin (BSA). Fifteen thousand events were collected using a Gallios flow cytometer (Beckman Coulter, Miami, FL) and analyzed with FlowJo software (Tree Star, Ashland, OR). Uninfected cells and cells infected with MVA-Venus were used as controls. GFP expression was analyzed for confirming MVA infection since all of the constructs contain a GFP expression cassette (36, 62).

Cell-to-cell spread inhibition assay. NAb ability to inhibit cell-to-cell spread and/or syncytium formation was evaluated on EpC using TB40/E and TR. ARPE-19 cells were seeded on a black 96-well plate (Costar) and infected 24 h later with HCMV TB40/E or TR (MOI of 1 or 0.05, as titrated on ARPE-19). After incubation for 24 h, cells were extensively washed with PBS and 2-fold serial dilutions of each NAb were added to the wells in a total volume of 200 μl. After 8 days of incubation, the plates were imaged with a Zeiss Axiovert fluorescence microscope (Carl Zeiss, Jena, Germany), and the cellular GFP was quantified by using ImagePro Premier Software (Media Cybernetics, Silver Spring, MD). The percentage of spread inhibition (IC) for each dilution was calculated as: IC = [1 − (fluorescence in infected wells incubated with NAb)/(fluorescence in infected wells without NAb)] × 100. The 50% cell-to-cell spread inhibition (IC_{50}) was calculated by determining the linear slope of the graph plotting IC versus NAb dilution by using the next higher and lower IC values that were closest to 50%.

Antibody binding affinity. Antibodies binding affinity was determined as described elsewhere (67). Briefly, 10 mg of purified NAb were biotinylated using EZ-Link NHS-PEG4-biotin biotinylation kit (Thermo Scientific/Pierce) according to the manufacturer's instructions. BHK-21 cells were infected with MVA-PC at an MOI of 5. After an incubation of 4 h at 37°C, the cells were dispensed at a concentration of 10^5 cells/well in a 96-well V-bottom plate, followed by 2 h of incubation at 4°C with 2-fold serial dilutions of the biotinylated NAb in PBS–0.1% BSA. Dilutions ranged from 500 μg/ml to 0.1 ng/ml. The cells were washed twice with PBS–0.1% BSA and incubated for 1 h at 4°C in the presence of streptavi-

din-DyLight 650 (Thermo Scientific) diluted 1:500 in PBS–0.1% BSA. After two washes, the cells were fixed with 4% paraformaldehyde. Fifteen thousand events were acquired with the Gallios flow cytometer and analyzed with FlowJo software. The equilibrium binding constant (K_d) was derived by plotting fluorescence as a function of the logarithm of NAb concentration to obtain a sigmoidal curve analyzed using the four-parameter logistic (4PL) nonlinear regression model (Prism 6; GraphPad Software, San Diego, CA).

Competition assay. NAb competition was evaluated as follows. BHK-21 cells were infected with MVA-PC at an MOI of 5 and 4 h later treated with Cytofix/Cytoperm. Approximately 10^5 cells were incubated for 2 h with 20- to 100-fold excess unlabeled competitor NAb (from 100 to 200 μg/ml). After a washing step with Perm/Wash buffer the cells were incubated for 2 h in the presence of 1 to 5 μg of biotinylated NAb/ml. For every NAb, cells in which the unlabeled competitor was not added to the biotinylated NAb were used to determine maximum binding. Cells were washed once with Perm/Wash buffer and incubated for 1 h with streptavidin-DyLight 650 diluted 1:500. After a final washing step, cells were resuspended in PBS–0.1% BSA, acquired with Gallios flow cytometer, and analyzed with FlowJo software. For every antibody pair, the percentage of inhibition was calculated as: 100 − [(% fluorescent cells with competitor NAb/% fluorescent cells without competitor NAb) × 100]. The complete prevention of binding of a biotinylated NAb by its unlabeled counterpart was used as a validation of the assay.

NAb variable heavy and light chain sequence characterization. Total RNA was extracted from hybridomas using the SV total RNA isolation system (Promega, Madison, WI). cDNA was generated by random hexamers (Qiagen GmbH, Hilden, Germany) and Superscript III reverse transcriptase (Life Technologies) according to the manufacturer's instruction. The kappa variable genes were characterized by a 5′RACE (rapid amplification of cDNA ends) PCR in which the cDNA was tailed with poly-dGTP by terminal transferase (New England BioLabs, Ipswich, MA). A 3′ reverse gene-specific primer located in the kappa constant region near the variable region (TGGATGGTGGGAAGATGGATAC AGT) was adopted, together with poly-dCTP to amplify the kappa variable genes. For the gamma variable genes, a protocol from Fields et al. (68) was followed. V_H and V_L genes were amplified by Phusion high-fidelity DNA polymerase (Thermo Scientific) and cloned into pCR4Blunt-TOPO vector (Life Technologies) according to the manufacturer's instructions. Three clones derived from each V_H/V_L genes were sequenced.

Immunoblotting. Immunoblotting to determine NAb binding to denatured gH was performed using lysates from cells infected with a gH-expressing adenoviral vector (Ad-gH) as previously described (36). Anti-gH Ab AP86 (59), 18F10, 21E9, 62-11, 62-100, and 2-80 were used at a dilution of 10 μg/ml. Anti-MEK1/2 (Cell Signaling Technology, Danvers, MA) was diluted 1:1,000. Immunoblot to evaluate 18F10 and AP86 binding to lysates from cells infected with different HCMV strains was performed as described above with the difference that lysates consisted in 2.5×10^5 MRC-5 infected for 4 days with HCMV strain Davis, Towne, AD169, TB40/E, or TR at an MOI of 1. Anti-pp65 was used to show HCMV infection in all of the samples independently from the strain used.

Statistical analysis. To determine the correlation coefficient between NAb binding affinity and neutralizing potency and its statistical significance, a two-tailed Pearson correlation analysis was performed. Differences in binding affinity and neutralization potency between PC-specific and gH/gL-specific NAb was evaluated using an unpaired *t* test. Both analysis were performed using GraphPad Prism 6 software.

RESULTS

MVA-PC vaccine-derived NAb recognize epitopes of the PC and gH. PC-specific NAb isolated from chronically infected $HCMV^+$ individuals predominantly recognize conformational antigenic sites formed by UL130/131A and UL128/130/131A (32, 33). Only one human NAb has been published that recognizes an epitope within the UL128 subunit (32). We isolated a panel of NAb from mice immunized with the MVA-PC vaccine by conventional hybridoma technology combined with screening for neutralization against TB40/E on ARPE-19 EpC. In order to determine the antigen specificity of the vaccine-derived NAb, we evaluated intracellular flow cytometry (FC) staining of permeabilized BHK-21 cells infected with MVA expressing single subunits or combinations of two or more subunits of the PC. Consistent with human NAb, we identified four vaccine-derived PC-specific NAb that recognized quaternary epitopes formed by UL130/131A or UL128/130/131A, and one NAb (13B5) with UL128 specificity (Fig. 1A and Table 1). Staining with 1B2 and 12E2 NAb was observed with UL128/130/131A or all five PC subunits. Expression of single subunits or any PC subunit combination with only one or two of the UL128/130/131A subunits did not result in binding of 1B2 and 12E2. In contrast, NAb 54E11 and 21F6 showed binding with UL130/131A, the three UL128/130/131A subunits, or all five PC subunits. Single subunits or PC subunit combinations lacking UL130/131A failed to enable binding of 54E11 and 21F6. NAb 13B5 showed binding with UL128 alone or combined with other PC subunits, whereas binding of 13B5 was not observed in the absence of UL128. As anticipated based on the vaccine's ability to elicit both EpC/EnC- and FB-specific NAb (36), we also identified NAb with gH specificity. Staining by these NAb was confirmed with gH alone, in combination with gL, or together with all other four PC subunits. Binding of these antibodies was not observed when gH was missing (Fig. 1A and Table 1). Hence, MVA-PC elicits PC and gH-specific NAb that have antigen recognition patterns similar to human PC-specific NAb isolated from chronically infected $HCMV^+$ individuals.

MVA-PC-infected cells present PC- and gH-specific neutralizing epitopes at the cell surface. We previously reported that the five PC subunits expressed from MVA-PC assemble with each other intracellularly, though it remained unclear whether the complexes were transported to the cell surface and presented PC-specific neutralizing epitopes. To address this, we evaluated the vaccine-derived NAb for cell surface FC staining of live nonpermeabilized BHK-21 cells infected with MVA-PC compared to MVA vaccine vectors expressing single subunits or different subunit subset combinations of the PC. Compared to intracellular staining (Fig. 1A), we observed different cell surface recognition patterns with the vaccine-derived PC-specific NAb (Fig. 1B). Intensive cell surface staining by the PC-specific NAb was confirmed with all five PC subunits (MVA-PC), whereas no or only minimal cell surface staining by the NAb was observed with single subunits or subunit subsets of the complex (Fig. 1B). As confirmed for intracellular staining (Fig. 1A), we observed intense cell surface staining by all gH-specific NAb with gH alone, together with gL, or combined with all other four PC subunits. In contrast to intracellular staining, cell surface staining by the anti-gH NAb was more intense with all five PC subunits compared to gH alone or only gH/gL. In addition, compared to gH single expression, stronger binding of the anti-gH NAb was observed with gH/gL (Fig. 1B). These results demonstrate that the five PC subunits expressed from MVA-PC efficiently assemble with one another and present conformational neutralizing epitopes of the UL128/130/131A subunits and gH at the cell surface.

Vaccine-derived PC-specific NAb are more potent than gH-specific NAb in neutralizing HCMV. In order to determine whether the vaccine-derived NAb confer similar potency than

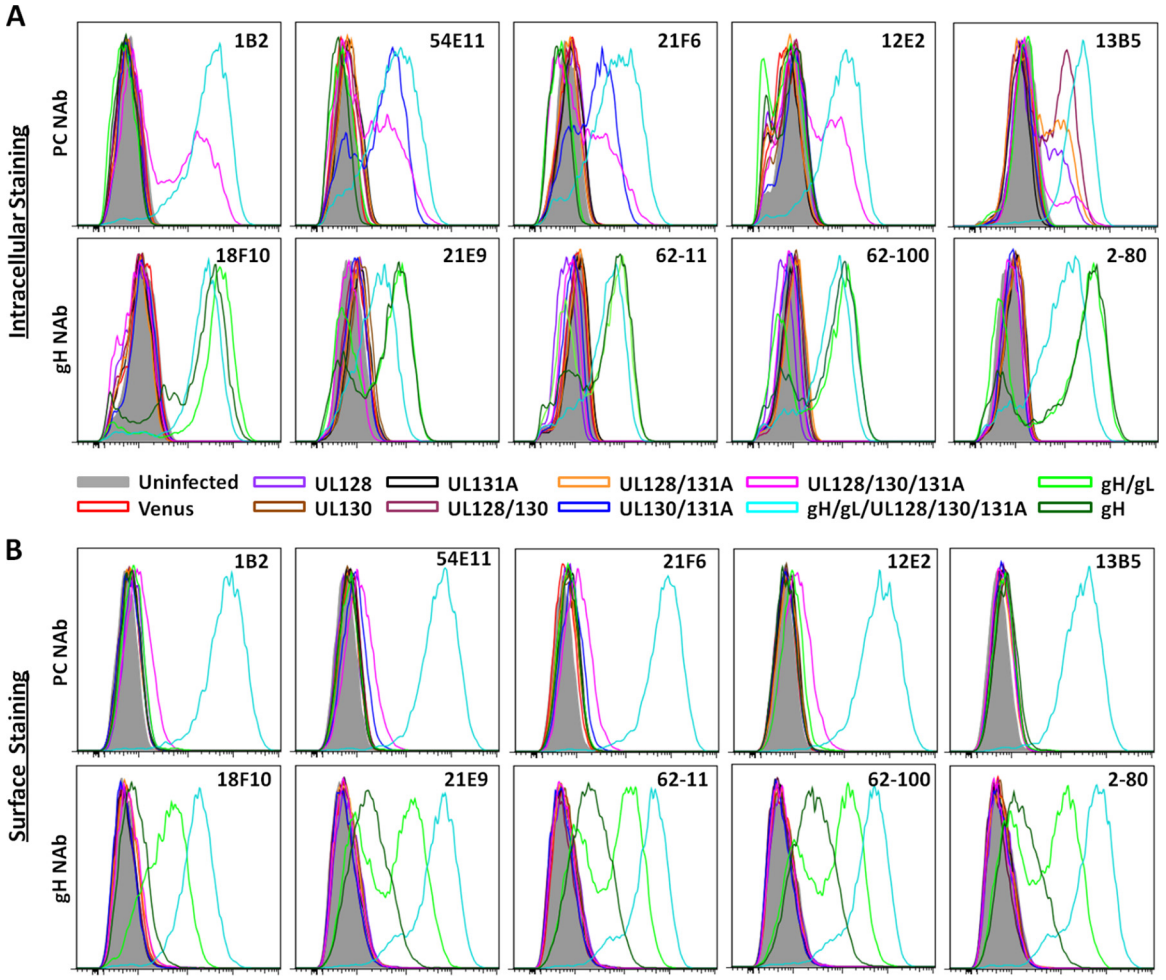

FIG 1 Intracellular and cell surface recognition of PC subunits by isolated NAb. FC staining by MVA-PC vaccine-derived NAb of permeabilized (A) and nonpermeabilized (B) BHK-21 cells infected with different MVA vectors expressing one or more subunits of the PC was assessed. Uninfected cells and cells infected with an MVA encoding the fluorescent protein Venus were used as negative controls.

previously described human NAb (32) to prevent host cell entry, we evaluated the inhibitory antibody concentration (IC_{50}) that blocked 50% HCMV infection of ARPE-19 EpC, HUVEC EnC, or MRC-5 FB using a standard microneutralization assay. We tested neutralization against HCMV strains TB40/E and TR to evaluate whether sequence variation in the gH component influences the potency of the NAb to neutralize HCMV (36, 69). Neutralization potency of HCMV hyperimmune globulin (CMV-HIG) was eval-

TABLE 1 NAb subunit recognition

	Subunit recognition															
NAb	UL128	UL130	UL131	UL128/130	UL128/131	UL130/131	UL128/130/131	gH	gH/gL	gH/gL/UL128	gH/gL/UL130	gH/gL/UL131	gH/gL/UL128/130	gH/gL/UL128/131	gH/gL/UL130/131	gH/gL/UL128/130/131
1B2	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	+
54E11	–	–	–	–	–	+	+	–	–	–	–	–	–	–	+	+
21F6	–	–	–	–	–	+	+	–	–	–	–	–	–	–	+	+
12E2	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	+
13B5	+	–	–	+	+	–	+	–	–	+	–	–	+	+	–	+
18F10	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+
21E9	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+
62-11	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+
62-100	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+
2-80	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+

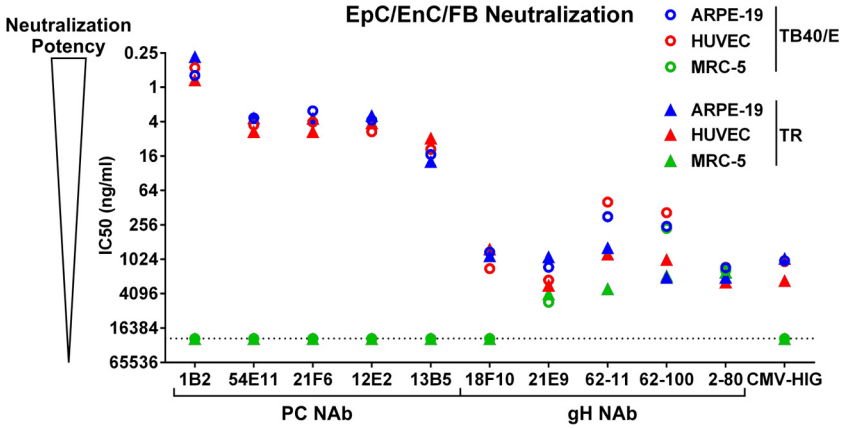

FIG 2 Neutralization potency of vaccine-derived NAb. NAb derived from MVA-PC immunized mice were used in a microneutralization assay to determine the antibody concentrations required to prevent 50% infection (IC_{50}) of ARPE-19 EpC, HUVEC EnC, and MRC-5 FB with HCMV strains TB40/E and TR. CMV-HIG was used as a reference. The dotted line indicates the highest antibody concentration used in the assay (25 μg/ml).

uated as a reference. As anticipated, all PC-specific NAb blocked TB40/E or TR infection of ARPE-19 cells and HUVEC with potency that significantly exceeded (on average more than 200-fold) that of anti-gH NAb or CMV-HIG (Fig. 2 and see Table S1 in the supplemental material). In contrast, most of the gH-specific NAb inhibited HCMV infection of all investigated cell types with comparable potency, albeit with much lower potency than the PC-specific NAb blocked ARPE-19/HUVEC entry. Neutralization potency of the anti-gH NAb was similar to that determined for CMV-HIG. We did not observe neutralization with CMV-HIG on MRC-5 FB at the highest investigated concentration (25 μg/ml), which is consistent with observations obtained by others (37, 70). Interestingly, one gH-specific NAb (18F10) demonstrated inhibition potency comparable to the other anti-gH NAb when measured on ARPE-19 cells and HUVEC, but it did not show ability to block HCMV infection of FB. Note that the IC_{50} values of the vaccine-induced PC and gH-specific NAb that we isolated were similar to published values determined for NAb isolated from $HCMV^+$ individuals (see Table S1 in the supplemental material) (32). In contrast to the other anti-gH NAb, two of the gH-specific NAb (62-11 and 62-100) blocked TR infection less potently than infection of TB40/E (Fig. 2), suggesting that anti-gH NAb 62-11 and 62-100 may target epitopes that are antigenically distinct in TB40/E and TR. In summary, these results show that the vaccine-derived PC and gH-specific NAb have neutralization potency comparable to that observed for NAb previously isolated from chronically HCMV-infected individuals.

PC-specific NAb limit HCMV spread in EpC more potently than anti-gH NAb. HCMV replication is highly cell-associated and the virus predominantly spreads from cell to cell (71, 72). Hence, inhibition of HCMV cell-to-cell spread and/or syncytium formation besides neutralization of cell-free HCMV entry may be an important antibody function to prevent dissemination. To test whether the MVA-PC vaccine-derived NAb can block spread of HCMV, we evaluated their potency to inhibit HCMV TB40/E or TR cell-to-cell spread and/or syncytium formation in ARPE-19 EpC. As noted for the neutralization potency (Fig. 2), we found significant differences in the potency of the PC- and gH-specific NAb to prevent HCMV cell-to-cell spread (Fig. 3 and see Table S1 in the supplemental material). All NAb specific for PC subunits

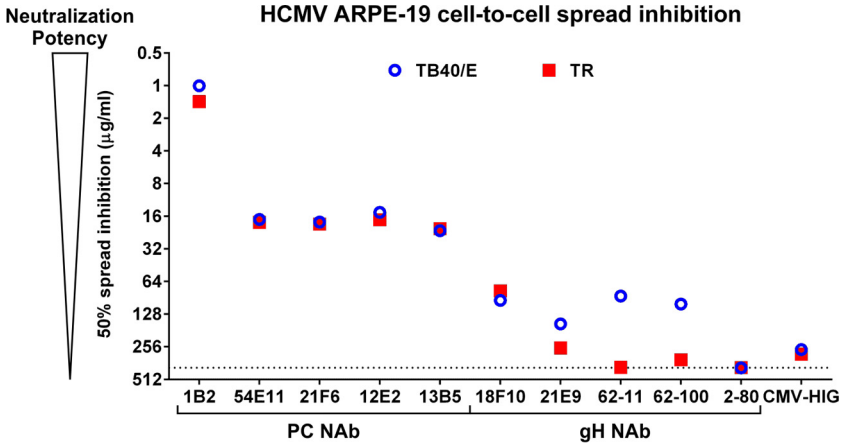

FIG 3 Inhibition of HCMV spread in EpC by NAb. ARPE-19 cells were infected with TB40/E or TR (MOI of 1), extensively washed 24 h later, and then incubated with serial dilutions of vaccine-derived NAb for 8 days. Cells were imaged for GFP quantification. The graph shows the NAb concentrations at which 50% reduction in the GFP-positive area (IC_{50}) in comparison to untreated controls was calculated. CMV-HIG was used as a control. The maximum evaluated antibody concentration is indicated by the dotted line.

blocked TB40/E and TR spread in ARPE-19 cells with potency that significantly exceeded those of the anti-gH NAb or CMV-HIG. In contrast, the anti-gH NAb demonstrated only very low spread inhibition potency or were even unable to prevent HCMV spread in ARPE-19 cells at a cutoff concentration of 400 μg/ml. Most of the anti-gH NAb demonstrated spread inhibition potency that was comparable to or only slightly higher than that of CMV-HIG. Notably, the ability of individual NAb to block viral spread in ARPE-19 EpC was similar to their property to neutralize HCMV infection of these cells, with the variation that higher antibody amounts (1,000-fold) were required to interfere with HCMV cell-to-cell spread than with HCMV entry (see Table S1 in the supplemental material). Consistent with the neutralization by the anti-gH NAb, we observed less potent spread inhibition of HCMV TR than of TB40/E by two of the anti-gH NAb (62-11 and 62-100), suggesting that sequence variation in the gH protein influences the ability of the NAb to prevent HCMV spread. Figures S1 and S2 in the supplemental material show the effects of potent PC-specific NAb 1B2 compared to anti-gH NAb and untreated controls to interfere with TB40/E and TR spread and/or syncytium formation in ARPE-19 cells following infection at a high or low MOI. Importantly, infection of the ARPE-19 cells with both TR and TB40/E remained focal, indicating virus transmission primarily from cell-to-cell without releasing large amounts of cell-free virus (see Fig. S2 in the supplemental material). These results indicate that PC-specific NAb are more potent than anti-gH NAb to prevent HCMV spread in EpC.

Neutralization potency of vaccine-induced NAb may correlate with antibody affinity. It has been shown that a reduced risk for HCMV transmission to the fetus is associated with high affinity, highly neutralizing antibody responses (42, 73). Consequently, we evaluated the affinity of the vaccine-induced NAb to bind cell surface PC on MVA-PC-infected BHK-21 cells and investigated whether the antibody affinity correlates with potency to neutralize HCMV. As shown in Fig. 4A, we determined a positive correlation between antibody affinity and neutralization potency taking into account all isolated NAb ($r = 0.743$, $P = 0.014$). Consistent with the neutralization potency, higher binding affinity was observed with the PC-specific NAb than with the anti-gH NAb (Fig. 4A and see Table S1 in the supplemental material). In addition, the highest affinity was observed with 1B2, which is the most potent NAb to block EpC/EnC entry that we have identified (see Table S1 in the supplemental material). However, despite a significant difference in neutralization potency between PC- and gH-specific NAb ($P = 0.0167$, Fig. 4C), the difference in binding affinity of the PC-specific NAb and anti-gH NAb was not significant ($P > 0.05$, Fig. 4B). Of importance, the EC_{50} values that we determined for the vaccine-derived NAb were in the range of published values for HCMV NAb observed by others (34). These findings provide evidence that the neutralization potency of the vaccine-derived PC and gH-specific NAb correlates with their ability to bind the PC.

Vaccine-derived PC and gH-specific NAb recognize different antigenic target sites. It has been reported that human NAb recognizing the PC target at least seven distinct antigenic sites (32, 33). In order to determine whether the vaccine-induced NAb bind overlapping or nonoverlapping target sites of the PC, we evaluated their ability to cross-compete for binding to PC expressed in MVA-PC-infected BHK-21 cells. As shown in Table 2 (see also Fig. S3 and S4 in the supplemental material), binding competition was

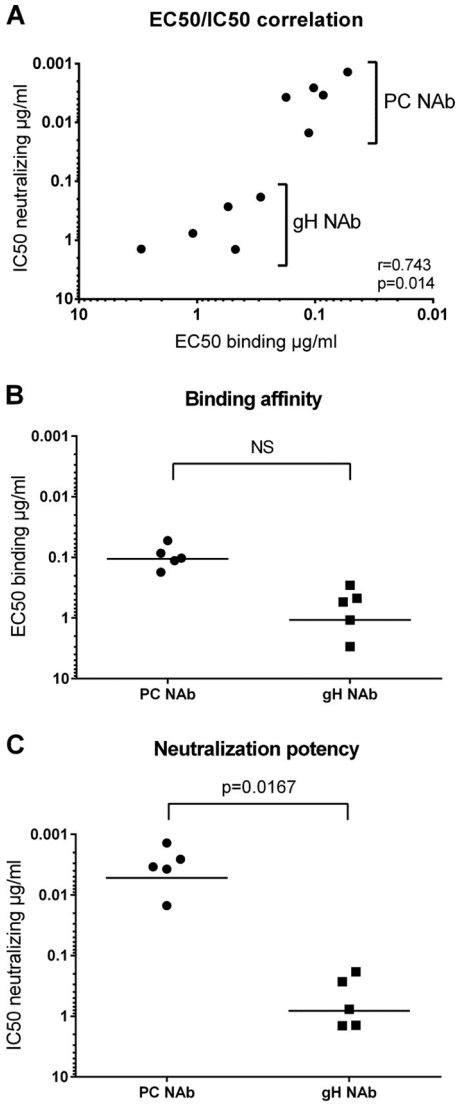

FIG 4 Correlation analysis between NAb binding affinity and neutralizing potency. (A) NAb EC_{50} and EpC IC_{50} values were plotted, and two-tailed Pearson analysis resulted in a positive correlation ($r = 0.7432$, $P = 0.014$). (B) NAb were grouped in PC-specific NAb and anti-gH NAb based on their subunit recognition (Fig. 1 and Table 1). No statistically significant difference between the two groups was found using an unpaired *t* test ($P > 0.05$). (C) The same groups as in panel B were analyzed based on their ARPE-19 IC_{50} values. As evaluated using an unpaired *t* test, the neutralizing potency of PC-specific NAb was significantly different ($P = 0.0167$) from that of gH NAb.

observed between the two PC-specific NAb 1B2 and 12E2, indicating that 1B2 and 12E2 recognize overlapping target sites formed by UL128/130/131A. The same result was obtained with the PC-specific NAb 54E11 and 21F6, demonstrating that these NAb target similar binding sites constituted by UL130/131A. UL128/130/131A-specific NAb and UL130/131A-specific NAb did not compete for binding with each other or with the anti-UL128 NAb 13B5. We also found binding competition between the anti-gH NAb 62-11 and 62-100 and between anti-gH NAb 21E9 and 2-80. Hence, 62-11/62-100 or 21E9/2-80 target similar antigenic sites on gH. In addition, 62-11 and 62-100 demonstrated ability to partially compete for binding with 21E9 and 2-80, sug-

TABLE 2 NAb competition for binding to the PC

NAb	Unlabeled Ab	Binding inhibition (%) of biotinylated Abs[a]											Antigenic site[b]
		1B2	54E11	21F6	12E2	13B5	18F10	21E9	62-11	62-100	2-80	AP86	
PC	1B2	100	0	0	100	0	0	ND	ND	ND	ND	ND	1UL
	54E11	0	100	100	0	0	0	ND	ND	ND	ND	ND	2UL
	21F6	0	100	100	0	0	0	ND	ND	ND	ND	ND	2UL
	12E2	100	0	0	100	0	0	ND	ND	ND	ND	ND	1UL
	13B5	0	0	0	0	100	0	ND	ND	ND	ND	ND	3UL
gH	18F10	0	0	0	0	0	100	0	0	0	0	100	1gH
	21E9	ND	ND	ND	ND	ND	0	100	32	28	100	0	2gH
	62-11	ND	ND	ND	ND	ND	0	46	100	100	54	0	3gH
	62-100	ND	ND	ND	ND	ND	0	51	100	100	57	0	3gH
	2-80	ND	ND	ND	ND	ND	0	100	43	40	100	0	2gH
	AP86	ND	ND	ND	ND	ND	100	0	0	0	0	100	1gH

[a] ND, not done.
[b] Antigenic site numbers are arbitrarily assigned based on cross-competition.

gesting that 62-11 and 62-100 share partially overlapping binding sites on gH with 21E9 and 2-80. In contrast to all other isolated gH NAb, NAb 18F10 was not able to compete with any of the gH-specific NAb. Overall, we could identify three antigenic sites on the UL128/130/131A subunits, and three antigenic sites on gH (Table 2 and see Fig. S3 and S4 in the supplemental material). Although some of the NAb competed for the same antigenic site, we determined unique variable heavy (V_H) and light (V_L) chain sequences for most of the NAb (see Table S2 in the supplemental material). Identical V_H and V_L sequences were only observed for the two UL130/131A-specific NAb 21F6 and 54E11. However, we confirmed that 54E11 and 21F6 have different isotypes (see Table S2 in the supplemental material), suggesting that these NAb were derived from the same centroblast B cell after class switch recombination (74). We identified a very limited number of point mutations in V_H and V_L sequences of the NAb compared to germ line sequences (see Table S2 in the supplemental material), suggesting that, at least in immunized mice, potent HCMV NAb are already encoded by the germ line with very low influence of affinity maturation. In summary, these data indicate that the vaccine-derived NAb recognize predominantly distinct antigenic target sites on the UL128/130/131A subunits or gH.

NAb 18F10 binds an immunodominant linear epitope on gH. Since all identified gH-specific NAb showed binding to gH by intracellular and cell surface staining even in the absence of gL (Fig. 1 and Table 1), we investigated whether the anti-gH NAb bind linear or conformational epitopes on the gH protein. Therefore, we tested the NAb to recognize gH expressed from adenoviral vectors (AdV) using immunoblot analysis under denaturing conditions. As a control, we used the well-characterized anti-gH antibody AP86, which is known to bind the linear immunodominant neutralizing epitope of gH (34-LDPHAFHLLL-43) (59). Compared to AP86, only anti-gH NAb 18F10 efficiently recognized denatured gH, while all other gH-specific NAb demonstrated only minimal ability to react with the linear form of gH (Fig. 5A). Based on this observation, we surmised that 18F10, like AP86, binds the linear immunodominant epitope of gH. To obtain further evidence for the similar antigen recognition properties of AP86 and 18F10, we determined the neutralization potency of 18F10 and AP86 to block TB40/E infection of MRC-5 FB or ARPE-19 EpC. In contrast to previous reports (59), although

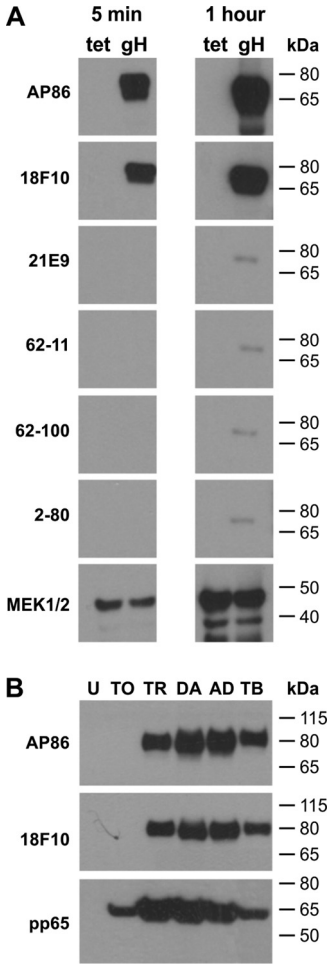

FIG 5 Recognition of linear gH by vaccine-derived gH-specific NAb. (A) Immunoblot detection of gH expressed from Ad vectors in infected ARPE-19 EpC using vaccine-derived anti-gH NAb and anti-gH antibody AP86. Cells infected with Ad-tet were analyzed for control. Chemiluminescence detection of gH was performed after short (5 min) or long (1 h) exposure of X-ray films to the immunoblot. MEK1/2 detection was performed as loading control. (B) Immunoblot detection of gH from HCMV strains Towne (TO), TR, Davis (DA), AD169 (AD), or TB40/E (TB) in infected FB using 18F10 and AP86. Uninfected cells (U) were used as a control. For control, samples were analyzed with anti-pp65 antibody (23-103). Mass markers (kDa) are shown next to each panel.

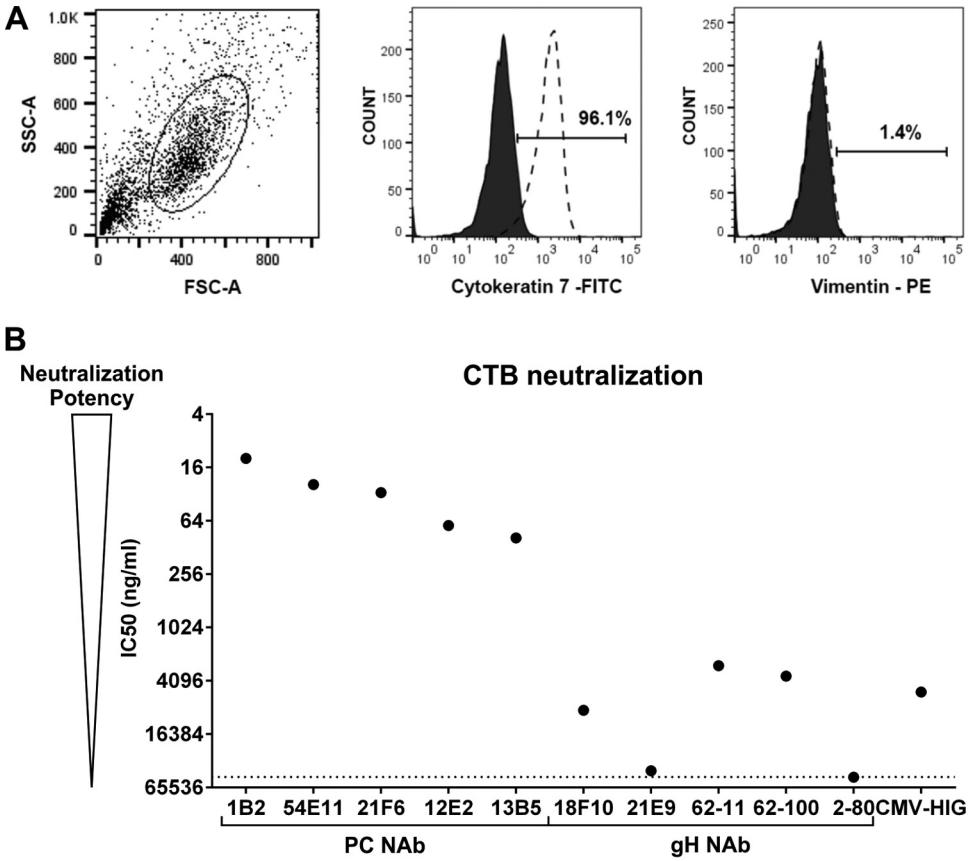

FIG 6 Neutralization of CTB infection by vaccine-derived NAb. (A) Primary CTB characterization. FSC versus SSC dot plot on the left indicates the gated population of CTB analyzed. Histograms represent cytokeratin-7 (center) and vimentin (right) expression of the gated CTB population. (B) NAb were tested for their ability to neutralize TB40/E infection of primary CTB isolated from term placentae. The IC_{50} values for each vaccine-derived NAb are shown. CMV-HIG was used as a control. A dotted line indicates the highest antibody concentration used in the assay (50 μg/ml).

comparable to what we observed for vaccine-derived anti-gH NAb 18F10, AP86 was unable to neutralize entry of TB40/E into MRC-5 FB (see Table S1 in the supplemental material). However, AP86 had comparable potency to 18F10 to prevent TB40/E entry into ARPE-19 cells (IC_{50} = 1 μg/ml; see Table S1 in the supplemental material). Since the AP86 epitope is present in most HCMV strains but not in Towne due to a gap and a point mutation (34-LD*KAFHLLL-43) (59), we evaluated 18F10 and AP86 for recognition of gH in Towne, TR, Davis, AD169, and TB40/E strain-infected MRC-5 cells via immunoblotting. As predicted, both 18F10 and AP86 bound to gH from TR, Davis, AD169, and TB40, but they did not bind to Towne gH (Fig. 5B). Finally, we evaluated cross competition of AP86 with 18F10 and, as a control, also with all other gH-specific NAb to recognize the PC in MVA-PC-infected BHK-21 cells. As anticipated, only 18F10 competed with AP86 for binding to the PC (Table 2 and see Fig. S4 in the supplemental material). These data indicate that the binding site of vaccine-derived anti-gH NAb 18F10 overlaps with the linear immunodominant epitope of HCMV gH and thus provide further evidence that NAb induced by MVA-PC are similar to those induced during natural HCMV infection.

PC-specific NAb are more potent than anti-gH NAb to block HCMV infection of placental CTB. CTB are thought to be the key placental cells HCMV utilizes to cross the fetal-maternal interface (40, 41, 46). In order to determine whether the vaccine-derived NAb can block HCMV infection of CTB, we used a standard microneutralization assay to evaluate their neutralization potency against TB40/E using freshly prepared primary CTB from term placentae. As shown in Fig. 6A, the prepared cell populations were almost exclusively positive for cytokeratin-7 and negative for vimentin, showing that almost all cells were primary CTB, whereas only minor proportions accounted for mesenchymal cells (57, 58, 75). Consistent with the potency to neutralize HCMV entry and cell-to-cell spread, the neutralization potency of all PC NAb measured on CTB was significantly higher than that of the NAb targeting gH or CMV-HIG (Fig. 6B and see Table S1 in the supplemental material). Compared to all NAb we isolated, the highest potency to block CTB infection was demonstrated with PC-specific NAb 1B2, which had also the most potent ability to inhibit HCMV entry into EpC and spreading in these cells. In contrast, anti-gH NAb showed lower level neutralization potency against TB40/E on CTB that was only comparable to that of CMV-HIG. Some anti-gH NAb were even unable to prevent TB40/E infection of CTB at the highest investigated antibody concentration (50 μg/ml). These data demonstrated that NAb specific for the UL128/130/131A subunits of the PC confer higher protection against HCMV infection of primary CTB from term placentae than NAb targeting gH or polyclonal Ab preparations from $HCMV^+$ individuals.

DISCUSSION

HCMV elicits high-titer NAb that block entry into EpC/EnC and lower titer NAb that prevent FB infection (37–39). These characteristic cell type-specific NAb titer arise from NAb to the major gC (gB, gH/gL, and gM/gN) that inhibit both EpC/EnC and FB entry and unusually potent NAb to conformational epitopes of the PC that specifically interfere with HCMV entry into EpC/EnC (32). The difference of the NAb titer observed on EpC/EnC and FB is likely caused by NAb targeting the PC. Our recently introduced vaccine concept based on the delivery of a membrane-tethered PC by an MVA poxvirus vector (MVA-PC) shows that the PC is sufficient as an immunogen to elicit prototypic EpC/EnC- and FB-specific NAb responses against HCMV (36). In the present study, we confirm that the NAb induced by MVA-PC have properties comparable to human PC- and gH-specific NAb from $HCMV^+$ individuals (32). In addition, we demonstrate that the vaccine-derived PC-specific NAb are significantly more potent than anti-gH NAb in preventing entry into placental CTB, cell types considered of critical importance for HCMV vertical transmission and infection of the fetus. These results further validate MVA-PC as a clinical vaccine candidate to prevent congenital HCMV infection.

Several findings confirm that the NAb isolated from MVA-PC immunized mice are similar to human NAb previously isolated from $HCMV^+$ individuals (32). Consistent with human NAb targeting the PC, the vaccine-derived PC-specific NAb recognize predominantly conformational epitopes constituted by UL130/131A and UL128/130/131A or an epitope specific for UL128. As with human NAb, the vaccine-derived PC-specific NAb are unable to prevent FB infection, although they are dramatically more potent than the anti-gH NAb that we have isolated to block EpC/EnC entry. Importantly, all vaccine-derived NAb either specific for the PC or gH have IC_{50} neutralization values that are well within the range of those determined for human NAb (32). Although we isolated only a limited number of NAb, we identified three nonoverlapping recognition sites for the vaccine-derived PC-specific NAb. Furthermore, similar to human NAb specific for gH, most of the vaccine-derived anti-gH NAb have ability to block HCMV infection of both FB and EpC/EnC, although they are significantly less potent than the PC-specific NAb. One of the anti-gH NAb derived from MVA-PC appears to recognize the immunodominant linear epitope of gH (59), further supporting that anti-gH NAb elicited by this vector have antigen recognition comparable to human Ab. These results strongly suggest that MVA-PC elicits NAb to linear and conformational epitopes of the UL128/130/131A subunits and gH that have antigen specificity and neutralization potency similar to NAb induced by HCMV during natural HCMV infection.

Our observations demonstrate that NAb induced by MVA-PC potently interfere with HCMV cell-to-cell spread and/or syncytium formation in EpC. These findings support results by Kabanova et al. (33), who investigated inhibition of HCMV cell-to-cell spread in ARPE-19 EpC by antisera that were raised against purified gB, gH/gL, or PC in mice. We demonstrate with MAb derived from MVA-PC immunized mice that NAb targeting the PC are significantly more potent than anti-gH NAb to interfere with EpC-associated replication of HCMV. As for many other studies that have reported HCMV spread inhibition (33, 50, 71, 72), a caveat of our study is that we did not use cell culture agarose overlays to obtain a more clear distinction between inhibition of infection by cell-associated and cell-free virus transmission. However, unlike HCMV infection in FB, which is largely driven by cell-free virus and where homogenous infection of cell culture monolayers can be observed following inoculation with a low MOI, HCMV infection in EpC/EnC is highly cell associated and remains primarily focal (71). We confirmed these HCMV replication properties in our spread inhibition experiment, indicating that the reduced infection in ARPE-19 EpC in the presence of the vaccine-derived NAb reflects primarily interference with HCMV cell-to-cell spread and only minimally inhibition of cell-free virus transmission. In addition, the 1,000-fold-higher Ab concentration to attain the IC_{50} neutralization values required for the NAb to prevent focal spread and/or syncytium formation suggest a different mechanism of interference than inhibition of HCMV entry by cell-free virus. Since the PC is thought to be involved in receptor-mediated entry of HCMV into EpC, our results showing that NAb targeting the PC are able to block HCMV cell-to-cell spread in EpC suggest that this mode of HCMV transmission must include an extracellular phase and does not occur by direct transfer from cell to cell. We speculate that the requirement of the extremely large amounts of NAb to inhibit HCMV cell-to-cell spread compared to the relatively low Ab amounts necessary to prevent HCMV entry in EpC is a consequence of the predominant cell-associated transmission mode of HCMV as opposed to low-level transmission by cell-free virus.

In contrast to our results are the findings by Jacob et al. (76), who investigated the inhibition of cell-to-cell spread by NAb using agarose overlays to exclude bias by cell-free virus. These authors demonstrated that monoclonal NAb targeting gB, gH, or the PC are unable to prevent HCMV spread in EpC. However, these authors used only very small amounts of Ab to evaluate HCMV spread inhibition, and thus their results are difficult to compare with our findings. We conclude that NAb in concentrations that dramatically exceed the Ab IC_{50} neutralization values are able to inhibit HCMV spread in EpC. In addition, our results show that NAb recognizing the PC are significantly more effective than anti-gH NAb to interfere with HCMV replication in these cells. In sum, these results demonstrate that PC-specific NAb induced by MVA-PC confer potent inhibition of HCMV entry and, in addition, they have potent ability to prevent HCMV spread and/or syncytium formation in EpC, suggesting that the anti-PC NAb elicited by MVA-PC may have the potential to limit cell-associated virus dissemination in the human host and transmission to the fetus.

Compared to the limited number of vaccine-derived NAb that we have isolated, two other groups isolated a much larger number of PC-specific NAb from animals immunized with either the UL128/130/131A-repaired HCMV laboratory strain AD169 or purified PC protein using high-throughput antibody production technologies (33, 34). As noted for the antibodies elicited by MVA-PC, NAb induced by the protein vaccine have properties that are remarkably similar to those of naturally induced human PC-specific NAb (33). Unfortunately, the study of the repaired AD169 virus does not provide a detailed analysis of the NAb subunit specificity, and it remains unclear whether antibodies elicited by the repaired virus recognize gH/gL or UL128/130/131A epitopes (34). Moreover, only two of the named "elite neutralizers" specifically interfere with the EpC/EnC route of HCMV entry, suggesting that only these two NAb recognize conformational

epitopes of the UL128/130/131A subunits. In contrast, we demonstrate that five out of the 10 MVA-PC derived NAb have PC-specific antigen recognition, and all of them have IC_{50} values higher than those of the "elite neutralizers" obtained with repaired AD169.

Consistent with other PC-based vaccine approaches (33–35), MVA-PC induces gH-specific NAb that have ability to prevent infection of both FB and EpC/EnC entry with comparable potency. These anti-gH NAb are likely induced by the gH component within the PC and recognize neutralizing epitopes that are shared by the PC and gH/gL (or gH/gL/gO), thereby interfering with both major entry routes. It may also be possible that anti-gH NAb are induced by MVA-PC because some of the gH expressed from the MVA-PC vaccine vector is presented to the immune system without being assembled with other PC subunits. However, similar cell surface staining intensity of MVA-PC-infected cells with the UL128/130/131A-specific NAb and anti-gH NAb (Fig. 1B) indicate that the individual PC subunits are only efficiently assembled with each other and transported to the cell surface when all five subunits are coexpressed. This suggests that gH expressed from MVA-PC is presented on the cell surface mainly associated with intact PC and in smaller amounts in other configurations (gH/gL). Consequently, anti-gH NAb induced by MVA-PC or other PC-based vaccine candidates may function as a surrogate for NAb targeting the major essential gC and provide broad host cell entry inhibition.

Our data suggest a positive correlation of the Ab neutralizing potency and binding affinity to cell surface PC for the PC- and gH-specific NAb that we have isolated. These findings are in contrast to observations by Freed et al. (34), who investigated a correlation of the Ab neutralization potency and binding affinity at the whole virion level for a large panel of MAb that were isolated from a single rabbit immunized with repaired AD169. We believe the major reason for the discrepancy between the two studies is likely a consequence of the different types of Ab that were included in the correlation analysis. We focused our correlation analysis exclusively on MAb with neutralizing activity, whereas Freed et al. included in their correlation analysis MAb with or without neutralizing activity. In fact, when we use the same test (Pearson test) that we have applied for the correlation analysis in our study to determine a correlation for the published neutralization potency and affinity values of MAb that were categorized as NAb in the study by Freed et al., the correlation between these two parameters is highly significant, even stronger than that determined for the NAb we have isolated. Hence, both studies suggest a relationship of the Ab neutralization potency and binding affinity when applied to MAb with potent neutralizing activity, while this is not the case when the analysis includes MAb with lesser potency or no ability to neutralize HCMV. However, in contrast to the difference in neutralization potency of the vaccine-derived PC-specific NAb and anti-gH NAb that we have isolated, the difference in binding affinity between these two groups of NAb was not significant. When associating these findings with the recent report describing protein amounts in HCMV virions (77), it is tempting to speculate about the potency of HCMV-specific NAb. We surmise that the significant difference in neutralization potency between PC-specific NAb and NAb targeting gH/gL (or gB) may reflect the relative small amount of the UL128/130/131A subunits in HCMV virions compared to gH/gL. Hence, much lower antibody concentrations are required to interfere with PC-mediated entry than with the fusion function of gH/gL (77). In contrast, the difference in neutralizing potency of individual NAb targeting the UL128/130/131A subunits of the PC may be a function of their binding affinity. Further studies are required to clarify whether the potency of HCMV NAb is dependent on the abundance of different gC in HCMV virions and on their affinity for their antigen target.

Interestingly, we also identified one NAb (18F10) with gH specificity that neutralized HCMV entry into EpC/EnC, although this antibody was unable to block FB infection, at least when determined with the "clinical strain-like" HCMV isolates TB40/E and TR. This is surprising since only NAb targeting the UL128/130/131A subunits are known to specifically interfere with EpC/EnC entry (32, 33), but an anti-gH NAb with cell type-specific inhibition property has not been reported. Compared to anti-gH antibody AP86 that targets the immunodominant linear epitope of gH (59), the vaccine-derived anti-gH NAb 18F10 has similar ability to recognize denatured gH of different HCMV strains with sequence variation in the AP86 epitope. In addition, AP86 and 18F10 compete for antigen recognition, showing that these two antibodies likely target overlapping binding sites. Whether they target identical binding sites and, hence, whether 18F10 recognizes the immunodominant linear gH epitope has to be confirmed by a peptide-based scanning procedure. The discovery of such an anti-gH NAb raises questions about the involvement of gH in receptor-mediated entry into EpC/EnC. It may be possible that 18F10 is binding to a sequence on gH that is directly or indirectly involved in the interaction of the PC with its receptor on EpC/EnC, but this binding position is not involved in FB entry. In addition, the binding similarity of 18F10 and AP86 indicates that the 18F10 epitope is likely localized on the gH N terminus, which is considered a receptor binding region based on the comparison with gH/gL complexes of other herpesviruses (29).

We demonstrated that PC-specific NAb isolated from MVA-PC-immunized mice show high potency to prevent HCMV entry into placental CTB, central players for HCMV placental transmission (10, 44, 46, 78). Previous reports by Zydek et al. demonstrated that entry into CTB progenitor cells obtained from first trimester placentae can be blocked by NAb to gB, although PC-specific NAb were unable to prevent infection of CTB progenitors (49). These observations suggest that gB-specific NAb, unlike NAb targeting the PC, may play a critical role in preventing HCMV from interfering with placental organ development and thus cause deregulation at the fetal-maternal interface during early stage of gestation (49, 50). Our results show that the MVA-PC-derived NAb targeting the PC are significantly more potent than the vaccine-derived anti-gH NAb that we have isolated to block HCMV entry into differentiating CTB from term placentae. This is the first evidence that NAb recognizing the PC have potent ability to block primary CTB infection. Consequently, PC-specific NAb may play a critical role in inhibiting HCMV from crossing the placenta or to cause placental developmental abnormalities at late stage of gestation when highest transmission rates of HCMV occur (79). Although the risk of vertical transmission is lower during early stages of gestation, the severity of disease outcome is higher (78, 80). Therefore, it is critical to investigate whether NAb targeting the PC can also block infection of CTB during the first trimester of pregnancy. Pereira et al. showed that *ex vivo* infection of villous explants from first trimester placentae can be efficiently prevented with PC-specific NAb, while gB NAb or CMV-HIG are 20 to 2,000 times, respectively, less potent to prevent infection of the villous

explants (81). This suggests that infection of CTB isolated from first trimester placentae may be as susceptible to PC-specific antibody-mediated neutralization as infection of CTB isolated from term placentae. Taken together, these results indicate that PC-specific NAb have differing abilities to neutralize CTB infection dependent on the state of CTB differentiation (49, 50).

Conclusions about the inhibitory potency of the vector-derived NAb that we have isolated should be considered with some reservations since they were only compared directly with CMV-HIG, which are purified polyclonal IgG preparations from $HCMV^+$ individuals that are composed of many different Ab types, with CMV-specific Ab in the minority. A direct comparison of our vector-derived NAb with NAb previously isolated from $HCMV^+$ individuals may add to a more precise understanding whether NAb induced by MVA-PC are as potent as those elicited by HCMV, in particular regarding prevention of cell-associated spread and CTB infection. However, this was difficult to conduct due to the unavailability of human PC-specific neutralizing MAb and challenges to derive such Ab. In this regard, the finding that MVA-PC stimulates NAb with overlapping binding sites to the immunodominant linear neutralizing epitope of gH may serve as more concrete evidence that Ab induced by MVA-PC and HCMV are of strong similarity. Despite the fact that we did not directly compare our NAb with human NAb isolated from $HCMV^+$ persons, our findings support the previously reported cell type-specific neutralization potency conferred by anti-PC and anti-gH NAb and provide evidence that PC-specific NAb are more potent than anti-gH NAb in inhibiting the cell-to-cell spread and infection of primary CTB (82, 83).

Since naturally induced Ab are not completely protective against HCMV transmission to the fetus, simply inducing immune responses similar to those induced by HCMV will likely not significantly alter the outcome of congenital infection, independent of HCMV seroprevalence (84). However, it has been demonstrated in two independent studies that an early Ab response to neutralizing epitopes of the PC or an early Ab response of high affinity during primary infection is associated with a reduced risk of HCMV vertical transmission (85, 86). These findings, combined with the absence of HCMV immune evasion pathways and immune diversion to less protective or nonprotective epitopes, when using a non-HCMV viral vaccine approach suggest that a subunit vaccine based on the PC such as MVA-PC could boost high affinity, highly neutralizing Ab responses that in protective magnitude and quality exceed those elicited by HCMV during natural infection. Hence, not only HCMV-seronegative women but also seropositive women may benefit from a subunit vaccine that has the primary property of eliciting humoral immunity based on antigenic forms of the PC. Furthermore, MVA provides the advantage over other subunit vaccines that it can easily accommodate additional immunodominant HCMV antigens such as gB, phosphoprotein 65, or IE1 to further improve the vaccine's ability to elicit robust humoral and cell-mediated immunity. Whether these assumptions translate into the induction of protective anti-HCMV immunity in humans can only be clarified by moving the vaccine candidates forward to clinical trials.

In summary, we confirm that MVA-PC induces potent mouse NAb that recognize different conformational and linear antigenic sites of the UL128/130/131A subunits or gH. The neutralization potency and subunit specificity of the vaccine-derived NAb are consistent with anti-PC and gH-specific NAb isolated from $HCMV^+$ individuals (32). In addition, the PC-specific NAb derived from MVA-PC have potent ability to prevent HCMV spread and/or syncytium formation in EpC and infection of placental CTB. These results further validate MVA-PC as a potential vaccine candidate to induce NAb that have properties similar to those induced by HCMV during natural infection for potent inhibition of HCMV host cell entry, cell-associated spread, and infection of key placental cells involved in HCMV fetal infection.

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